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Aspergillus welwitschiae: A Potential *amylases* Producer

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Abstract

Amylases, glycoside hydrolases widely used in several industrial processes, can be produced by many animals, plants, bacteria, and fungi. Fungal *amylases* from *Aspergillus* sp. hold remarkable importance in biotechnological applications for presenting a great catalysis efficiency in a wide range of pH and temperature. The production of *amylases* is mainly dependent on the genetic background of the species, i.e., *Aspergillus* strains, and abiotic factors. Among the major producers of *amylases* are the species of *Aspergillus* section *Nigri*, including *Aspergillus welwitschiae*. In this study, *Aspergillus welwitschiae* strains were evaluated for their ability to produce extracellular *amylases*. Among the 24 strains, wild *Aspergillus welwitschiae* UELAs 15.262 and mutant *A. welwitschiae* UELAs 15.262/35 strains showed greater potential for *amylases* production. The *A. welwitschiae* UELAs 15.262 produced more *amylases* (8645 U/mg) when compared to *A. welwitschiae* UELAs 15.262/35 (6666 U/mg). The *amylases* activity from partially purified crude enzymatic extract of *A. welwitschiae* UELAs 15.262 strain obtained at pH 5.5, 60 °C, resulted in 1.98-fold (3837 U/mg) increase in enzymatic activity. Likewise, the *amylases* activity from partially purified crude extract of *A. welwitschiae* UELAs 15.262/35 obtained at pH 5.0, 60 °C resulted in 2.2-fold (9077 U/mg) increase in *amylases* activity. The presence of metallic ions (Cu^{2+} and Fe^{3+}) also provided an increase of *amylases* activity for both strains. To our knowledge, this is the first study reporting the ability of *Aspergillus welwitschiae* strains in order to produce *amylases*.

Introduction

Amylases are of great biotechnological importance and can be applied in pharmaceutical, food, and even environmental industries [1, 2]. The *amylases* (EC 3.2.1.1) catalyze starch hydrolysis by breaking the α-1,4-O-glycosidic bonds of polysaccharides and are distributed into three major groups, the *alpha-amylases* (EC 3.2.2.1.1), the *beta-amylases* (EC 3.2.1.2), and the *glucoamylases* (EC 3.2.1.3) [3, 4].

This class of hydrolytic enzymes can be produced by several microorganisms, highlighting fungi such as *Aspergillus niger* widely used in industrial processes [2, 4]. However, the search for new enzyme-producing species like *A. welwitschiae* is an assiduous exercise and may also present potential for industrial and/or biotechnological applications.

The *Aspergillus welwitschiae* is inserted in the *Aspergillus* section *Nigri*. In this section, there are a group of species with very similar morphological characteristics that are difficult to identify, denominated as "*niger* aggregate," including *A. welwitschiae* [5]. This species has already been isolated from sources, such as onion bulbs, garlic, and other products [6–9]. Although the production of Fumonisin B2 and Ochratoxin A has been reported in *A. niger* and *A. welwitschiae*, part of these strains do not produce these mycotoxins [7, 9, 10] and can be used in biotechnological applications.

Approximately 60% of the industrial production of enzymes is made by filamentous fungi, as they have advantageous production characteristics, such as development in wide temperature and pH ranges, and mainly produce of large amounts of enzyme [2, 11, 12]. Traditionally, *amylases* are produced industrially by Submerged Fermentation (SmF) allowing the monitoring of the fermentation process, followed by easy extraction and separation of the mycelium.

Regarding to *amylases* production by filamentous fungi, there are still some limiting factors to their availability. A greater production of these enzymes can be achieved by the

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association between abiotic factors and genetic background of the strains and the maximum catalytic activity, which can be obtained by characterizing the *amylases*.

In this sense, strategies such as the use of mutant strains for the *amylases* production have been developed in order to obtaining large amounts of theses enzymes. The association between abiotic and biotic factors, such as mutant strains, has provided an increase in *amylases* production and activity [13, 14].

The characterization of pH, temperature, and metallic ions are among the main factors involved to obtain the maximum catalytic activity of *amylases*. Such associated parameters adequately provide the *amylases* with maximum and stable catalytic activity. Variations in temperature and pH can result in protonation or deprotonation of a specific side groups at the enzyme active site, changing its chemical features, altering the enzyme conformation, and causing decrease in substrate affinity or loss activity [15]. Most *amylases* are holoenzymes and therefore require metallic ions as cofactors for correct active site orientation and protein stabilization [16–18].

Therefore, in this study it demonstrated the production and partial characterization of *amylases* by wild selected and mutant *Aspergillus welwitschiae* strains. The results showed an increase of enzyme production after optimization of abiotic parameters and partial characterization of the crude enzyme extract obtaining the maximum *amylases* activity.

Materials and Methods

Biological Material

This study used 24 *Aspergillus* strains, isolated from garlic marketed in different Brazilian states. The strains were identified as *Aspergillus welwitschiae* and characterized as non-producing potential Ochratoxin A and Fumonisin B2 [9]. In addition, *Aspergillus welwitschiae* UELAs 15.262/35 strain was introduced in this study to evaluate the production and characterization of *amylases*. *Aspergillus welwitschiae* UELAs 15.262/35 is a mutant strain for citric acid production obtained from the *A. welwitschiae* UELAs 15.262 wild strain, by random mutation induced by ultraviolet light [19].

Selection of *Aspergillus welwitschiae* Strains for *amylases* Production

A. welwitschiae strains were inoculated punctually in Petri dishes, containing Czapeck-Dox agar medium (soluble starch 20 g/L; $NaNO_3$ 1 g/L; K_2HPO_4 1 g/L; $MgSO_4.7H_2O$ 1 g/L, $FeSO_4$ 0.01 g/L, Agar 15 g/L and pH 4.5) for selection of the potential *amylases* production. Petri dishes were then incubated at 28 ± 2 °C for 5 days and stained with iodine (KI 1 g/100 mL; I_2 0.5 g/100 mL) and then the Petri dishes were incubated again for 10 days under the same conditions. The potential *amylases* production was evaluated by the Enzymatic Index (EI) expressed by the relationship between the diameter of the halo of enzymatic degradation + colony growth and the diameter of the growth of the colony [20].

The 24 *A. welwitschiae* strains were also evaluated for the potential for *amylases* production in liquid Czapeck-Dox medium. Suspensions of 10^5 conidia/mL from each *A. welwitschiae* strain were prepared and they were inoculated in 5 mL of Czapeck-Dox medium, at 28 ± 2 °C for 4 days. After, the Crude Enzymatic Extract (CEE) was collected and the potential *amylases* production was indirectly evaluated by quantifying reducing sugars by 3,5-dinitrosalicylic acid (DNS), according to Miller [21]. All the *A. welwitschiae* strains were performed in experimental triplicate, the standard deviation was performed with the R software.

The *A. welwitschiae* strain that showed higher EI and production of reducing sugars were selected for further evaluations.

Evaluation of Abiotic Parameters for the Production of *amylases* by *Aspergillus welwitschiae* Strains

The *A. welwitschiae* strain selected were for evaluation of abiotic parameters of temperature and pH for *amylases* production according to the Rotational Central Compound Design (RCCD). The evaluated temperatures and pHs were 28 °C, 30 °C, 35 °C, 40 °C, and 42 °C and 4.2, 5.0, 7.0, 9.0, and 9.8, respectively, in liquid Czapeck-Dox medium for 4 days. All analyses were performed in experimental triplicate and the standard deviation, RCCD, Analysis of Variance (ANOVA), and Tukey test ($P < 0.01$) were performed with the R software.

Amylases Production by *A. welwitschiae* Under Submerged Fermentation

A. welwitschiae strain selected under abiotic parameters for *amylases* production were subjected to *amylases* production kinetics, under SmF, in Czapeck-Dox medium. The *A. welwitschiae* UELAs 15.262/35 mutant strain was also submitted to the *amylases* production kinetics assays using the same abiotic conditions of *A. welwitschiae*. Each day, a portion (a flask with 5-mL Czapeck-Dox medium and the repetition) was removed to determine *amylases* activity, until the eighth day. *A. welwitschiae* strain was performed in experimental triplicate, and the standard deviation was performed with the R software.

Determination of *amylases* Activity of *A. welwitschiae* Strains

After each fermentation period (kinetics of *amylases* production), the cultivation was interrupted by filtration (Whatman paper no 1) to obtain the Crude Enzymatic Extract (CEE). The *amylases* activity was determined according to Sperotto [22].

The determination of *amylases* activity was carried out by enzymatic reaction, consisting of 200 µL of citrate–phosphate buffer 0.05 M, pH 6.0 (citric acid 1.05 g/100 mL; NaH_2PO_4 1.38 g/100 mL), 300 µL of soluble starch (1%), and 100 µL of the CEE of each *A. welwitschiae* strain. The reactional mixture was incubated for 30 min at 40 °C. To interrupt the enzymatic reaction, 1.5 mL of DNS was added to each sample, followed by incubation at 100 °C for 5 min and subsequent addition of 17.9 mL of distilled water. The amount of reducing sugars was evaluated at A_{550nm} (Biochrom Libra S22). Under such conditions, one unit (U) of *amylases* activity is defined as the amount of enzyme that releases 1 µmoL of reducing sugars per mL of sample per minute. Protein content was determined by the method of Lowry et al. [23].

Partial Purification of the Crude Enzymatic Extract of *A. welwitschiae* Strains

In a flask, a total of 680 mL of each CEE obtained by the selected *A. welwitschiae* strain and *A. welwitschiae* UELAs 15.262/35 mutant strain were added, which were precipitated with 80% (1:1 v/v) ammonium sulfate saturated solution, under shaking for 2 h at 4 °C. The resulting precipitate was collected at 9050 g (Hettich, Universal 320 R), by 15 min at 4 °C. The precipitate was solubilized with 12-mL citrate–phosphate buffer (0.05 M, pH 6.0). The solubilized precipitate was dialyzed for 24 h, with three changes of distilled water for obtaining Partially Purified Crude Enzymatic Extract (PPCEE).

Analysis of Partially Purified Crude Enzymatic Extract in Sodium Dodecyl Sulfate–Polyacrylamide gel electrophoresis (SDS-PAGE)

The PPCEE of *A. welwitschiae* strains were mixed with an equal volume of 2 × protein loading buffer and boiled for 95 °C for 10 min. The samples were subjected to SDS-PAGE using 4% stacking gels and 12% resolving gels in an electrophoresis mini-system. The gels were visualized using Coomassie-Brilliant Blue R-250 0.1% (w/v), methanol 50% (v/v), and glacial acetic acid 7% (v/v). The standard molecular weight protein marker (AMRESCO® Mid/Low-Range Protein Molecular Weight Marker) in a range of 14.4 – 97.4 KDa were used.

Characterization of *amylases* of the Partially Purified Crude Enzymatic Extract of *A. welwitschiae* Strains

The influence of pH on the *amylases* production of (PPCEE) was evaluated by incubating 100 µL of *A. welwitschiae* strain PPCEE for 30 min, at 40 °C in 0.05 M citrate–phosphate buffer at pHs 5.5, 6.0, 6.5, and 7.0 and Tris/HCl buffer at pHs 7.5 and 8.0. The *amylases* activity was determined [22]. The pH that provided the increased *amylases* activity for each *A. welwitschiae* strain was used for further evaluations.

Likewise, the influence of temperature on *amylases* activity was performed using 100 µL of PPCEE. The temperature range was 30 °C, 35 °C, 40 °C, 45 °C, 50 °C, 55 °C, 60 °C, 65 °C, and 70 °C for 30 min. The *amylases* activity was determined [22].

Under conditions of pH and temperature favorable to increased *amylases* activity, the thermal stability of PPCEE was analyzed. The *amylases* reaction containing 700 µL of PPCEE was incubated for different time periods (0, 20, 40, 60, and 100 min). For each period of reaction, the *amylases* activity was analyzed [22].

The influence of metal ions on the *amylase* activity of *A. welwitschiae* strains was also evaluated. So, 500 µL of PPCEE were incubated with different solutions of metallic salts (1:1 v/v) of cations: Ca^{2+}, K^{+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Fe^{3+}, and Hg^{2+} at 5 mM at 30 °C for 1 h. Aliquots of 100 µL were collected and *amylases* activity was evaluated [22]. The characterization of *amylases* of the PPCEE of *A. welwitschiae* strains was performed in experimental triplicate, and the standard deviation was performed with the R software.

Partially Purified Crude Enzymatic Extract zymogram of *amylases*

The zymographic analysis of the PPCEE of *A. welwitschiae* strains were loaded in 10% denaturing gel run. Previously, the PPCEE were mixed with an equal volume of 2 × protein loading buffer (without β-mercaptoethanol) and boiled at 95 °C for 10 min. Gel was incubated for 1 h in 50 mM sodium acetate buffer pH 5.5. So, the gel was incubated again for 12 h at 4 °C in a solution containing 50 mM sodium acetate pH 5.5 and 0.5% starch. The gel was rinsed with water and incubated for 2 h at 37 °C in 50 mM sodium acetate buffer and treated with a solution of 1.3% I_2/3% KI to stop the reaction and stain the unreacted starch background.

Results

Selection and evaluation of abiotic parameters of *Aspergillus welwitschiae* strains for *amylases* production

The 24 *A. welwitschiae* strains showed potential for *amylases* production (Table 1). The *A. welwitschiae* UELAs 15.262 strains were able to produce *amylases*, both in solid and liquid Czapeck-Dox medium (Table 1). Due to the higher production in both conditions *A. welwitschiae* UELAs 15.262 strain was selected for further evaluations.

The significant abiotic parameters for *amylases* production were obtained at 40 °C, pH 5.0–8.5 mg/mL and 35 °C, pH 7.0–7.8 mg/mL (Table 2). ANOVA showed a significant influence of pH (Supplemental Fig. S1) and considering future applications, the abiotic parameter of 35 °C, pH 7.0 for the production of *amylases* by *A. welwitschiae* UELAs 15.262 for kinetic of *amylases* production was selected.

Table 2 Abiotic parameters for *amylases* production by *A. welwitschiae* UELAs 15.262

A. welwitschiae strain	Temperature (°C)	pH	Amount of reducing sugar (mg/mL)
UELAs 15.262	35	4.2	6.0^{bc}
	40	**5.0**	**8.5^{a}**
	30	5.0	6.4^{bc}
	35	9.8	1.7^{d}
	40	9.0	1.9^{d}
	30	9.0	1.3^{d}
	35	**7.0**	**7.8^{a}**
	42	7.0	5.7^{c}
	28	7.0	6.7^{b}

The analysis was performed in experimental triplicate, using 4 mL of Czapeck-Dox medium, incubated for 4 days, 10^5 conidia/mL. The abiotic parameters in bold were significant by the Tukey test 1% level

Kinetics of *amylases* Production by *A. welwitschiae* in Submerged Fermentation

The kinetics of *amylases* production was evaluated by Smf using the previously selected abiotic parameters. At this stage under the same conditions, we introduced the *A. welwitschiae* UELAs 15.262/35 mutant strain, because this strain was previously selected for citric acid production.

There was an increase in the *amylases* production by *A. welwitschiae* UELAs 15.262 throughout the fermentation process, with higher production (951 U/mL, corresponding to 8645 U/mg and 0.11 mg/mL of total protein) of these enzymes on the seventh day. For *A. welwitschiae* UELAs 15.262/35 strain, the increase of *amylases* production was observed, only until the sixth day of fermentation, (580 U/mL, corresponding to 6666 U/mg and 0.087 mg/mL), according to Fig. 1.

Characterization of *amylases* from Partially Purified Crude Enzymatic Extract of *A. welwitschiae*

The PPCEE visualized on SDS-PAGE revealed up-regulated distinct proteins for both *A. welwitschiae* strains. Two protein bands with molecular weight of approximately 66 kDa and 100 kDa were observed in PPCEE of *A. welwitschiae*

Table 1 Selection of *amylases* production potential by *A. welwitschiae* strains

A. welwitschiae strains	Enzymatic Index (EI)	Amount of reducing sugar (mg/mL)	*A. welwitschiae* strains	Enzymatic Index (EI)	Amount of reducing sugar (mg/mL)
UELAs 15.262	**1.61**	**7.50**	UELAs 26.365	1.39	0.84
UELAs 20.290	1.50	1.59	UELAs 32.459	1.39	1.01
UELAs 1.05	1.47	0.69	UELAs 29.432	1.39	1.24
UELAs 11.225	1.45	0.68	UELAs 11.228	1.38	1.06
UELAs 7.182	1.43	0.90	UELAs 28.422	1.38	0.81
UELAs 12.247	1.42	0.70	UELAs 27.405	1.37	0.65
UELAs 25.348	1.41	1.22	UELAs 28.430	1.37	1.44
UELAs 28.425	1.41	0.62	UELAs 7.200	1.36	0.85
UELAs 24.232	1.41	0.52	UELAs 6.144	1.36	0.97
UELAs 28.411	1.40	0.86	UELAs 12.233	1.35	1.11
UELAs 34.262	1.40	1.69	UELAs 2.14	1.34	1.16
UELAs 6.136	1.40	0.74	UELAs 27.237	1.28	0.68

The *A. welwitschiae* strain in bold was selected because it has the highest EI value and quantity of reducing sugars. Average Enzyme Index is obtained in experimental triplicates, and the Petri dishes were incubated at 28 °C for 4 days. The amount of reducing sugar generated was evaluated in liquid Czapeck-Dox medium, incubated at 28 °C for 4 days with 10^5 conidia/mL

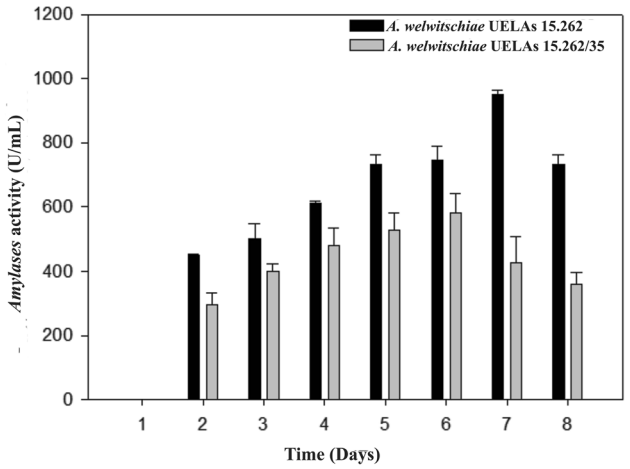

Fig. 1 Kinetics of *amylases* production by *Aspergillus welwitschiae* UELAs 15.262 and *Aspergillus welwitschiae* UELAs 15.262/35 in Submerged Fermentation. The data were expressed using Mean ± standard deviation

UELAs 15.262 and two bands of approximately 40 kDa and 50 kDa in *A. welwitschiae* UELAs 15.262/35 (Fig. 2A).

The highest *amylases* activity of *A. welwitschiae* UELAs 15.262 was obtained with pH 5.5 (798.7 U/mL), while for UELAs 15.262/35 the highest enzymatic activity was observed in pH 5.0 (908.7 U/mL), as shown in Fig. 2B.

The highest *amylases* activity was obtained at 55 °C (1477 U/mL), 60 °C (1587 U/mL), and 65 °C (1510 U/mL) by *A. welwitschiae* UELAs 15.262. Furthermore, for *A. welwitschiae* UELAs 15.262/35 strain, the highest *amylases* activity was obtained at 60 °C (1982 U/mL) and 65 °C (1973 U/mL). At 70 °C there was 66% reduction in *amylases* activity from *A. welwitschiae* UELAs 15.262. In contrast, only 10% reduction in *amylases* activity by *A. welwitschiae* UELAs 15.262/35 (Fig. 2C).

Under the conditions previously established, there was decrease in *amylases* activity over the time. The *amylases* activity of *A. welwitschiae* UELAs 15.262 strain was more stable in the first 40 min, when compared to *amylases* of *A. welwitschiae* UELAs 15.262/35 strain. However, *amylases* activity from the *A. welwitschiae* UELAs 15.262/35 strain showed more thermal stability between 60 and 100 min, as shown in Fig. 2D.

The Cu^{2+} and Fe^{3+} ions provided greater increase on the *amylases* activity for both *A. welwitschiae* UELAs 15.262 (2410 U/mL and 2324 U/mL, respectively) and *A. welwitschiae* UELAs 15.262/35 (2976 U/mL and 3150 U/mL, respectively) strains (Fig. 2E). The other metallic ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, Hg^{2+}) were not efficient as cofactors. So, the *amylases* activity from PPCEE of *A. welwitschiae* UELAs 15.262 strain obtained at pH 5.5, 60 °C in the presence of Cu^{+2} ion was 2410 U/mL or 3837 U/mg and 0.628 mg/mL of the total protein, while *A. welwitschiae* UELAs 15.262/35 strain at pH 5.0, 60 °C in the presence of Fe^{+3} ion was 3150 U/mL or 9077 U/mg and 0.347 mg/mL of the total protein.

The zymogram was performed to confirm the activity of *amylases* produced by *A. welwitschiae* strains. The starch degradation activity of the separated protein was visualized by the presence of a light band on the iodine-stained gel (Fig. 2F) indicating *amylases* activity of PPCEE from *A. welwitschiae* strains.

Discussion

The increase of *amylases* production by microorganisms is dependent on the genetic background of strains and/or species and abiotic factors during the fermentation process, such as temperature, pH, and type of substrate among others [2, 11, 24]. The selection of strains with genetic characteristics that favor the increase of *amylases* production has been of great importance. *Aspergillus* strains are described as good *amylases* producers, among which *Aspergillus* section *Nigri* [25, 26]. *A. niger* has been the most studied species of *Aspergillus* section *Nigri* regarding the production of *amylases* [27, 28]. However, in this study we have detected that *A. welwitschiae* is a potential *amylases* producer. To our knowledge, this is the first study reporting the production of *amylases* by *A. welwitschiae* strains.

In addition, *A. niger* and *A. welwitschiae* harbor potentially producing strains of Ochratoxin A and Fumonisin B2 [29, 30]. This fact highlights the importance of using strains capable of producing *amylases*, which are safe in terms of the inability to produce Ochratoxin A and Fumonisin B2. In this sense, all *A. welwitschiae* strains in this study were evaluated as genotype no-producing both mycotoxins [9] and all *A. welwitschiae* strains *amylases* produced.

A. welwitschiae UELAs 15.262 strain stood out in terms of production of these enzymes. The screening of *Aspergillus* strains regarding the potential for *amylases* production has also been reported [31].

Besides the genetic background of the strains, the abiotic factors interfere in the increase of *amylases* production [32]. Temperature and pH are crucial factors that have provided the *amylases* production. The *amylases* production by *A. welwitschiae* UELAs 15.262 strain was favored at slightly acidic pH (pH 5.0) and temperature between 35 °C and 40 °C. These data are according to reports of *amylases* production by species of *A. niger*, pH 4.0, 30 ± 2 °C [33] and *Aspergillus* sp., pH 5.5, 30 °C [34].

Under such specific abiotic conditions for *amylases* production, *A. welwitschiae* UELAs 15.262 and *A. welwitschiae* UELAs 15.262/35 strains produced the greatest amounts of *amylases* in the period of seven and six days of fermentation, respectively. In fact, studies have reported the highest *amylases* production by *Aspergillus* strains

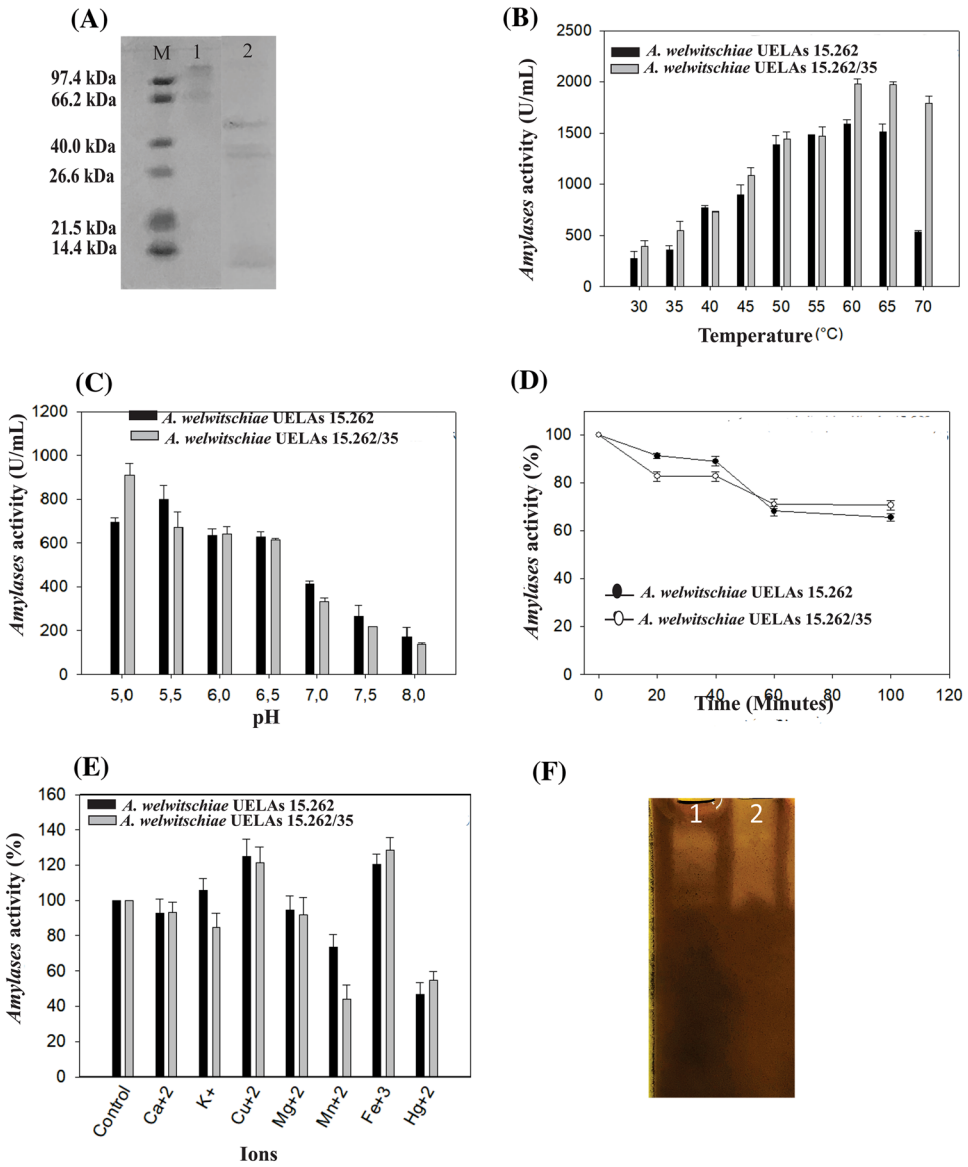

Fig. 2 Characterization of *amylases* activity from Partially Purified Crude Enzymatic Extract of *A. welwitschiae* UELAs 15.262 and UELAs 15.262/35 strains. **A** SDS-PAGE of partially purified crude enzymatic extract of *amylases* (1- UELAs 15.262, 2- UELAs 15.262/35 strains); **B** influence of pH on *amylases* activity; **C** influence of temperature on *amylases* activity; **D** influence of thermal stability on *amylases* activity; **E** influence of metallic ions on *amylases* activity; **F** zymographic analysis of *amylases* (1- UELAs 15.262/35; 2- UELAs 15.262 strains). In the **B**, **C**, **D**, and **E**, the data were expressed using Mean ± standard deviation

in periods of time longer than four days of Smf (502 U/mL—6 days – 30 °C – 180 rpm [35]; 1780 U/mL—4 days – 40 °C – pH 4,5 – under shaking [36]), under specific conditions. *Aspergillus* strains under Smf up to four days produced less *amylases* (33.52 U/mL—35 °C—4 days [37], 70.29 U/mL—30 °C—88 h—200 rpm [38], 230 U/mL—35 °C—3 days—200 rpm [39], 0.48 U/mL—28 °C—2 days—200 rpm [4]).

On the other hand, *A. welwitschiae* UELAs 15.262/35 strain showed ability to increase *amylases* production in a shorter time, under favorable conditions to future applications. It well known that the use of mutant strains has contributed to increase the production of metabolites, such as *amylases* [14]. The *A. welwitschiae* UELAs 15.262/35 mutant strain was selected for the highest accumulation of citric acid, and according to Hu et al. [39], strains that accumulate citric acid from starch materials consequently up-regulate the production of *amylases*.

The fact that *A. welwitschiae* UELAs 15.262 and UELAs 15.262/35 presented considerable *amylases* production rates, and the characterization of PPCEE was relevant. The proteins detected in *A. welwitschiae* UELAs 15.262/35 may correspond to *alpha-amylases*, whose molecular weight in *Aspergillus* sp. has been reported to be between 45 and 65 kDa [40, 41], whereas proteins detected in *A. welwitschiae* UELAs 15.262 may correspond to fungal *glucose oxidase* [42]. According to Pel et al. [43], *A. niger* (a species highly related to *A. welwitschiae*) contains four putative extracellular *amylases*—*Amy*A, *Amy*B, *Amy*C, and *Aam*A. All these *amylases* are also found in the *A. welwitschiae*

genome with high similarity. In this study, we possibly have different *amylases* being expressed in *A. welwitschiae* UELAs 15.262 and UELAs 15.262/35.

Enzyme activity is generally higher post-purification, even if partially purified, due to the larger contact surface. And in fact, in this study, the *amylases* activity after semi-purification reached 2410 U/mL and 3150 U/mL, corresponding to 3837 U/mg and 9077 U/mg in *A. welwitschiae* UELAs 15.262 and UELAs 15.262/35, respectively.

The *amylases* activity from PPCEE of *A. welwitschiae* UELAs 15.262 strain obtained at pH 5.5, 60 °C provided 1.98-fold increase in *amylases* activity. Similarly, the *amylases* activity from PPCEE of *A. welwitschiae* UELAs 15.262/35 strain obtained at pH 5.0, 60 °C increased 2.2-fold the activity of these enzymes. Most of the *amylases* produced by *Aspergillus* species have good enzymatic activity with pH around 4.5 to 6.0, between 50 °C and 65 °C [44, 45].

Another factor that influences enzymatic activity is the thermal stability of the enzyme, one of the main requirements for its application. The reduction of 35% and 30% in the *amylases* activity of *A. welwitschiae* UELAs 15.262 and *A. welwitschiae* UELAs 15.262/35 strains, respectively, at the end of 100 min allows the use of *amylases* produced by *A. welwitschiae* in future applications. Studies that evaluated the *amylases* activity produced by *Aspergillus* sp. obtained between 40 and 70% of reduction in the activity of these enzymes, in a period between 50 and 180 min and between 50 °C and 60 °C [4, 46].

Still regarding the PPCEE *amylases* activity of *Aspergillus* strains, there was increase of 25% and 20% for *amylases* activity of *A. welwitschiae* UELAs 15.262 strain and 21% and 29% for *A. welwitschiae* UELAs 15.262/35 strain, in the presence of Cu^{+2} and Fe^{+3} ions, respectively. Selim [47], evaluating *amylases* activity from *A. niger*, obtained increase in *amylases* activity of 53% using the Cu^{2+} ion and 38% with Fe^{3+} ion. Xian and Feng [48] using the ions of Cu^{2+} and Fe^{3+} (5 mM) obtained increase of 14% and 23%, respectively, in the enzymatic activity of *amylases* produced by the species *A. tritici* WZ99.

The association between pH, temperature, and metallic ions allows to obtain maximum catalytic activity, a factor of great relevance in the different sectors that use *amylases*, since one of the limiting obstacles to the applications of these enzymes is the poor catalytic activity. It is also noted that maximum catalytic activity provides better use of the substrate and consequent cost reduction. Several studies have shown that variations in catalysis parameters contribute to achieving the maximum catalysis activity of *amylases* [49, 50].

Thus, the set of abiotic factors together with the strains genetic background provide greater *amylases* production and similarly, specific parameters provide maximum *amylases* activity. This is the first report of the production and characterization of *amylases* from *A. welwitschiae* UELAs 15.262 and *A. welwitschiae* UELAs 15.262/35, which demonstrated that both strains have greater potential for *amylases* production, under the established conditions. The results showed the greater *amylases* production of *A. welwitschiae* UELAs 15.262 in relation to *A. welwitschiae* UELAs 15.262/35. On the other hand, under such conditions, the greater *amylases* activity by *A. welwitschiae* UELAs 15.262/35 is slightly higher compared to *A. welwitschiae* UELAs 15.262, suggesting that both strains could be used in future applications.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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References

1. Gopinath SCB, Anbu P, Arshad MKM, Lakshmipriya T, Voon CH, Hashim U, Chinni SV (2017) Biotechnological processes in microbial amylase production. BioMed Res Int. https://doi.org/10.1155/2017/1272193
2. Sundarram A, Murthy TPK (2014) α-amylase production and applications a review. J Appl Environ Microbiol 2(4):166–175. https://doi.org/10.12691/jaem-2-4-10
3. Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B (2003) Microbial α-amylases: a biotechnological perspective. Proc Biochem 38:1599–1616. https://doi.org/10.1016/S0032-9592(03)00053-0

4. Sethi S, Gupta S (2015) Isolation, characterization and optimization of cultural conditions for amylase production from fungi. J Biosci 4(9):3356–3363
5. Samson RA, Visagie CM, Houbraken J, Hong SB, Hubka V, Klaassen CH, Frisvad JC (2014) Phylogeny, identification and nomenclature of the genus *Aspergillus*. Stud Mycol 78:141–173. https://doi.org/10.1016/j.simyco.2014.07.004
6. Massi FP, Sartori D, Ferranti LS, Iamanaka BT, Taniwaki MH, Vieira MLC, Fungaro MHP (2016) Prospecting for the incidence of genes involved in ochratoxin and fumonisin biosynthesis in Brazilian strains of *Aspergillus niger* and *Aspergillus welwitschiae*. Int J Food Microbiol 221:19–28. https://doi.org/10.1016/j.ijfoodmicro.2016.01.010
7. Massi FP, Iamanaka BT, Barbosa RL, Sartori D, Ferrranti L, Taniwaki MH, Fungaro MHP (2020) Molecular analysis of *Aspergillus* section *Nigri* isolated from onion samples reveals the prevalence of *A. welwitschiae*. Braz J Microbiol. https://doi.org/10.1007/s42770-020-00390-2
8. Gherbawy Y, Elhariry H, Kocsubé S, Bahobial A, Deeb BE, Altalhi A, Varga J, Vágvölgyi C (2015) Molecular characterization of black *Aspergillus* species from onion and their potential for Ochratoxin A and Fumonisin B2 production. Foodborne Pathog Dis 12(5):414–423. https://doi.org/10.1089/fpd.2014.1870
9. Vanzela DOA, Massi FP, Oliveira ALM, Fungaro MHP, Sartori D (2020) Isolation and Identification of *Aspergillus* Section *Nigri*, and Genotype Associated with Ochratoxin A and Fumonisin B_2 Production in Garlic Marketed in Brazil. Curr Microbiol 77:1150–1158. https://doi.org/10.1007/s00284-020-01915-6
10. Palumbo JD, O'keeffe TL, (2014) Detection and discrimination of four *Aspergillus* section Nigri species by PCR. Lett Appl Microbiol 60(2):188–195. https://doi.org/10.1111/lam.12358
11. Saini R, Saini HS, Dahiya A (2017) *Amylases*: Characteristics and industrial applications. J Pharmacogn Phytochem 6(4):1865–1871
12. Melnichuk N, Braia MJ, Anselmi PA, Meini MR, Romanini D (2020) Valorization of two agroindustrial wastes to produce alpha-amylase enzyme from *Aspergillus oryzae* by solid-state fermentation. Waste Manag 106:155–161. https://doi.org/10.1016/j.wasman.2020.03.025
13. Abdullah R, Ikram-Ul-HAQ, (2015) Purification and characterization of α-amylase produced by mutant strain of *Aspergillus oryzae* EMS-18. Nat Prod Res 29(8):710–716. https://doi.org/10.1080/14786419.2014.982648
14. Abdullah R, Ikram-Ul-HAQ MJ (2011) Optimization of cultural conditions for the production of alpha amylase by wild and mutant strain of *Aspergillus oryzae* in stirred fermenter. Pak J Bot 43(1):715–723
15. Devasena T (2010) Enzymology, 1st edn. Oxford University Press, New Delhi, pp 9–12
16. Kellis J, Todd R, Arnold F (1991) Protein stabilization by engineered metal chelation. Biotechnol 9:994–995. https://doi.org/10.1038/nbt1091-994
17. Saboury AA, Karbassi F (2000) Thermodynamic studies on the interaction of calcium ions with alpha-amylase. Thermochim Acta 362:121–129. https://doi.org/10.1016/S0040-6031(00)00579-7
18. Sarojini R, Deepika K, Rangabhashiyam S (2012) Production, characterization, kinetic studies of glucoamylase through solid-state fermentation by *Aspergillus niger* using agricultural residues as substrate. Int J Curr Rsrch 4:198–201
19. Sartori D, Ribeiro MM, Castilho POS, Bossa LF, Amador IR (2021) Produção de Ácido Cítrico a partir de linhagem mutante de *A. welwitschiae* UELAs 15.262/35. 2021, Brasil. Patente: Privilégio de Inovação. Número do registro: BR10202100913, título: "Produção de Ácido Cítrico a partir de linhagem mutante de *A. welwitschiae* UELAs 15.262/35", Instituição de registro: INPI - Instituto Nacional da Propriedade Industrial. Depósito: 11/05/2021
20. Hankin L, Anagnostakis SL (1975) The use of solid media for detection of enzyme production by fungi. Mycologia 67:597–607. https://doi.org/10.2307/3758395
21. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31(3):426–428. https://doi.org/10.1021/ac60147a030
22. Sperotto RA (2014) Protocolos e métodos de análise em laboratórios de biotecnologia agroalimentar e de saúde humana. Editora Univates, Lajeado
23. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with folin phenol reagent. J Biol Chem 193:265–275
24. Ahmad MA, Isah U, Raubilu IA, Muhammad SI, Ibrahim D (2019) An overview of the enzyme: Amylase and its industrial potentials. Bayero J Pure Appl Sci 12(1):352–358. https://doi.org/10.4314/bajopas.v12i1.53S
25. Saranraj P, Stella D (2013) Fungal amylase—a review. Int J Microbiol Res 4(2):203–211. https://doi.org/10.5829/idosi.ijmr.2013.4.2.75170
26. Uguru GC, Akinayanju JÁ, SanI A (1997) The use of Yam peel for growth of locally isolated *Aspergillus niger* and amylase production. Enzyme Microb Technol 21:48–51. https://doi.org/10.1016/S0141-0229(96)00225-6
27. Hernández MS, Rodríguez MR, Guerra NP, Rosés RP (2006) Amylase production by *Aspergillus niger* in submerged cultivation on two wastes from food industries. J Food Eng 73(1):93–100. https://doi.org/10.1016/j.jfoodeng.2005.01.009
28. Weerasooriya MKB, Piyarathne SAPM (2019) Production of extracellular amylase by *Aspergillus niger* under submerged fermentation using jack fruit rag as the carbon source. Indian J Tradit Knowl 19(1):158–163
29. Abarca ML, Bragulat MR, Castella G, Cabanes FJ (1994) Ochratoxin A production by strains of *Aspergillus niger var. niger*. Appl Environ Microbiol 60(7):2650–2652. https://doi.org/10.1128/aem.60.7.2650-2652.1994
30. Frisvad JC, Smedsgaard J, Samson RA, Larsen TO, Thrane U (2007) Fumonisin B2 production by *Aspergillus niger*. J Agric Food Chem 55:9727–9732. https://doi.org/10.1021/jf0718906
31. Saleem A, Ebrahim MKH (2014) Production of amylase by fungi isolated from legume seeds collected in Almadinah Almunawwarah. Saudi Arabia J Taibah Univ Sci 8(2):90–97. https://doi.org/10.1016/j.jtusci.2013.09.002
32. Sudharhsan S, Senthilkumar S, Ranjith K (2007) Physical and nutritional factors affecting the production of amylase from species of *Bacillus* isolated from spoiled food waste. Afr J Biotechnol 6:430–435
33. Spier MR, Woiciechowski AL, Vandenberghe LPS, Soccol CR (2006) Production and characterization of *amylases* by *Aspergillus niger* under solid state fermentation using agro industrials products. Int J Food Eng. https://doi.org/10.2202/1556-3758.1116
34. Chimata MK, Sasidhar P, Challa S (2010) Production of extracellular amylase from agricultural residues by a newly isolated *Aspergillus* species in solid state fermentation. Afr J Biotechnol 9(32):5162–5169
35. Suleimenova ZB, Saduyeva ZK, Rakhmetova ZK (2016) Alpha-amylase production from *Aspergillus oryzae* in submerged fermentation. Biotechnol Acta 9(4):77–82. https://doi.org/10.15407/biotech9.04.077
36. Sreelakshmi SN, Paul A, Vasanthi NS, Saravanan D (2014) Low-temperature acidic *amylases* from *Aspergillus* for desizing of cotton fabrics. J Text Inst 105(1):59–66. https://doi.org/10.1080/00405000.2013.810019
37. Ali E, El-Nagdy M, Al-Garni S, Ahmed M, Rawaa A (2017) Enhancement of alpha amylase production by *Aspergillus flavus* AUMC 11685 on mandarin (*Citrus reticulata*) peel using submerged fermentation. Eur J Biol Res 7(3):154–164. https://doi.org/10.5281/zenodo.818271

38. Ratnasri PV, Lakshmi BKM, Devi KA, Hemalatha KPJ (2014) Isolation, characterization of *Aspergillus fumigatus* and optimization of cultural conditions for amylase production. Int J Res Eng Technol 3(2):457–463
39. Hu W, Li W, Chen H, Liu J, Wang S, Chen J (2017) Changes in transcript levels of starch hydrolysis genes and raising citric acid production via carbono ion irradiation mutagenesis of *Aspergillus niger*. PLoS ONE 12(6):e0180120. https://doi.org/10.1371/journal.pone.0180120
40. Li S, Zuo Z, Niu D, Singh S, Permaul K, Prior BA, Shi G, Wang Z (2011) Gene cloning, heterologous expression. and characterization of a high maltose-producing α-amylase of *Rhizopus oryzae*. Appl Biochem Biotechnol 164:581–592. https://doi.org/10.1007/s12010-011-9159-5
41. Sidkey N, Abo-Shadi M, Balahmar R, Sabry R, Badrany G (2011) Purification and characterization of α-amylase from a newly isolated *Aspergillus flavus* F 2 Mbb. Int Res J Microbiol 2:96–103
42. Derakshan FK, Darvishi F, Dezfulian M, Madzak C (2017) Expression and characterization of glucose oxidase from *Aspergillus niger* in *Yarrowia lipolytica*. Mol Biotechnol 59:307–314. https://doi.org/10.1007/s12033-017-0017-8
43. Pel HJ, de Winde JH, Archer DB, Dyer PS, Hofmann G, Schaap PJ, Turner G, de Vries RP, Albang R, Albermann K, Andersen MR, Bendtsen JD, Benen JA, van den Berg M, Breestraat S, Caddick MX, Contreras R, Cornell M, Coutinho PM, Danchin EG, Debets AJ, Dekker P, van Dijck PW, van Dijk A, Dijkhuizen L, Driessen AJ, d'Enfert C, Geysens S, Goosen C, Groot GS, de Groot PW, Guillemette T, Henrissat B, Herweijer M, van den Hombergh JP, van den Hondel CA, van der Heijden RT, van der Kaaij RM, Klis FM, Kools HJ, Kubicek CP, van Kuyk PA, Lauber J, Lu X, van der Maarel MJ, Meulenberg R, Menke H, Mortimer MA, Nielsen J, Oliver SG, Olsthoorn M, Pal K, van Peij NN, Ram AF, Rinas U, Roubos JA, Sagt CM, Schmoll M, Sun J, Ussery D, Varga J, Vervecken W, van de Vondervoort PJ, Wedler H, Wösten HA, Zeng AP, van Ooyen AJ, Visser J, Stam H (2007) Genome sequencing and analysis of the versatile cell factory *Aspergillus niger*. Nat Biotechnol 25(2):221–231. https://doi.org/10.1038/nbt1282
44. Aisien ET, Igbinosa IH (2019) Production, purification, and characterization of α-amylase from *Aspergillus niger, Aspergillus flavus* and *Penicillium expansum* using cassava peels as substrate. Niger J Biotechnol 36(2):114–126. https://doi.org/10.4314/njb.v36i2.12
45. Oluwabunmi MA, Adesola AA, Grace IO (2019) Production and characterization of partially purified α-amylase from *Aspergillus niger*. J Phys. https://doi.org/10.1088/1742-6596/1378/4/042077
46. Wang J, Zhang Y, Wang X, Shang J, Li Y, Zhang H, Lu F, Liu F (2018) Biochemical characterization and molecular mechanism of acid denaturation of a novel α-amylase from *Aspergillus niger*. Biochem Eng J 137:222–231. https://doi.org/10.1016/j.bej.2018.06.004
47. Selim M (2016) Optimization of glucoamylase production by local isolate of A*spergillus niger* using agro-industrial substrates under solid state fermentation. J Agric Chem Biotechnol 7(12):303–309. https://doi.org/10.21608/jacb.2016.41143
48. Xian L, Feng JX (2018) Purification and biochemical characterization of a novel mesophilic glucoamylase from *Aspergillus tritici* WZ99. Int J Biol Macromol 107:1122–1130. https://doi.org/10.1016/j.ijbiomac.2017.09.095
49. Ezugwu AL, Ottah VE, Eze SOO, Chilaka FC (2016) Effect of pH, various divalent metal ion and diferente substrates on glucoamylase activity obtained from *Aspergillus niger* using amylopectin from tiger nut starch as carbon source. Afr J Biotechnol 15(21):980–988. https://doi.org/10.5897/AJB2015.14886
50. Okwuenu PC, Agbo KU, Ezugwu AL, Eze SO, Chilaka FC (2017) Effect of divalent metal ions on glucoamylase activity of glucoamylase isolated from *Aspergillus niger*. Ferment Technol 6:141. https://doi.org/10.4172/2167-7972.1000141

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