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Biodegradable and bioactive CGP/PVA film for fungal growth inhibition

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ABSTRACT

In this study, chitinolytic enzymes produced by *Trichoderma asperellum* were immobilized on a biodegradable film manufactured with a blend of cashew gum polysaccharide (CGP) and polyvinyl alcohol (PVA), and tested as a fungal growth inhibitor. The film was produced by casting a blend of CGP and PVA solution on glass molds. The CGP/PVA film showed 68% water solubility, tensile strength of 23.7 MPa, 187.2% elongation and 52% of mass loss after 90 days in soil. The presence of *T*-CWD enzymes immobilized by adsorption or covalent attachment resulted in effective inhibition of fungal growth. *Sclerotinia sclerotiorum* was the most sensitive organism, followed by *Aspergillus niger* and *Penicillium* sp. SEM micrograph showed that the presence of immobilized *T*-CWD enzymes on CGP/PVA film produced morphological modifications on vegetative and germinative structures of the microorganisms, particularly hyphae disruption and changes of spores shape.

1. Introduction

In recent years, the increasing demand for eco-friendliness packaging and the emphasis of growing environmental awareness stimulated the search for alternative products obtained from renewable sources. One of the many approaches to produce eco-friendly material was to blend biodegradable plastics with natural polymers as reinforcement agent (Lopez-Rubio, Gavara, & Lagaron, 2006). In spite of biodegradability, the introduction of natural polymers results in a material with lower mechanical properties. However, to become commercially competitive with existing thermoplastics, the biodegradable material should have similar thermo-mechanical and visual characteristics (Follain, Joly, Dole, & Bliard, 2005). In this scenario, the search for biodegradable plastics remains an open research area.

Recently, a new generation of biodegradable materials has innovated food-packaging concept, introducing the active biodegradable materials. These active packaging interacts with food extending the self-live, restricting the growth of microorganisms and maintaining food safety, even for non-sterile foods (Lee, Han, & Seo, 2008; Scannell, Hill, Ross, Marx, Hartmeier, & Arendt, 2000).

In a previous study, a bioactive thermoplastic material was obtained using a pool of *Trichoderma asperelum* cell wall degrading enzymes (*T*-CWDE) immobilized in CS-PBAT film (Silva, Ulhoa, Batista, Yamashita, & Fernandes, 2011). This film was successfully used to inhibit *Penicillium* sp., *Aspergillus niger* and *Sclerotinia sclerotiorum* growth. Nevertheless, the opacity of CS-PBAT film restricted the use of this material as active packaging, once the direct visualization of the product was compromised.

Among natural polymers, cashew gum polysaccharide (CGP) obtained from *Anacardium occidentale* L. exudates is an inexpensive, non-toxic, hydrophilic, biocoMPatible, biodegradable polymer, with good rheological properties. These factors enable the use of CGP in biotechnological fields such as pharmacological formulations, edible films and immobilization matrix (Carneiro-da-Cunha, Cerqueira, Souza, Souza, Teixeira, & Vicente, 2009; Gowthamarajan, Komar, Gaikwad, & Suresh, 2011; Silva, Santiago, Purcena, & Fernandes, 2010).

Polyvinyl alcohol (PVA) is a highly used thermoplastic material that assembles good biocoMPatible properties and excellent film forming capacity. Due to the characteristics such as easy preparation, chemical resistance, and mechanical properties, the PVA has been used combined with natural polymers in many biomaterial applications (Srinivasa, Ramesh, Kumar, & Tharanathan, 2003; Tripathi, Mehrotra, & Dutta, 2010).

In this research, a biodegradable film was manufactured by blending CGP and PVA. The physical and chemical properties

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of CGP/PVA film were analyzed. Following, this film was used as support for *T*-CWDE immobilization and tested as bioactive material to prevent *Penicillium* sp., *A. niger* and *S. sclerotiorum* growth.

2. Materials and methods

2.1. Biodegradable film production

The biodegradable film was composed of a blend of cashew gum polysaccharide (CGP) and polyvinyl alcohol (PVA) as follows. The blend was produced adding to 20 mL of 3% (w/v) PVA solution, 2.0 mL of 0.1 mol L^{-1} sodium metaperiodate, 3% (w/v) of CGP and 2.0 mL of 0.1 mol L^{-1} H_2SO_4 as catalyst. The films were produced by casting the solution on glass molds, followed by evaporation of water at room temperature (25 °C) for 24 h. The dry films were stored in plastic bags before all following characterization procedures.

2.2. Film characterization

2.2.1. Fourier transform infrared spectroscopy (FTIR)

Transmission infrared spectra of all films were recorded at room temperature using FTIR (Spectrum 400Series FT-IR/FT-NIR) spectrophotometer in the range from 4000 to 400 cm^{-1} during 12 scans, with 5 cm^{-1} resolution. The film was grinded and analyzed as KBr pellets.

2.2.2. Thermo gravimetric analysis (TGA)

TGA of CGP/PVA films were performed using a Shimadzu Simultaneous TGA/DTA Analyzer (DTG-60H). The samples (10 mg) were heated to 800 °C at a rate of 10 °C min^{-1} under nitrogen atmosphere. The results were analyzed using the PA 60 software (Shimadzu).

2.2.3. Water solubility

The film water solubility was carried out according to Gontard, Guilbert and Cuq (1992). The dry matter was determined by heating CGP/PVA film at 105 °C for 24 h. 500 mg of dried film were immersed in 50 mL of distilled water and incubated at 20 °C under stirring for 24 h. After that, the mixture was filtered and the material retained in the filter was dried at 105 °C for 24 h. The film solubility was expressed as percentage of soluble mass in relation to the initial mass according following equation:

$$\text{Solubility}(\%) = \frac{(\text{sample initial weight(mg)} - \text{weight of dried material retained in the filter(mg)})}{\text{sample initial weight(mg)}} \times 100$$

2.2.4. Soil burial degradability

Soil burial degradation was performed as described by Guohua, Ya, Culian, Min, Caiqiong and Zonfdao (2006) with slight modifications. The substrate was composed by a 1:1 mixture of perlite and savanna soil (red latosol) taken from a culture field at Universidade Federal de Goiás (Goiânia, Brazil). The garden pots with approximate capacity of 100 mL were filled with the substrate. The plastic samples were cut into 10 mm × 20 mm pieces and buried in the soil. The pots were placed in an uncovered gazebo. The soil was kept moist by sprinkling water at a regular time interval to maintain 20–40% humidity. The excess water was drained through the hole at the bottom of the pot. The degradation of the CGP/PVA film was determined at a regular time interval (15 days). The content of the pots was passed through a sieve (0.5 mm) and the retained residues were gently washed with distilled water. The residue of CGP/PVA film was dried under vacuum until a constant weight was obtained. Weight loss was used to indicate degradation rate in the soil burial test, according following equation:

$$\%B_m = \frac{W_f}{W_i} \times 100,$$

where B_m is the biodegradable matter; W_f is the final weight; and W_i is the initial weight.

2.2.5. Film thickness and morphology

The film thickness was determined using a manual micrometer (Mitutoyo, São Paulo, Brazil). Final thickness was determined as means of 20 random determinations in all film area. Scanning electron micrographs (SEMs) observations were performed using JEOL JSM-7001F equipment. Prior to observation, samples were covered with gold (30 nm) using an ionic BAL-SCD 050 sputter.

2.2.6. Mechanical properties

Mechanical properties were measured by using a Texture Analyzer TA.TX2 (Stable Micro Systems, Surrey, UK), with a 50 N load cell equipped with tensile grips (A/TG model). Sample films were cut into 20 mm wide and 40 mm long strips, according to the ASTM D-638M-93 standard (ASTM, 1995). Grip separation was set at 25 mm, with a cross-head speed of 500 mm min^{-1}. Tensile strength (TS) and percentage of elongation (%*E*) at break were evaluated. Sample used were previously inspected and those having any defect such as air bubbles, holes, tears or having average thickness variation superior than 5% were rejected.

2.3. Enzyme immobilization

The immobilization was conducted by adsorption or covalent binding. Adsorption of the chitinolytic enzymes was carried out by immersing strips (4 cm × 0.5 cm) of CGP/PVA film in a 0.1% (w/v) enzyme solution for 30 min, at room temperature. Alternatively, immobilization by covalent binding was conducted by immersing the CGP/PVA strips in a 0.1 mol L^{-1} sodium metaperiodate solution for 30 min, following by immersion in the 0.1% (w/v) enzyme solution for 30 min, at room temperature (25 °C). To verify the immobilization efficiency, strips with 0.25 cm^2 were tested for total immobilized protein and for chitinase activity as described by Silva et al. (2011). The amount of immobilized protein and enzyme were determined according to the following equations:

$$\text{Immobilized protein(mg)} = \text{Protein offered} - \text{Protein in the supernatant}$$
$$\text{Immobilized enzyme(EU)} = \text{EU offered} - \text{EU in the supernatant}$$

Blanch proofs were done with CGP/PVA film without enzyme and CGP/PVA film treated with sodium metaperiodate followed by immersion in 0.1 mol L^{-1} glycine solution. Total protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

2.4. Bioactivity of CGP/PVA film

The bioactivity of the CGP/PVA film was determined by evaluating the growth of *A. niger*, *Penicillium* sp. and *S. sclerotiorum* in CGP/PVA film in the presence or absence of chitinolytic enzymes. CGP/PVA strips (4 cm × 0.5 cm) containing immobilized chitinolytic enzymes were placed over PDA medium in Petri dishes and then

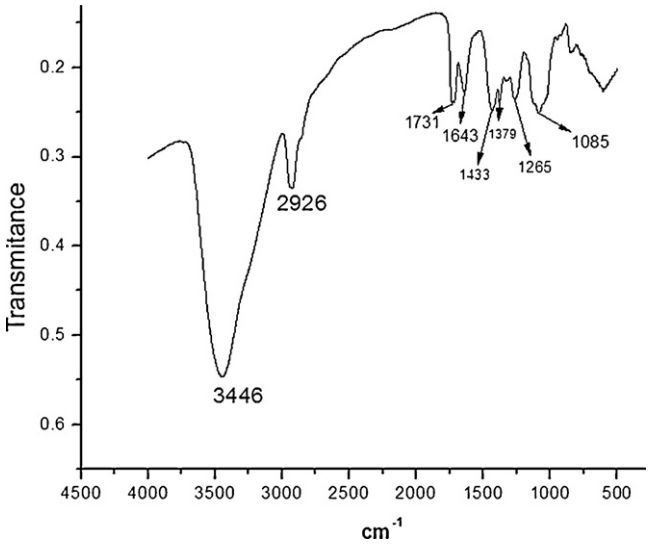

Fig. 1. FTIR spectra of CGP/PVA film as KBr pellets.

20 μL of 10^6 spores mL^{-1} of *A. niger* and *Penicillium* sp. or a plug of *S. sclerotiorum* were dropped at film. Inoculums were incubated at 37 °C for 4 days and effect of chitinolytic enzymes in microorganism growth was analyzed by SEM.

2.5. Statistical analysis

All experiments were performed in triplicate with replicate and the results were subjected to statistical analysis by Student's *t*-test, with alpha level set at 0.05.

3. Results and discussion

3.1. Film characterization

3.1.1. Infrared spectroscopy (FTIR) and TGA

The FTIR spectrum of CGP/PVA film showed the characteristics bands of CGP and PVA (Fig. 1). Two bands are common to PVA and CGP – the broad strong band at 3446 cm^{-1} assignable to the stretching vibrations of hydroxyl group (from PVA chain and hexoses from CGP) and the band at 2926 cm^{-1} relative to CH stretching from sp^3 carbon. The band at 1647 cm^{-1} related to secondary amine deformations, and the peaks in the region from 1350 to 1400 cm^{-1}, related to folding of CH_3 and CH_2 groups, are provided from CGP. Strong peaks at 1153, 1081 and 1029 cm^{-1} are due to the stretching vibrations of C—O—C from glucosidic bonds and stretching vibrations of O—H bending from pyranosidic structures of the sugars or OH from PVA (Pavia, Lampman, Kriz, & Vyvyan, 2010). The amino groups of the CGP are commonly assigned to primary amino sugar derivatives of the CGP. However, the presence of proteins associated with CGP was reported by Silva et al. (2010). Therefore, the characteristic band at 1647 cm^{-1}, related to secondary amine groups are probably due to the presence of such structures in the side chain of amino acids. The band at 1731 cm^{-1}, only present in the spectrum of CGP/PVA film, is characteristic of this blended material. This band is characteristically assigned to carbonyl group and allows proposing a reaction route for film production involving hydroxyl group of PVA with a carboxyl group from galacturonic acid generated by the oxidative action of sodium metaperiodate (Fig. 2).

TGA showed that CGP/PVA film presented high thermal stability. TGA profile presents three degrading zones (Fig. 3). The first in the range from 238 to 279 °C is related to polysaccharide chain degradation, as reported by Silva et al. (2010), resulting in 49% of weight loss in this interval. The second weight loss, observed from 430 to 447 °C, is probably associated to degrading of PVA chains, representing 11% of weight loss. In the last degrading zone, from 510 to 532 °C, a weight loss of 18% was observed, corresponding to mineralization of the film material. Two important observations can be extracted from TG analysis: increasing the PVA content in the CGP/PVA film probably will increase thermal stability of the blended film. The second observation is related to the high mineral content of the film (ca. 18%). According to Coutinho, Mello and Santa-Maria (2003), CGP presents high content of calcium, magnesium, potassium and sodium salts.

Table 1
Mechanical properties of CGP/PVA film.

	CGP/PVA film
Tensile strength (mPa)	23.7 ± 2.8
Elongation (%)	187.2 ± 9.3
Solubility (%)	68.0 ± 3.24

3.1.2. Thickness and morphology

The CGP/PVA films presented thickness values around 170 μm (±2.07). The produced film presented no fractures or breaks, were flexible and easy to use. The coloration was uniform, presenting high transparency and brightness.

3.1.3. Mechanical properties and water solubility

Analyses of mechanical properties of films are very important because they allow previewing film behavior considering flexibility, break resistance, elasticity and therefore applications (Matzinos, Tserki, Giamolouris, Pavlidou, Panayiotou, 2002). Table 1 shows mechanical properties of the CGP/PVA films. Tensile strength (TS) was 23.7 MPa (±2.8), value higher than 5–16 MPa reported by Zhou, Ma, Ren, Tong, Liu and Xie (2009) for TPS/PVA film; 13.9–20.6 MPa for starch/PVA/glycerol films founded by Yoon, Park and Byun (2012); and 10.2–18.3 MPa for PVA/starch film reported by Xiong, Tang, Tang and Zhou (2008). TS is a very important property that expresses the maximum stress developed in a film and defines the capacity of resistance to rupture when material is submitted to pressure force (Yamashita, Nakagawa, Veiga, Mali & Grossmann, 2005). In the case of CGP/PVA film, the improvement in TS may be attributed to metaperiodate cross-linking CGP and PVA which resulted in a blended material linked by covalent bound.

In addition, elongation percentage (%*E*) of CGP/PVA films (Table 1) were higher than PVA (105.47%) (Srinivasa et al., 2003), presenting high malleability comparable to polypropylenes (Thomason, Vlug, Schipper, & Krikor, 1996) and starch/PVA/glycerol films (Yoon et al., 2012). On the other hand, CPV/PVA films presented elongation values higher than other films blending PVA and polysaccharides (Khan, Bhattacharia, Kader, & Bahari, 2006; Shi, Bi, Zhang, Zhu, Chen, Zhou, Zhang, & Tian, 2008), and PVA/protein (Yang, Li, & Nie, 2007).

Evidence that sodium metaperiodate plays a main role in the stability of the tridimensional network of CGP/PVA was obtained in the tests of water solubility (Table 1). In the absence of sodium metaperiodate, the blended material was quite unstable, dissolving rapidly in the presence of water. However, cross-linking produced by metaperiodate stabilized the tridimensional network without compromising the CGP/PVA biodegradability.

CGP/PVA film presented 68% water solubility, value higher than presented by biopolymers films such as xylan/wheat gluten films (Kayserilioglu, Bakir, Yilmaz, & Akkas, 2003); soy protein isolate films (González, Strumia, & Igarzabal, 2011); and oxidized cassava starch biodegradable films (Pauli, Quast, Demiate, & Sakanaka,

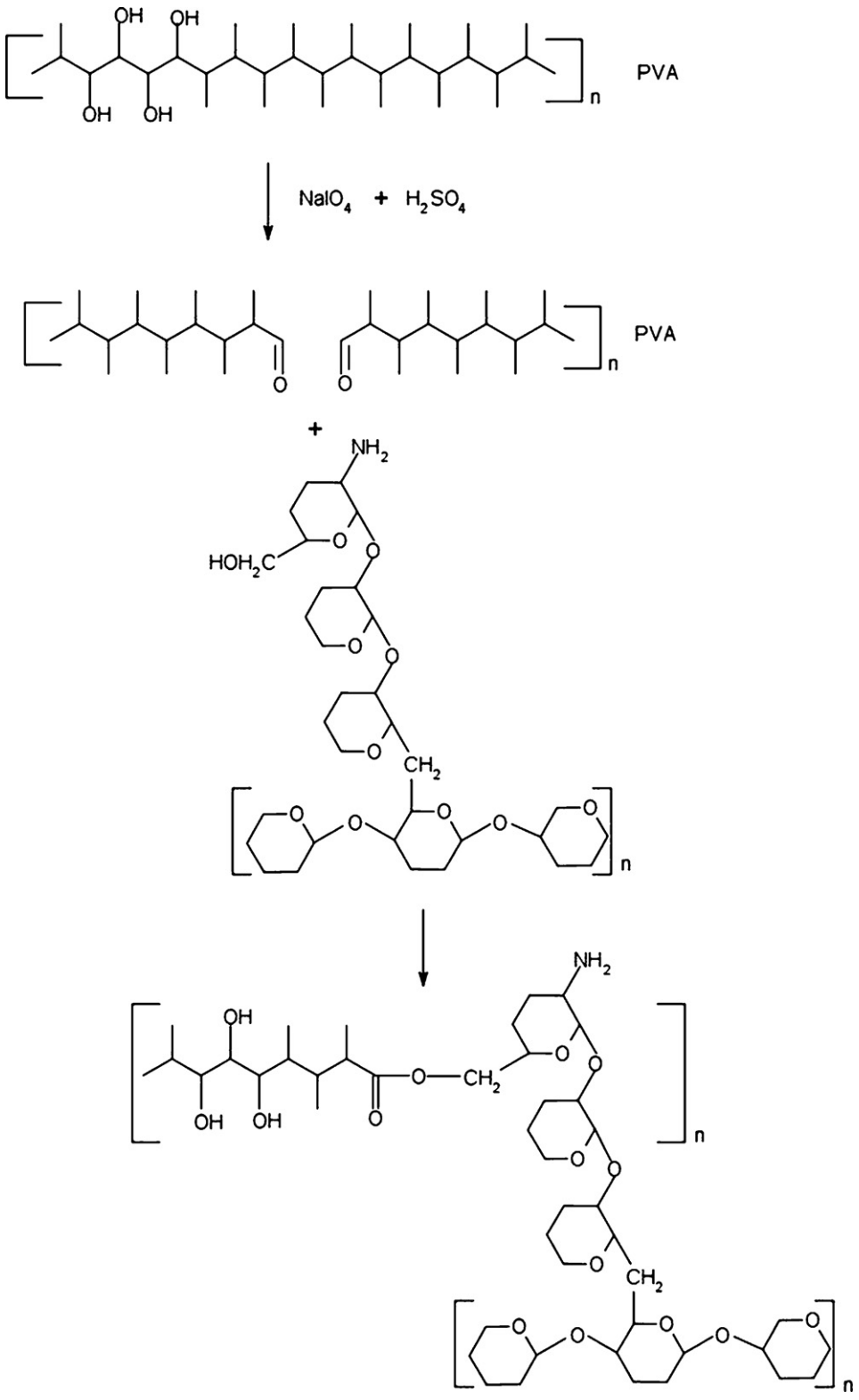

Fig. 2. Scheme representing the mechanism of CGP/PVA reaction.

2011). The water solubility is related to content of free hydroxyl groups in polymeric matrix, which allow the establishment of hydrogen interactions between film and water (McHugh & Krochta, 1994). High solubility is a desirable characteristic for biodegradable films because the increase in the solubility leads to increased biodegradability. According Bourtoom and Chinnan (2008), films presenting high solubility are advantageous in situations when these materials will be consumed with a product and may also be a relevant factor that determines the application as packaging wrap.

3.1.4. Soil burial biodegradability

Soil burial degradability of polymers is a critical parameter for their application. Soil burial test was carried out to evaluate the degradation of CGP/PVA film in natural environment. The soil burial

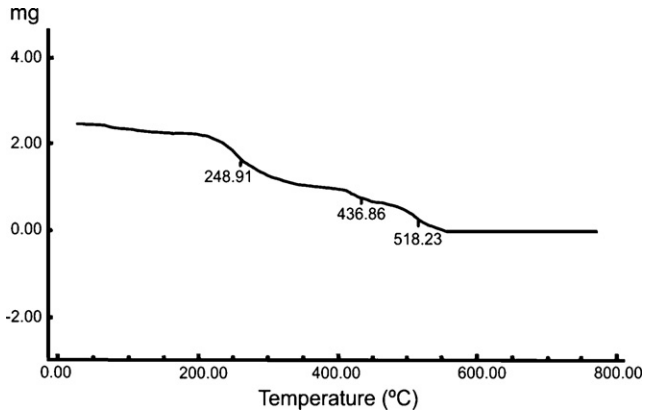

Fig. 3. TGA curve of CGP/PVA film under nitrogen at indicated heating rates.

test is an outdoor experiment that provides a realistic environment where soil humidity, temperature and microbial community are in less control and change with season.

As can be seen in Fig. 4, after 90 days the remaining CGP/PVA film presented 48% of initial weight. The diffusion of water into films resulted in swelling and allowed the growth of microorganisms. Enzymatic and non-enzymatic degradation are responsible by weight loss and disruption of the film samples. In addition, since both CGP and PVA had overall hydrophilic properties, the dissolution of CGP, PVA and their partially degraded products would be another factor related to weight loss.

3.1.5. Scanning electron micrograph (SEM)

Scanning electron micrographs (SEMs) of CGP/PVA are shown in Fig. 5. The SEM showed the formation of continuous film. The surface presented good structural integrity, without any pores or cracks. CGP are dispersed within PVA matrix of the film with very good interfacial adhesion between the two components.

3.2. Enzyme immobilization

In order to verify the capacity of CGP/PVA film as support for enzyme immobilization tests were conducted using *T*-CWDE. The immobilization tests were carried out by adsorption or covalent binding methodologies.

Table 2 shows the results of *T*-CWDE immobilization on CGP/PVA film. Immobilization via covalent binding was twice more efficient than via adsorption. The higher amount of immobilized *T*-CWDE via covalent binding may be due to the introduction of additional binding sites at the surface of CGP/PVA film produced by sodium metaperiodate treatment (Silva et al., 2011).

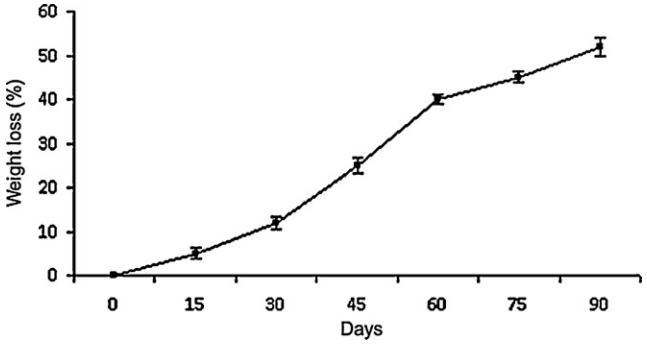

Fig. 4. Weight loss (%) of CGP/PVA film in soil burial test.

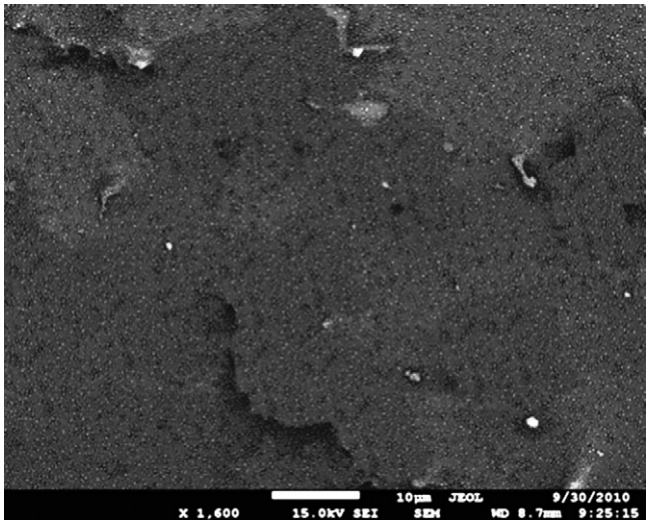

Fig. 5. SEM micrographs of PVA/CGP film.

3.3. Bioactivity of CGP/PVA film

Results for bioactivity tests of CGP/PVA film containing immobilized *T*-CWDE were showed in Fig. 6. The growth of all microorganisms was normal in control films, but markedly compromised in the presence of immobilized *T*-CWDE. Furthermore, pores were observed in the matrix of the film. This film degradation can be due to the activity of β→1,3-glucanases, enzymes capable to hydrolyze β→1,3 linkages present in the cashew gum.

As can be observed in Fig. 6IA, the spores of *A. niger* are germinating, and hyphae are spread in the film's surface. However, in CGP/PVA film containing immobilized *T*-CWDE, the spores initially inoculated did not germinated and was not observed hyphae formation (Fig. 6IB). *Penicillium* sp. was the lowest sensitive microorganism to the *T*-CWDE present in CGP/PVA film. In control CGP/PVA film was observed intense growth, with intense hyphae formation (Fig. 6IIA). Nevertheless, in the presence of *T*-CWDE, the germination of *Penicillium* spores was lower than in control film and the hyphae formation was less expressive. Moreover, it was observed some hyphae disruption (Fig. 6IIB).

In the case of *S. sclerotiorum*, an expressive hyphae growth was observed in the control film (Fig. 6IIIA). However, an evident decrease in hyphae growth was observed in the CGP/PVA film containing *T*-CWDE. Moreover, those few hyphae that were able to growth still showed severe structural damage (Fig. 6IIIB).

The different profiles of microbial growth may be related to cell wall composition of these fungi. Although different effects have been observed to each microorganism, the enzyme immobilization was effective in reducing proliferation in the film matrix. Additionally, the capacity of CGP/PVA film to successfully retain active *T*-CWDE enables it as a bioactive material very attractive to use in packaging. Other possible applications include coverage for fruits and vegetables, preservation of historical documents, coating cardboard boxes, and preservation of seeds.

Table 2
Amounts of *T*-CWD enzymes immobilized by absorption and covalent binding.

	Adsorption	Covalent binding
Total immobilized protein (mg)	$14.34 \pm 0.012^{*}$	$33.02 \pm 0.019^{**}$
Immobilized chitinase units (mU)	$198.32 \pm 0.018^{*}$	$394.68 \pm 0.011^{**}$
Chitinase activity (mU cm^{-2})	793.28*	1578.72**

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

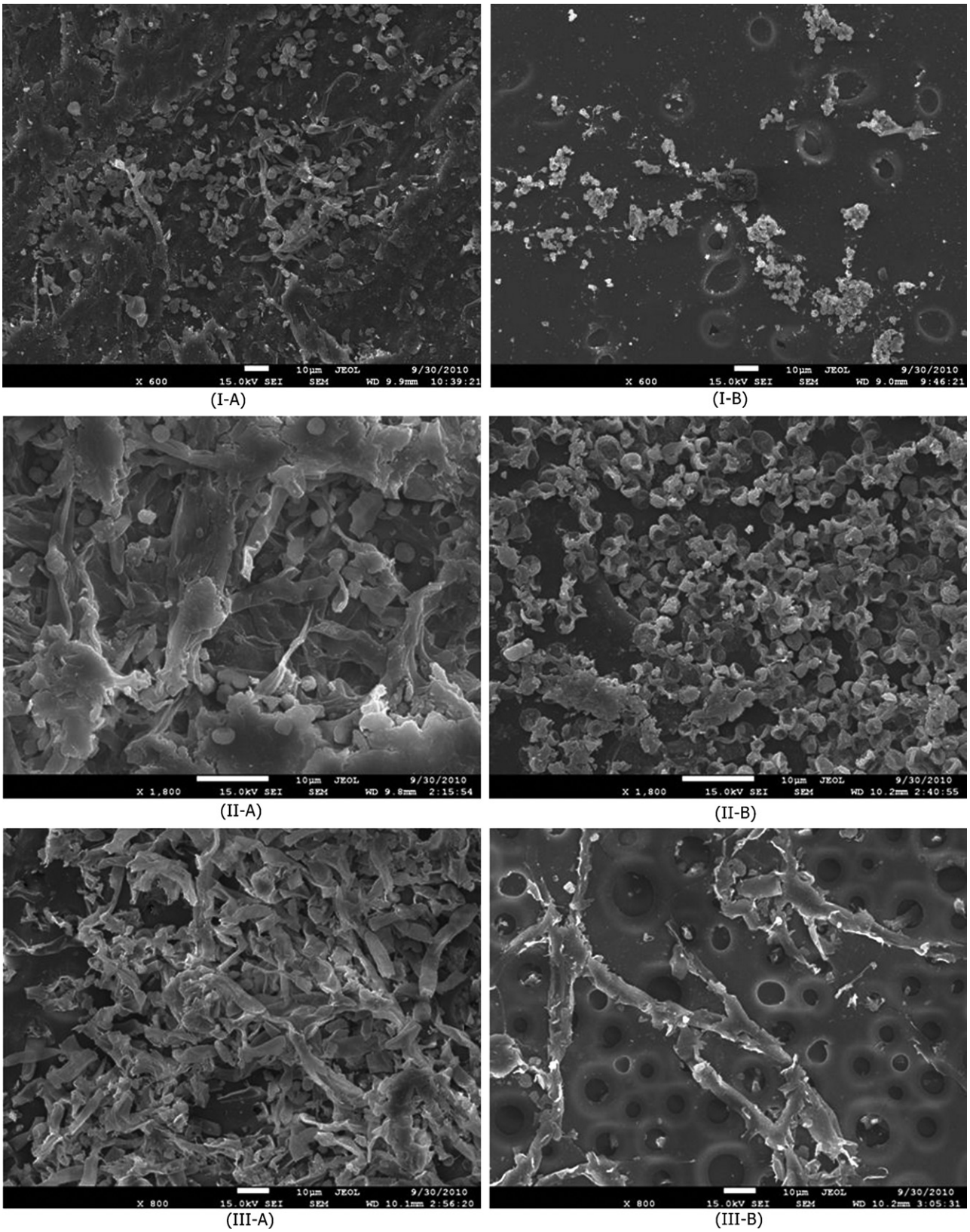

Fig. 6. SEM micrograph of microorganisms growing in the presence or absence of *T*-CWD enzymes. Lane A, control; lane B, CGP/PVA film with *T*-CWD enzymes immobilized by covalent binding. I, *A. niger*; II, *Penicillium* sp.; III, *S. sclerotiorum*.

Allied to bioactivity acquired by *T*-CWDE immobilization, CGP/PVA film presents mechanical properties and biodegradability very interesting, which enable this material as an alternative ecofriendly film.

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