# Potential Fungal Inhibition by Immobilized Hydrolytic Enzymes from *Trichoderma asperellum*

Bárbara Dumas S. Silva,[†] Cirano J. Ulhoa,[§] Karla A. Batista,[†] Fábio Yamashita,[#] and Kátia F. Fernandes*,[†]

[†]Departamento de Bioquímica e Biologia Molecular, Laboratório de Química de Proteínas, Universidade Federal de Goiás, Cx. Postal 131, 74001-970, Goiânia, GO, Brazil

[§]Departamento de Tecnologia de Alimentos e Medicamentos, Laboratório de Tecnologia, Universidade Estadual de Londrina, Cx. Postal 6001, CEP 86051-990, Londrina, PR, Brazil

[#]Departamento de Bioquímica e Biologia Molecular, Laboratório de Enzimologia, Universidade Federal de Goiás, Cx. Postal 131, 74001-970, Goiânia, GO, Brazil

**ABSTRACT:** The use of cell wall degrading enzymes from *Trichoderma asperellum* immobilized on biodegradable support is an alternative for food packaging. In this study, hydrolytic enzymes produced by *T. asperellum* were tested as a fungal growth inhibitor, in free form or immobilized on a biodegradable film composed of cassava starch and poly(butylene adipate-*co*-terephtalate) (PBAT). The inhibitory activity was tested against *Aspergillus niger*, *Penicillium* sp., and *Sclerotinia sclerotiorum*, microorganisms that frequently degrade food packaging. The use of chitin as carbon source in liquid medium induced *T. asperellun* to produce *N*-acetylglucosaminidase, $\beta$-1,3-glucanase, chitinase, and protease. The presence of *T. asperellun* cell wall degradating enzymes (*T*-CWD) immobilized by adsorption or covalent attachment resulted in effective inhibition of fungal growth. The enzymatic activity of *T*-CWD was stronger on *S. sclerotiorum* than on the *Aspergillus* or *Penicillum* isolates tested. These results suggest that *T*-CWD can be used in a free or immobilized form to suppress fungi that degrade food packaging.

**KEYWORDS:** cell wall degrading (CWD) enzymes, antifungal agents, immobilization, biodegradable film, PBAT, cassava starch

## INTRODUCTION

Some species of the genus *Trichoderma* are economically important because of their production of antibiotics and industrial enzymes (e.g., cellulases and hemicellulases) and because of their action as biocontrol agents.[1,2] Although relatively less studied than other species, *Trichoderma asperellum* is also an effective biological control agent.[2−5] This biocontrol activity involves synthesis of hydrolytic enzymes such as cell wall degrading (CWD) enzymes, which include chitinases, glucanases, and proteases.[6] The CWD enzymes that attack the polymers in other microorganism cell walls offer an interesting possibility for consumers searching for minimally processed and pesticide-free foods or artificial chemical-free foods.[7] These enzymes may play important roles as biocidal or biostatic agents when incorporated into packaging in their free or immobilized form.

Consumers are also concerned with the eco-friendliness of packaging, and for the past 10 years, new classes of biodegradable polymers have been undergoing development.[8] A good example of these polymers is poly(butylene adipate-*co*-butylene terephthalate) (PBAT). This material has been distributed since 1998 under the trade name Ecoflex (BASF Chemical Co.) for versatile applications, such as use in compost bags, agricultural materials (mulch film), lamination materials, and films for wrapping food. The inclusion of cassava starch in PBAT resulted in a blended polymer with very high biodegradability, enlarging its applicability.[8−11]

Active packaging is one of the innovative food packaging concepts that have been introduced as a response to continuous changes in current consumer demands and market trends.[12] This packaging interacts with food, not just separating it from the environment but also helping in restricting the growth of microorganisms and reducing the rate of deterioration, prolonging shelf life, and maintaining food safety, even for nonsterile foods.[13,14]

In this work, we tested the inhibition properties of a pool of enzymes produced by *T. asperellum* (*T*-CWD) on fungal growth, in free form or immobilized in a biodegradable film manufactured with a blend of cassava starch/PBAT (60:40). The growth inhibition activity of the free and immobilized *T*-CWD enzymes was tested against *Aspergillus niger*, *Penicillium* sp., and *Sclerotinia sclerotiorum*. *A. niger* and *Penicillium* present great importance due to their ability to produce mycotoxins. *Sclerotinia* is a fungus that can attack more than 400 plants, particularly dicots.[15−17] These microorganisms were chosen because they frequently attack food packaging, causing food decay.

## MATERIALS AND METHODS

**Biodegradable Film Production.** The biodegradable film was manufactured with a blend of cassava starch (CS) and PBAT (Ecoflex; BASF Chemical Co.) by blow extrusion as described by Yamashita et al.[18] Briefly, the blended film was prepared using a proportion of 60% crude cassava starch, 40% PBAT, and 1.0% glycerol as plasticizer. The film presented good mechanical properties as reported by Yamashita et al.[18] The CS−PBAT film was cut in strips of 4.0 cm × 0.5 cm and

~150 $\mu$m thickness and stored in closed vessels until use for immobilization experiments.

**Organisms: Strain Origin and Culture Conditions.** *T. asperellum* (strain T 00) was obtained from the Laboratory of Enzymology's culture collection at the Universidade Federal de Goiás and maintained in PDA medium, at room temperature. The *T. asperellum* (strain T00) identification was made by amplification of the nuclear rDNA region containing the ITS1 and ITS2 and the 5.8S rRNA gene using the primer combination SR6R and LR1, following the protocol described by White et al.[19] Sequence analysis of the ITS amplicon was performed using TrichOKEY 2.0.[20,21]

For production of the *T*-CWD enzymes, *T. asperellun* (1 × 10$^7$ spores mL$^{-1}$ of culture medium) was grown in 250 mL of TLE liquid medium [0.1% bactopeptone; 0.03% urea; 0.2% KH$_2$PO$_4$; 0.14% (NH$_4$)$_2$SO$_4$; 0.03% MgSO$_4$·7H$_2$O; 0.03% CaCl$_2$·6H$_2$O; 1 mL of 0.01% trace elements solution (Fe$^{2+}$, Mn$^{2+}$, Zn$^{2+}$, and Co$^{2+}$), 0.1% glucose], with constant shaking (180 rpm) at 28 °C for 72 h.

The fungi *Penicillium* sp. and *A. niger* strains were kindly donated by the Laboratório de Microbiologia of the Universidade Federal de Santa Catarina. The *S. sclerotiorum* strains were obtained from the Laboratório de Enzimologia of Universidade Federal de Goiás. *A. niger*, *Penicillium* sp., and *S. sclerotiorum* were maintained in PDA medium, at room temperature (25 °C).

**Conditions for Enzyme Induction and Production.** For enzyme induction experiments, *T. asperellum* spores were transferred to an Eppendorff flask and then washed with sterile 0.9% (w/v) NaCl solution. The spores (1 × 10$^7$ spores mL$^{-1}$) were transferred to 1000 mL Erlenmeyer flasks containing 250 mL of TLE medium, containing 0.02% (w/v) glucose and 0.5% (w/v) chitin. The culture was incubated in a rotary shaker (180 rpm), at 28 °C, for 6 days and harvested by filtration with Whatman no. 1 filter paper. The culture filtrate containing *T*-CWD enzymes was dialyzed in cellulose acetate membrane with cutoff at 10 kDa. Both dialyzed (retained inside dialysis tube) and dialysate (low molecular material released in the dialysis procedure) were concentrated by lyophilization (−40 °C, 250−300 $\mu$Hg, 8 h; Enterprise 1, Terroni) and then used for growth inhibition tests. Only *T*-CWD enzymes (retained material) presented growth inhibitory activity and were used in immobilization.

**Enzyme Immobilization.** The immobilization was conducted by adsorption or covalent binding. Adsorption of the *T*-CWD enzymes was carried out by immersing strips of CS−PBAT film in an enzyme solution (0.1% w/v) for 30 min, at room temperature. Alternatively, immobilization by covalent binding was conducted by immersing the CS−PBAT strips in a 0.1 mol L$^{-1}$ sodium periodate solution for 30 min followed by immersion in the 0.1% (w/v) *T*-CWD enzyme solution for 30 min, at room temperature (25 °C). After immobilization, the strips were placed on PDA medium. The control tests were conducted using untreated film strips, as well as strips treated with sodium periodate followed by immersion in a 0.1 mol L$^{-1}$ glycine solution, to prevent the presence of free reactive groups on the strips' surface.

To verify the film's enzymatic activity, strips (0.25 cm$^2$) were prepared and analyzed for total immobilized protein and for chitinase activity as described above. The amounts of immobilized protein and enzyme were determined according to the following equations:

immobilized protein (mg)

= total protein offered − protein in the supernatant      (1)

immobilized enzyme (EU)

= EU offered − EU in the supernatant      (2)

**Enzyme Activity Assays.** *T. asperellum* CWD enzyme production was periodically evaluated during 10 days. Total protein concentration was determined according to the method of Bradford[22] using bovine serum albumin as standard.

*N*-Acetylglucosaminidase activity was assayed by monitoring the rate of formation of *p*-nitrophenol from *p*-nitrophenol-$\beta$-*N*-acetylglucosamine (pNglcNAc) according the method of Yabuki et al.[23] One unit of enzyme activity (U) was defined as the amount of enzyme that releases 1 $\mu$mol of *p*-nitrophenol min$^{-1}$ at 37 °C.

$\beta$-1,3-Glucanase activity was assayed as described by Noronha and Ulhoa[24] in a reaction containing 0.25% (w/v) laminarin (Sigma). The mixture was incubated at 40 °C for 30 min, and the amount of reducing sugar formed was then determined spectrophotometrically at 550 nm. One unit of $\beta$-1,3-glucanase activity (U) was defined as the amount of enzyme that produced 1 $\mu$mol of reducing sugar min$^{-1}$ under the above conditions.

Protease activity was determined according Arnon[25] using 1% (w/v) casein solution as substrate. The reaction was performed for 20 min, at 37 °C, and stopped by the addition of trichloroacetic acid (TCA). After centrifugation at 3300$g$, the supernatant was analyzed spectrophotometrically at 280 nm. One enzyme unit (U) was defined as the amount of enzyme necessary to produce an elevation of 0.1 in optical density (OD) after 10 min.

Chitinase activity of free and immobilized chitinase was determined according to the method of Molano et al.,[26] using colloidal chitin, prepared by the methodology of Berger and Reynolds,[27] with modifications as described. Crude chitin (10 g) was suspended in concentrated HCl, and the mixture was left in an ice bath for 4 h under soft stirring. The colloid was slowly filtered over cold ethanol (95,5%) and left to precipitate at −4 °C for 18 h. The precipitate was separated by centrifugation at 3300$g$ for 10 min and then thoroughly washed with distilled water to remove excess acid. The colloid obtained was lyophilized and stored at 5 °C. The colloidal chitin was prepared by suspending 0.75 g of chitin powder in 50 mM acetate buffer, pH 5.5. The colloid was stirred for 15 min and filtered, and the solution obtained was used as substrate for chitinase. The product of chitinase activity was revealed after incubation with *p*-dimethylaminobenzaldehyde (DMAB). One unit of enzyme (U) was defined as the amount of enzyme necessary to produce 1 $\mu$mol of reducing sugar in 1 h at 40 °C.

**Inhibitory Activity of the *T*-CWD Enzymes.** To verify the inhibitory activity of the enzymes, the microorganisms *A. niger*, *Penicillium* sp., and *S. sclerotiorum* were grown in liquid medium at two different enzyme concentrations: 0.1% and 0.5% (w/v). Twenty microliters of 10$^6$ spores mL$^{-1}$ of *A. niger* and *Penicillium* sp. was inoculated in 2 mL of Sabouraud liquid medium (1% bactopeptone; 4% glucose; 1% CuSO$_4$) supplemented with 20 $\mu$L of 0.1 or 0.5% *T*-CWD enzyme solution. *S. sclerotiorum* (samples with OD$_{750}$ = 0.15) was grown in 2 mL of PD liquid medium supplemented with 20 $\mu$L of *T*-CWD enzyme solution (0.1 or 0.5%). All cultures were incubated under constant shaking (180 rpm) at 28 °C for 24 h. Aliquots were withdrawn at 2 h intervals for 12 h starting 2 h after inoculums were made. The growth curve was constructed using a microbalance (Shimadzu AUW-220-D) for dry weight measurement. The control tests were performed in the absence of *T*-CWD enzymes.

The growth inhibitory activity of the *T*-CWD enzymes immobilized (adsorbed and covalently bounded) on CS−PBAT film was determined by evaluating the growth inhibition of *A. niger*, *Penicillium* sp., and *S. sclerotiorum* in solid medium. The CS−PBAT strips containing immobilized *T*-CWD enzymes were placed over PDA medium in Petri dishes, and then 20 $\mu$L of 10$^6$ spores mL$^{-1}$ of *A. niger* and *Penicillium* sp. or *S. sclerotiorum* (samples OD$_{750}$ = 0.15) was dropped on the strip. Inoculums were incubated at 37 °C for 4 days. Growth inhibition was analyzed by SEM and macroscopic observations. SEM analysis was performed at 15 kV (JEOL JSM 7001F).

**Statistical Analysis.** All experiments were performed in triplicate with replicate, and the results were subjected to statistical analysis by Student's *t* test, with the $\alpha$ level set at 0.05.
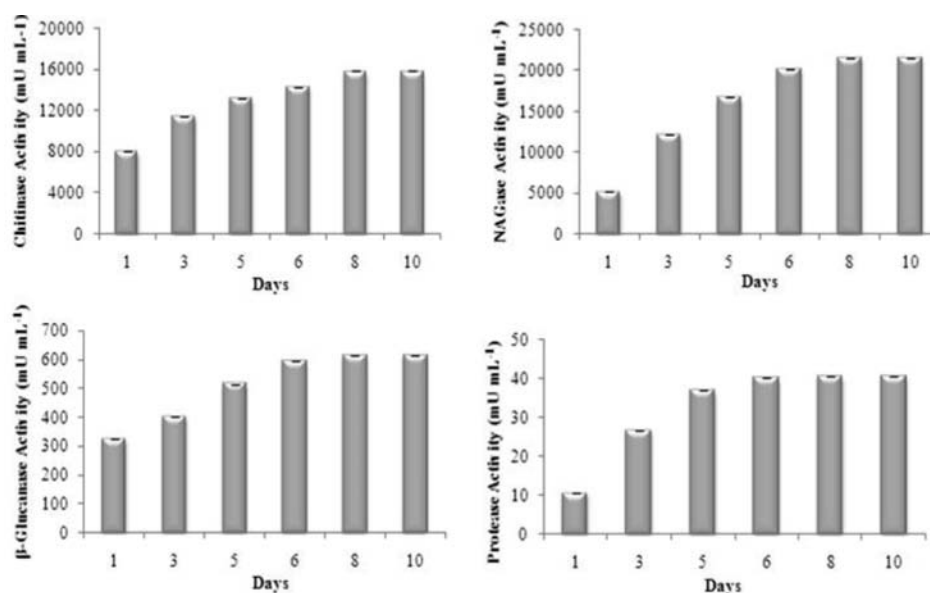
**Figure 1.** Course of chitinase, $N$-acetylglucosaminidase (NAGase), $\beta$-1,3-glucanase, and protease production by $T.\ asperellum$ (T 00) in liquid medium containing 0.5% chitin, under stirring (180 rpm), at 28 °C.

**Table 1. Enzyme Concentrations in Culture Medium with $T.$ $asperellum$ after 6 Days of Incubation**

| enzyme | activity (mU mL$^{-1}$) | specific activity (mU mg$^{-1}$ protein) |
|---|---|---|
| $\beta$-1,3-glucanase | 514.8 | 9.75 |
| NAGase[a] | 16 828 | 318.59 |
| chitinase | 13 216 | 250.21 |
| protease | 37.2 | 0.49 |

[a] NAGase, $N$-acetylglucosaminidase.

**Table 2. Amounts of $T$-CWD Enzymes Immobilized for Adsorption and Covalent Binding[a]**

| immobilization method | total immobilized protein (mg) | immobilized chitinase units (mU) | chitinase specific activity (mU mg$^{-1}$) |
|---|---|---|---|
| adsorption | 18.86 ± 0.015* | 203.51 ± 0.018* | 10.79 |
| covalent bonding | 30.30 ± 0.029** | 375.85 ± 0.021** | 12.40 |

[a] Results are the mean of three determinations ± SD. Within columns, means with same number of asterisks are not significantly different ($p > 0.05$).

## RESULTS AND DISCUSSION

**Enzyme Activity.** A number of factors, including the species and strain used in the culture as well as the substrate used for growth, influence fungal CWD enzyme production.[28,29] These enzymes are frequently produced and secreted into the culture medium by a wide variety of fungi in response to the presence of chitin.[24,30] In this study, $T.\ asperellum$ produced substantial amounts of CWD enzymes in the culture medium containing 0.5% chitin as a carbon source (Figure 1). Moreover, the presence of chitin in TLE medium induced $T.\ asperellum$ to produce $N$-acetylglucosaminidase, $\beta$-1,3-glucanase, and protease in a very similar pattern, as shown in Figure 1.

As can be observed, the production of chitinase and $N$-acetylglucosaminidase was very expressive. On the first day, the level of chitinase produced was 8046 mU mL$^{-1}$, and this stabilized by the seventh day at 15656 mU mL$^{-1}$, representing an increase of nearly 2-fold. Similarly, the expression of $N$-acetylglucosaminidase began at the first day. Although the initial amount produced was lower (5219 mU mL$^{-1}$) than that of chitinase, at the seventh day the production had increased to 21541 mU mL$^{-1}$, an increase of almost 4-fold.

The relationship between the production of chitinase and $N$-acetylglucosaminidase can be explained by the catalytic mechanism these enzymes. Chitinase releases dimers of $N,N'$-diacetyl-chitobiose (GlcNAc)$_2$ that are converted to monomers of $N$-acetylglucoseamine by $N$-acetylglucosaminidase.[31] In this way, while the substrate of chitinase is decreasing in the culture medium, the substrate of $N$-acetylglucosaminidase is increasing for a certain time period, as result of chitinase action.

The production of $\beta$-1,3-glucanase was lower than that of chitinase and $N$-acetylglucosaminidase. On the first day, the production was 326 mU mL$^{-1}$, stabilizing by the sixth day at 596 mU mL$^{-1}$. The lower production of this enzyme may be a consequence of its catalytic activity on chitin, because $\beta$-1,3-glucanase acts over branching points in the polymeric chain, which are lower in amount than linear linkage.

The activity of protease also increased from the first to the sixth day (40.2 mU mL$^{-1}$), representing an increase of 3.72 times. It is well established that fungal cell walls present high protein content.[32] Suarez et al.[32] showed that proteases produced by $T.\ harzianum$ were important for cell wall degradation. Although production of proteases of $T.\ asperelum$ was lower than that observed with $T.\ harzianum$, these enzymes probably contributed to cell wall degradation and fungal growth inhibition.

The production of CWD enzymes stabilized after around 7 days. For the immobilization assays, enzymes from the sixth day were utilized. The total protein amount present in the culture medium at day 6 was 52.82 mg mL$^{-1}$. The amount of each enzyme and its specific activity at day 6 are shown in Table 1.
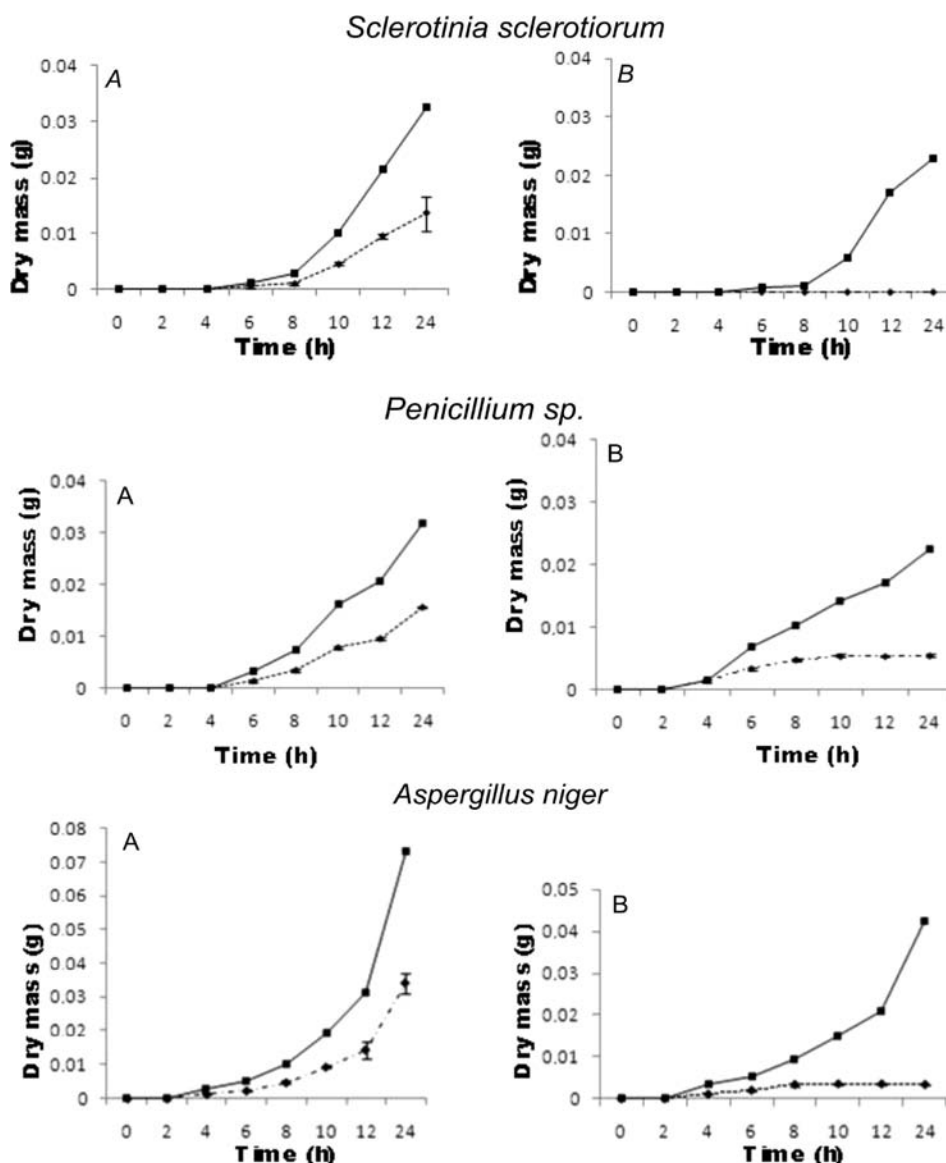
**Figure 2.** Fungal growth inhibition by T-CWD enzymes: (A) 0.1% (w/v) enzyme solution; (B) 0.5% (w/v) enzyme solution; (—■—) control; (···◆···) test.

**Enzyme Immobilization.** The results obtained from assays using CS−PBAT film immobilized total protein and chitinase are presented in Table 2. Immobilization via covalent binding was 20% more efficient than via adsorption. The higher amount of immobilized T-CWD via covalent bonding may be due to the reaction of sodium periodate with the starch component in the CS−PBAT blend leading to the appearance of sites for enzyme binding, probably through amino groups from the lateral chain of lysine.[33]

**Inhibitory Activity of the T-CWD Enzymes.** The inhibition was pronounced in all treatments, and increasing the concentration of T-CWD enzymes in the culture medium from 0.1 to 0.5% resulted in enhanced growth inhibition for all microorganisms tested (Figure 2).

The least sensitive organism was *Penicillium* sp., which presented 56 and 64.7% reduction in growth compared to controls using Sabouraud medium supplemented with 0.1 and 0.5% T-CWD enzymes, respectively, as measured by dry weight after 12 h of growth. An intermediate suppression was observed for *A. niger*, with dry weight reductions of 57% and 92.3% observed after 12 h of growth in the presence of 0.1 and 0.5% T-CWD, respectively. The most sensitive microorganism was *S. sclerotiorum*, with dry weight reduced by 64% after 12 h of growth in PDA medium supplemented with 0.1% T-CWD enzymes, and a total absence of growth in 0.5% supplemented medium.

For the growth inhibition tests using the CS−PBAT-immobilized T-CWD enzymes, a concentration of 0.1% was used, as the severe fungal growth reduction observed using a concentration of 0.5% T-CWD enzymes would make it impossible to determine the inhibitory effect. In the untreated film (control), the microorganisms grew normally using the starch of the film and the nutrients of the culture medium for maintenance of their metabolism. However, the presence of T-CWD enzymes immobilized by adsorption or covalently bonded resulted in inhibition of fungal growth (Figure 3), more pronounced for the covalently bonded enzymes.
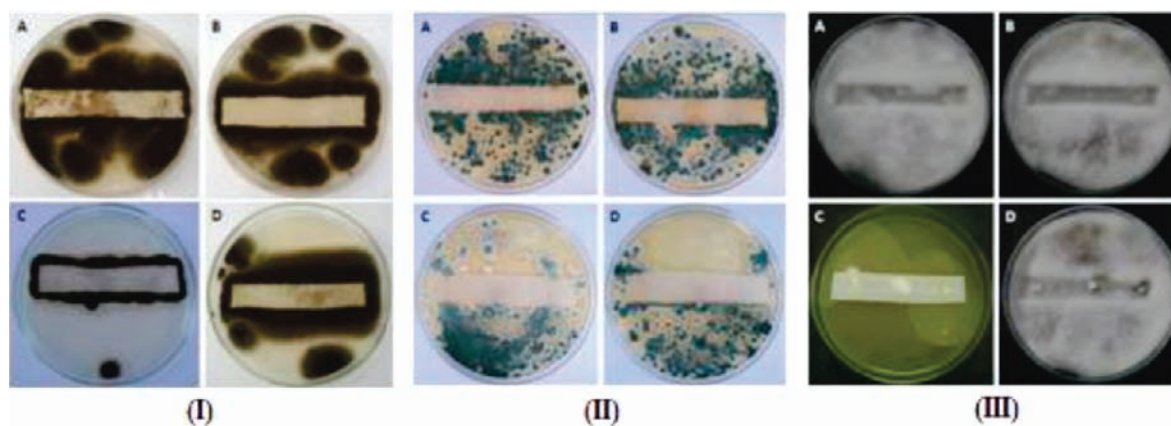
**Figure 3.** Fungal growth inhibition by immobilized *T*-CWD enzymes after 4 days of growth at 37 °C: (I) *A. niger*; (II) *Penicillium* sp.; (III) *S. sclerotiorum*; (A) untreated biodegradable film strips; (B) immobilization by adsorption; (C) immobilization by covalent binding; (D) immobilization control with sodium periodate and glycine solution.
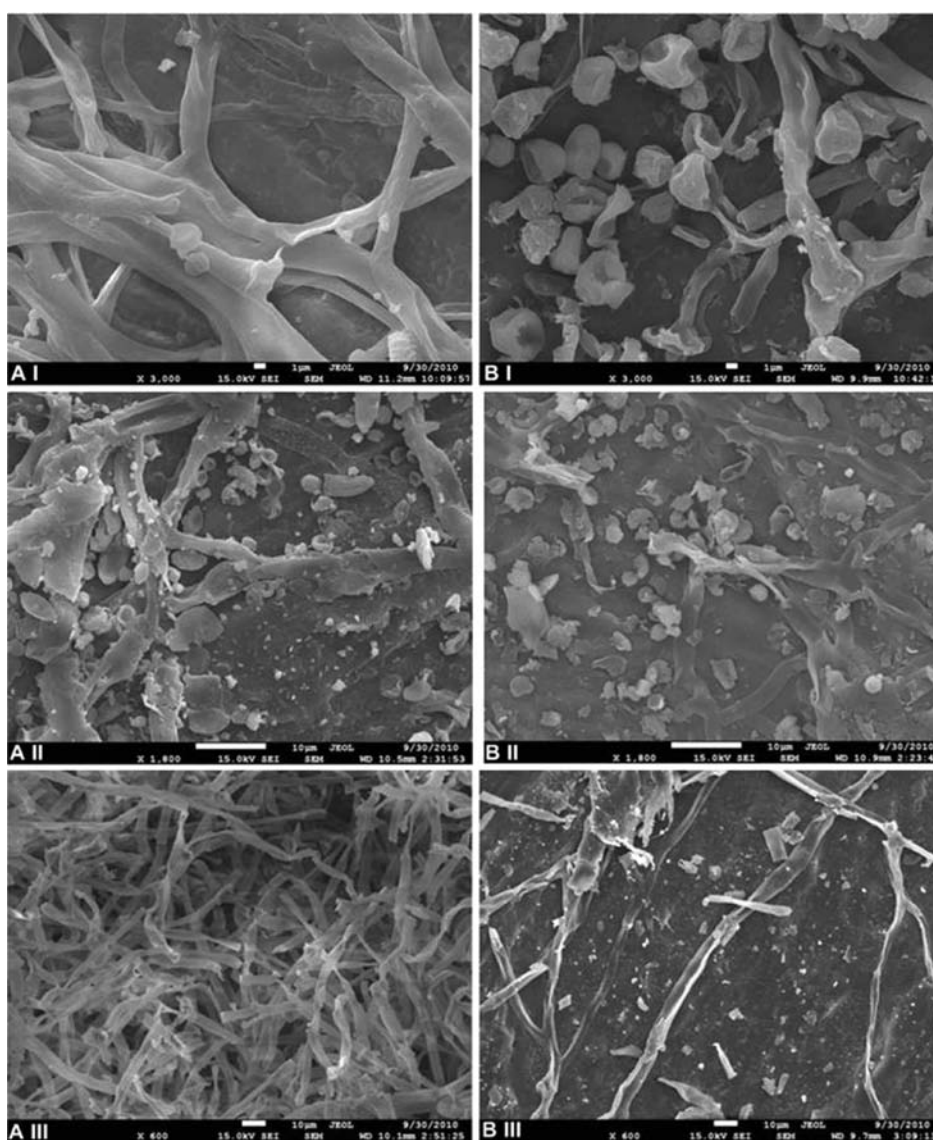


**Figure 4.** SEM micrograph of microorganisms growing in the presence or absence of *T*-CWD enzymes: (A) control; (B) CS−PBAT with *T*-CWD enzymes immobilized by covalent bounding; (I) *A. niger*; (II) *Penicillium* sp.; (III) *S. sclerotiorum*.

8152

dx.doi.org/10.1021/jf2009815 |*J. Agric. Food Chem.* 2011, 59, 8148–8154

Immobilization by adsorption was effective in the inhibition of growth for all microorganisms, mainly in the region of the film strip. However, the reduction in growth was even more evident using immobilization by covalent bonding via sodium periodate. In this case, the growth was reduced on the strip and in the culture plate in general. As observed in the liquid medium, *S. sclerotiorum* was the more sensitive organism, followed by *A. niger*, whereas *Penicilium* sp. showed the lowest sensitivity to *T*-CWD enzymes. All microorganisms showed growth in the control plates.

The inhibitory effect of *T*-CWD enzymes on the microorganism's growth was more evident in the SEM (Figure 4). The growth of all microorganisms was normal in control films (panels A), but markedly compromised in the presence of immobilized *T*-CWD enzymes (panels B). The hyphae of *A. niger* presented structural disruption and apparent decrease in turgor (B I). In addition, the spores initially inoculated did not germinate, showing an alteration in morphology from that normally oval to an biconcave shape. *Penicillium* sp. was the microorganism least sensitive to the *T*-CWD enzymes, free or immobilized. Changes promoted by the presence of *T*-CWD enzymes were less expressive than that observed for *A. niger* (Figure 4, panels A II and B II. Although less susceptible, the germination of *Penicillium* spores inoculated in CS−PBAT containing immobilized *T*-CWD enzymes was lower than in control and some disruption of hyphae can be observed. On the other hand, the highest sensitivity of *S. sclerotiorum* against immobilized T-CWD enzymes was confirmed by SEM (Figure 4, panels A III and B III). An evident decrease in hyphae growth in the presence of *T*-CWD enzymes was observed here, and those few hyphae that were able to grow showed severe structural damage.

The difference of microbial growth may be related to cell wall composition in different fungi. Although it is known that the main constituents of the fungal cell wall are polysaccharides (chitin and α- and β-glucans), protein, and lipids, the distributions of these compounds across the cell walls of *A. niger*, *Penicillium* sp., and *S. sclerotiorum* may be very different,[34] resulting in different hydrolysis levels.

These results suggest that the enzymes produced by *T. asperellum* are effective inhibitors of fungal growth and have potential for the packaging industry, considering the cost and ease of production. The use of both free and immobilized enzymes resulted in effective inhibition of microbial growth. The inhibition mechanism is associated with disruption of microbial structures and probably the morphological changes of spores, which prevent their germination. The use of bioactive natural compounds such CWD enzymes and a biodegradable material as support for the immobilization may be an attractive alternative for packaging, compatible with the worldwide preoccupation with environmental issues.

## ■ AUTHOR INFORMATION

**Corresponding Author**
*E-mail: katia@icb.ufg.br. Phone: +55 (62) 3521 1492. Fax: +55 (62) 3521 1190.

## ■ REFERENCES

(1) Kullnig-Gradinger, C. M.; Szakacs, G.; Kubicek, C. P. Phylogeny and evolution of the genus *Trichoderma*: a multigene approach. *Mycol. Res.* **2002**, *106*, 757–767.

(2) Tondje, P. R.; Roberts, D. P.; Bon, M. C.; Widmer, T.; Samuels, G. J.; Ismaiel, A.; Begoude, A. D.; Tchana, T.; Nyemb-Tshomb, E.; Ndoumbe-Nkeng, M.; Bateman, R.; Fontem, D.; Hebbar, K. P. Isolation and identification of mycoparasitic isolates of *Trichoderma asperellum* with potential for suppression of black pod disease of cacao in Cameroon. *Biol. Control* **2007**, *43*, 202–212.

(3) Marcello, C. M.; Steindorff, A. S.; Silva, S. P.; Silva, R. N.; Bataus, L. A. M.; Ulhoa, C. J. Expression analysis of the exo-β-1,3-glucanase from the mycoparasitic fungus *Trichoderma asperellum*. *Microbiol. Res.* **2008**, *165*, 75–81.

(4) Deberdt, P.; Mfegue, C. V.; Tondje, P. R.; Bon, M. C.; Ducamp, M.; Hurard, C.; Begoude, B. A. D.; Ndoumbe-Nkeng, M.; Hebbar, P. K.; Cilas, C. Impact of environmental factors, chemical fungicide and biological control on cacao pod production dynamics and black pod disease (*Phytophthora migakarya*) in Cameroon. *Biol. Control* **2008**, *44*, 149–159.

(5) Samuels, G. J.; Ismaiel, A.; Bon, M. C.; De-Respinis, S.; Petrini, O. *Trichoderma asperellum* sensu lato consists of two cryptic species. *Mycologia* **2010**, *102*, 944–966.

(6) Benítez, T.; Rincón, A. M.; Limón, M. C.; Codón, A. C Biocontrol mechanisms of *Trichoderma* strains. *Int. Microbiol.* **2004**, *7*, 249–260.

(7) Azeredo, H. M. C. Nanocomposites for food packaging applications. *Food Res. Int.* **2009**, *42*, 1240–1253.

(8) Witt, U.; Einig, T.; Yamamoto, M.; Kleeberg, I.; Deckwer, W. D.; Müller, R. J. Biodegradation of aliphatic−aromatic copolyesters: evaluation of the final biodegradability and ecotoxicological impact of degradation intermediates. *Chemosphere* **2001**, *44*, 289–299.

(9) Kasuya, K.; Ishii, N.; Inoue, Y.; Yasawa, K.; Tagaya, T.; Yotsumoto, T.; Kazahaya, J.; Nagai, D. Characterization of a mesophilic aliphatic-aromatic copolyester-degrading fungus. *Polym. Degr. Stab.* **2009**, *94*, 1190–1196.

(10) Kijchavengkul, T.; Auras, R.; Rubino, M.; Ngouajio, M.; Fernandez, R. T. Assesment of aliphatic-aromatic copolyester biodegradable mulch films. Part I: field study. *Chemosphere* **2008**, *71*, 942–953.

(11) Yamashita, F.; Nakagawa, A.; Veiga, G. F.; Mali, S.; Grossmann, M. V. E. Active packaging for West Indian cherry fruits. *Braz. J. Food Technol.* **2006**, *9*, 95–100.

(12) Vermeiren, L.; Devlieghere, F.; Van Beest, M.; Kruijf, N.; Debevere, J. Developments in the active packaging of foods. *Trends Food Sci. Technol.* **1999**, *10*, 77–86.

(13) Han, J. H. Antimicrobials and package system design. Annual Metting and Food Exposition, Anaheim, CA, 2002; http://ift.confex.com/ift/2002/techprogram/paper_9793.htm (accessed Aug 3, 2009).

(14) Scannell, A. G. M.; Hill, C.; Ross, R. P.; Marx, S.; Hartmeier, W.; Arendt, E. K. Development of bioactive food packaging materials using immobilised bacteriocins Lacticin 3147 and Nisaplin®. *Int. J. Food Microbiol.* **2000**, *60*, 241–249.

(15) Bae, Y. S.; Knudsen, G. R. Effect of sclerotial distribution pattern of *Sclerotinia sclerotiorum* on biocontrol efficacy of *Trichoderma harzianum*. *App. Soil Ecol.* **2007**, *35*, 21–24.

(16) Clemmensen, L. H.; Hansen, M. E.; Frisvad, J. C.; Ersbóll, B. K. A method for comparison of growth media in objective indentification of *Penicillium* based on multi-spectral imaging. *J. Microbiol. Methods* **2007**, *69*, 249–255.

(17) González-Salgado, A.; Patiño, B.; Vázquez, C.; González-Jaén, M. T. Discrimination of *Aspergillus niger* and other *Aspergillus* species belonging to section *Nigri* by PCR assays. *FEMS Microbiol. Lett.* **2005**, *245*, 353–361.

(18) Yamashita, F.; Nakagawa, A.; Veiga, G. F.; Mali, S.; Grossmann, M. V. E. Biodegradable active for minimally processed fruits and vegetables. *Braz. J. Food Technol.* **2005**, *8*, 335–343.

8153

dx.doi.org/10.1021/jf2009815 |*J. Agric. Food Chem.* 2011, 59, 8148–8154

(19) White, T. J.; Bruns, T.; Lee, S.; Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: a Guide to Methods and Applications*; Innis, M. A., Gelfand, D. H., Sninsky, J. J., White, T. J., Eds.; Academic Press: New York, 1990; pp 315—322.

(20) Druzhinina, I. S.; Kopchinskiy, A. G. TrichOKEY v. 2 — a DNA oligonucliotide BarCode program for the identification of multiple sequences of *Hypocrea* and *Trichoderma*. *Proceedings of the 8th International Mycological Congress*, Cairns, Australia, Medimond: Pianoro, Italy, **2006**.

(21) Druzhinina, I. S.; Kopchinskiy, A. G.; Komon, M.; Bissett, J.; Szakacs, G.; Kubicek, C. P. An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. *Fungal Genet. Biol.* **2005**, *42*, 813–828.

(22) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein—dye binding. *Anal. Biochem.* **1976**, *72*, 680–685.

(23) Yabuki, M.; Mizushima, K.; Amatatsu, T.; Anda, A.; Fujii, T.; Shimada, M.; Yamashida, M. Purification and characterization of chitinase and chitobiase produced by *Aeromonas hydrophyla* subsp. *Anaerogenes* A 523. *J. Gen. Appl. Microbiol.* **1986**, *2*, 25–38.

(24) Noronha, E. F.; Ulhoa, C. J. Characterization of a 29-kDa $\beta$-glucanase from *Trichoderma harzianum*. *FEMS Microbiol. Lett.* **2000**, *183*, 119–23.

(25) Arnon, R. Papain. In *Methods in Enzymology*; Perlmann, G., Loran, D. L., Eds.; Academic Press: New York, 1970; pp 226—244.

(26) Molano, J.; Duram, A.; Cabib, E. A rapid and sensitive assay for chitinase using tritriated chitin. *Anal. Biochem.* **1977**, *83*, 648–656.

(27) Berger, L. R.; Reynolds, D. M. The chitinase system of a strain of *Streptomyces griseus*. *Biochim. Biophys. Acta* **1958**, *29*, 552–634.

(28) Harman, G. E.; Howell, C. R.; Viterbo, A.; Chet, I.; Lorito, M. *Trichoderma* species — opportunistic, avirulent plant symbionts. *Nat. Rev. Microbiol.* **2004**, *2*, 43–56.

(29) Howell, C. R. Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. *Plant Dis.* **2003**, *87*, 4–10.

(30) Dahiya, N.; Tewari, R.; Hoondal, G. S. Biotechnological aspects of chitinolytic enzymes: a review. *Appl. Microbiol. Biotechnol.* **2006**, *71*, 773–782.

(31) Seidl, V. Chitinases of filamentous fungi: a large group of diverse proteins with multiple physiological functions. *Fungal Biol. Rev.* **2008**, *22*, 36–42.

(32) Suarez, M. B.; Sanz, L.; Chamorro, M. I.; Rey, M.; González, F. J.; Llobell, A.; Monte, E. Proteomic análisis of secreted proteins from *Trichoderma harzianum* — identification of a fangal cell wall-induced aspártico protease. *Fungal Gen. Biol.* **2005**, *42*, 924–934.

(33) Papas, A. C.; Stalikas, C. D.; Fiamengos, Y. Ch.; Karayannis, M. I. Determination of hydrogen peroxide by using a flow injection system with immobilized peroxidase and long pathlenght capillary spectrophotometry. *Anal. Chim. Acta* **2002**, *455*, 305–313.

(34) Escott, G. M.; Heam, V. M.; Adams, D. J. Inducible chitinolytic system of *Aspergillus fumigatus*. *Microbiology* **1988**, *144*, 1575–1581.