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# Production of Invertases by Anamorphic (*Aspergillus nidulans*) and Teleomorphic (*Emericela nidulans*) Fungi under Submerged Fermentation Using Rye Flour as Carbon Source

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## ABSTRACT

The production of invertases by anamorph (*A. nidulans*) and teleomorph (*E. nidulans*) was investigated. The best level of extracellular enzymatic production for anomorph was obtained in Khanna medium containing sucrose as carbon source, whereas for teleomorph the best production was archived using M5 medium containing inulin as carbon source. Despite this, rye flour was selected as carbon source. The extracellular enzyme production was higher for teleomorph than that observed for anomorph for all carbon sources used. The enzyme production was inhibited by the addition of fructose and glucose in the medium containing rye flour as carbon source. The best conditions to recover the higher enzymatic activity were temperature of 54˚C - 62˚C and pH of 4.8 - 5.6 for both enzymes determined by experimental design (CCRD). The stability of the temperatures at 40˚C and 50˚C were similar for both enzymes. The invertases from the anomorph and teleomorph were activated by $Mn^{2+}$, but the response of each one towards the presence of this cation was different with best activation observed for the anomorph enzyme (+80%). The extracellular enzymes were able to hydrolyze inulin, sucrose and raffinose. However, the affinity was higher for sucrose than inulin. In conclusion, the carbon source assimilation and the invertase production, as well as the enzymes properties, were different for the anomorph and teleomorph mycelia.

**Keywords:** Invertase; Fructofuranosidase; *Aspergillus*; *Emericela*

## 1. Introduction

Invertases (EC 3.2.1.26) are enzymes which are able to catalyze the hydrolysis of $\beta$ 1-2 bonds from sucrose molecule producing an equimolar mixture of D-glucose and D-fructose named invert sugar. The first study on the invertase activity was in 1828 using yeast cells. Nowadays, many works showing the production, characterization and application of these enzymes have been performed. Among the invertases sources, microorganisms deserve attention, especially filamentous fungi as *Aspergillus casepitosus* [1], *Aspergillus niger* [2], *Paecylomyces variotti* [3], *Fusarium oxysporum* [4] and *Rhizopus* sp. [5], among others. Generally, the fungal invertases have attracted the attention of different sectors of the industry because of their biotechnological potential. The invert sugar, for example, can be used in food and beverage industries. Additionally, some invertases with fructosyltransferase activity can be used to produce fructooligosaccharides (FOS), as 1-kestose, nystose and fructofuranosyl nystose, which have important functional properties such as reduction of cholesterol and ammonia in the blood, and stimulation of *Bifidobacterium* growth in the human colon [6]. Despite the great number of filamentous fungi investigated as producers of invertases, a comparative study on the enzyme production considering both asexual phase and sexual phase has not been considered, especially for invertase. This aspect is important to understand the differences on the metabolism at different stages of the life cycle of the fungi. A filamentous fungus can receive two Latin names corresponding to the

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anomorph (asexual stage) and teleomorph (sexual stage) [7]. The anomorph is characterized by the production of spores by mitosis while the teleomorph is characterized by the presence of specific reproductive structure where meiosis occurs. According to Geiser (2009) [8], the sexual phase for 60% of the *Aspergillus* species is not observed. The species that show both stages are mentioned as pleomorphic [7]. In this article, in a comparative way, the production and characterization of extracellular invertases from anomorph (*A. nidulans*) and teleomorph (*E. nidulans*) filamentous fungi are described, using an agro-industrial residue as carbon source.

## 2. Material and Methods

### 2.1. Microorganisms and Culture Conditions

The strains *Aspergillus nidulans* (anamorph) and *Emericela nidulans* (teleomorph) were obtained from the soil, identified by the Laboratory of Microbiology from Universidade Federal de Pernambuco, Pernambuco, Brazil and deposited in the Culture Collection from Laboratory of Microbiology, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, University of São Paulo, Brazil. Both strains were maintained in PDA (Potato Dextrose Agar) medium previously autoclaved at 120˚C, 1.5 atm for 30 minutes. The cultures were maintained at 4˚C for 30 days.

The submerged fermentation cultures were obtained by the inoculums of 1 mL of aqueous spore suspension ($10^6$ spores/mL) in 25 mL of Khanna medium [9] and M5 [10] in 125 mL Erlenmeyer flasks, pH 6.0, for anamorphic and teleomorphic strains, respectively. The media were added with different carbon sources (1% w/v for oligosaccharides and complex sources, and 2% w/v for monosaccharides) and autoclaved at 120˚C, 1.5 atm for 30 minutes. Afterwards, the cultures were maintained for different periods under agitation (100 rpm) at 30˚C.

### 2.2. Influence of Different Compounds on Enzyme Production

The culture media for both strains were added (1% w/v) with organic (yeast extract and peptone) and inorganic [$(NH_4)_2SO_4$; $NH_4H_2PO_4$ and $(NH_4)_2HPO_4$] nitrogen sources or $NaH_2PO_4$, $Na_2HPO_4$, $KH_2PO_4$ and $K_2HPO_4$ as phosphate sources, separately according to that defined for each experiment.

The influence of different concentrations (0% - 5% w/v) of glucose and fructose added to the media on invertase production was also analyzed. The microorganisms were also grown in culture media containing 1% (w/v) glucose as carbon source at 30˚C, for 24 h under agitation (100 rpm). Then, the mycelia obtained through vacuum filtration were washed with distilled water and transferred to a new media containing 1% (w/v) glucose, 1% (w/v) sucrose or 1% (w/v) rye flour as carbon sources at 30˚C, for 96 h under agitation (100 rpm).

### 2.3. Obtainment of the Extract Containing Invertase

The submerged cultures were harvested under vacuum filtration using Whatman filter paper No. 1. The free cell filtrate was dialyzed against distilled water overnight at 4˚C and used as source of extracellular invertase. The mycelia were washed with distilled water, dried using filter paper, disrupted using acid clean sand, ressuspended using distilled water and centrifuged at 23,000 g for 15 minutes at 4˚C. The supernatant obtained was dialyzed against distilled water at 4˚C overnight and used as source of intracellular invertase.

### 2.4. Partial Purification of the Extracellular Enzymes

The extracellular crude extracts containing the invertase were loaded in DEAE-Cellulose chromatographic column (14.5 × 1.0 cm) previously equilibrated with 10 mM Tris-HCl buffer pH 7.5. Fractions of 3.0 mL were collected at flow rate of 1.0 mL/min. The fractions containing invertase activity were eluted using a linear gradient of NaCl (0 - 1.5 M) in the same buffer cited above. These fractions were pooled, dialyzed against distilled water overnight at 4˚C, lyophilized, ressuspended in 50 mM Tris-HCl buffer pH 7.5 + 50 mM NaCl and loaded in Sephacryl S-200 chromatographic column (180.0 × 1.0 cm) previously equilibrated using the same buffer. Fractions of 1.2 mL were collected at flow rate of 0.3 mL/min. The fractions containing invertase were pooled, dialyzed against distilled water overnight at 4˚C and used for enzymatic assays.

### 2.5. Enzyme Assay and Protein Quantification

The invertase activity was determined using 2% sucrose as substrate in sodium acetate buffer (100 mM, pH 5.0). The reducing sugars after hydrolysis were quantified using DNS according to Miller *et al.* (1959) [11] at 540 nm. One unit of enzyme activity was defined as the amount of enzyme necessary to produce 1 μmol of glucose per minute under the assay condition.

The protein was quantified using the Lowry method [12] using BSA as standard.

### 2.6. Optimization of Temperature and pH of Activity and Enzyme Stability

The influence of the temperature and pH (independent variables) on the extracellular invertase activities (dependent variable) from both strains was performed using a Rotational Central Composite Design with 11 trials

with 3 repetitions at the central point and 2 axial points. The software Statistica V8.0 (StatSoft, USA) was used to perform the analysis of variance (ANOVA) with 95% of significance level and to generate the surface response plots.

The thermo stability was determined by the incubation of the samples of enzymes aqueous solution at different temperatures (40˚C - 70˚C) for 60 minutes and then assayed for invertase activity as described above. The pH stability was determined by the incubation of the enzyme samples in different pH values (sodium acetate buffer pH 3.7 - 5.7; Tris-HCl buffer pH 7.0 - 8.0; CAPS buffer pH 9.0 - 10.0) for 60 minutes and then used for invertase assay as described above.

### 2.7. Influence of Different Compounds on Invertase Activity

The influence of 1 mM of different salts and $\beta$-mercaptoethanol added to the reaction medium on invertase activity was analyzed.

### 2.8. Hydrolysis of Different Substrates and Kinetic Parameters

The hydrolytic activity from the extracellular invertases on different substrates was analyzed using 0.5% (w/v) of inulin, rafinose and sucrose in 100 mM sodium acetate buffer pH 5.0. Mixtures (1:1 v/v) of inulin plus rafinose, inulin plus sucrose and sucrose plus rafinose were also analyzed. The kinetic parameters $K_m$ and $V_{max}$ were determined for sucrose as substrate (0.1 - 20 mM) using Lineweaver-Burk plot obtained with Origin Graph software (OriginLab Corporation).

## 3. Results and Discussion

### 3.1. Enzyme Production

The *Aspergillus* genus comprises many fungal species recognized as anamorph, which are classified in different teleomorph genera as, for example, *A. nidulans*, which is part of the teleomorph genus *Emericela* [13]. *Aspergillus* spp. has been mentioned as a good producer of different enzymes that act on polymers to obtain nutrients. In addition, these enzymes, according to their properties, have biotechnological potential for many industrial purposes. Among these enzymes, invertases deserve attention. *A. nidulans* was selected because it has been mentioned as a model for production of different extracellular enzymes with biotechnological potential [14]. In addition, the teleomorph *E. nidulans* was observed as an important enzyme producer in our laboratory. So far, the comparison between invertase production by anamorph and teleomorph filamentous fungi has not been elucidated.

The growth of filamentous fungi and, consequently, the enzyme production, are influenced by the nutrients used, including the carbon source. As it can be observed in the **Table 1**, the highest enzymatic production for both intra and extracellular invertases by *E. nidulans* was achieved using inulin as substrate. On the other hand, the sucrose was the best substrate for extracellular enzyme production by *A. nidulans*. The fungi *Aspergillus niger* and *A. nidulans* were mentioned as models for enzyme secretion, since invertase production is induced by sucrose [15]. Considering the best conditions obtained for each one, the enzyme production by *E. nidulans* was around 8-fold higher than that observed for intra and extracellular enzymes from *A. nidulans*. This fact shows that the carbon source assimilation by filamentous fungi is related to the life cycle period. The extracellular enzyme production by the teleomorph was higher than that observed for the anamorph for all carbons sources tested. The second best carbon source for both *E. nidulans* and *A. nidulans* was rye flour. Considering this agroindustrial product, the extracellular production was around 10-fold higher for *E. nidulans* if compared to the *A. nidulans* and similar for the intracellular enzymes. Agroindustrial residues/products are low-cost substrates that can be used to produce enzymes with industrial application. In addition, this type of carbon source is similar to the carbon sources found in the natural environment of the filamentous fungi, which are able to hydrolyze different polymers.

**Table 1. Influence of different carbon sources on production of intra and extracellular invertases by *A. nidulans* (anamorph) and *E. nidulans* (telomorph) using Submerged Fermentation.**

| Carbon sources | Invertase activity (total *U*) | | | |
|---|---|---|---|---|
| | Teleomorph | | Anamorph | |
| | Extra | Intra | Extra | Intra |
| Without | 182.9 ± 32.4 | 5.4 ± 1.2 | 7.8 ± 3.4 | 1.7 ± 0.7 |
| Corncob | 188.1 ± 13.0 | 17.5 ± 5.8 | 0.22 ± 0.4 | 9.0 ± 1.6 |
| Crushed corn | 106.4 ± 19.0 | 50.1 ± 10.8 | 1.2 ± 0.2 | 4.8 ± 1.3 |
| Fructose | 64.4 ± 7.0 | 21.7 ± 3.6 | 3.5 ± 1.0 | 26.0 ± 7.3 |
| Glucose | 28.3 ± 8.4 | 30.1 ± 2.3 | 0 | 12.8 ± 0.7 |
| Inulin | 714.4 ± 100.3 | 224.4 ± 6.5 | 11.4 ± 2.6 | 23.5 ± 4.5 |
| Oat meal | 279.8 ± 24.8 | 42.5 ± 2.4 | 19.0 ± 11.4 | 16.3 ± 4.5 |
| Rice straw | 204.3 ± 11.9 | 2.5 ± 1.5 | 6.2 ± 1.5 | 3.1 ± 0.4 |
| Rye flour | 361.9 ± 30.6 | 28.3 ± 2.3 | 33.6 ± 8.3 | 30.0 ± 5.0 |
| Sucrose | 244.3 ± 17.0 | 30.0 ± 4.1 | 93.0 ± 12.3 | 28.3 ± 10.0 |
| Sugar cane bagasse | 103.8 ± 5.0 | 16.6 ± 0.1 | 13.7 ± 2.0 | 31.9 ± 7.3 |

The fungi *A. nidulans* and *E. nidulans* were cultured in Khanna and M5 media, respectively, with initial pH 6.0, maintained at 30˚C, under orbital agitation for 72 h.

The production of the extracellular enzyme by both anamorph and teleomorph was inhibited when the monosaccharides glucose and fructose were used as supplement in the media containing rye flour as the main carbon source (**Figure 1**). However, the influence of fructose on extracellular enzyme production by *E. nidulans* was less pronounced than that observed for glucose. Considering the production of the intracellular enzyme, it is possible to see an increment for *A. nidulans* invertase production at 0.5% (w/v) and 1.0% (w/v) of glucose, differing from that observed for *E. nidulans*, with a reduction of enzyme production in all glucose concentrations analyzed. The use of 1.0% (w/v) of fructose promoted an increase in the intracellular enzyme production by *A. nidulans*, but not for *E. nidulans*. On the other hand, these monosaccharides are related with the regulation of enzyme secretion at a range of 0.5% - 1.0% for *A. nidulans*, since there was an increment in the level of intracellular enzyme and reduction in the level of extracellular enzyme.

High concentrations of glucose and fructose promoted a reduction in the production of intracellular enzyme. This kind of inhibition is recognized as catabolic repression by carbon source. When the microorganisms were grown for 24 h using glucose as carbon source and transferred to new media containing glucose, sucrose or rye flour, the best induction of enzyme production (intra and extracellular forms) was achieved with the latter (**Figure 2**). For anamorph, the extracellular invertase activity was not detected when the fungus was grown in presence of sucrose, a natural inducer, but the intracellular form was observed in presence of both glucose and sucrose. These saccharides are transported into the cell by sugar transporters and, consequently, the sucrose can be hydrolyzed by intracellular enzymes. When the anamorph is directly cultured in presence of sucrose, it needs to hydrolyze this carbon source in the extracellular medium to obtain nutrients for spore germination, growth and the mycelia formation, what is not necessary when the mycelium mass was previously obtained in glucose containing medium. For invertase production by the fungus *F. oxysporum*, the monosaccharides did not have a repressive effect [4]. It is important to remember that these sugars are products from the sucrose hydrolysis and can act as regulators for enzyme production. In some filamentous fungi, the products of hydrolysis of sucrose can act as inducers [16]. The expression of *SUC*2 gene, that encodes two enzymes with different cellular localization in *Saccharomyces cerevisiae*, is controlled by glucose [17] and fructose repression [18]. The synthesis of invertase was not induced by glucose and fructose in *A. niger* cultures [19] while fructose acted as inducer in *Penicillium glabrum* cultures [16].

The influence of organic and inorganic nitrogen sources, and phosphate sources on invertase production,

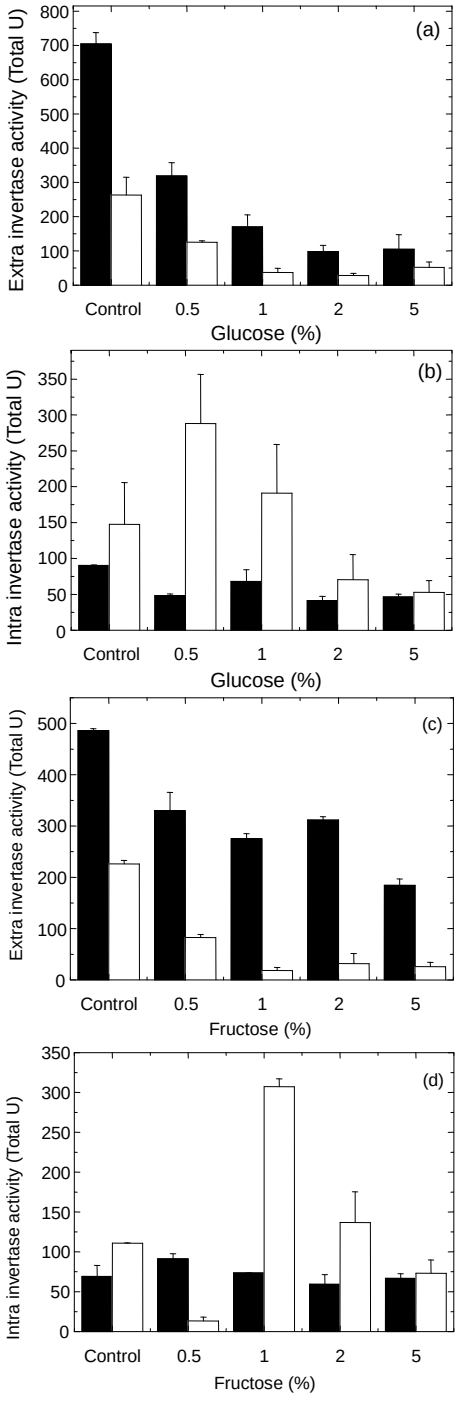



**Figure 1. Influence of different concentrations of glucose ((a) and (b)) and fructose ((c) and (d)) added to the culture medium containing rye flour as the main carbon source on extracellular ((a) and (c)) and intracellular ((b) and (d)) invertases from *A. nidulans* (□) and *E. nidulans* (■).**

was also investigated (data not shown). In this case, the production of extracellular invertase by *A. nidulans* was improved 2.6-fold in the presence of peptone and yeast extract compared to the control while for *E. nidulans* extracellular enzyme production was improved 2-fold in

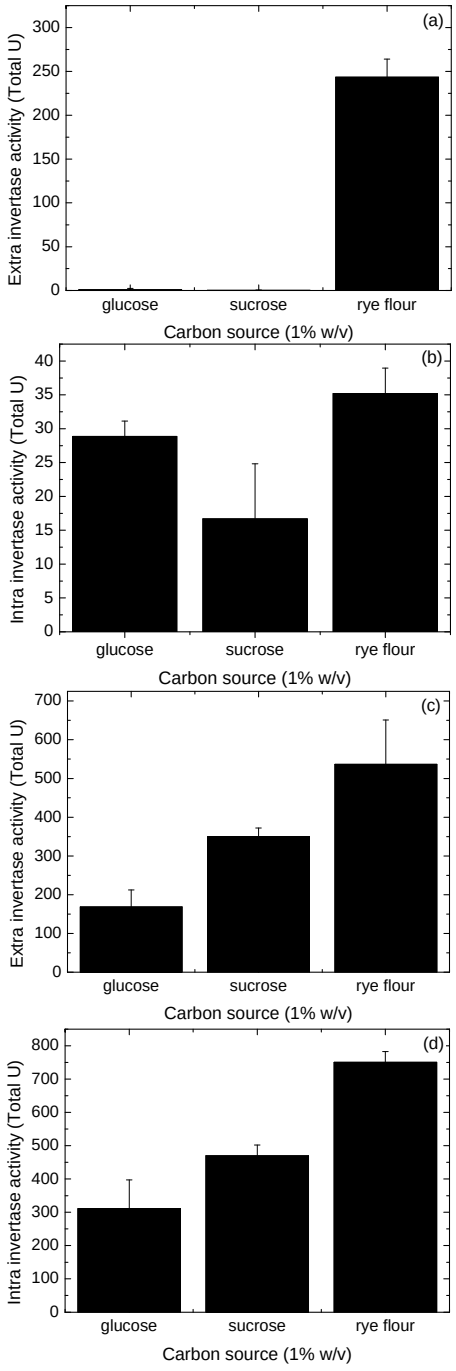



**Figure 2. Influence of glucose, sucrose and rye flour on extracellular ((a) and (c)) and intracellular ((b) and (d)) invertases production by *A. nidulans* ((a) and (b)) and *E. nidulans* ((c) and (d)) pre-cultured by 24 h in M5 and Khanna media, respectively, containing 1% (w/v) glucose.**

the presence of $(NH_4)_2SO_4$ as also observed for intracellular enzyme. The invertase production by other fugal strains in the presence of peptone and yeast extract has been mentioned [20-22]. Considering the phosphate sources, the production of intra and extracellular invertases by *E. nidulans* was not significantly affected, while the extracellular enzyme from *A. nidulans* was reduced. On the other hand, the production of intracellular form was not significantly affected by the phosphate sources used. This fact indicates that the influence of the phosphate source is not on enzyme production, but on the secretion. Production of invertases by *A. caespitosus* was influenced positively in the presence of $Na_2HPO_4$ and $KH_2PO_4$ [1] as also observed for *S. cerevisiae* GCB-K5 [23].

Another aspect that should be considered is the mycelium morphology. It is recognized that the mycelium is heterogeneous considering the gene expression and, consequently, affecting the growth and secretion. The heterogeneity has been mentioned for peripheral and central zones of the colony. Under submerged fermentation, three types of growth can be observed: 1) disperse, 2) as clumps and 3) as pellets. These possibilities have considerable impact on enzyme production [13]. For both anamorph and teleomorph stages, development as pellets was observed (data not shown), indicating that the differential enzyme production is not associated with the type of growth. The differential expression of *SUC* genes for anamorph and teleomorph stages can be hypothesized, probably related to nutrient obtainment for development of vegetative hyphae and reproductive structures.

## 3.2. Influence of Temperature and pH on Extracellular Invertase Activity

The influence of the independent variables temperature (X1) and pH (X2) on the extracellular activity (dependent variable) was determined using factorial design (**Table 2**). According to the ANOVA (**Table 3**) for enzyme activity from *E. nidulans*, the quadratic temperature, linear and quadratic pH effects were significant ($\alpha < 0.05$) with $R^2$ value of 0.92. For the enzyme activity from *A. nidulans*, the linear temperature and pH, and quadratic pH were significant ($\alpha < 0.05$) with $R^2$ value of 0.94. For both enzymes, the values of $F_{calc.}$ were higher than the value of $F_{tab}$, showing that the models are significant and predictive (data not shown). The equations (Equation (1)) and (Equation (2)) represent the models for *E. nidulans* and *A. nidulans* extracellular invertase activities, respectively.

$$\text{Total } U = 828.8 + 79.3X_1 - 132.7X_1^2 - 267.5X_2 - 271.5X_2^2 - 103.8X_1X_2 \quad (1)$$

$$\text{Total } U = 242.8 + 32.1X_1 + 2.3X_1^2 - 77.7X_2 - 69.7X_2^2 - 2.3X_1X^2 \quad (2)$$

The surface responses for both extracellular invertase activities (*E. nidulans* and *A. nidulans*) are presented in **Figure 3**. The best conditions to recover the higher enzymatic activity were temperature of 54˚C - 62˚C and pH

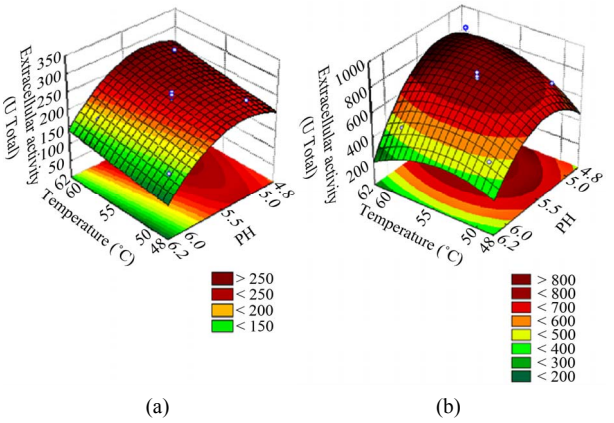
**Table 2. Experimental design for influence of the independent variables temperature and pH on the extracellular invertase activities from *A. nidulans* (anamorph) and *E. nidulans* (teleomorph).**

| Run | Encoded (real) values | | Extracellular invertase activity (total U) | |
|---|---|---|---|---|
| | X1 (˚C) | X2 (pH) | Anamorph | Teleomorph |
| 1 | 1 (60) | 1 (6.0) | 172.2 ± 13.9 | 457.7 ± 13.7 |
| 2 | 1 (60) | −1 (5.0) | 271.6 ± 20.9 | 927.0 ± 35.3 |
| 3 | −1 (50) | 1 (6.0) | 140.6 ± 7.8 | 461.4 ± 24.1 |
| 4 | −1 (50) | −1 (5.0) | 235.4 ± 7.4 | 723.1 ± 41.3 |
| 5 | 0 (55) | 0 (5.5) | 227.8 ± 50.0 | 805.9 ± 60.3 |
| 6 | 0 (55) | 0 (5.5) | 254.5 ± 69.7 | 823.0 ± 55.1 |
| 7 | 0 (55) | 0 (5.5) | 246.1 ± 14.3 | 857.6 ± 45.6 |
| 8 | 1.41 (62) | 0 (5.5) | 270.7 ± 2.5 | 721.9 ± 14.6 |
| 9 | −1.41 (48) | 0 (5.5) | 227.8 ± 1.64 | 639.1 ± 6.9 |
| 10 | 0 (55) | 1.41 (6.2) | 136.0 ± 23.9 | 421.8 ± 86.9 |
| 11 | 0 (55) | −1.41 (4,8) | 218.5 ± 17.2 | 661.6 ± 21.5 |

**Table 3. ANOVA for the CCRD with temperature and pH as independent variable for the extracellular invertase activities from *A. nidulans* (anamorph) and *E. nidulans* (teleomorph).**

| **Anamorph** | | | | | |
|---|---|---|---|---|---|
| Effects | SQ | GL | QM | Fcalc. | *p* value |
| Temp. (L) | 2063.25 | 1 | 2063.25 | 8.13062 | 0.035764* |
| Temp. (Q) | 7.41 | 1 | 7.41 | 0.02922 | 0.870981 |
| pH (L) | 12081.32 | 1 | 12081.32 | 47.60872 | 0.000979* |
| pH (Q) | 6856.18 | 1 | 6856.18 | 27.01808 | 0.003473* |
| 1 × 3 | 5.38 | 1 | 5.38 | 0.02121 | 0.889897 |
| Pure error | 1268.81 | 5 | 253.76 | | |
| Total | 23077.51 | 10 | | | |
| SQ: Some of Square; GL: Liberty degree; QM: Medium square. $R^2$: 0.94; $^*\alpha = 0.05$ | | | | | |
| **Teleomorph** | | | | | |
| Temp. (L) | 12587.2 | 1 | 12587.2 | 2.68976 | 0.161919 |
| Temp.(Q) | 24868.6 | 1 | 24868.6 | 5.31419 | 0.069317* |
| pH (L) | 143165.1 | 1 | 143465.1 | 30.59306 | 0.002649** |
| pH (Q) | 104066.3 | 1 | 104066.3 | 22.23800 | 0.005263** |
| 1 × 3 | 10770.3 | 1 | 10770.3 | 2.30151 | 0.189697 |
| Pure error | 23398.3 | 5 | 4679.7 | | |
| Total | 298306.9 | 10 | | | |
| SQ: Some of Square; GL: Liberty degree; QM: Medium square. $R^2$: 0.92; $^*\alpha = 0.1$; $^{**}\alpha = 0.05$ | | | | | |

Temp.: temperature; 1 × 3: Temp.(L) × pH(L).




(a) (b)

**Figure 3. Surface response for the influence of independent variables, temperature and pH on extracellular invertase activities from (a) *A. nidulans* and (b) *E. nidulans*.**

of 4.8 - 5.6 for both enzymes. The range of temperature obtained is in agreement with other observations as, for example, 60˚C for enzymes from *A. niger* [20] and *A. niveus* [24], among others. However, the pH value for *A. niger* invertase activity was lower than that observed for both *E. nidulans* and *A. nidulans* enzymes [19].

The extracellular invertases from *E. nidulans* and *A. nidulans* were stable at 40˚C and 50˚C for 1 h, while at 60˚C, 22-minute half-life ($T_{50}$) was observed for the former and 30 minutes for the latter (**Figure 4**). At 70˚C, the stability was drastically reduced. The thermal stability observed for both enzymes was higher than that observed for the invertases from *A. flavus* [21] and *A. caespitosus* [1]. The extracellular enzyme produced by *E. nidulans* was fully stable at all pH analyzed for 1 h, differing from the results observed for extracellular enzyme from *A. nidulans*, which was stable only at pH 3.0 and from pH 7.0 to 10.0. The pH stability of invertases produced by different strains of *A. niger* has been described. The enzyme produced by the strain PSSF21 was stable at a pH range from 3.5 to 4.5 [20]. For strain IMI 303386, it was stable from pH 4.0 to 8.0 and for strain AS0023, the enzyme was stable from pH 4.0 to 11.0 [25].

## 3.3. Influence of Different Compounds on Invertase Activity

The influence of different salts on extracellular invertases from *E. nidulans* and *A. nidulans* was analyzed (**Table 4**). Among all salts used, the $MnCl_2$ promoted the highest activation for both enzymes from *E. nidulans* (+36%) and *A. nidulans* (+80%). The $Zn^{2+}$ and $K^+$ were also good stimulators of *A. nidulans* enzyme activity, while $Ca^{2+}$ and $Ba^{2+}$ were the best stimulators for *E. nidulans* enzyme. The enzyme activity was drastically inhibited by $Hg^{2+}$ and $Pb^{2+}$ considering the anamorph, while $NH^{4+}$ reduced drastically the enzyme activity from the teleomorph. The $MgCl_2$, $CuCl_2$, $NH_4Cl$ and $Ag_2SO_4$ reduced

**Table 4. Effect of different salts and compounds on the extracellular invertases activities from *A. nidulans* (anamorph) and *E. nidulans* (teleomorph).**

| Salts and compounds (1 mM) | Relative invertase activity (%) | |
|---|---|---|
| | Anamorph | Teleomorph |
| Without | 100 | 100 |
| $Ag_2SO_4$ | 64.8 ± 14.1 | 94.3 ± 4.2 |
| $AlCl_3$ | 99.7 ± 0.1 | 100.4 ± 20.8 |
| $BaCl_2$ | 106.7 ± 13.7 | 122.1 ± 24.4 |
| $CaCl_2$ | 108.0 ± 12.1 | 132.8 ± 17.6 |
| $CoCl_2$ | 110.0 ± 15.2 | 112.5 ± 21.1 |
| $CuCl_2$ | 89.4 ± 45.3 | 80.5 ± 23.7 |
| $FeCl_3$ | 106.5 ± 47.9 | 109.3 ± 20.2 |
| $HgCl_2$ | 2.3 ± 0.7 | 82.2 ± 1.2 |
| KCl | 128.5 ± 31.0 | 77.0 ± 13.2 |
| $MgCl_2$ | 92.2 ± 6.4 | 74.0 ± 13.5 |
| $MnCl_2$ | 180.0 ± 57.3 | 136.4 ± 16.4 |
| NaCl | 110.1 ± 33.7 | 109.6 ± 16.6 |
| $NH_4Cl$ | 85.0 ± 14.4 | 20.0 ± 1.1 |
| $Pb(C_2H_3O_3)_2$ | 23.5 ± 8.4 | 65.0 ± 9.0 |
| $ZnCl_2$ | 120.2 ± 29.7 | 102.3 ± 5.5 |
| $\beta$-mercaptoethanol | 107.0 ± 40.5 | 123.4 ± 24.5 |
| EDTA | 100.5 ± 53.6 | 111.9 ± 24.8 |

the activity of both enzymes. The $\beta$-mercaptoethanol promoted an activation of 6% - 23% in the enzyme activities. The invertases from both strains showed different behaviors in the presence of the salt solutions used, despite the activation by $Mn^{2+}$, but at a different proportion. The best enzyme activation by $Mn^{2+}$ was observed when we used 25 mM and 10 mM of $MnCl_2$ for invertases from *A. nidulans* and *E. nidulans*, respectively (data not shown). These data reinforce that the enzymes have different properties according to the period of life cycle. Activation of the enzyme activity by $Mn^{2+}$ was also observed for invertases from *Aspergillus niveus* [24], *Aspergillus phoe*nicis [26] and *Aspergillus ochraceus* [27]. Metallic ions can affect the aminoacid residues through the induction of charge modifications promoting an increase or decrease in the enzyme activity (sometimes due to structural distortion).

## 3.4. Hydrolysis of Substrates and Kinetic Parameters

The extracellular enzymes from anamorph and teleomorph fungi were able to catalyze the hydrolysis of all substrates analyzed and their combinations (**Table 5)**. The high hydrolytic activity using only one substrate for

**Table 5. Hydrolysis of different substrates by the extracellular invertases from *A. nidulans* (anamorph) and *E. nidulans* (teleomorph).**

| Substrates | Specific activities (U·mg$^{-1}$ of protein) | |
|---|---|---|
| | Anamorph | Teleomorph |
| Inulin | 14.5 ± 3.5 | 7.3 ± 3.6 |
| Raffinose | 138.8 ± 0.7 | 51.0 ± 8.9 |
| Sucrose | 106.7 ± 7.8 | 193.0 ± 12.3 |
| Inulin + sucrose | 443.5 ± 7.8 | 232.3 ± 5.7 |
| Raffinose + sucrose | 659.5 ± 9.9 | 256.6 ± 1.1 |
| Inulin + raffinose | 432.5 ± 2.1 | 51.2 ± 1.4 |

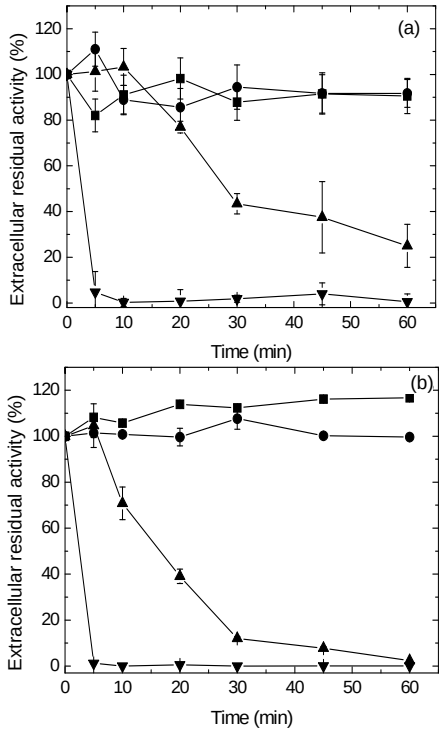



**Figure 4. Thermal stability at 40˚C (-■-), 50˚C (-●-), 60˚C (-▲-) and 70˚C (-▼-) for extracellular invertases produced by (a) *E. nidulans* and (b) *A. nidulans*.**

anamorph was observed with raffinose (138.8 U/mg of protein), and sucrose (193.0 U/mg of protein) for teleomorph. However, the activities obtained with raffinose plus sucrose were 4.7 and 1.3-fold higher than that reported above, respectively. Additionaly, the S/I value was 7.3 and 26.5 for extracellular enzymes from *A. nidulans* and *E. nidulans*, respectively. The S/I value is used as a parameter to help determine the real nature of the enzymes, *i.e.*, invertase or inulinase. Nevertheless, this parameter cannot be considered isolated and other aspects as kinetic parameters and structural characterization should be performed. One of the factors that can influence the S/I value is the inulin source, which can be

obtained from chicory and dahlia tubers among others.

The kinetic parameters for the partially purified extracellular enzymes from both *A. nidulans* (purification of 9.3-fold and yield of 10.9%) and *E. nidulans* (purification of 16.6-fold and yield of 32.1%) were determined using sucrose as substrate. In this case, the *A. nidulans* invertase showed higher affinity ($K_m$ = 2.0 mM) for the substrate than the *E. nidulans* invertase ($K_m$ = 4.8 mM) despite the $V_{max}$ value of 66.7 U/ mg of protein for the former and 117.6 U/mg of protein for the latter. Both enzymes showed best affinity for the substrate than the enzymes produced by *A. niveus* [24] and *A. phoenicis* [27]. The ratio $V_{max}/K_m$ values were 32.6 U/mg of protein $mM^{-1}$ and 24.5 U/mg of protein $mM^{-1}$ for extracellular enzymes from *A. nidulans* and *E. nidulans*, respectively, showing that the *A. nidulans* invertase is more efficient to hydrolyze the substrate sucrose.

## 4. Conclusion

The invertase production was distinct from anamorph (*A. nidulans*) and teleomorph (*E. nidulans*) stages of the filamentous fungus life cycle, indicating that this enzyme has different participation in the fungal metabolism of nutrient uptake. This fact is confirmed by some distinct properties of each enzyme such as substrate affinity and ion influence. Although the different properties of both enzymes showed biotechnological potential, future investigation to elucidate the true relation among invertase synthesis, metabolism and life cycle of filamentous fungi is a challenge that only has begun.

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