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Production, partial purification, and characterization of a glucoamylase from *Epicoccum nigrum*

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ABSTRACT. Starch processing industries use amylases, accounting for approximately 30% of the world's enzyme market. Previously, an amylase-producing strain of *Epicoccum nigrum* was isolated from maize grains. Although *E. nigrum* amylase production is already reported in the literature, no published data on production optimization or characterization of the produced enzyme exists. The objectives of this work were to improve the amylase production by the *E. nigrum* PG 16 strain and to purify and characterize the produced enzyme. The *E. nigrum* PG 16 amylase production best conditions in submerged culture were: inoculum of 4% (v v^{-1}) of a five-days-old stationary culture homogenate, agitation at 100 rpm, 25°C, natural light, 72 hours of incubation, starch as the carbon source, and an initial medium pH of 7.0. A molecular exclusion chromatography profile has shown the production of only one amylase, which was partially purified with ammonium precipitation and dialysis. The enzyme optima pH and temperature are 6.0 and 50°C, respectively. The partially purified enzyme lost its activity when incubated for 30 min in temperatures above 40°C, presenting a T_{50} of 46.25°C. The K_M and V_{max} of the partially purified enzyme are 1.72 mg mL^{-1} of starch and 0.15 mg min^{-1} of degraded starch, respectively. The ion Ca^{2+} slightly activated the studied enzyme. The ions Cu^{2+}, Zn^{2+}, and Fe^{3+} and the detergents SDS and Tween 80 acted as inhibitors of the studied enzyme. The partially purified enzyme released glucose from *p*-nitrophenyl α-D-glucopyranoside (*p*-NPG). Glucose was the enzyme's main product from starch hydrolysis, as evidenced by thin-layer chromatography. The *E. nigrum* PG 16 studied enzyme is a glucoamylase and represents an alternative for enzymatic starch hydrolysis.

Keywords: starch; glucoamylase; *Epicoccum nigrum*; characterization.

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Introduction

Enzyme production is an expanding area that moves billions of dollars annually (Singh, Kumar, Mittal, & Mehta, 2016; Paul, Gupta, Beliya, Tiwari, & Jadhav, 2021). Hydrolases are the most widely used class of enzymes (~75% of the total), and among them are amylases, which catalyze starch hydrolysis and account for approximately 25-30% of the enzyme market (Chapman, Ismail, & Dinu, 2018; Gurung, Ray, Bose, & Rai, 2013; Läufer, 2017; Singh et al., 2016).

Starch has two glucose homopolysaccharides, linear amylose, and the branched amylopectin. Amylases are classified according to their mechanism. They can be divided into endoamylases and exoamylases, which catalyze the hydrolysis of glycosidic bonds within and from the non-reducing end of the starch molecules, respectively (Singh et al., 2016). The main amylases are (a) α-amylases (endoamylases) which hydrolyze α-1,4 glycosidic bonds, transforming starch molecules into high molecular weight α-dextrins (Gupta, Gigras, Mohapatra, Goswami, & Chauhan, 2003; Mehta & Satyanarayana, 2016); (b) β-Amylases (exoamylases) that hydrolyze α-1,4 glycosidic bonds from non-reducing ends in the starch molecules, releasing maltose (Pandey et al., 2000); (c) glucoamylases (exoamylases) that hydrolyze α-1,4 and α-1,6 glycosidic bonds in the starch molecules, releasing free glucose (Karim & Tasnim, 2018; Kumar & Satyanarayana, 2009; Marín-Navarro & Polaina, 2011; Norouzian, Akbarzadeh, Scharer, & Young, 2006); and (d) debranching enzymes, which hydrolyze only the α-1,6 glycosidic bonds in the branches, transforming amylopectin into linear dextrins (Pandey et al., 2000).

For some industrial uses, starch must be degraded to maltodextrins, maltose, and glucose. Enzymatic hydrolysis is preferable to acidic hydrolysis because it produces fewer residues and yields higher (Läufer, 2017; Singh et al., 2016). Glucoamylase's main commercial applications are to produce high-glucose syrups in food

industries and free glucose in fermentation industries, such as in beverage industries and biofuel production (Gurung et al., 2013; Karim & Tasnim, 2018; Kumar & Satyanarayana, 2009; Läufer, 2017; Marín-Navarro & Polaina, 2011; Norouzian et al., 2006). In such industries, the starch is first gelatinized by heating and then liquefied by bacterial α-amylases to small dextrins (Kumar & Satyanarayana, 2009; Läufer, 2017). In the next phase, glucose is produced from the dextrins through saccharification carried out by glucoamylases (Kumar & Satyanarayana, 2009; Läufer, 2017).

Most enzymes in the market are of microbial origin (Chapman et al., 2018; Gupta et al., 2003). These enzymes have high selectivity for the substrate and the product. They are also comparatively more stable than enzymes extracted from plants and animals and exhibit easier production and simultaneous purification, in addition to being seasonally independent (Gurung et al., 2013; Singh et al., 2016; Souza & Magalhães, 2010). Among the enzyme-producing microorganisms, filamentous fungi are preferred, as they secrete large amounts of proteins in solid or submerged culture media and can grow in different environments and on the most varied substrates, which facilitates their cultivation for large-scale production and industrial biotechnological application of the produced enzymes (Gurung et al., 2013; Singh et al., 2016; Souza & Magalhães, 2010). Amylase-producing microorganisms are widely distributed in nature, with fungi of the genus *Aspergillus* and *Rhizopus* and bacteria of the genus *Bacillus* being the primary producers (Gupta et al., 2003; Gurung et al., 2013; Paul et al., 2021). The production of amylolytic enzymes is generally influenced by strain, medium composition, and culture conditions (Gupta et al., 2003).

The microbiological biodiversity exploration to find new organisms able to produce enzymes with diverse characteristics is a good prospect for the scientific and commercial areas. *Epicoccum nigrum* Link strain PG 16 was previously isolated from rotten maize grains (Faria, Abe, Silva, Tessmann, & Barbosa-Tessmann, 2011). This strain was molecularly identified by DNA barcoding and was shown to produce amylase in solid medium culture (Abe et al., 2015). The species *E. nigrum* is a saprophyte with worldwide distribution and is well-known for its use in the biological control of many plant pathogens and its production of several secondary metabolites (Elkhateeb & Daba, 2019; Fávaro, Melo, Aguilar-Vildoso, & Araújo, 2011; Mims & Richardson, 2005; Schol-Schwarz, 1959). This species is also reported to produce amylase in a solid medium (Fávaro et al., 2011). However, there are no reports in the literature of studies on the production studies or characterization of the produced enzyme. Therefore, this work aimed to optimize the amylase production by *E. nigrum* PG 16 in submerged culture and characterize the produced enzyme.

Material and methods

Microorganism and amylase production

The *E. nigrum* PG 16 strain was stored in PDA with transfers cultured every six months and preserved in PDA slants under mineral oil. Macro- and microscopic characteristics were observed in five-day-old colonies obtained in potato dextrose agar (PDA) in 9 cm Petri dishes, at 25°C, with a 12 hours dark-light photoperiod because of the microorganism circadian cycle. For amylase production analysis in solid medium, a 0.5 cm diameter plug from the PDA culture plate was transferred to the center of a 9 cm Petri dish containing nutrient agar with starch (3 g L^{-1} yeast extract, 5 g L^{-1} peptone, 15 g L^{-1} agar, 2 g L^{-1} soluble starch). After three days of culture at 25°C, with a photoperiod of 12 hours, the plate was stained with the iodine reagent (2% KI (w v^{-1}); 0.2% I_2 (w v^{-1})) and distaining with distilled water.

Culture conditions for the enzyme production

The culture liquid medium initially used contained: 3 g L^{-1} yeast extract, 5 g L^{-1} peptone, 50 mM phosphate buffer (21 mM NaH_2PO_4, 29 mM $Na_2HPO_4 \cdot 7H_2O$), pH 7.0, and 0.2% (w v^{-1}) soluble starch (Lago, Santos, Bueno, Oliveira, & Barbosa-Tessmann, 2021). The inoculum was prepared by transferring and smashing a 1 cm^3 fragment of a PDA culture to a 125 mL Erlenmeyer flask containing 25 mL of the liquid media just described. This flask was incubated without agitation for five days at 25°C, with a photoperiod of 12 hours. The obtained mycelia were homogenized through a stainless-steel mesh and sterile syringe with a needle (18G, 40 × 12 mm). One ml of the resulting homogenate was used as an inoculum (4% v v^{-1}) for new 125 mL vials containing 25 mL of the same sterile liquid medium. These flasks were then incubated for three days (72 hours), at 25°C, under orbital shaking at 100 rpm. After this incubation period, cultures were filtered on filter paper to retain the mycelia, and enzyme activity was evaluated in the filtrate. The mycelia retained on the filter paper were dried at 50°C until constant weight, which was used to evaluate the microorganism growth.

Culture conditions optimization for the amylase production by *E. nigrum*

The optimization was performed by modifying the culture medium (Lago et al., 2021) and the culture conditions described above. The culture's agitation effect was initially evaluated for inoculum and enzyme production. For this, the inoculum production culture was incubated at 25ºC, for three days (72 hours), with orbital agitation of 100 rpm and natural light or stationary and with a photoperiod of 12 hours of light. After inoculation, the cultures for amylase production were also incubated under agitation or not (stationary), in the same manner, for three days (72 hours), at 25°C. The inoculum production culture was stationary for all the subsequent experiments, and the amylase production culture was shaken. Finally, however, the inoculum culture was incubated for five days (120 hours) at 25°C, with a photoperiod of 12 hours, considering that a more prolonged incubation resulted in a higher mycelium yield due to the slow fungus growth.

A fungus growth curve was performed to evaluate the best cultivation time for enzyme production. For that, 12 Erlenmeyer flasks of 125 mL containing 25 mL of the liquid medium were inoculated and incubated at 25°C, with a photoperiod of 12 hours. Then, at each 24 hours after inoculation, three culture flasks were removed from incubation, and the culture was filtered in filter paper. The enzyme was assayed in the filtrate, and the mycelia dry weight was used to estimate the fungus growth.

The phosphate buffer composition of the culture medium was changed to result in initial pHs of 5.0, 6.0, 7.0, and 8.0. In addition, changes in the inoculum amount (4, 8, 12, and 16%, v v^{-1}) were also made. Cultures were also carried out at 25 or 30°C to test for the best temperature for enzyme production.

The best carbon source for the *E. nigrum* PG 16 amylase production was studied by replacing the 0.2% starch in the medium described above with 0.2% lactose, sucrose, cellulose, arabinose, galactose, glucose, maltose, raffinose, or cellobiose. Increases in starch concentration (0.2-1.0%) were also tested to study the enzyme production profile. In order to test the effect of micronutrients and detergents in the culture medium on the amylase production by *E. nigrum* PG 16, the used medium was the same as described above but added individually with 10 μM of $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $MnSO_4 \cdot H_2O$, and $CaCl_2 \cdot 2H_2O$, 1.5 mM $MgSO_4 \cdot 7H_2O$, 25 mM KCl, 10 mM sodium citrate, or 0.2% (v v^{-1}) Tween 80. Variations in the concentration of KCl (5-25 mM) were also tested to evaluate the production of amylase by the fungus. All tests described were performed in triplicates.

Statistical analyzes were performed by calculating the result's mean and standard deviation. Where indicated, data were submitted to analysis of variance (ANOVA) and compared by the student (t) test ($\alpha = 0.01$ or 0.05, when indicated) using the SASM-Agri program (Canteri, Althaus, Virgens Filho, Giglioti, & Godoy, 2001).

Enzyme assay

Unless otherwise stated, the starch-iodine complex distaining method (Palanivelu, 2001) was used to measure the amylase activity in all the experiments. For this, a 500-μL sample aliquot was added to 1.0 mL of 0.1 M phosphate buffer, pH 7.0, and 500 μL of 0.1% (w v^{-1}) soluble starch prepared in the same buffer. The reaction was incubated for 10 min at 50°C and then stopped with 500 μL of 0.1 M HCl. In order to evaluate the remaining starch, 500 μL of iodine solution [2.0% KI; 0.2% I_2] was added, followed by absorbance measurement at 690 nm in a spectrophotometer. The blank was prepared with 2.0 mL of 0.1 M phosphate buffer, pH 7.0. A control reaction to access the initial starch concentration was performed with a 500 μL aliquot of the 0.1% soluble starch solution and 1.5 mL of 0.1 M phosphate buffer, pH 7.0. Before reading the absorbance, the blank and control reactions were also added of 500 μL of 0.1 M HCl and 500 μL of the iodine reagent. The control's absorbance was decreased from the sample's absorbance. One enzyme unit was defined as the enzyme's amount that caused an absorbance reduction of 20% per min under the assay conditions. The residual starch in the culture filtrate was evaluated in reactions with 500 μL of the filtrate and 1.5 mL of 0.1 M sodium phosphate buffer, pH 7.0. The obtained absorbance was decreased from the sample's absorbance.

Alternatively, the enzymatic activity was assayed with *p*-nitrophenyl α-D-glucopyranoside (*p*-NPG, Sigma-Aldrich cat. N1377, USA) as substrate. For that, samples (100 μL) were transferred to tubes containing 900 μL of the substrate solution (4 mM *p*-NPG in 50 mM phosphate buffer, pH 7.0). After incubation for 30 min at 50°C, 1.0 mL of 2% (w v^{-1}) sodium carbonate was added, and the absorbance was read at 410 nm. One enzyme unit was defined as the amount of enzyme liberating 1.0 μmol of *p*-nitrophenol $min.^{-1}$.

Partial purification of the enzyme and protein concentration determination

The fungus was cultured, as described above, for three days, in 10 flasks (125 mL) containing 25 mL of the culture medium described above, pH 7.0, with 0.2% starch and 10 mM KCl. The culture's filtrates were

combined, and ammonium sulfate was added to a final concentration of 90% (w v^{-1}) for protein precipitation. Precipitated proteins were collected by centrifugation (21,000*g*, 5 $min.^{-1}$) and dialyzed (dialysis membrane with 14,000 cut-offs, Sigma D-9777) in 50 mM phosphate buffer, pH 7.0. The protein quantification was performed using the method of Bradford (1976), using bovine serum albumin as a standard.

The dialysate was applied in a Sephacryl HR-200 column (100 × 1.5 cm), which was equilibrated and eluted with 50 mM phosphate buffer pH 7.0. The column flux was 0.5-0.6 mL $min.^{-1}$, and fractions of 4.0 mL were collected. Absorbance at 280 nm in each fraction was used to estimate protein elution, and the starch-iodine complex distaining method was used to estimate the amylase elution. Proteins aliquots of the fractions containing amylase activity were TCA precipitated (1350 μL + 150 μL of 100% TCA), collected by centrifugation (10 min., 12,000 g), washed with absolute ethanol twice using centrifugation (5 min., 12,000 g), and resuspended in sample dilution buffer [20% (v v^{-1}) glycerol, 1% (w v^{-1}) SDS, 0.03 mg mL^{-1} bromophenol blue, 125 mM tris-base, pH 6.8, 0.72 M β-mercaptoethanol]. After boiling for 10 min., proteins were loaded into a 7.5% polyacrylamide resolving SDS-PAGE gel, pH 8.9, with a 4.5% stacking gel, pH 6.8, in the Tris-glycine buffer system (Laemmli, 1970). The resulting gel was stained with Coomassie blue R-250.

Biochemical characterization of the partially purified enzyme

The optimum pH was determined in the enzymatic assay of starch-iodine complex color disappearance in 0.1 M Britton and Robinson buffer (0.1 M boric acid, 0.1 M acetic acid, 0.1 M phosphoric acid), which pH was corrected with NaOH to 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0.

The optimum temperature was determined using the enzymatic starch-iodine complex color disappearance assay described above with 0.1 M phosphate buffer, pH 6.0, and with replacement of the phosphate buffer by the Britton and Robinson 0.1 M buffer, pH 6.0, at different temperatures.

The thermal stability was evaluated by incubating the enzyme for 30 min. in 0.1 M phosphate buffer, pH 6.0, at 30, 40, 50, and 60°C and then subjected to enzymatic assay for starch-iodine complex distaining. The temperature at which the *E. nigrum* amylase loses 50% of its activity (T_{50}) after incubation for 30 min. was calculated with the obtained equation of the second-order polynomial regression curve.

Kinetic parameters, Michaelis-Menten constant (K_M), and maximum reaction speed (V_{max}) were estimated by nonlinear regression using the GraphPad Prism program. The starch concentration effect, ranging from 0.5 to 2 mg mL^{-1}, on a fixed amount of *E. nigrum* partially purified amylase (7.9 μg) was evaluated in the enzymatic starch-iodine complex distaining assay. The residual starch concentration after enzyme action was calculated using a starch calibration curve. The initial velocities were expressed as mg of starch consumed per min.

The effect of potential inhibitors and activators was tested with the partially purified enzyme in the enzymatic starch-iodine complex distaining assay with 0.1 M Tris buffer, pH 6.0, but with the individual addition of 5 mM and 10 mM of $MnSO_4 \cdot H_2O$, $CuSO_4 \cdot 5H_2O$, $CaCl_2 \cdot 2H_2O$, $CoCl_2 \cdot 6H_2O$, $FeCl_3$, $ZnSO_4 \cdot 7H_2O$, $MgSO_4 \cdot 7H_2O$, SDS, and Tween 80. The activity was expressed as a percentage of the activity observed in the absence of any compound.

Analysis of the enzymatic products by Thin Layer Chromatography (TLC)

A 250 μL (130 μg; 2.8 U) aliquot of the partially purified enzyme was incubated with 750 μL of 1.0% (w v^{-1}) highly pure grade soluble starch from potato (Cat. no: 33615; Sigma-Aldrich) prepared in 50 mM phosphate buffer (pH 7.0) in a screw-capped microtube, for 16 h at 50°C, in a rotatory mixer (10 rpm). A control reaction was prepared with 750 μL of the soluble starch 1.0% solution with 250 μl of 50 mM phosphate buffer, pH 7.0, and was incubated similarly. The obtained hydrolysis solutions (20 μL) were spotted on a thin-layer chromatography plate (Silica gel, Sigma-Aldrich, Germany), along with 10 μL of the 0.1 M standards: glucose and maltose and 20 μL of the control reaction (only starch). The solvent system in the ascending chromatography was butanol/ethanol/water in 5:3:2 proportions. After air-drying the plate, the spots were developed by spraying them with H_2SO_4 and methanol (1:9) containing 0.2% (w v^{-1}) orcinol, followed by heating at 100°C (Benassi, Pasin, Facchini, Jorge, & Polizeli, 2014).

Results and discussion

The *E. nigrum* PG 16 morphological characteristics and amylase production

The E. *nigrum* PG 16 strain used in this study presented this species' cultural characteristics (Figure 1A) with red pigmentation in PDA (Figure 1B). At four days of culture in PDA, the microscopic characteristics

showed hyphae with parallel walls and septa and absence of spores or conidiophores (not shown). When the *E. nigrum* PG 16 was cultured in a solid medium containing soluble starch and stained with iodine, it produced a hydrolytic zone around and inside the colony, confirming its amylase production (Figure 1C). The species *E. nigrum* presents two genotypes, group 1 and group 2. Strains of group 1 are reported to produce amylase in solid medium (Fávaro et al., 2011). Therefore, considering the amylase production in the solid medium (Figure 1C), the *E. nigrum* PG 16 strain used in this work belongs to group 1 of the *E. nigrum* species.

In the starch-iodine complex color distaining method, the *E. nigrum* PG 16 amylase production was 0.7 U mL of filtrate (11.6 U mg^{-1} of protein) in a 3-day-old culture. In the *p*-NPG method, the amylase production was 2.5 U mL^{-1} of filtrate (41 U mg^{-1} of protein) in the same 3-day-old culture. Using *p*-NPG as a substrate characterizes the produced enzyme as a glucoamylase, releasing glucose from a non-reducing end of the starch molecule.

Culture conditions optimization for enzyme production

Commercial biocatalysis needs substantial amounts of glucoamylases; therefore, optimizing its production is imperative (Kumar & Satyanarayana, 2009). Glucoamylases are generally produced by submerged fermentation in shaken cultures (Karim & Tasnim, 2018; Kumar & Satyanarayana, 2009). The media agitation and its intensity cause an excellent mixture and oxygen transfer rates for several fungi, influencing the mycelium morphology and enzyme production (Gopinath et al., 2017). Thus, it is unsurprising that the highest enzyme production and mycelial growth were obtained when the *E. nigrum* PG 16 inoculum production and amylase production cultures were under agitation. However, there was no significant difference (α = 0.01) when the inoculum production culture was shaken or not in combination with the shaken amylase production culture (Figure 1D). Because of this, in all subsequent analyses, the *E. nigrum* PG 16 inoculum production cultures were performed stationarily. Moreover, the amylase production cultures were carried out with agitation.

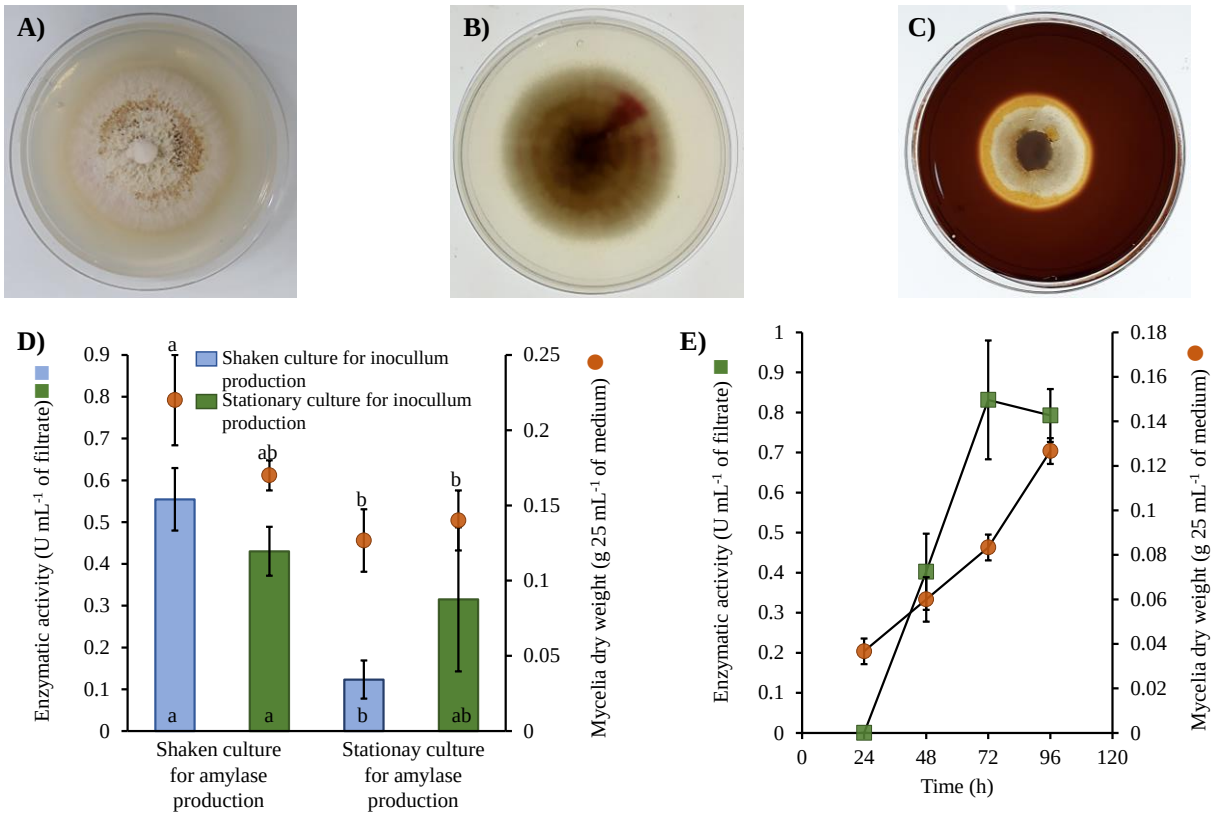

Figure 1. The *Epicoccum nigrum* PG 16 morphology and amylase production optimization. A) and B) Front and reverse, respectively, of a 9 cm Petri dish containing a five-day-old colony of the *E. nigrum* PG 16 isolate in PDA. C) Amylase production detection in nutrient agar with starch stained with the iodine solution. D) Agitation effect on the *E. nigrum* PG 16 culture for inoculum and amylase production. Means followed by identical letters (a and b) are not different by the student test (α = 0.01). Cultures were carried out at 25°C for three days. E) *E. nigrum* PG 16 growth curve and amylase production. The enzyme production was evaluated in shaken liquid culture (100 rpm) at 25°C. Data in D) and E) represent means and standard deviations of triplicates.

The culture time to achieve a fungus enzyme maximum production is based on the microorganism's growth rate. The *E. nigrum* PG 16 growth and amylase production curve showed that the highest enzyme production occurred at 72 hours of culture, with no residual starch in the culture medium. After 72 hours of culture, there was a decrease in enzyme production (Figure 1E). However, the mycelia growth did not follow the enzyme production, not reaching a stationary phase at 72 hours of culture (Figure 1E). This performance may be explained by the formation of secondary metabolites and starch depletion in the medium, which may have inhibited enzyme formation (Gupta et al., 2003). Accordingly, the best culture time for amylase production by *Chrysosporium asperatum* in submerged culture was 72 hours (Sanghvi, 2011).

Aspergillus niger, a leading producer of glucoamylase, is reported to secrete 137 U ml^{-1} of this enzyme in submerged fermentation (Wang et al., 2008). The *E. nigrum* PG 16 produced approximately 1.0 U mL^{-1} of amylase after three days of culture (Table 1). The species *E. nigrum* is reported as an endophytic fungus (Fávaro et al., 2011), which is reported to produce hydrolytic enzymes and other bioproducts (Choudhary, Gupta, Dhar, & Kaul, 2021). Other endophytic fungi are reported to have a wide range of amylase production, such as *Acremonium* sp., which is reported to produce 67.3 U mL^{-1} of a raw sago starch degrading enzyme (Marlida, Saari, Hassan, & Radu, 2000), and *Cladosporium cladosporioides*, which is reported to produce 0.639 U mL^{-1} of an α-amylase (Mushimiyimana, Umuhozariho, & Maniraho, 2019).

Higher amylase production by *E. nigrum* PG 16 occurred when the submerged culture was carried out with a culture medium with initial pH of 7.0 (Figure 2A). The culture medium´s neutral initial pH is also reported as the best for other amylases production, such as the ones by *A. niger* (Varalakshmi et al., 2009). However, there are reports in the literature that the culture medium with more acidic initial pHs is the best for amylase production by *Acremonium* sp. and *C. asperatum* (Marlida et al., 2000; Sanghvi, 2011).

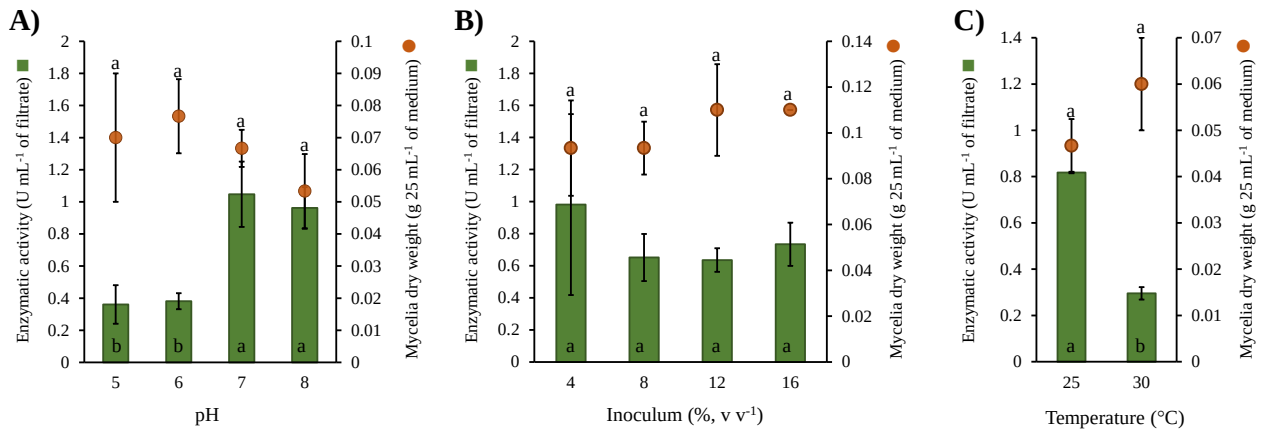

Figure 2. Effect of pH, inoculum concentration, and temperature on the *Epicoccum nigrum* PG 16 amylase production. A) The used liquid medium initial pH effect. B) The inoculum concentration effect. C) The culture temperature effect. Cultures were carried out at 25°C for three days with an agitation of 100 rpm. Means followed by identical letters (a and b) are not different by the student test (α = 0.01). Data represent the mean and standard deviation of triplicates.

High inoculum levels are generally inhibitory for amylase production. For example, Lago et al. (2021) reported that high inoculum levels inhibit *Aspergillus wentii* glucoamylase production. However, there was no statistical difference in growth and amylase production by *E. nigrum* PG 16 when different inoculum concentrations were used (Figure 2B). *E. nigrum* has a slow growth rate, which may impact its enzyme production. Therefore, the 4% (v v^{-1}) inoculum was chosen for the subsequent experiments, as it was the smallest inoculum with equal enzyme production.

The *E. nigrum* PG 16 amylase production was higher at 25°C than at 30°C (Figure 2C). However, the fungus growth was not affected (Figure 2C). Filamentous fungi grow best and produce amylase at a wide range of temperatures. For instance, for the fungus *P. minima* (Zaferanloo, Bhattacharjee, Ghorbani, Mahon, & Palombo, 2014), the amylase production was performed at 25°C, and for *Acremonium* sp. and *C. asperatum*, the best temperature for enzyme production was 30°C (Marlida et al., 2000; Sanghvi, 2011).

Starch, cellulose, and simple sugars can be used as carbon sources for fungi glucoamylase production (Karim & Tasnim, 2018; Kumar & Satyanarayana, 2009). However, monosaccharides can also inhibit glucoamylase production by fungal-specific species or strains (Karim & Tasnim, 2018; Kumar & Satyanarayana, 2009). According to this, starch was the best carbon source for amylase production by *E.*

nigrum PG 16 (Figure 3A), with statistical significance (α = 0.01). In agreement, starch was also the best carbon source for high amylase productivity by the thermophilic fungus *Thermomyces lanuginosus* (Kunamneni, Permaul, & Singh, 2005). On the other hand, simple sugars had little effect in promoting amylase production by *E. nigrum* PG 16 (Figure 3A). For instance, galactose led to low enzyme production and was also the worse substrate for the fungus growth (Figure 3A). Cellulose seems to have increased the *E. nigrum* PG 16 growth. However, it is insoluble and has added weight to the dry mycelia (Figure 3A). All the other used carbohydrates could support the *E. nigrum* PG 16 growth (Figure 3A).

The best starch concentration for the *E. nigrum* PG 16 amylase production was 0.2-0.4%, and higher starch concentrations could not induce amylase production. However, they improved the *E. nigrum* PG 16 growth (Figure 3B). Contrarily, the glucoamylase production by *C. asperatum* was best with 3.0% of potato starch in the liquid culture medium (Sanghvi, 2011). The fact that fungi use different starch concentrations to give maximum extracellular enzymes has been reviewed elsewhere (Karim & Tasnim, 2018). Therefore, a soluble starch concentration of 0.2% was employed in the subsequent experiments because it induced the most negligible *E. nigrum* PG 16 growth with the highest amylase production.

Supplementing the culture medium with certain salts can improve the *E. nigrum* PG 16 growth and enzyme production. For example, adding 25 mM KCl to the culture medium significantly (α = 0.01) increased the *E. nigrum* PG 16 amylase production and growth, and this ion may be necessary for this fungus metabolism. (Figure 3C). Variations in KCl concentration showed that 10 mM was the lowest concentration that resulted in better enzyme production (α = 0.05) (Figure 3D), and this KCl concentration was subsequently used in all experiments. Sodium citrate and the ions Zn^{2+}, Ca^{2+}, and Mn^{2+} did not change the amylase production by the *E. nigrum* PG 16 (Figure 3C). However, they had a different effect on this fungus growth (Figure 3C). On the other hand, the ions Mg^{2+} and Cu^{2+} and the detergent Tween 80 inhibited the amylase production and the fungus growth (Figure 3C).

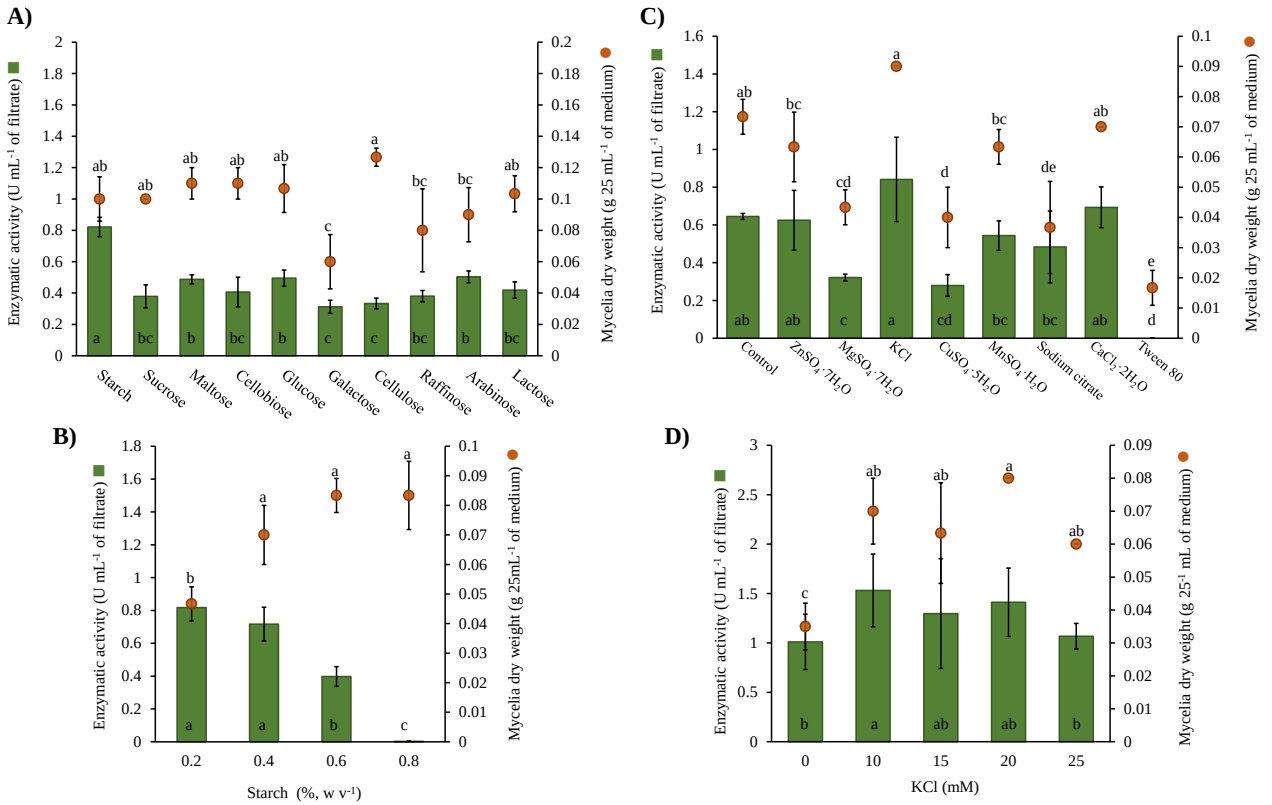

Figure 3. The carbon source, micronutrients, and detergent effects on the *Epicoccum nigrum* PG 16 amylase production. A) Different carbon sources (0.2%, w v^{-1}) were used in the fungus-submerged culture medium, and the amylase production was monitored. B) Different starch concentrations were used in the culture medium for enzyme production. C) Several ions and a detergent were added to the culture medium at the concentrations described in the Materials and Methods section, and the enzyme production was evaluated. D) The effect of different KCl concentrations in the culture medium for amylase production. Data represent the mean and standard deviation of triplicates. Means followed by the same letter (a, b, c, d, and e) are not different by the student test (α = 0.01 for the carbon source, ions, and detergents tests, and α = 0.05 for the KCl concentrations test).

The *Epicoccum nigrum* PG 16 amylase partial purification

In the *E. nigrum* PG 16 enzyme purification process, the molecular exclusion chromatography showed the elution profile with a single activity peak (Figure 4), indicating that the fungus produced only one amylase. However, the SDS-PAGE analysis of the fractions containing amylase activity did not show a strong band of purified protein, demonstrating that the amount of purified enzyme was insufficient for the subsequent characterization (not shown). For that reason, a partial purification with precipitation and dialysis was performed. The protein was recovered with similar profiles in all partial purification repetitions that were performed. The data from a significant experiment is shown in Table 1. The enzyme was partially purified with a recovery of 37-38% after dialysis and had a total increase of 1.5 times in the specific activity compared with the crude extract when the activity was assayed by both the starch-iodine and *p*-NPG methods (Table 1). The one-peak profile in the exclusion chromatography and the increase in the enzyme's specific activity through its purification (Table 1) indicate that the partially purified glucoamylase is probably the only amylase secreted by the *E. nigrum* PG 16.

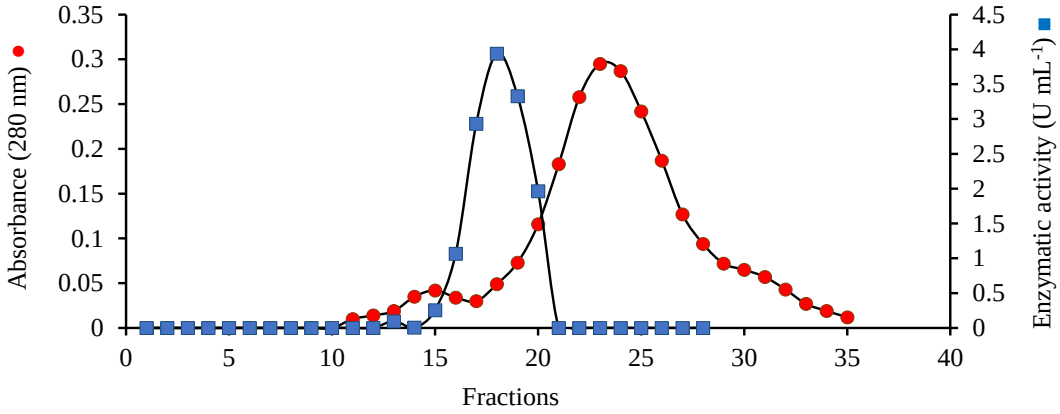

Figure 4. Size exclusion chromatography. The dialysate was applied in a Sephacryl HR-200 column (100 × 1.5 cm), which was equilibrated and eluted with 50 mM phosphate buffer, pH 7.0. The column flux was 0.5-0.6 mL min.$^{-1}$, and fractions of 4.0 mL were collected. The fractions absorbance at 280 nm was used to estimate the protein elution, and the starch-iodine method was used to estimate the amylase elution.

Table 1. The *Epicoccum nigrum* PG 16 amylase partial purification.

	Volume (mL)	Total enzymatic activity (U)*	Protein (mg)*	Specific activity (U mg^{-1} of protein)*	Yield (%)
Starch-iodine complex distaining method					
Culture filtrate	190	136.6 ± 0.48	11.7 ± 0.23	11.7 ± 0.27	
Dialysate	5	50.8 ± 5.64	2.8 ± 0.18	17.7 ± 0.84	37.19
p-NPG method					
Culture filtrate	190	481.6 ± 117.13	11.7 ± 0.23	41.2 ± 10.8	
Dialysate	5	187.2 ± 18.27	2.8 ± 0.18	65.1 ± 2.22	38.87

*Data show means and standard deviation of duplicates.

Biochemical characterization of the partially purified amylase

pH markedly affects enzyme activity, as substrate binding to the enzyme's catalytic site often depends on component charge distributions. Moreover, pH changes may influence the enzyme structure in the environment. For example, fungi glucoamylases have been reported as most active at acidic pH but also have a wide range of optimum pH (Karim & Tasnim, 2018; Norouzian et al., 2006). Not surprisingly, the *E. nigrum* PG 16 partially purified glucoamylase has an optimum pH of 6.0 with the 0.1 M Britton and Robinson buffer (Figure 5A), which agrees with the *A. wentii* glucoamylase optimum pH of 5.0-6.0 (Lago et al., 2021).

The *E. nigrum* PG 16 glucoamylase optimal temperature of 50°C (Figure 5B), at pH 6.0, in both used buffers (0.1 M phosphate and Britton and Robinson), is in the same range as other fungi glucoamylases optimum temperatures (40-60°C) (Karim & Tasnim, 2018; Kumar & Satyanarayana, 2009; Norouzian et al., 2006). This optimum temperature is not as low as the *Tetracladium* sp. glucoamylase optimum temperature of 30°C nor as high as the one from *Chaetomium thermophilum* of 65°C (Carrasco, Alcaíno, Cifuentes, & Baeza, 2017; Chen, Li, Zhang, & Zhou, 2005).

The partially purified *E. nigrum* PG 16 glucoamylase was stable when incubated for 30 min. at 40°C but lost 50% of its activity (T_{50}) when incubated at 46.25°C, and maintained only 7% of its activity when incubated at 50°C, for the same amount of time (Figure 5C). The thermal stability of glucoamylases among fungi is quite variable (Kumar & Satyanarayana, 2009; Norouzian et al., 2006). In agreement, the *E. nigrum* PG 16 glucoamylase is less thermal stable than the *C. thermophilum* glucoamylase, which lost 50% of its activity when incubated for 1 hour at 65°C (Chen et al., 2005). However, the *E. nigrum* PG 16 glucoamylase had about the same thermal stability as the *Tetracladium* sp. glucoamylase, which maintained 10% of its activity after incubation for 1 hour at 50°C (Carrasco et al., 2017).

Bacterial α-amylases are more thermophilic and thermostable than glucoamylases (Gupta et al., 2003; Kumar & Satyanarayana, 2009). Glucoamylases are used in starch saccharification at acidic pH and mild temperature (60°C). Considering this, the *E. nigrum* PG 16 glucoamylase optimal pH and temperature, and thermal stability make it an excellent enzyme for this application.

The *E. nigrum* PG 16 partially purified amylase enzymatic reaction dependence ratio on starch at pH 6.0 and 50°C followed the Michaelis–Menten kinetics (Figure 5D). The calculated value of *K*M was 1.72 mg ml-1 of starch, and *V*max was 0.152 mg min-1 of degraded starch. K_M values for starch from other fungal glucoamylases range from 0.11 to 4.16 mg mL^{-1} (Kumar & Satyanarayana, 2009) and the obtained *E. nigrum* PG 16 glucoamylase K_M value for starch falls in this range. However, data for fungal glucoamylases V_{max} are rare and use different units, making comparing data difficult.

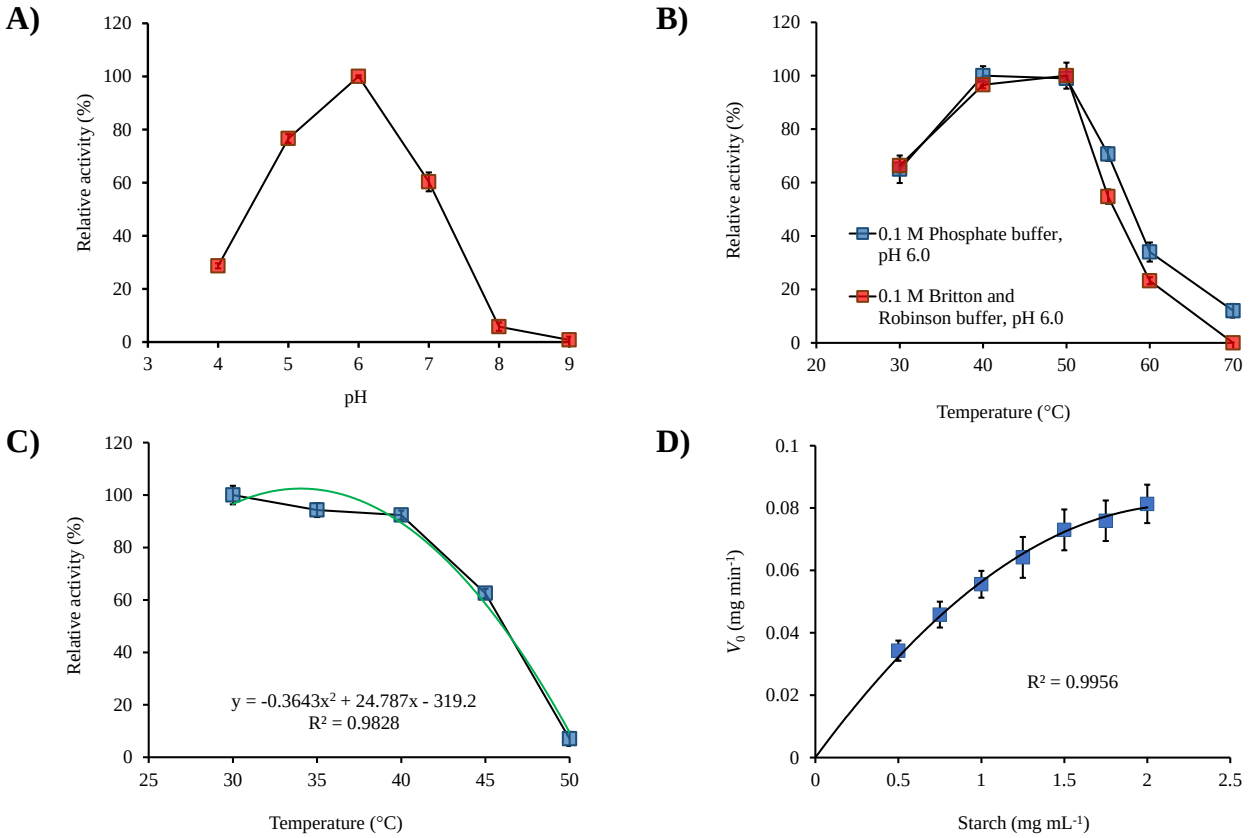

Figure 5. Biochemical characterization of the partially purified *Epicoccum nigrum* PG 16 glucoamylase. A) Optimum pH. The optimum pH was determined with the enzymatic assay carried out in 0.1 M Britton and Robinson buffer at different pHs in the enzyme assay at 50°C. B) Optimum temperature. The optimum temperature was determined by carrying out the enzymatic assay at different temperatures at pH 6.0 in two different buffers. C) Thermal stability. The enzyme was incubated for 30 minutes at different temperatures and then submitted to the enzymatic assay with 0.1 M phosphate buffer, pH 6.0. D) Determination of V_{max} and K_M. The starch-iodine complex distaining enzymatic assay was carried out with different starch concentrations with a fixed amount of the partially purified enzyme (7.9 µg). In A), B,) and C), the results are averages and standard deviations of duplicates. In D), the results represent averages and standard deviations of three experimental data.

Mg^{2+} and Mn^{2+} did not dramatically change the *E. nigrum* PG 16 glucoamylase activity at 5 or 10 mM (Table 2). Ca^{2+} has slightly activated the enzyme activity by 5% at 5 and 10 mM (Table 2). The role of Ca^{2+} in activating and maintaining the α-amylase's stability is well understood (Gupta et al., 2003). However,

glucoamylases are not always dependent on Ca^{2+} (Kumar & Satyanarayana, 2009). For instance, Ca^{2+} has increased the activity of *C. thermophilum* glucoamylase (Chen et al., 2005) and *Aspergillus phoenicis* (Benassi et al., 2014) but was not required for the activity of *Fusarium solani* and *Aspergillus oryzae* glucoamylases (Bhatti et al., 2007; Wang, Yang, Luo, Tang, & Wang, 2020). Cu^{2+}, Zn^{2+}, and Fe^{3+} have inhibited the *E. nigrum* PG 16 glucoamylase at 5 mM and Co^{2+} at 10 mM (Table 2). In agreement, Cu^{2+} and Zn^{2+} have also inhibited the glucoamylase from *C. thermophilum*, and Fe^{3+} inhibited the *F. solani* glucoamylase (Chen et al., 2005; Bhatti et al., 2007). SDS, an anionic detergent, and Tween 80, a neutral detergent, have inhibited the *E. nigrum* PG 16 glucoamylase activity (Table 2). The detergent SDS has also inhibited the glucoamylase activity of *A. wentii* (Lago et al., 2021).

Table 2. Residual relative activity of the *Epicoccum nigrum* PG 16 partially purified glucoamylase in the presence of potential inhibitors and activators.

	5 mM# (% of remaining activity)	10 mM# (% of remaining activity)
Control	100.0 ± 1.69	100.0 ± 1.69
$MnSO_4{\cdot}H_2O$	96.8 ± 0	95.5 ± 3.86
$CaCl_2{\cdot}2H_2O$	105.7 ± 0.55	105.04 ± 0
$MgSO_4{\cdot}7H_2O$	86.1 ± 0.59	91.9 ± 1.61
$CoCl_2{\cdot}6H_2O$	99.1 ± 9.20	78.9 ± 2.19
$ZnSO_4{\cdot}7H_2O$	54.5 ± 1.72	40.4 ± 0.57
$CuSO_4{\cdot}5H_2O$	20.5 ± 2.62	6.8 ± 2.93
$FeCl_3$	70.6 ± 4.26	*
SDS	7.6 ± 0.69	10.9 ± 0.73
Tween 80	57.08 ± 9.43	*

The enzyme assay was carried out with 0.1 mM Tris buffer, pH 6.0. #The data are averages and standard deviations of duplicates and represent the residual activity compared with the control, where no substance was added. *Non-determined. At 10 mM $FeCl_3$ increased the reaction's yellow color and Tween 80 increased the reaction's turbidity.

The TLC analysis showed glucose as the *E. nigrum* PG 16 glucoamylase starch hydrolysis main product after 24 hours and only 1 hour of reaction (Figure 6). In agreement, glucose was the only starch hydrolysis product by the *A. phoenicis* glucoamylase (Benassi et al., 2014). The TLC results and the partially purified enzyme's ability to degrade *p*-NPG (Table 1) validate the *E. nigrum* PG 16 amylase as a glucoamylase.

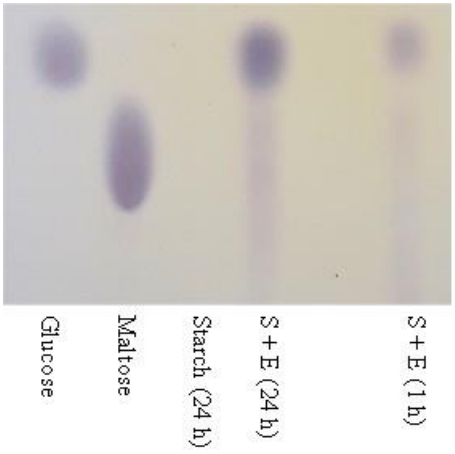

Figure 6. Products of the *Epicoccum nigrum* PG 16 amylase starch hydrolysis. The hydrolysis reactions were spotted in a thin layer plate. Glucose and maltose were used as standards. Starch alone was used to indicate that there were no hydrolysis products without the enzyme's digestion. Starch + enzyme (S + E) corresponds to the glucoamylase reaction on starch for 24 hours and also for 1 hour.

Availability of data and material (data transparency)

The data supporting this study's findings are freely available as Supporting Information Material attached to this article and permanently stored as Open Science Framework data available at https://osf.io/bvcwq/?view_only=85d0e55bc0f746c58c9f19bc7d72d424

Conclusion

The best growth conditions for the *E. nigrum* PG 16 glucoamylase production were standardized, and the biochemical characteristics of the partially purified enzyme were established. The *E. nigrum* PG 16

glucoamylase is the only secreted amylase, and its biochemical characteristics allow its use in starch saccharification. Considering that *E. nigrum* PG 16 does not produce spores in the culture medium at the best culture time for the studied amylase production and that this species is not considered a mycotoxin producer, a crude culture filtrate of this fungus could be used in several industrial processes as a source of glucoamylase. In addition, the enzyme efficiency in releasing glucose with only one hour of hydrolysis could be helpful for several saccharification processes, representing an advance in the field of glucoamylases.

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