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# Purification, Characterization and Antifungal Activity of the *Aspergillus niveus* Chitinase Produced Using Shrimp Shells

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**Abstract:** Chitinases are biotechnologically relevant enzymes that can be applied in such different sectors as pharmaceutical, food, environmental management, the biocontrol of pests and in the paper and cellulose industry. Microorganisms as filamentous fungi are the most important source of these biomolecules. The fungus *Aspergillus niveus* produces extracellular chitinase when cultured under submerged fermentation using shrimp shells, a residue generated by the fish industry, as a carbon source, for 96 h at 30 °C and 100 rpm. The particle size and concentration of the shrimp shells affected enzyme production. The chitinase was purified until electrophoretic homogeneity through the use of a Sephadex G-100 chromatographic column. It is a monomeric glycoprotein with a molecular mass of 47 kDa estimated using SDS-PAGE and 49.3 kDa determined using gel filtration. The carbohydrate content was 22.8%. The best temperature and pH for enzyme activity were 65 °C and 6.0, respectively. Approximately 80% of the enzymatic activity was preserved at pH 4.0 and 5.0 for 48 h, and the half-life ($t_{50}$) was maintained for 48 h at 40 °C. Salts, EDTA and β-mercaptoethanol did not affect chitinase activity significantly, but organic solvents reduced it. The kinetic parameters determined using p-NPGlycNac were $K_m$ of 2.67 mmol $L^{-1}$, $V_{max}$ of 12.58 U mg of protein$^{-1}$, Kcat of 2.47 $s^{-1}$ and K cat/$K_m$ of 0.93 $s^{-1}$ mmol $L^{-1}$. The *A. niveus* chitinase inhibited the growth of all fungal strains used, especially *Trichoderma harzianum* (MIC = 22.4 μg $mL^{-1}$) and *Penicillium purpurogenum* (MIC = 11.2 μg $mL^{-1}$). The chitinase produced by *A. niveus* presented interesting characteristics that indicate its potential of application in different areas.

**Keywords:** antifungal activity; *Aspergillus*; chitin; fungal fermentation



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## 1. Introduction

Chitin is the second most abundant polysaccharide on the planet and it can be found in different organisms such as insects, crustaceans and fungi with structural roles [1]. Structurally, it is constituted by N-acetylglucosamine units linked through β-1,4 glycosidic linkages with a OH reducing end at C1 and a non-reducing end at C4. The polymeric chains of chitin are organized as sheets grouped in parallel and/or anti parallel mode, giving different polymorphic structures named α, β and γ chitin [2]. Chitin is a biodegradable polymer that can be applied in several sectors such as textiles, cosmetics, paper and agriculture, among others [3]. Its polymeric structure can be enzymatically hydrolyzed by the action of chitinases to recover monomeric units, allowing for better collection of residues containing chitin, such as those produced by the fishing industry, such as shrimp, and minimizing the negative impact of residue accumulation in the environment.

Chitinases, enzymes that are biotechnologically relevant, are classified as glucosyl hydrolases (GH 18, 19 and 20 families) which act on the β-1,4 linkages of the N-acetylglucosamine units of chitin. In general, according to the pattern of cleavage of the chitin polymer, these enzymes can be divided into endo-chitinases (EC 3.2.1.14) and

exochitinases. The former randomly breaks down the β-1,4 linkage of chitin and chitodextrins to release N-acetylglucosamine and low molecular mass oligomers of β-1,4 N-acetylglucosamine such as chitobiose, chitotriose and chito-oligosaccharides (CHOS). The exochitinases are subdivided into two categories, the chitobiases (EC 3.2.1.29), promoting diacetylchitobiose from the non-reducing end from the chitin microfibrils, and β(1,4)N-acetylglucosaminidase (EC 3.2.1.30), acting on oligomeric products obtained by the action of the endochitinases and chitobiases, providing monomers of N-acetylglucosamine [3,4].

These enzymes can be found in organisms from different kingdoms with diverse roles. Among the microorganisms, the presence of chitinases is well documented in bacteria and fungi [5]. The fungal chitinases belong to the GH 18 family and present different physiological roles such as fungal growth, cell wall remodeling, mycoparasitism, autolysis and the use of chitin for nutritional and energy demands [3]. The fungal chitinases can be separated into three subgroups depending on the cleavage pattern (exo-/endo-), substrate-binding cleft and the presence of carbohydrate-binding modules (CBMs). The enzymes allocated to subgroup A present exo-activity, a molecular mass varying from 40 to 60 kDa, one catalytic domain and the absence of CBMs. They can be intra- or extracellular enzymes. Subgroup B comprises chitinases with a molecular mass from 30 to 90 kDa and with an endo-activity pattern and the presence of the CBM at the C-terminal end. Differing from the previous subgroups, chitinases belong to subgroup C and are recognized as larger proteins with a molecular mass from 120 to 200 kDa and the presence of various CBMs. The similarity of the substrate-binding site to that of subgroup A suggests an exo-activity pattern [3]. The potential of different fungal strains to produce chitinases using both submerged (SbmF) and solid-state fermentation (SSF) has been explored as observed for *Trichoderma asperellum* PQ34 [1] and *Humicola grisea* [6] using SbmF and *Penicillium ochrochloron* MTCC 517 [7] and *Fusarium oxysporum* CFR 8 [8] using SSF.

Biotechnologically, chitinases can be applied in several sectors such as the food and pharmaceutical industries, medicine, agriculture and environmental management [9,10]. Nowadays, environmental quality is an important aspect considered for the preservation of biodiversity and the life quality of humans. In this context, the treatment of residues containing chitin such as shrimp, lobster and crab shells allows for the obtainment of products with added value such as CHOS and minimizes the impact on the natural environment [10]. Considering all of the positive aspects of the use of enzyme technology for the hydrolysis of chitin-rich residues as well as the potential bioactivity of chitinases, our aim was to investigate the production of extracellular chitinase by *Apergillus niveus* under SbmF using shrimp shells as a carbon source and then purifying and characterizing it. Antifungal potential was also determined.

## 2. Materials and Methods

### *2.1. Microorganism and Culture Conditions*

The filamentous fungus *Aspergillus niveus* LH0306 was deposited in the culture collection of the Laboratory of Biotechnology and Enzymology of Filamentous Fungi (LaBEFF) of the Faculty of Philosophy, Sciences and Letters of Ribeirão Preto. It was maintained in PDA (potato dextrose agar) slants previously autoclaved at 120 °C for 30 min and stored at 4 °C in a refrigerator until use. For the obtainment of the spore suspension, the fungal growth was scraped and distilled water was added.

For enzyme production, the fungus was cultured in minimal medium (MM) [11] using 1% (*m*/*v*) shrimp residues (shells and heads) of *Xiphopenaeus kroyeri* (seven-beard shrimp) and *Farfantepenaeus brasiliensis* (pink shrimp) and silkworm chrysalis, separately as carbon sources, with an initial pH of 6.0, for different periods (24–144 h) as determined for each experiment, at 30 °C under orbital agitation (100 rpm). The media were previously sterilized at 120 °C for 30 min, cooled at room temperature and inoculated with 1 mL spore suspension ($10^6$ spores/mL). The shrimp residues were washed extensively with tap water, dried at 50 °C for 48 h, manually crushed and separated using 1, 2 and 10 $mm^2$ sieves.

### 2.2. Obtainment of the Enzymatic Extract

After cultivation, the cultures were harvested under vacuum using filter paper No. 1 in a Buchner funnel. The free cell filtrate obtained was dialyzed against distilled water for 24 h at 4 °C. The filtrate was used for the assessment of extracellular chitinase activity and the purification procedure. The mycelium was disrupted in a mortar using sea sand previously treated with nitric acid, suspended with sodium acetate buffer (100 mmol $L^{-1}$, pH 5.0) and centrifuged at 10,000× *g* for 10 min at 4 °C. The supernatant was used for the determination of intracellular chitinase activity, and the residues were discarded.

### 2.3. Assessment of Chitinase Activity

Chitinase activity was determined using 4-nitrophenil-β-D-N-acetylglucosaminide (pNP-GlcNAc) (Sigma-Aldrich, St. Louis, MO, USA) as synthetic substrate using McIlvaine buffer pH 5.6. The mixture of the reaction was constituted by 200 µL of the substrate solution and 200 µL of the enzymatic sample. The reaction was performed for different periods (5 and 10 min), under different temperatures (30–80 °C) and stopped by the addition of 1 mL of saturated solution of sodium borate. The phenolate obtained was monitored at 405 nm. One unit of enzyme activity was defined as the amount of enzyme necessary to hydrolyze 1 µmol of substrate per minute under the assay conditions.

### 2.4. Quantification of the Protein and Carbohydrates

The protein concentration was estimated according to Bradford (1976) [12], using Coomassie Blue G-250 and expressed as mg per mL of sample. Bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) was used as standard, and absorbance was determined at 595 m.

The total carbohydrate content was estimated using phenol–sulfuric methodology [13] and expressed as mg of carbohydrate per ml of sample. Mannose (Merck, Darmstadt, Germany) was used as standard.

### 2.5. Purification

The free cell filtrate was lyophilized, suspended in 20 mmol $L^{-1}$ McIlvaine buffer, pH 5.6, 100 mmol $L^{-1}$ KCl was added and it was loaded in a Sephadex G-10 chromatographic column (1 × 60 cm) previously equilibrated using the buffer mentioned. Fractions of 1 mL were collected at a flow rate of 150 µL $min^{-1}$ and used for protein monitoring at 280 nm and enzymatic assay. Fractions with chitinase activity were pooled, dialyzed against distilled water for 24 h at 4 °C and used for enzyme characterization. The molecular mass markers (Sigma-Aldrich, St. Louis, MO, USA) were β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa) and carbonic anhidrase (29 kDa). The void ($V_0$) was 42 mL as determined using Blue Dextran 2000.

### 2.6. Electrophoresis

The crude filtrate and purified fraction were submitted to 10% SDS-PAGE [14]. The run was performed for 90 min using the energy source PowerPac (Bio-Rad Laboratories, Inc., Hercules, CA, USA) adjusted to 100 V and 40 mA. After the run, the gel was stained using Coomassie blue silver G-250 [15]. Precision Plus Protein Kaleidoscope Standards (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were used as the molecular mass marker.

### 2.7. Influence of Temperature and pH on Chitinase Activity

The thermal stability of the *A. niveus* chitinase was assayed, and the enzymatic samples were maintained in aqueous solution for different periods (5–280 min) at 40, 50 and 60 °C. For each period, samples were taken, kept in an ice bath and used afterward for the assessment of enzyme activity.

For pH stability, the enzyme was maintained in 100 mmol $L^{-1}$ McIlvaine buffer with a pH of 3.0 to 8.0 for different periods (0.5–24 h). For each period, samples were taken and assayed for chitinase activity.

### *2.8. Influence of Different Compounds on Chitinase Activity*

The effect of different salts (1 mol $L^{-1}$: $AgNO_3$, $BaCl_2$, $CaCl_2$, $CoCl_2$, $CuCl_2$, $CuSO_4$, $FeCl_3$, KCl, $KH_2PO_4$, $MgCl_2$, $MgSO_4$, $MnCl_2$, NaBr, NaCl, $NaNO_3$, $NH_4Cl$, $NH_4NO_3$, $Zn(NO_3)_2$ and $ZnSO_4$), 1 mmol $L^{-1}$ of β-mecaptoethanol, SDS and EDTA, 0.05% Triton X-100 and Tween-20 and 1% (*v*/*v*) acetone, acetonitrile, ethanol, isopropanol, methanol and butanol on chitinase activity was analyzed. The results are expressed as relative activity (%).

### *2.9. Assessment of Kinetic Parameters*

The kinetic parameters Michaelis–Menten constant ($K_M$), maximal velocity ($V_{max}$), turnover number ($K_{cat}$) and catalytic efficiency ($K_{cat}/K_M$) were assessed using 4-nitrophenil-β-D-N- acetylglucosaminide (pNP-GlcNAc) (Sigma-Aldrich, St. Louis, MO, USA) as substrate. The $K_M$ and $V_{max}$ values were calculated using SigrafW 2.2 software [16].

### *2.10. Evaluation of Antifungal Activity*

The antifungal potential of the chitinase was evaluated considering different fungal strains (*Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus phoenicis*, *Rhizopus microsporus var. microsporus*, *Cladosporium herbarium*, *Paecilomyces variotii*, *Fusarium verticillioides*, *Fusarium lateritium*, *Penicillium purpurogenum* and *Trichoderma harzianum*) as described by Mania et al. (2010) [17], with modification. All fungal strains were deposited in the culture collection of the Laboratory of Biotechnology and Enzymology of Filamentous Fungi (LaBEFF) of the Faculty of Philosophy, Sciences and Letters of Ribeirão Preto. Briefly, a 96-well plate was used, and to each well, 100 µL of MM (Hill and Kapper, 2001) [11] supplemented with 1% (*m*/*v*) dextrose was added, followed by the addition of 100 µL of purified chitinase considering different protein concentrations (0.7, 1.4, 2.8, 5.6, 11.2, 22.4, 44.7 and 89.4 µg $mL^{-1}$) or for each antifungal agent (amphotericin B—(Merck, Darmstadt, Germany; and fluconazole—Sigma-Aldrich, St. Louis, MO, USA) as positive controls (0.6, 1.3, 2.5, 5, 10, 20, 40 and 80 µg $mL^{-1}$). As a negative control, only the medium without spore inoculation was used. Thereafter, the $10^4$ spores of each fungus were inoculated, separately, in the wells, and the plate was maintained at 30 °C for 120 h. After incubation, resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide) (Sigma-Aldrich, St. Louis, MO, USA) at 100 µmol $L^{-1}$ was added to each well, and they were maintained for 2 h in dark conditions and monitored at 490 nm. The minimal inhibitory concentration (MIC) was defined as the concentration of the antifungal agent able to impair the conversion of resazurin to resorufin.

## 3. Results

### *3.1. Enzyme Production*

The filamentous fungus *A. niveus* produced both extracellular and intracellular chitinase in the presence of all carbon sources analyzed (Figure 1). Considering the extracellular form, the best production was obtained in the presence of residues of seven-beard shrimp (1:1 *m*/*m* of shells and heads) for 96 h of cultivation (Figure 1A), while for the intracellular form, cultivation for 96 h with powder silkworm chrysalis allowed for the best production (Figure 1B). Considering the extracellular form, enzyme production using seven-beard shrimp overtook the production with pink shrimp from 48 to 96 h.

The influence of the use of the shells and heads of seven-beard shrimp, separately, and their respective particle size on enzyme production by *A. niveus* was also verified (Figure 2). The best enzyme production was achieved using shrimp shells with particle sizes between 1 and 2 $mm^2$. For the particle sizes smaller than 1 $mm^2$ and for those greater than 2 $mm^2$, enzyme production in the presence of heads was similar but reduced considering the use of the shells (Figure 2A). The quantity of shrimp shells used as a carbon source also impacted enzyme production (Figure 2B). The best enzymatic level was obtained using 1% (*m*/*v*) of the carbon source, while major quantities reduced it.

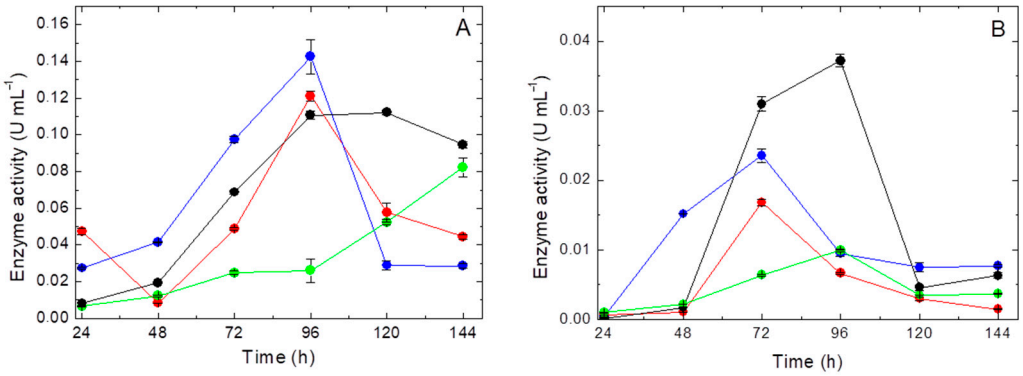


**Figure 1.** Production of extracellular (**A**) and intracellular (**B**) chitinases by the fungus *A. niveus* in SbmF using pink shrimp (●), seven-beard shrimp (●), crushed chrysalis (●) and powder chrysalis (●) according to the cultivation period.

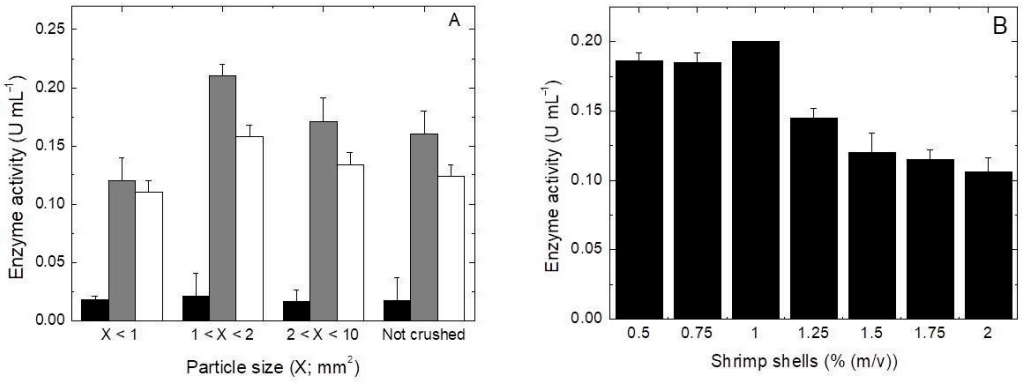


**Figure 2.** Influence of the particle size (**A**) of the shrimp heads (■), shells (■) and heads + shells (□) and the shrimp shell quantity (**B**) added to the culture medium on the production of extracellular chitinase by *A. niveus*.

### *3.2. Purification and Molecular Mass Estimation*

Figure 3 illustrates the chromatographic profile for the enzymatic extract loaded in the Sephadex G-100 column. Only a single peak of chitinase activity was eluted, allowing for a 40-fold purification factor and recuperation of 14% (Table 1). The fraction purity was confirmed by the SDS-PAGE analysis (Figure 4), presenting a single protein band with 47 kDa, near the native molecular mass of 49.3 kDa estimated using gel filtration, indicating a monomeric form of the *A. niveus* chitinase.

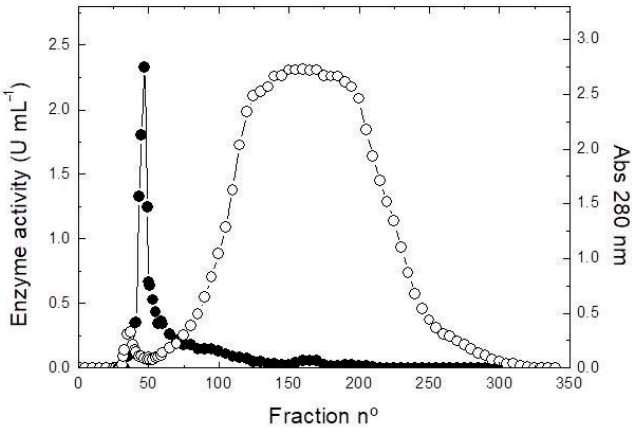


**Figure 3.** Sephadex G-100 chromatographic profile for the elution of the extracellular chitinase produced by *A. niveus*. Symbols: (●) chitinase activity; (○) absorbance 280 nm.

**Table 1.** Purification of the extracellular chitinase produced by *A. niveus* cultured in SbmF containing shrimp shells as a carbon source.

| Step | Volume (mL) | Activity (Total U) | Protein (Total mg) | Specific Activity (U/mg prot) | Yield (%) | Purification Factor (Fold) |
|---|---|---|---|---|---|---|
| Crude filtrate | 100 | 140 ± 25 | 128.1 ± 10 | 1.1 ± 0.3 | 100 | 1 |
| Sephadex G-100 | 13 | 20.2 ± 3.4 | 0.45 ± 0.05 | 44.3 ± 2.9 | 14.3 ± 0.1 | 40.3 ± 5.9 |

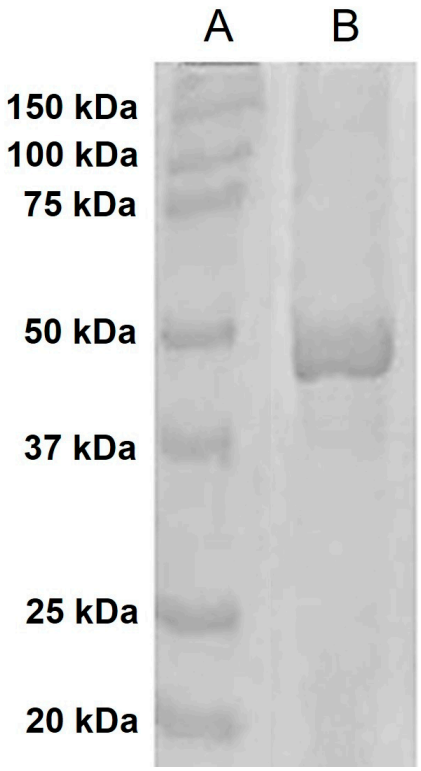


**Figure 4.** The 10% SDS-PAGE profile for the purified fraction containing chitinase (**B**) after Sephadex G-100 chromatography: (**A**) molecular mass markers.

### *3.3. Influence of Temperature and pH on Enzyme Activity*

The influence of temperature and pH on chitinase activity is presented in Figure 5. As can be observed, high enzymatic activity was obtained at 65 °C (Figure 5A) and pH 6.0 using McIlvaine buffer. The buffer's composition also influenced the enzymic activity (Figure 5B). The half-life of the *A. niveus* enzyme was longer than 48 h when maintained at 30 °C. At 40 °C, the $t_{50}$ was 24 h, and at 50 °C, enzyme activity was drastically reduced (Figure 5C). Considering pH stability, the *A. niveus* chitinase maintained approximately 70% of its initial activity when incubated at pH 4.0 and 5.0 for 48 h. At pH 3.0 and 6.0, the $t_{50}$ was 12 h and 4 h, respectively. Incubation of the chitinase at pH 7.0 and 8.0 reduced its activity drastically (Figure 5D).

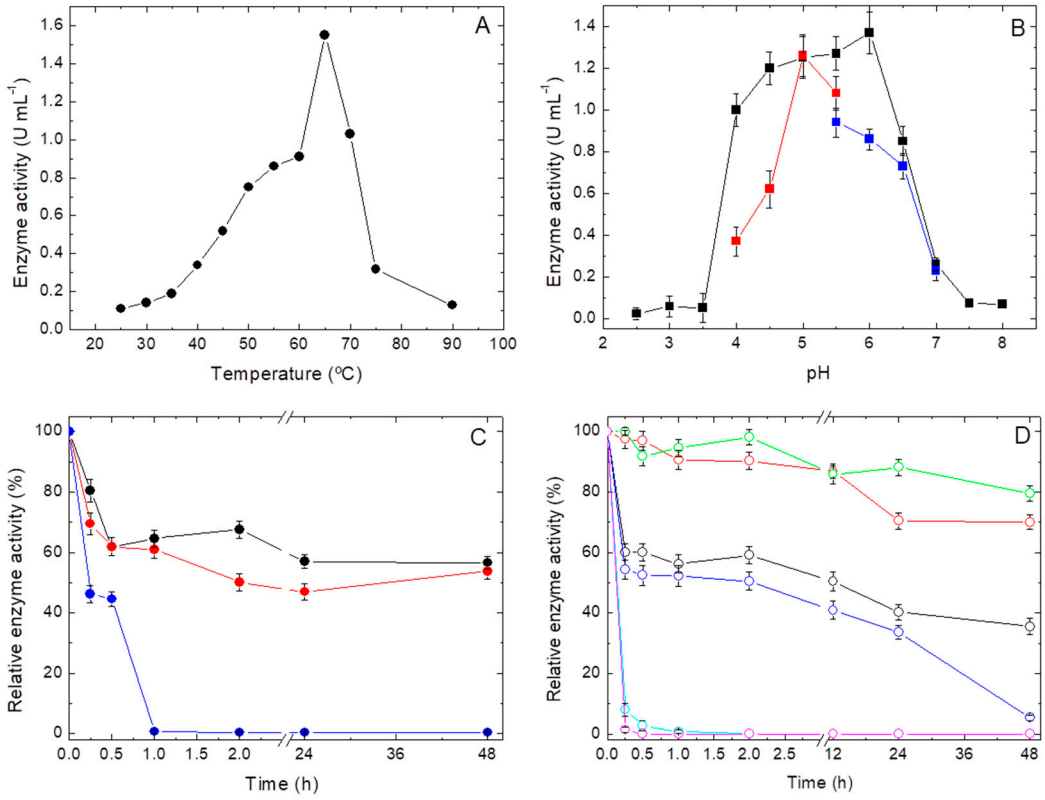


**Figure 5.** Influence of temperature (**A**) and pH (**B**) (symbols: ■—McIlvaine; ■—sodium acetate; ■—phosphate buffers), thermostability (**C**) at 30 °C (●), 40 °C (●) and 50 °C (●) and stability to pH (**D**) for the purified chitinase from *A. niveus*. For pH stability: pH 3.0 (○), 4.0 (○), 5.0 (○), 6.0 (○), 7.0 (○) and 8.0 (○). The 100% activity corresponds to 1.7 U mL$^{-1}$.

### *3.4. Influence of Different Compounds on Enzyme Activity*

Enzyme activity can be affected by other factors such as the presence of salts, detergents and solvents, among others. Interestingly, the *A. niveus* chitinase was not affected drastically by the salts added to the reaction mixture (Table 2). No activation of enzyme activity was observed. However, the addition of organic solvents promoted the reduction in enzymatic activity. The use of 0.05% of the detergents Tween-20 and SDS did not significantly affect enzyme activity. EDTA and β-mercaptoethanol minimally reduced enzyme activity.

**Table 2.** Influence of different compounds on enzyme activity.

| Compounds | Relative Activity (%) | Compounds | Relative Activity (%) |
|---|---|---|---|
| Salts (1 mmol L$^{-1}$) | | $NH_4Cl$ | 72.1 ± 0.3 |
| $AgNO_3$ | 74.0 ± 0.1 | $NH_4NO_3$ | 77.5 ± 0.4 |
| $BaCl_2$ | 78.6 ± 0.2 | $Zn(NO_3)_2$ | 73.6 ± 0.3 |
| $CaCl_2$ | 68.4 ± 0.1 | $ZnSO_4$ | 88.7 ± 0.2 |
| $CoCl_2$ | 86.1 ± 0.6 | Solvents (1%, *v*/*v*) | |
| $CuCl_2$ | 73.6 ± 0.1 | Acetone | 38.3 ± 0.1 |
| $CuSO_4$ | 86.0 ± 0.3 | Acetonitrile | 38.9 ± 0.1 |
| $FeCl_3$ | 74.5 ± 0.2 | Butanol | 29.2 ± 0.1 |
| $FeSO_4$ | 86.9 ± 0.5 | Ethanol | 35.9 ± 0.2 |
| KCl | 98.0 ± 0.5 | Isopropanol | 32.2 ± 0.2 |
| $KH_2PO_4$ | 75.7 ± 0.3 | Methanol | 35.3 ± 0.4 |

**Table 2.** *Cont.*

| Compounds | Relative Activity (%) | Compounds | Relative Activity (%) |
|---|---|---|---|
| $MgCl_2$ | 79.3 ± 0.3 | Detergents (0.05%, *v*/*v*) | |
| $MgSO_4$ | 85.6 ± 0.1 | Tween-20 | 96.1 ± 0.2 |
| $MnCl_2$ | 80.1 ± 0.2 | Triton X-100 | 74.4 ± 0.1 |
| $MnSO_4$ | 86.0 ± 0.4 | SDS (5 mmol $L^{-1}$) | 93.3 ± 0.2 |
| NaBr | 82.9 ± 0.3 | Others (5 mmol $L^{-1}$) | |
| NaCl | 85.0 ± 0.3 | EDTA | 88.7 ± 0.3 |
| $NaNO_3$ | 82.9 ± 0.3 | β-mercaptoethanol | 92.3 ± 0.2 |

The 100% relative activity corresponds to the assay without compounds in the reaction mixture.

### *3.5. Kinetic Parameters*

The kinetic parameters $K_m$, $V_{max}$ and Kcat were estimated as 2.67 mmol $L^{-1}$, 12.58 U mg prot.$^{-1}$ and 2.47 $s^{-1}$, respectively, using 4-nitrophenil-N-acetil-β-D-glucosaminide as substrate. Catalytic efficiency (Kcat/$K_m$) was calculated as 0.93 $s^{-1}$ mmol $L^{-1}$.

### *3.6. Antifungal Activity*

The chitinase produced by *A. niveus* inhibited the growth of different fungal strains, especially *Penicillium purpurogenum* with a MIC value of 11.2 μg $mL^{-1}$, while the values for amphotericin and fluconazole were 40 μg $mL^{-1}$ (Table 3). The MIC value obtained considering *Trichoderma harzianum* was 22.4 μg $mL^{-1}$, which is lower than the value observed for the use of fluconazole but higher than that obtained with amphotericin B. For the other fungal strains, the MIC values were higher when compared to the antifungal agents used as controls.

**Table 3.** Minimal inhibitory concentration (MIC) for the use of purified chitinase from *A. niveus* considering different fungal strains.

| Fungal Strains | MIC (μg $mL^{-1}$) | | |
|---|---|---|---|
| | Chitinase | Amphotericin | Fluconazole |
| *Aspergillus flavus* | 44.7 | 5 | 20 |
| *Aspergillus fumigatus* | 89.4 | 10 | 20 |
| *Aspergillus niger* | 5.6 | 0.6 | 1.3 |
| *Aspergillus phoenicis* | 89.4 | 10 | 40 |
| *C. herbarum* | 11.2 | 10 | 20 |
| *Fusarium lateritium* | 22.4 | 10 | 20 |
| *Fusarium verticillioides* | 11.2 | 1.3 | 2.5 |
| *Paecilomyces variotii* | 89.4 | 5 | 5 |
| *Penicillium purpurogenum* | 11.2 | 40 | 40 |
| *Trichoderma harzianum* | 22.4 | 10 | 40 |

Fixed concentrations: purified chitinase (0.7, 1.4, 2.8, 5.6, 11.2, 22.4, 44.7 and 89.4 μg $mL^{-1}$); amphotericin B and fluconazole (0.6, 1.3, 2.5, 5, 10, 20, 40 and 80 μg $mL^{-1}$).

## 4. Discussion

Chitinases are enzymes with biotechnological potential that can be obtained from different microbial sources, including filamentous fungi. In general, these enzymes can be applied in chito-oligosaccharide production, for waste management, as biocontrol agents and for medical applications, among others [18]. The identification of fungal producers of chitinases and enzyme characterization are important steps to finding biomolecules with attractive properties for biotechnological application. Differing from Alves et al. (2018) [19], who reported that the production of chitinase by *A. niveus* through the use of pure crab chitin, the use of fish residue, such as shrimp shells and heads, is described in the present article. The best collection of different types of residues in order to obtain products with high added value, such as enzymes, is a global demand, contributing to reducing the environmental impact resulting from their inadequate disposal.

In this context, the production of chitinases by different strains of filamentous fungi has been reported using both submerged (SbmF) and solid-state (SSF) fermentation in the presence of carbon sources/substrates containing chitin [7,19,20]. The filamentous fungus *A. niveus* produced both extracellular and intracellular chitinase in the presence of chitin-rich residues obtained from the fishing industry and silkworm chrysalis as carbon sources. For the extracellular form, the best production was obtained in the presence of residues of seven-beard shrimp (1:1 *m*/*m* of shells and heads), while for the intracellular form, the powder silkworm chrysalis was better. The different results observed for enzyme production using seven-beard shrimp and pink shrimp can be explained by the differences in shell composition, since it can vary according to the shrimp species, season and location of the animals [21], affecting the nutritional properties of the culture medium and, consequently, fungal development and enzyme production. Additionally, the lowest enzyme production observed using shrimp heads is explained by the presence of other organic constituents that have not been removed during the manual cleaning of this substrate. The fungus preferentially uses these organic components that are more easily accessible than chitin.

The period of cultivation was the same for both enzymatic forms. The enzymatic level of the extracellular chitinase was four-fold higher than that observed for the intracellular enzyme. Both shrimp and chrysalis present at least 20% of their dried mass as being constituted by chitin [22,23], representing important carbon sources for the induction of chitinase production. Residues containing chitin have been used for the production of chitinases by fungi as demonstrated for the *A. niger* LOCK62 [20] and *A. terreus* [24]. The production of chitinase in the presence of crab chitin by *A. niveus* was previously reported by Alves et al. (2018) [19]. Colloidal chitin was used for enzyme production by the fungi *Trichoderma viride* AUMC 13021 [25] and *A. flavus* AUMC 13576 [26]. The collection of residue produced by the fishing industry for enzyme production by fungi, as presented in this article, allows for a reduction in the environmental impact generated by this type of activity.

Considering the influence of the use of the shells and heads of seven-beard shrimp, separately, and their respective particle size on enzyme production by *A. niveus*, the maximal enzyme production was achieved using shrimp shells with particle sizes between 1 and 2 $mm^2$, 10-fold higher than that obtained with shrimp heads and overcoming the value observed for the use of the mixture (shells + heads). When using particle sizes smaller than 1 $mm^2$ and greater than 2 $mm^2$, enzyme production was reduced. Substrates with high superficial area, as obtained with small particles, favor both fungal growth and enzymatic action. However, for substrates with very small particle sizes, such as those smaller than 1 $mm^2$, compaction hinders enzymatic access [27]. The quantity of shrimp shells used as a carbon source also impacted enzyme production. The best enzymatic level was obtained using 1% (*m*/*v*) of the carbon source, while major quantities reduced it. A reduction in the carbon source concentration can negatively impact enzyme production through a reduction in microbial growth. On the other hand, excess carbon sources can promote microbial stress because of the amount of nutrients, such as proteins and minerals, that adhere to the shrimp shells [27,28], impacting enzyme production negatively. Hassan and Ismail also used 1% (*m*/*v*) powder shrimp shells for the production of chitinase by *Penicillium* sp. SSW2, while Atalla et al. (2019) [29] used 1% shrimp shells supplemented with dextrose for enzyme production by *Penicillium chrisogenum* MH745129.

The purified extracellular chitinase from *A. niveus* was characterized as a monomeric glycoprotein and its molecular mass was in agreement with other fungal chitinases. The *Eremothecium ashbyii* [30] and *Rhizomucor mihei* [31] chitinases were also described as monomers. Fungal chitinases belong to the GH18 family, with them showing a molecular mass from 30 to 200 kDa according to the microbial source [3]. For example, the molecular mass observed for the enzyme produced by *Aspergillus terreus* was estimated as 60 kDa [24], while the chitinases from different *B. bassiana* strains presented molecular masses varying from 34 to 49 kDa [32]. According to Menghiu et al. (2019) [33], the glycosylation of

chitinases can stabilize the enzyme and also improve recognition, specificity and affinity with the substrate.

The analysis of the influence of temperature and pH on chitinase activity showed that the best conditions were achieved at 65 °C and pH 6.0. Considering the temperature of the activity, the value found was higher than that observed for the chitinases produced by *Penicillium chrysogenum* [29], *Aspergillus terreus* [24] and *Aspergillus flavus* AUMC 13576 [26], with the best enzymatic activity at 40, 50 and 60 °C, respectively. The temperature increment impacts the reaction rate, with it overcoming the reaction activation energy until the point of thermal stability. At high temperatures, different modifications (rapid and slow) occur in the enzyme conformation, impacting positively or negatively on its activity [34]. The half-life of the *A. niveus* enzyme was higher than 48 h when maintained at 30 °C. The stability observed at 30 °C is an important characteristic when aiming for the application of chitinase as a fungicide and bio-insecticide in plantations. For example, the temperature in sugar cane cultures is approximately 29 °C in the state of São Paulo, Brazil [35].

The best chitinolytic activity for the enzymes produced by *A. terreus*, *Trichoderma viride* AUMC 13021 and *Thermothelomyces heterothalicus* PA2S4T was found at pH 5.6, 6.5 and 4.5, respectively [24,25,36]. Considering pH stability, the *A. niveus* chitinase remained more stable than the other fungal chitinases such as that produced by the *A. terreus* enzyme, which kept 80% of its initial activity when maintained at a pH range from 5.0 to 8.0 for 1 h [24]. Pommer et al. (2021) [36] reported that the *T. heterothallicus* Pa2S4T chitinase was stable from pH 4.0 to 5.0.

Enzyme activity can be affected by other factors such as the presence of salts, detergents and solvents, among others. Interestingly, the *A. niveus* chitinase was not affected drastically by the salts added to the reaction mixture. No activation of enzyme activity was observed, differing from the results for the *Trichoderma viride* AUMC 13021 chitinase, which had its activity increased in the presence of $Ca^{2+}$ and $Mn^{2+}$ [25], and the *A. flavus* AUMC 13576 enzyme, activated by $Mn^{2+}$ and $Fe^{2+}$ [26]. On the other hand, the chitinolytic activity of the enzyme produced by *A. niveus* was inhibited mainly in the presence of $Ca^{2+}$ (−30%). Ions can interact with amino acid residues, modifying the global charge of the protein and also inducing conformational changes and reducing or increasing the catalytic activity. The addition of the organic solvents promoted the reduction in enzymatic activity, as also observed for the chitinase from *A. terreus*, inhibited in the presence of acetone [24]. These solvents can interact with the apolar groups of the proteins, reducing the number of hydrophobic interactions and, consequently, affecting their conformation and promoting denaturation [37]. The use of 0.05% of the detergents Tween-20 and SDS did not significantly affect enzyme activity, differing from the result obtained in the presence of Triton X-100, which promoted a reduction of 26% in chitinase activity. EDTA and β-mercaptoethanol minimally reduced enzyme activity.

The kinetic parameters $K_m$, $V_{max}$ and Kcat were estimated as 2.67 mmol $L^{-1}$, 12.58 U mg prot.$^{-1}$ and 2.47 $s^{-1}$, respectively, using 4-nitrophenil-N-acetil-B-D-glucosaminide as substrate. Catalytic efficiency (Kcat/$K_m$) was calculated as 0.93 $s^{-1}$ mmol $L^{-1}$. Different substrates were used for the determination of these parameters. The $K_m$ and $V_{max}$ values for the chitinase from *T. viride* AUMC 12021 were 6.66 mg $mL^{-1}$ and 90.8 U $mL^{-1}$ using colloidal chitin as substrate [25].

The chitinase produced by *A. niveus* inhibited the growth of different fungal strains, especially *Penicillium purpurogenum*. The MIC value was lower than that observed for amphotericin and fluconazole. The MIC value obtained considering *Trichoderma harzianum* was lower than the value observed for the use of fluconazole but higher than that obtained with amphotericin B. For the other fungal strains, the MIC values were higher when compared to the antifungal agents used as controls. The differences in the level of growth inhibition among the fungal strains used can be explained, probably, by the different chitin content of the cell wall of each fungus. Chitin is the main component of fungal cell walls, providing resistance and mechanical stability [38], and it can be hydrolyzed by chitinase. The fungal cell wall is not only constituted by chitin but also other components such as

glucans, proteins and other polysaccharides [39]. This composition varies according to species, hindering access of the enzyme to chitin, which can explain the different levels of fungal development. The action mechanisms of amphotericin and fluconazole differ from chitinase. The former acts on the ergosterol present in the cell membrane, modifying its permeability, and the latter inhibits ergosterol synthesis, affecting cell membrane structure [40]. The ergosterol content in the membrane is also different for each fungal strain [38]. The antifungal properties of other fungal chitinases have been reported. For example, the antifungal activity of the *A. niveus* chitinase on different fungal strains was reported by Alves et al. (2018) [19]. However, in the present study, antifungal activity was evaluated on some species different from those reported by Alves et al. (2018) [19], such as *C. herbarium*, *P. pupurogenum*, *R. microsporus*, *F. lateritium*, *F. verticillioides* and *T. harzianum*. The *Trichoderma* chitinase was able to act against *Fusarium* sp., *Aspergillus* sp. and *Rhizoctonia* sp. [41]. *Trichoderma asperellum* PQ34 inhibited the growth of the *Colletotrichum* sp. and *Sclerotium rolfsii* [1]. Chitinases can be used as biopesticides against fungal strains that attack different plantations of economic importance and also in combination with antifungal drugs to treat different fungal infections [18].

## 5. Conclusions

In conclusion, the filamentous fungus *A. niveus* produced an extracellular chitinase, using fishing residue like shrimp shells as a carbon source. The potential of this type of low-cost residue to obtain products with greater added value has been seldom explored, with it being an alternative that can reduce production costs, resulting in more accessible value for the consumer. Also, it contributes to the reduction in environmental impact and cost reduction for the recovery of degraded environments. Additionally, the enzyme presented interesting biochemical properties such as temperature and pH activity, thermal and pH stability and tolerance to the presence of salts, highlighting its biotechnological potential of application in different areas such as an antifungal agent to inhibit the fungal growth of phytopathogen species.

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