Brazilian Journal of Microbiology (2024) 55:2267–2277
https://doi.org/10.1007/s42770-024-01432-9

BIOTECHNOLOGY AND INDUSTRY - RESEARCH PAPER

Fungal endo and exochitinase production, characterization, and application for *Candida* biofilm removal

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Received: 9 February 2024 / Accepted: 18 June 2024 / Published online: 1 July 2024
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Abstract

Chitinases are promising enzymes for a multitude of applications, including chitooligosaccharide (COS) synthesis for food and pharmaceutical uses and marine waste management. Owing to fungal diversity, fungal chitinases may offer alternatives for chitin degradation and industrial applications. The rapid reproduction cycle, inexpensive growth media, and ease of handling of fungi may also contribute to reducing enzyme production costs. Thus, this study aimed to identify fungal species with chitinolytic potential and optimize chitinase production by submerged culture and enzyme characterization using shrimp chitin. Three fungal species, *Coriolopsis byrsina*, *Trichoderma reesei*, and *Trichoderma harzianum*, were selected for chitinase production. The highest endochitinase production was achieved in *C. byrsina* after 168 h cultivation (0.3 U mL^{-1}). The optimal temperature for enzyme activity was similar for the three fungal species (up to 45 and 55 ºC for endochitinases and exochitinases, respectively). The effect of pH on activity indicated maximum hydrolysis in acidic pH (4–7). In addition, the crude *T. reesei* extract showed promising properties for removing *Candida albicans* biofilms. This study showed the possibility of using shrimp chitin to induce chitinase production and enzymes that can be applied in different industrial sectors.

Keywords Chitin residue · Bioeconomy · Chitinolytic enzymes · Microbial enzymes · Submerged culture

Introduction

The constant increase in the industrial shellfish processing generates approximately 10^{12}–10^{14} tons chitinous waste per year [1]. Chitin is the most common biopolymer in the marine environment and its disposal, whether through ocean dumps, incineration, or landfills, contributes to natural resource wastage, economic losses, and environmental pollution [2].

Chitin is found in crustacean shells, insects, fungal cell walls, and mollusks. Crustaceans (shrimp and crabs) are the most critical sources of chitin and usually contain proteins (30–40%), $CaCO_3$ (30–50%), chitin (20–30%), and several other compounds. Insects contain > 30% chitin in their exoskeletons and may be an alternative source of chitin [1, 3].

Chitin is a polysaccharide composed of β(1,4)-N-acetylglucosamine units. Chitinases catalyze the cleavage of glycosidic bonds in chitin [4]. They comprise a diverse group of enzymes with varied structures and mechanisms that determine their activity and suitability for applications such as chitooligosaccharide (COS) synthesis for use in the food and pharmaceutical industries, marine waste management, biocontrol of insect pests and pathogenic fungi, and biofuel production [5].

Currently, examples of commercial chitinases include Chitinase from *Aspergillus niger* (food grade)/Creative Enzymes®, Native *Trichoderma viride* Chitinase/Creative Enzymes®, Native *Streptomyces griseus* Chitinase/Creative Enzymes®, Chitinase from *Streptomyces griseus*/Merck (Sigma-Aldrich) and Chitinase (*Clostridium thermocellum*)/ Megazyme [6]. Despite their significant biotechnological potential, chitinases have not been extensively exploited commercially to the same extent as other glycosidases. This limited commercial utilization is due to factors such as the

Responsible Editor: Julio Santos.

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low number of organisms that show high chitinase production rates, low enzyme activity and stability, and high cost of production [7].

Understanding the effect of culture medium composition on microbial growth is essential to optimize enzyme production. Considering the importance of bioconverting chitin-rich biomasses and the role of chitinases in developing new value-added products, in this work, we evaluated the composition of the submerged medium for chitinase production by fungi, in addition to biochemical studies of these enzymes and their ability to remove *Candida* biofilms.

Materials and methods

Microorganisms

The fungi used for chitinase production, *Trichoderma reesei*, *Trichoderma harzianum*, and *Coriolopsis byrsina*, were obtained from a collection of microorganisms at the Laboratory of Biochemistry and Applied Microbiology, UNESP, São José do Rio Preto/SP, Brazil.

Colloidal chitin preparation

To prepare colloidal chitin, 10 g shrimp chitin (Sigma Aldrich) was added to 200 mL 36–37% HCl and constantly agitated for 4 h at 4 ºC. Then, 4 L cold distilled water was added and the mixture was centrifuged at 4 ºC and 10,000 rpm for 10 min. Several washing and centrifugation cycles were performed until the pH 7.0 was reached. The chitin was dried completely at 80 °C, crushed into a powder, sieved through a 425 mm sieve to remove larger granules, and subsequently stored at −20 ºC.

Submerged culture

First, to obtain biomass for seeding the submerged media, a pre-inoculum of each fungus was used. The fungi were inoculated in 125 mL Erlenmeyer flasks containing 30 mL of potato dextrose agar (PDA) medium and incubated at 30 ºC for 5 days. Next, 12 mL sterile distilled water was added to the PDA flasks. Using a Neubauer chamber for spore counting, 1×10^{7} spores mL^{-1} submerged medium were added to the fermentative flasks.

The optimal composition of the culture medium was evaluated and four different formulations were tested. The minimal medium contained 0.7% KH_2PO_4, 0.2% K_2HPO_4, 0.01% $MgSO_4$, 0.01% $CaCl_2$, and 0.5% colloidal chitin: 1- Minimal medium (C + minimal medium); 2- Minimal medium with 0.1% yeast extract (C + Y); 3- Minimal medium with 0.1% yeast extract and 0.1% peptone (C + Y + P); 4- Minimal medium with 0.1% yeast extract and 0.1% ammonium sulfate (C + Y+$(NH_4)_2SO_4$). Where C (chitin), Y (yeast extract), and P (peptone).

The volume of the culture medium was 50 mL (250 mL Erlenmeyer flask), pH 6.0, and the flasks were incubated at 30 ºC for 10 days (240 h). One flask was harvested every 24 h, filtered using Whatman® qualitative filter paper (grade 1), and the fermentative extract was used to quantify the chitinolytic activity (endo and exochitinase). After determining the best conditions for enzyme production, another larger-scale culture (1 L) was performed to produce enough enzymatic extract for biochemical characterization and application.

The extract was concentrated using a tangential membrane filtration system with a 10 kDa cut-off filter membrane, surface area 420 cm^2 (Hollow Fiber cartridge, Model UFP-10-E-4MA, GE Healthcare).

Enzyme activity assay

The reaction mixture for measuring endochitinase activity (0.01 g colloidal chitin, 100 µL enzyme, and 1 mL 0.2 mol L^{-1} sodium acetate buffer, pH 5.0) was incubated at 45 °C for 20 h with stirring at 180 rpm. Next, 100 µL reaction mixture was added to 100 µL dinitrosalicylic acid (DNS) solution and incubated at 95 °C for 10 min to quantify the reducing sugars. The absorbance at 540 nm was measured using a spectrophotometer. A standard curve with N-acetyl-D-glucosamine as the reference was used for determining the activity. One unit of enzyme activity was defined as the amount of enzyme required to produce 1.0 µmoL of N-acetyl-D-glucosamine mL^{-1} h^{-1} under the assay conditions.

We used *p*-nitrophenyl-N-acetyl-β-D-glucosaminide as the substrate for measuring the exochitinase activity; the reaction mixture comprised 5 µL enzyme and 90 µL 4 mmol L^{-1} substrate dissolved in 0.2 mol L^{-1} sodium acetate buffer (pH 5.0). The reaction was carried out in a microplate for 15 min at 45 ºC. Then, 100 µL 2 mol L^{-1} sodium carbonate was added and the enzymatic activity was measured at 410 nm. A standard curve with *p*-nitrophenol as the reference was used to determine the activity. One unit of enzyme activity was defined as the amount of enzyme required to produce 1.0 µmoL of *p*-nitrophenol mL^{-1} min^{-1} under the assay conditions.

Functional biochemical characterization of the crude extract

Effect of pH and temperature on enzyme activity and stability

The optimal pH for enzyme activity was determined at 45 °C using 0.2 mol L^{-1} acetate (pH 4.0 and 5.0), MES (pH 5.5, 6.0, and 6.5), HEPES (pH 7.0 and 7.5), bicine (pH 8.0, 8.5, and 9.0), and CAPS (pH 9.5 and 10.0) buffers. The effect of temperature on enzyme activity was investigated at 30–60 ºC.

The thermal stability of the extracts was studied after incubating them at 30–70 ºC for 1 h. While the effect of pH on stability was studied after incubating the extracts at pH 4.0–10.0 for 1–24 h at 4 °C. In both cases, after incubation, the enzyme reaction was carried out at the optimum pH and temperature for determining their activity.

Effect of metal ions on enzyme activity

The enzyme activity in the presence of 5 mmol L^{-1} (final concentration) iron III ($FeCl_3$), cadmium ($CdCl_2$), barium ($BaCl_2$), calcium ($CaCl_2$), cobalt ($CoCl_2$), lithium (LiCl), magnesium ($MgCl_2$), manganese ($MnCl_2$), nickel ($NiSO_4$), and zinc ($ZnSO_4$) was determined. In all the tests, the enzymes were pre-incubated with their respective salts for 5 min at room temperature. The reactions were performed under optimal pH and temperature conditions for each extract.

Hydrolysis of different substrates

In addition to chitinase activities, β–xylosidase, β–glucosidase, and α–L-arabinofuranosidase activities were determined using 10 µL enzyme extract with 90 µL 0.2 mol L^{-1} sodium acetate buffer solution (pH 5.0) with 4 mmol L^{-1} dissolved substrate (*p*-nitrophenyl-β-D-xylopyranoside [*p*NPX; Sigma Aldrich], *p*-nitrophenyl-β-D-glucopyranoside [*p*NPG], and *p*-nitrophenyl-α-L-arabinofuranoside [*p*NPA, Sigma Aldrich] for β–xylosidase, β–glucosidase, and α–L-arabinofuranosidase, respectively). The reaction mixtures were incubated at 40 °C for 30 min and then interrupted with 100 µL 2 mol L^{-1} Na_2CO_3. The released *p*-nitrophenol was quantified spectrophotometrically at 410 nm. A standard curve with *p*-nitrophenol as the reference was used for determining enzyme activity. One unit of enzyme activity was defined as the amount of enzyme required to produce 1.0 µmoL of *p*-nitrophenol mL^{-1} min^{-1} under the assay conditions.

For carboxymethylcellulose (CMC) and β-1,3-glucan, the DNS method was used to quantify reducing sugars. The reaction mixture, comprising 10 µL enzyme extract and 90 µL substrate (1% in 0.2 mol L^{-1} acetate buffer pH 5.0), was incubated for 30 min at 40 ºC. Then, the reaction mixture was incubated with 100 µL DNS at 95 °C for 10 min. The amount of reducing sugar released was quantified at 540 nm using a spectrophotometer (SPECTRAmax Plus 384). A standard curve with D-glucose as the reference was used to determine the reducing sugar released. One unit of enzyme activity was defined as the amount of enzyme required to produce 1.0 µmoL of glucose mL^{-1} h^{-1} under the assay conditions.

To evaluate the caseinolytic activity, 50 µL enzyme extract was incubated with 500 µL 1% casein diluted in 0.2 mol L^{-1} sodium phosphate buffer (pH 6.5) and incubated at 40 °C for 30 min. Next, the reaction was interrupted by adding 300 µL 10% of trichloroacetic acid (TCA). Blank tubes were prepared by adding 10% TCA to the reaction mixture before adding the substrate. The reaction tubes and blank tubes were then centrifuged at 10,000× *g* for 10 min at 25 °C, the supernatant was collected, and the absorbance was measured spectrophotometrically at 280 nm. Caseinolytic activity was expressed as U mL^{-1}, where one unit of enzyme activity (U) was defined as the amount of enzyme required to increase the absorbance (A_{280nm}) by 0.01 per minute under the assay conditions [8].

Biofilm removal

The experiment was conducted as described by Menezes et al. [9] with some modifications. The yeast *Candida albicans* ATCC 90028 (initial inoculum: 0.1 at A_{600nm}) was cultivated in a sterile 6-well polystyrene plate containing 1.5 mL yeast extract peptone dextrose (YPD) at 30 ºC for 5 days under static conditions for biofilm formation. Then, the culture medium was removed, the plates were washed twice with sterile water, and 2 mL enzyme extract from the three fungal species and 1 mL 0.2 mol L^{-1} sodium acetate buffer (pH 5.0) was added. The enzyme extracts were previously filtered using 0.22 µm syringe filter.

The enzyme solution used for biofilm removal was a pool of the fermentative extract collected at the best enzyme production time for each species; in this experiment the sample presented 490 µg mL^{-1} total protein and 0.85 U mL^{-1} (chitinolytic activity); an experiment containing the heat-inactivated enzyme was used as a control. Then, we removed the enzyme solution, washed the plates with water, added 3 mL 1% (w/v) crystal violet, and incubated for 20 min at 25 ºC. Next, we removed the stain, washed the plates with water, and dried them by incubation for 1 h at 40 ºC. We imaged biofilm removal and quantified the biofilm by adding 3 mL 30% (v/v) acetic acid solution, followed by measuring the

absorbance in a spectrophotometer at 595 nm. The control experiment (denatured enzyme) was considered as 100%.

Total protein quantification

This test was performed using the Bradford method [10] to quantify the microbial extract proteins in. A standard curve with bovine serum albumin was used to determine the protein concentration at 595 nm.

Data analysis

All measurements were performed with at least three independent replicates, and the control experiment was performed under the same conditions. Experimental results were expressed as the mean of replicate determinations and standard deviations (mean ± SD).

For the ion interference tests, statistical significance was assessed using one-way analysis of variance (ANOVA), followed by Dunnett's test. Results were considered statistically significant at $P \leq 0.05$. Statistical analyses were performed using IBM SPSS Statistics 20 and GraphPad Prism 9 softwares.

Results

Endo and exochitinase production

We tested four different culture media formulations to evaluate the profile of fungal chitinase production. After 168 h of cultivation (Fig. 1a), *C. byrsina* showed a remarkable increase in endochitinase production in $C+Y+(NH_4)_2SO_4$, C + Y + P, and C + Y (0.3 U mL^{-1}). The best production

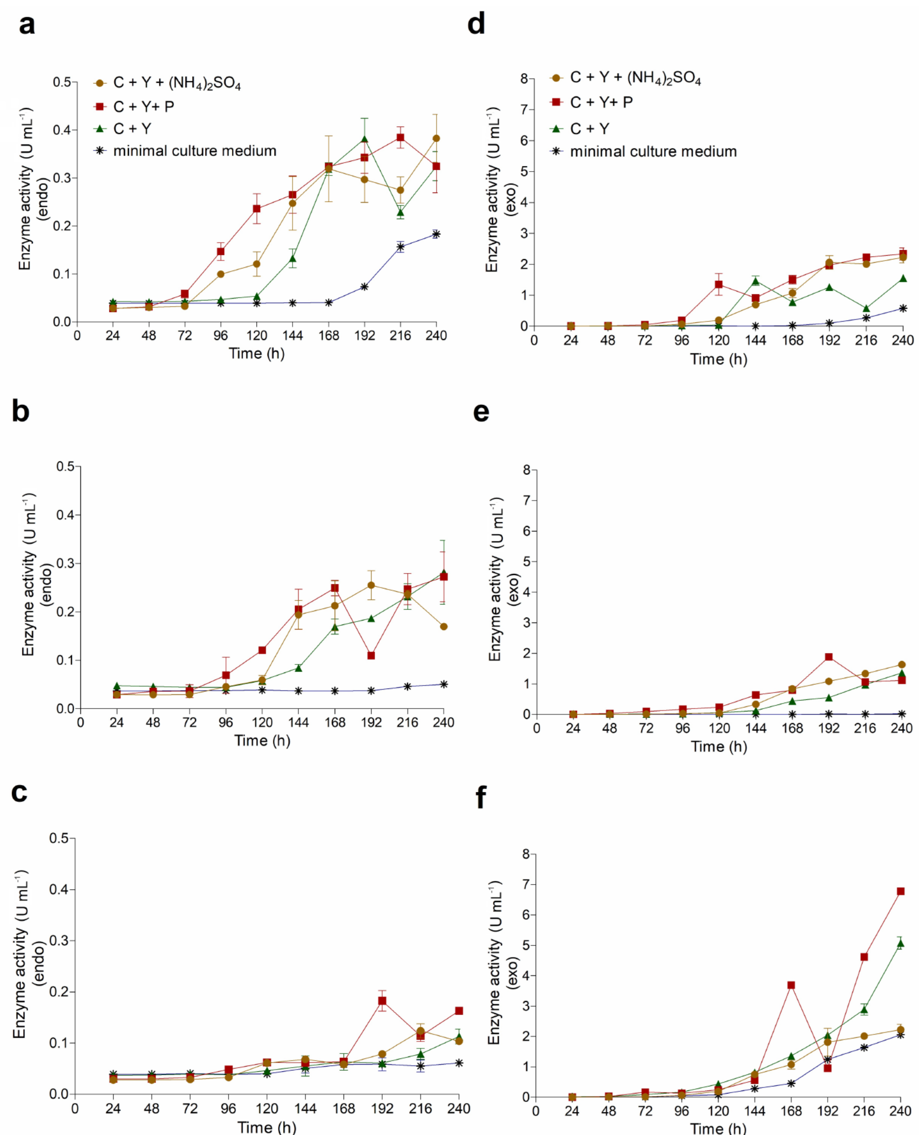

Fig. 1 Submerged culture with colloidal chitin as a substrate in different culture media. a, b, and c show graphs for endochitinase activity, whereas d, e, and f show graphs for exochitinase activity. *Coriolopsis byrsina*: **(a)** endochitinase activity, **(d)** exochitinase activity; *Trichoderma harzianum*: **(b)** endochitinase activity, **(e)** exochitinase activity; *Trichoderma reesei*: **(c)** endochitinase activity, and **(f)** exochitinase activity

time for C + minimal medium was 240 h (0.179 U mL^{-1}), which was considerably different from that for the other media used.

For the four culture media tested, the production of endochitinase by *C. byrsina* was low between 24 h and 72 h, increasing considerably from 120 h for C + Y + P and C+$(NH_4)_2SO_4$, while for C + Y, enzyme production only increased after 144 h. After 168 h, chitinase production remained stable until 240 h in C + Y + P and C + Y+$(NH_4)_2SO_4$(Fig. 1a). We observed that exochitinase production gradually increased with fungal growth, with the highest activity at 240 h (Fig. 1d). The enzyme activity profile was similar for C + Y + P and C + Y+$(NH_4)_2SO_4$, these had the best enzyme productivity (2 U mL^{-1}).

T. harzianum produced the maximum endochitinase (0.280 U mL^{-1}) in C + Y + P after 240 h (Fig. 1b). The minimum medium showed a low endochitinase yield (0.05 U mL^{-1}). This value was also lower than that of *C. byrsina* and similar to that observed of *T. reesei*.

Compared to the other fungi, *T. reesei* showed the lowest endochitinase activity. A similar profile was observed in all media, with the maximum activity observed at 192 h (0.183 U mL^{-1}) in C + Y + P (Fig. 1c). It showed strong exochitinase activity in all tested media, reaching approximately 7 and 5 U mL^{-1} in C + Y + P and C + Y, respectively, at 240 h (Fig. 1f).

Functional biochemical characterization of endo and exochitinases

We used the *C. byrsina* extract cultivated in C + Y + P for 168 h. The maximum endochitinase activity was observed between pH 5 and 6.5. The activity decreased as the pH increased, and the activity reached 50% at pH 7, gradually decreasing at alkaline pH levels (Fig. 2a). The endochitinase remained stable at all pH levels when incubated for 1–24 h. The optimum temperature for activity was noted between 40 ºC and 45 °C. The enzyme activity sharply dropped at higher temperatures and was not detected at 55 ºC. Endochitinase was stable up to 45 ºC, considering that its activity at 50 ºC was reduced to less than 30% (Fig. 2d).

We used the *T. harzianum* extract cultivated in C + Y + P for 144 h. *T. harzianum* endochitinase was more active between pH 5 and 6.5 and 45 ºC. The enzyme remained stable from pH 5 to 10, with more than 70% and 60% residual activity after 1 and 24 h, respectively (Fig. 2b). The endochitinase was also thermostable up to 45 ºC, with approximately 70% residual activity. After incubation at 50 ºC for 1 h, the enzyme lost approximately 90% activity (Fig. 2e).

We used the *T. reesei* extract cultivated in C + Y + P for 192 h. The optimum pH of the *T. reesei* endochitinase was the most acidic. Maximum activity was reached at pH 4.5-5. Enzyme stability decreased as pH increased under the two tested conditions (1 and 24 h). The highest stability was observed at pH 4–5 and 61% activity was retained at pH 10 (Fig. 2c). The endochitinase was stable up to 45 ºC, with > 80% activity, but the enzyme performance drops abruptly at 50 ºC, as with the other endochitinases studied (Fig. 2f).

The maximum exochitinase activity for *C. byrsina* extract was detected at pH 5.5–7. Exochitinases remained stable over a wide pH range (5–10), with > 60% residual activity at both 1 and 24 h (Fig. 3a). Under the effect of temperature (Fig. 3d), a sharp peak for activity at 55 ºC and stability up to 50 ºC (100% residual activity) were observed. However, the enzyme did not tolerate prolonged exposure to temperatures above 50 ºC, which considerably decreased the enzyme activity at 55 ºC; no activity was detected above 60 ºC (Fig. 3d).

The exochitinase activity of *T. harzianum* extract was more pronounced at 55 ºC and between pH 5.5–7. The enzyme remained stable over pH 4–10, maintaining > 80% and > 60% activity for 1 and 24 h, respectively (Fig. 3b). The enzyme was stable up to 45 ºC with relative activity above 80%; after incubating for 1 h at 55 ºC, it had < 10% activity (Fig. 3e).

The optimal enzyme activity of *T. reesei* extract was detected at 55 ºC and between pH 4.5–5.5. Moreover, the enzyme remained stable between pH 4–9, with > 70% activity after incubation for both 1 and 24 h (Fig. 3c). The enzyme maintained 40% activity up to 40 ºC, gradually decreasing at other temperatures, with no activity at 70 ºC (Fig. 3f).

Interference of metal ions on the enzyme activity

The highest positive modulation of endochitinase activity was observed with Mn^{2+}. Ions Mg^{2+}, Fe^{3+}, Li^{+}, Zn^{2+}, Ca^{2+} and Ni^{2+} did not significantly affect the endochitinase activity of *C. byrsina* and *T. harzianum* extracts (Table 1). However, Co^{2+}, Ba^{2+} and Mn^{2+} improved the endochitinase activity of *C. byrsina* extract (by 19%, 14%, and 78%, respectively) and *T. harzianum* extract (by 15%, 11%, and 32%, respectively). Almost all metal ions tested, except Mg^{2+} and Fe^{3+}, positively modulated the endochitinase activity of *T. reesei* extract (Zn^{2+}: +9%; Ni^{2+}: +12%; Ca^{2+}: +15%; Cd^{2+}: +21%; Ba^{2+}: +23%; Li^{+}: +26%; Co^{2+}: +30%; and Mn^{2+}: +56%).

The main positive and negative modulation of the exochitinase activity of *C. byrsina* extract was caused by Fe^{3+} (+ 53%) and Li^{+} (− 16%), respectively (Table 2). Cd^{2+} (+ 7%), Mn^{2+} (+ 9%), and Ba^{2+} (+ 16%) also improved exochitinase activity. Fe^{3+} and Ni^{2+} decreased (approximately 18%), while Mg^{2+} (+ 26%), Co^{2+} (+ 34%), and Ba^{2+} (+ 18%) increased the catalytic activity of *T. harzianum* fermentative extract. The exochitinase activity of *T. reesei*

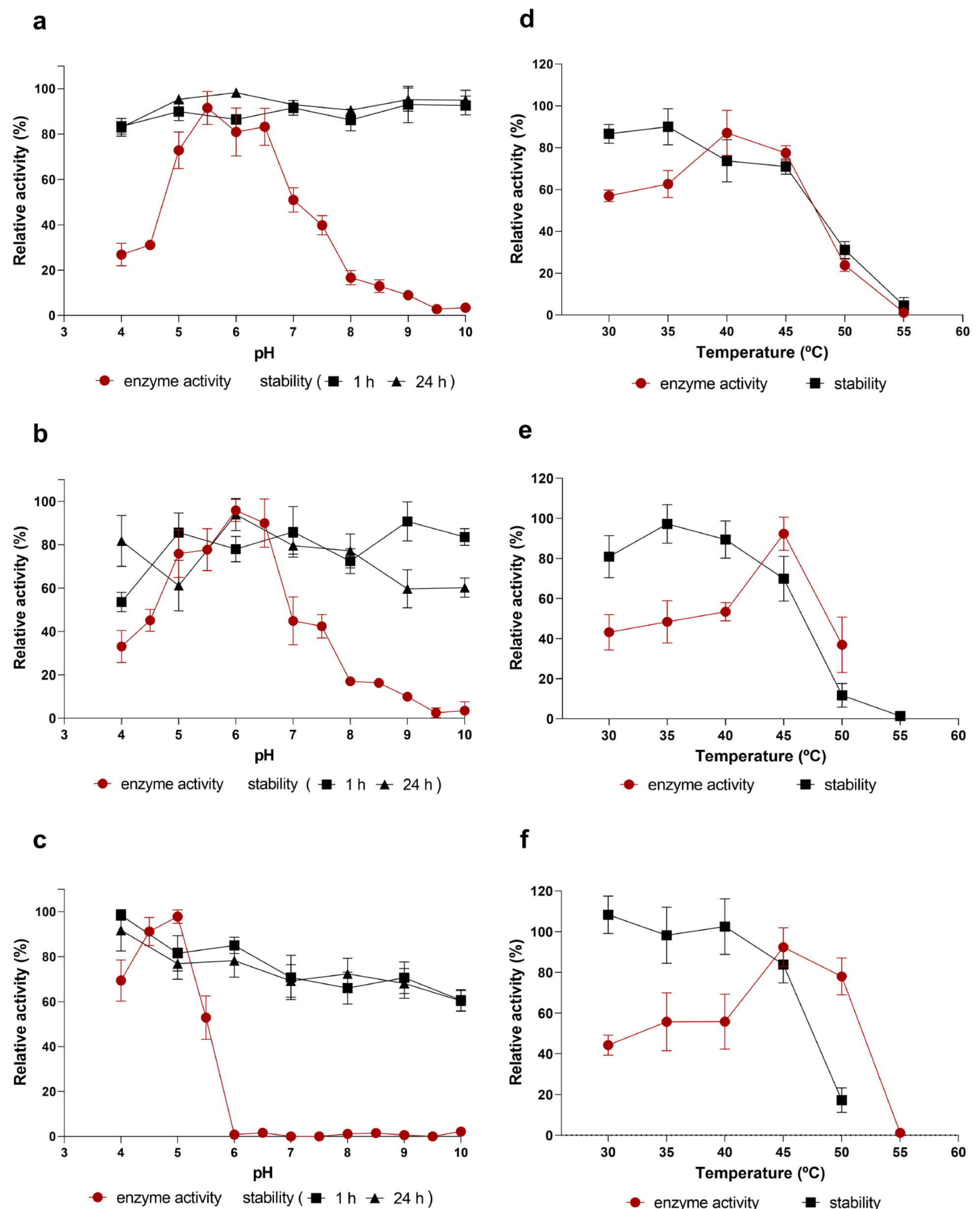

Fig. 2 Effect of **(a–c)** pH and **(d–f)** temperature on the endochitinase activity and stability of **(a** and **d)***Coriolopsis byrsina*, **(b** and **e)***Trichoderma harzianum*, and **(c** and **f)***Trichoderma reesei.* The red and black lines represent enzyme activity and stability, respectively

extract was considerably reduced in the presence of Zn^{2+} (−20.5%) and increased in the presence of Cd^{2+} (+12%), Co^{2+} (+21%), Ni^{2+} (+33%), Mg^{2+} (+55%), Li^{+} (+66%), and Fe^{3+} (+190%).

Hydrolysis of different substrates

Enzyme activities of *C. byrsina, T. harzianum*, and *T. reesei* fermentative extracts on different substrates are summarized in Table 3. For comparison, we also tested the activity of the commercial *T. harzianum* enzyme (Sigma Aldrich). In addition to chitinase activity, we mainly detected *p*NP-arabinofuranoside, *p*NP-Glucopyranoside, β-1,3-Glucan, Carboxymethylcellulose and casein hydrolysis.

The *T. reesei* extract did not show significant activity against *p*NPA, *p*NPX or *p*NPG, but it showed the best exochitinase activity (11.53 U mg^{-1}). The *T. harzianum* enzyme extract exhibited the highest activity against casein and colloidal chitin (121.04 and 27.12 U mg^{-1}, respectively).

C. albicans biofilm removal

Given that the fermentation extracts of the three fungal species exhibited significant activities on various substrates, including *p*NP-Glucopyranoside, *p*NP-Arabinofuranoside, *p*NP-Glucosaminide, β-1,3-Glucan, Carboxymethylcellulose, colloidal chitin and casein, we subsequently assessed the effectiveness of these fermentative extracts in dispersing

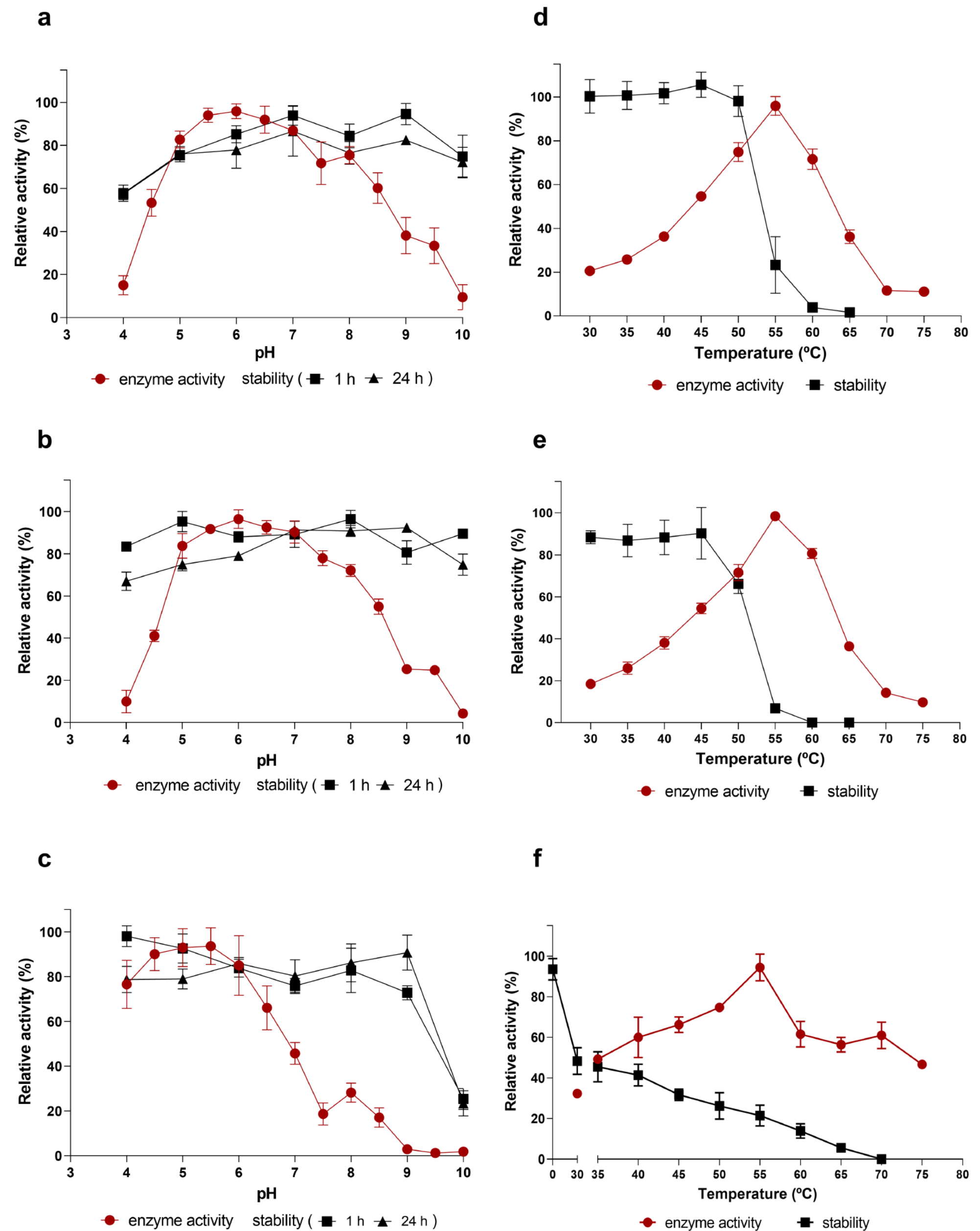

Fig. 3 Effect of **(a–c)** pH and **(d–f)** temperature on the exochitinase activity and stability of **(a** and **d)** *Coriolopsis byrsina*, **(b** and **e)** *Trichoderma harzianum*, and **(c** and **f)** *Trichoderma reesei*. The red and black lines represent enzyme activity and stability, respectively

Candida albicans biofilm. Only the extract from the *T. reesei* showed the ability to remove biofilms. In plates treated with the inactive enzyme (control), we observed intense crystal violet coloration, while in the presence of active enzymes we observed a decrease in staining intensity. After 5 h of incubation, the biofilm removal was 46%, and at 20 h, it was approximately 79%. In the Fig. 4 it is possible to visualize the removal of the biofilm after enzymatic treatment. The mean triplicate values and SD are shown.

Discussion

Chitinases have recently gained increasing attention due to their wide application potential in various fields, particularly chitin bioconversion and biocontrol in agriculture [11]. Among chitinase-producing microorganisms, *Trichoderma* spp. are of great industrial interest and have long been used in agriculture as biological controls [12].

Since phytopathogens and insects are considered the greatest threats to important crops (wheat, rice, corn, and potatoes), the abusive use of chemical pesticides against these agricultural pests has been practiced for years [13]. This has caused worldwide concern regarding soil and

Table 1 Effect of metal ions (5 mmol L^{-1}) on the endochitinase activity for the three fungal culture extracts

Metal ions	C. byrsina extract	T. harzianum extract	T. reesei extract
$MgCl_2$	104.4 ± 3.9	98.84 ± 17.7	90.41 ± 6.7
$FeCl_3$	98.72 ± 7.2	106.75 ± 11.7	97.64 ± 10.9
$CdCl_2$	97.36 ± 8.3	85.82 ± 13.2	121.69 ± 8.3
$CoCl_2$	119.4 ± 5.05	115.75 ± 17.3	130.67* ± 15.0
LiCl	96.93 ± 7.7	96.8 ± 11.8	126.79* ± 10.3
$BaCl_2$	114.2 ± 14.8	111.66 ± 5.5	123.59 ± 13.4
$ZnSO_4$	93.86 ± 4.7	103.41 ± 7.3	109.74 ± 6.7
$MnCl_2$	178.6* ± 19.6	132.24* ± 6.2	156.16* ± 16.4
$CaCl_2$	103.8 ± 10.3	105.93 ± 8.9	115.37 ± 9.2
$NiSO_4$	104.9 ± 14.7	96.93 ± 8.7	112.79 ± 7.1
Control	100.0 ± 3.1	100.0 ± 3.8	100.0 ± 4.5

Values represent means of 3 replicates, and ± standard deviations are reported. *Value significantly different from control ($p < 0.05$)

Table 2 Effect of metal ions (5 mmol L^{-1}) on the exochitinase activity for the three fungal culture extracts

Metal ions	C. byrsina extract	T. harzianum extract	T. reesei extract
$MgCl_2$	100.25 ± 3.4	125.97* ± 10.8	155.28* ± 10.8
$FeCl_3$	153.43* ± 19.7	81.63 ± 18.5	289.7* ± 5.8
$CdCl_2$	107.52 ± 1.2	102.22 ± 3.0	112.47 ± 5.4
$CoCl_2$	98.05 ± 2.2	134.36* ± 3.2	121.68 ± 7.6
LiCl	84.56 ± 0.6	102.06 ± 1.2	166.12* ± 9.4
$BaCl_2$	116.89 ± 11.1	118.92 ± 10.0	110.3 ± 18.0
$ZnSO_4$	96.24 ± 4.0	62.87* ± 6.7	79.4 ± 7.6
$MnCl_2$	109.72 ± 6.0	96.36 ± 3.2	98.1 ± 13.9
$CaCl_2$	92.33 ± 2.9	93.03 ± 6.3	94.31 ± 5.6
$NiSO_4$	101.25 ± 7.2	82.11 ± 1.5	133.33 ± 17.6
Control	100 ± 7.3	100.00 ± 5.2	99.7 ± 8.0

Values represent means of 3 replicates, and ± standard deviations are reported

*Value significantly different from control ($p < 0.05$)

groundwater contamination as well as human health [14, 15].

However, suitable ecofriendly, biodegradable, and economical bioproducts can be found presently. The presence of chitin in insects, fungi, and nematodes makes them logical targets for chitinases, which can function as biopesticides and are harmless to plants and vertebrates that lack chitin in their tissues [13].

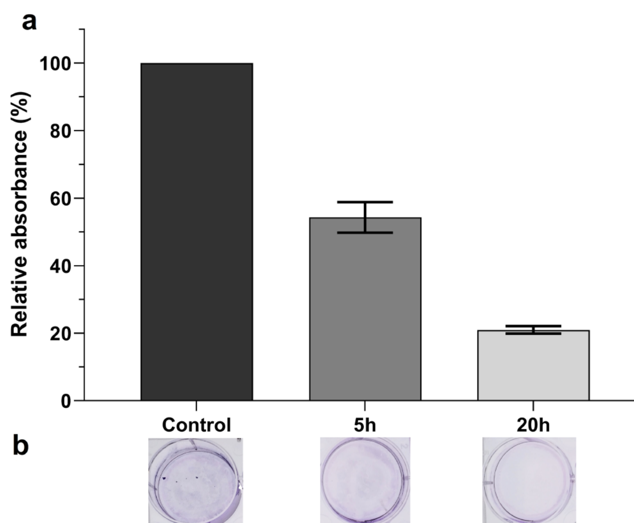

Fig. 4 *Candida albicans* biofilm dispersion after treatment with *Trichoderma reesei* enzyme extract (490 µg mL^{-1} final protein concentration and 0.85 U mL^{-1} chitinolytic activity) for 5 h and 20 h. Relative absorbance (595 nm) of biofilms stained with 1% crystal violet solubilized with 30% glacial acetic acid. **(a)** Mean triplicate values and standard deviation (SD) are shown, and **(b)** visualization of biofilm removal after enzyme treatment

Further studies are needed to enhance chitinase production at the laboratory scale. Optimizing the culture medium for producing specific enzymes is essential to not only maximize yield, but also minimize production costs, as the culture medium components can enhance growth and accelerate microbial metabolism [16].

Colloidal chitin has been widely used as an inducer for chitinase production in several studies, including the expression of chitinases from *Acremonium* sp. YS2-2, *Trichoderma asperellum* PQ34, *Myxococcus fulvus* UM01, and *Achromobacter xylosoxidans* in response to 1% colloidal chitin [17]. In addition to chitin, other nitrogen sources

Table 3 Hydrolysis of different substrates

Enzymes	pNP-Glucop.[1] U mg^{-1} of protein	pNP-Xylop.[2] U mg^{-1} of protein	pNP-Arab.[3] U mg^{-1} of protein	pNP-Glucosa.[4] U mg^{-1} of protein	β- 1,3 -Glucan U mg^{-1} of protein	CMC [5] U mg^{-1} of protein	Chitin U mg^{-1} of protein	Casein U mg^{-1} of protein
Commercial *T. harzianum* enzyme	1.01 ± 0.07	0.01 ± 0	0.17 ± 0	2.30 ± 0.1	0.3 ± 0	0.5 ± 0.1	0.91 ± 0.1	65.11 ± 1.1
C. byrsina extract	0.16 ± 0	0.03 ± 0	0.12 ± 0	6.21 ± 0.1	1.7 ± 0.1	0.8 ± 0.02	11.73 ± 0.1	39.28 ± 0.8
T. harzianum extract	0.30 ± 0	0.07 ± 0	0.30 ± 0	7.14 ± 0	0.33 ± 0	0.3 ± 0.01	27.12 ± 0	121.04 ± 0.1
T. reesei extract	0.09 ± 0	0.01 ± 0	0.02 ± 0	11.53 ± 1.2	0.4 ± 0.1	0.3 ± 0.01	4.66 ± 0.10	29.13 ± 0.5

[1]*p*NP-Glucopyranoside, [2]*p*NP-Xylopyranoside, [3]*p*NP-Arabinofuranoside, [4]*p*NP-Glucosaminide, [5]Carboxymethylcellulose

increased the production of chitinases by *Glutamicibacter uratoxydans* up to eight times [18].

Although yeast extract is an alternative vitamin, mineral, and amino acid sources, culturing *Aspergillus niveus* in minimal medium with crab shell chitin (96 h, 30 °C, 100 rpm), without yeast extract, had the best production of chitinase (6.5 U mg^{-1} protein). In minimal medium (in the absence of yeast extract), fungal metabolism is directed toward chitin degradation, which is the only available carbon/nitrogen source [19]. In another study, the production of chitinase by *Aspergillus terreus* was also influenced by the source of nitrogen incorporated into the medium, and the highest chitinase activity (6.28 U mg^{-1} of protein) was recorded from growth in a medium containing ammonium sulfate at 1% [20].

Typically, chitinase activity is optimal under acidic conditions. The optimal temperature and thermal stability of mesophilic fungi are similar to those found in this report (40–65 ºC). The optimal endochitinase and exochitinase activities described for the three fungal species are between pH 4–7 and up to 45 and 55 °C for endochitinases and exochitinases, respectively.

Recent reports on chitinases from different fungal species have focused on producing and determining the functional characteristics of chitinases toward colloidal chitin, including the enzyme from *Thermomyces lanuginosus*, which exhibited maximum activity at pH 3–4 and 50 °C, with gradual decreased activity after pH 6.0 [21]. The maximum activity for recombinant chitinase expressed in *Escherichia coli* (gene from *Aspergillus fumigatus* df347) was at pH 5 and 45 °C [22]. The chitinase purified from *Penicillium oxalicum* k10 showed maximum activity (100%) at pH 5 and 40 °C. The enzyme was stable up to 40 °C, with > 90% activity at 40 °C for 60 min [17]. Study with thermostable and acidic chitinase from *Paecilomyces thermophila* J18 (expressed in *Pichia pastoris*) reported optimal enzyme activity at pH 5.5 and 60 °C, and stability within pH 3.5–9.0 at 45 °C for 30 min (more than 70% residual activity) and approximately 90% residual activity up to 55 °C for 30 min [23].

Metal ions play a significant role in biological catalysis by forming complexes with proteins and interfering with enzyme structure and activity. Endochitinase activity mainly increased in the presence of $MnCl_2$ in all three fungal species. Chitinase activity of *Aspergillus niveus* also increased in the presence of $MnCl_2$ (by approximately 122%) than that in the control [19]. In the presence of 10 mM Zn^{2+} and Mn^{2+}, the activity of *A. terreus* chitinase decreased to 46.81% and increased by 25.62%, respectively [24]. Deng et al. [25] reported that Zn^{2+} ions decreased the enzymatic activity of *Trichoderma harzianum* GIM 3442 by 49.4%. In contrast, the enzyme activity of chitinase from *Penicillium oxalicum* k10 increased by Zn^{2+} and K^+, while Ag^+ and Fe^{2+} decreased the chitinolytic activity to 65.9% and 63.9%, respectively. The addition of Cu^{2+} decreased chitinolytic activity by 60% [17].

The enzyme activity profile of the three fungal culture extracts highlights the synergistic role of chitinases, in addition to the presence of other enzymes, particularly proteases and β-1,3-glucanases, in trace amounts. A broad spectrum of enzymatic actions may also play an important role in biocontrol mechanisms [26]. In the context of fungal biofilms, chitinases are capable of degrading the chitin present in fungal cell walls [17]. When used in conjunction with proteolytic enzymes, which degrade proteins that are components of extracellular polymeric substances (EPS), this cocktail of enzymes is potentially effective in reducing the adhesion of microbial cells to surfaces and improving the accessibility of antimicrobial agents to the biofilm [9, 27].

In the study with *Metschnikowia* species tested as biocontrol agents against postharvest fungal decays on lemons, different enzymes such as chitinase, protease, pectinase and β-1,3 glucanase were detected. Among the tested yeasts, *Metschnikowia aff. fructicola* was the most antagonistic against the phytopathogens *Penicillium digitatum* and *P. expansum* [28].

In addition to their biocontrol applications in agriculture, enzymes are also promising agents for biofilm dispersion. We must highlight that *Candida* spp. infections are emerging as major health problems, with high mortality rates and increasing medical costs for governments and hospitalized patients. Mortality can be attributed to the increasing incidence of invasive systemic candidiasis and septicemia, particularly in immunocompromised patients [29].

We found that *T. reesei* fermentative extract was useful for removing *C. albicans* biofilms. Commercial enzymes, including chitinases, lipases, proteases, DNAse and lyticase (final enzyme concentration: 50 µg mL^{-1}) were also tested for removing *C. albicans* biofilms [27]. The same study evaluated *C. tropicalis* biofilm removal for 2 h at 37 or 25 °C. Under these conditions, biofilm detachment by chitinase treatment was approximately 23% for *C. albicans* and 29% for *C. tropicalis.* Lyticase exhibited the best biofilm removal with 85% and 54% biofilm dispersion for *C. albicans* and *C. tropicalis*, respectively. In future studies, tests combining *T. reesei* extract with other enzymes, such as DNAse and lyticase, as well as antibiotics, should be carried out to improve the effect on biofilm removal.

Conclusions

The chitinases secreted by the fungal species *T. reesei*, *T. harzianum* and *C. byrsina* are promising for chitin degradation, mainly due to their stability at different pH levels, temperatures up to 45 °C, and in the presence of various metallic ions. Chitinases from *Trichoderma* are well described, but we did not find any reports on chitinases from *C. byrsina*.

Fungal chitinases are proficient candidates for producing value-added chitin degradation products such as COS and GlcNAc. We also demonstrated the potential of the fermentative extract of *T. reesei* for removing *Candida* biofilms, which is an important finding given the emerging need to study environment-friendly compounds capable of removing biofilms.

Funding Fundação de Amparo à Pesquisa do Estado de São Paulo-FAPESP (process 2017/06399-3).

Declarations

Competing interests The authors declare no competing interests.

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