RESEARCH LETTER

An anamorph of the white-rot fungus *Bjerkandera adusta* capable of colonizing and degrading compact disc components

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Abstract

A *Geotrichum*-like fungus isolated from a biodeteriorated compact disc (CD) was able to degrade *in vitro* the components of different CD types. The fungal hyphae inside the CD fragments grew through the aluminium layer and produced the solubilization of this metal. Furthermore, examination of CDs by scanning electron microscopy showed that the fungus was able to destroy the pits and lands structures grooved in the polycarbonate layer, confirming degradation of this aromatic polymer. The fungus secretes aryl-alcohol oxidase and Mn^{2+}-oxidizing peroxidase, two kinds of oxidoreductases characteristic of ligninolytic basidiomycetes. Analysis of the ITS region of ribosomal DNA, as well as the morphological characteristics, the lack of sexual forms and the profile of enzymes secreted in liquid medium identified the fungus as a *Geotrichum*-like anamorph of *Bjerkandera adusta* (Willd.) P. Karst.

Introduction

Compact discs (CDs) are among the most popular optical supports used to store digital data. Audio CDs (CD-A) were first developed for storing digital music. These CDs are made from a disc of polycarbonate (~1.2 mm), usually poly(bisphenol A carbonate), with a 12 cm diameter, where the information is grooved as binary information (as pits and lands), coated with a thin layer of a reflective metal, usually aluminium (~50–100 nm), a film of lacquer (~10 μm) protecting the metal from the external environment and, on the surface, an optional labelling ink (Harley *et al.*, 1988). Recordable CDs (CD-R) show similar structure but they contain the data-recording layer between the poly(bisphenol A carbonate) and the metal layer. This layer consists of an organic dye (cyanine, azo, or phthalocyanine dyes) that can be modified by a laser. The new digital supports, named digital versatile discs (DVD), present a structure with similar components because they are made of two discs of poly(bisphenol A carbonate).

The poly(bisphenol A carbonate) is essentially an amorphous polymer of 2,2-bis(4-hydroxyphenyl)propane linked by carbonate ester linkages. CDs and DVDs utilize the transparency, low birefringence, and mechanical and dynamic stability properties of this aromatic polymer. In addition to the optical media formats, there are many other important products based on this thermoplastic substrate, such as eyeglass lenses, medical devices, packaging materials, etc. Some studies have reported the thermal degradation of poly(bisphenol A carbonate) into low-molecular-weight compounds (Jang & Wilkie, 2004) but little is known about its biological degradation.

Recently a fungus was isolated from a biodeteriorated CD-A (stored at ~30 °C, ~90% humidity) (García-Guinea *et al.*, 2001), found in Belize on the western Caribbean coast. The analysis of the CD-fragments by transmitted and reflected light microscopy, X-ray diffraction, and scanning electron microscopy (SEM) revealed important alterations in the metal and polycarbonate layers, in zones where fungal hyphae were detected. From different surface-sterilized CD-fragments, a unique fungus was isolated and reported as a *Geotrichum* Link species based on its morphological characteristics, the cell wall polysaccharide composition and lack of sexual forms (García-Guinea *et al.*, 2001).

In this work, the ability of this fungus to degrade *in vitro* the different CD-components, the enzymatic activities secreted in liquid medium, and its identification by molecular

techniques are reported. The possibility of using this fungus in different environmental applications is discussed.

Materials and methods

Fungal strain and culture media

The fungus isolated from the deteriorated CD-A from Belize (García-Guinea *et al.*, 2001) has been deposited in the IJFM culture collection of the 'Centro de Investigaciones Biológicas' (Madrid, Spain) with the reference A757.

The *in vitro* degradation experiments were carried out on 2% malt extract agar (w/v) Petri dishes, homogeneously colonized by the fungus (28 °C), and glucose–peptone–yeast extract liquid medium (Kimura *et al.*, 1990) (28 °C, 150 r.p.m.). All experiments were performed using at least three replicates.

Identification of the fungus by sequencing the ITS region

For DNA extraction, the fungus was grown in a 250 mL flask containing 50 mL of glucose–peptone–yeast extract medium for 5 days. Mycelium was harvested by vacuum filtration, washed with sterile Milli-Q water, and lyophilized. Then 2.5 mL of HSE solution (10 mM HEPES, pH 6.9, 0.5 M sucrose and 20 mM EDTA, pH 8.0) and 250 μL of 10% sodium dodecyl sulphate were added to 0.1 g of the powdered mycelium. After incubation at 65 °C for 15 min, 2.5 mL of TEA solution (50 mM Tris-HCl and 20 mM EDTA, pH 8.0) were added, followed by three extractions with 5 mL of phenol/chloroform/isoamyl alcohol (25 : 24 : 1). Then a final chloroform/isoamyl alcohol (24 : 1) extraction was made. 0.5 mL of 3 M sodium acetate and 3 mL isopropanol were added to the remaining aqueous phase and the mixture stored at room temperature for 30 min. The precipitate was collected by centrifugation (12 000 ***g***, 10 min, 4 °C), washed with ethanol (70%), dried, and dissolved in TEB solution (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). RNAase A (0.1 μg μL^{-1}) was added to the suspension and incubated at 37 °C for 30 min. Then, 0.2 mL of 3 M sodium acetate and 1.2 mL isopropanol were added and the mixture centrifuged (5000 r.p.m., 5 min, 4 °C). The pellet was washed with ethanol (70%), desiccated, and dissolved in sterile Milli-Q water (González *et al.*, 1992).

The extracted DNA was used as the template in a PCR to amplify the ITS1 and ITS2 regions and the 5.8S rRNA gene. The primers used for the amplification were ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCG CTTATTGATATGC-3′) (White *et al.*, 1990). PCR was performed in 1 × PCR amplification buffer (Applied Biosystems) with 1 mM $MgCl_2$ (Applied Biosystems), 1 μM of each primer, 50 μM of each deoxynucleoside triphosphate (Promega), 0.2 μg of DNA template, and 1.2 U of Taq DNA polymerase (Applied Biosystems), in a final volume of 50 μL, using a GeneAmp PCR System 2400 (Perkin Elmer). Cycling parameters were 95 °C for 3 min followed by 35 cycles of 94 °C for 1 min, 52 °C for 40 s, and 72 °C for 1 min, and the final extension at 72 °C for 10 min. Control reactions lacking template DNA were performed in parallel. Amplified fragments were visualized on 1% agarose gels stained with ethidium bromide and subsequently purified using a GeneClean kit (Q-BIOgene). Products were then sequenced using the two PCR primer, the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems), and the automated ABI Prism 3730 DNA sequencer (Applied Biosystems). The sequence was compared by BLAST search (Altschul *et al.*, 1997) in GenBank.

Reproduction of CD alterations

The CDs were commercially available (CD-A or CD-R) or obtained at different stages of the manufacturing process (CD-A), kindly provided by a CD manufacturer (Madrid, Spain). The recording layer of the CD-R was a phthalocyanine-type dye. The CDs were cut into suitable pieces using a manual paper cutter. These pieces were sterilized at 110 °C for 30 min (for liquid-medium experiments), or immersed in 70% (v/v) ethanol for 3 min (for experiments on agar-malt plates).

The malt agar plates were inoculated with two plugs of mycelium and, after 10 days of incubation at 28 °C, the CDs (complete CD-A or CD-R) were deposited with the lacquer layer in contact with the agar surface. The lacquer layer was previously scratched in different zones to accelerate the fungal alteration process. Incubation was performed in a closed chamber at room temperature (22–24 °C) and with ~90% humidity. The same conditions were used to incubate fragments of CD-A (cracked in eight parts) at different stages of manufacture: (1) polycarbonate grooved, (2) polycarbonate grooved and aluminium, and (3) polycarbonate grooved, aluminium, and lacquer. Controls on uninoculated plates were carried out in parallel. Progression of degradation was monitored every week and photographed with a digital camera (Kodak DC290).

Studies of polycarbonate degradation by SEM

Fragments (~2.5 mm × 2.5 mm × 1 mm) of CDs were incubated in 250 mL flasks containing 50 mL of the glucose–peptone–yeast extract medium. The flasks were inoculated with homogenized 7-day-old precultures and incubated at 28 °C in a rotary shaker at 150 r.p.m. These fragments were obtained using a manual paper cutter from commercial CD-A (complete CD), or CDs in the first stage of production (grooved polycarbonate fragments). Uninoculated flasks were used as controls.

Samples were taken after 1 and 2 months of incubation. The liquid was separated from the mycelium and CD fragments by decantation. CD samples were collected with tweezers, fixed to an aluminium stub using double-sided carbon tape, dried under vacuum (1 Torr), and sputter coated with gold using a SC510 sputter machine (Bio-Rad) for 1 min. SEM images were obtained with a FEI Quanta 200 environmental microscope using a secondary electron detector operating at high vacuum, with an accelerating voltage of 27 kV, and at a working distance of 9.6 or 9.8 mm.

Studies of aluminium solubilization by inductively coupled plasma optical emission spectrometry (ICP-OES)

Aluminium content was determined in the CD-fragments incubated in liquid cultures under the conditions described previously. Controls were carried out at the pH of the culture (pH 7 and 8). The CD fragments (~2.5 mm × 2.5 mm × 1 mm), polycarbonate grooved with the aluminium layer or including the lacquer layer, were added after 10-day incubation.

The liquid, mycelium, and pieces of CD were separately collected after 20 days, as described previously, and lyophilized. The mycelium was hydrolysed with 6 N HCl (65–70 °C for 4 h) and, after acid evaporation on a boiling water bath, the residue was dissolved in 10% nitric acid. The liquid culture and the CD fragments were resuspended directly in 10% nitric acid. After overnight incubation at room temperature, all the samples were diluted to 1% nitric acid with distilled water, centrifuged (12 000 ***g***, 10 min), filtered (7–9 μm), and the aluminium determined in each fraction by ICP-OES using an Optima 4300 DV Perkin Elmer system.

Detection of organic acids by HPLC

Organic acids in the liquid cultures were analysed by HPLC with a Supelcogel C-610H column (Sigma-Aldrich) at 30 °C. Phosphoric acid (15 mM) was used as the mobile phase at a flow rate of 0.5 mL min^{-1} and the compounds were monitored at 210 nm. Standards consisting of various organic acids were used.

Enzyme assays, proteins, and reducing sugars

Production of extracellular enzymes was studied in the glucose–peptone–yeast extract medium under the conditions described above. The cultures were supplemented with 500 μM $MnSO_4$ to study its effect on peroxidase production. Samples were taken daily from three replicate flasks and the mycelium separated from the culture liquid by centrifugation at 11 337***g*** and 4 °C for 10 min.

Aryl-alcohol oxidase (AAO) activity was measured spectrophotometrically by monitoring the oxidation of 10 mM veratryl (3,4-dimethoxybenzyl) alcohol to veratraldehyde ($\varepsilon_{310} = 9300\ M^{-1}\ cm^{-1}$) in 100 mM phosphate buffer (pH 6) (Guillén *et al.*, 1992). Laccase activity was determined by 10 mM 2,6-dimethoxyphenol oxidation to coerulignone, in 100 mM acetate buffer, pH 5 ($\varepsilon_{469} = 27\,500\ M^{-1}\ cm^{-1}$, referred to substrate) (Muñoz *et al.*, 1997). Mn^{2+}-oxidizing peroxidase activity was determined by the formation of Mn^{3+}-tartrate complexes ($\varepsilon_{238} = 6500\ M^{-1}\ cm^{-1}$) during the oxidation of 0.1 mM $MnSO_4$ in 100 mM tartrate buffer (pH 5) in the presence of 0.1 mM H_2O_2 (Martínez *et al.*, 1996). Lignin peroxidase activity was measured using 2 mM veratryl alcohol in 100 mM tartrate buffer (pH 3) supplemented with 0.4 mM H_2O_2 (Tien & Kirk, 1984). Controls without H_2O_2 were included. Esterase activity was assayed by *p*-nitrophenol ($\varepsilon_{346} = 4800\ M^{-1}\ cm^{-1}$) release from *p*-nitrophenyl butyrate (*p*NPB) in 100 mM Tris-HCl buffer (pH 7) (Calero-Rueda *et al.*, 2002). All enzyme assays were performed at room temperature. The enzymatic activities were expressed as international units (U), defined as the amount of enzyme that releases 1 μmol product min^{-1}.

Protein concentration was determined by the method of Bradford (1976) and reducing sugars by the method of Somogyi–Nelson (Somogyi, 1945) using albumin and glucose as standards, respectively.

Results and discussion

Reproduction of the fungal CD alteration

The isolated *Geotrichum*-like fungus was studied here to analyse *in vitro* its capacity to colonize different CD types (CD-A and CD-R) in malt agar plates. After 6 months of incubation, at room temperature and high humidity, the biodeterioration path in the CD-A was clearly visible to the naked eye (Fig. 1a), similar to that reported in the original CD found in Belize (García-Guinea *et al.*, 2001). The fungus grew through the aluminium layer from the fractures that were produced in the lacquer layer to accelerate the alteration process.

In the case of CD-R the degradation signs appeared earlier. Figures 1b and c show a CD-R after 3 months of incubation. In these CDs, the access was also facilitated by fractures in the lacquer layer. In the area colonized by the fungus, mobilization of the reflective metal and removal of the phthalocyanine dye present in the data-recording layer were observed. Synthetic dyes are widely used in several industries and about 10–15% of these dyes are found in wastewaters (Spadaro *et al.*, 1992). It has been reported that basidiomycetes fungi, including different *Bjerkandera* P. Karst. species, are able to degrade dyes and can play an important role in different biotechnological applications related to wastewater treatment (Heinfling *et al.*, 1997; Moreira *et al.*, 2000). These fungi have an unspecific system, including oxidoreductases, low molecular mass metabolites,

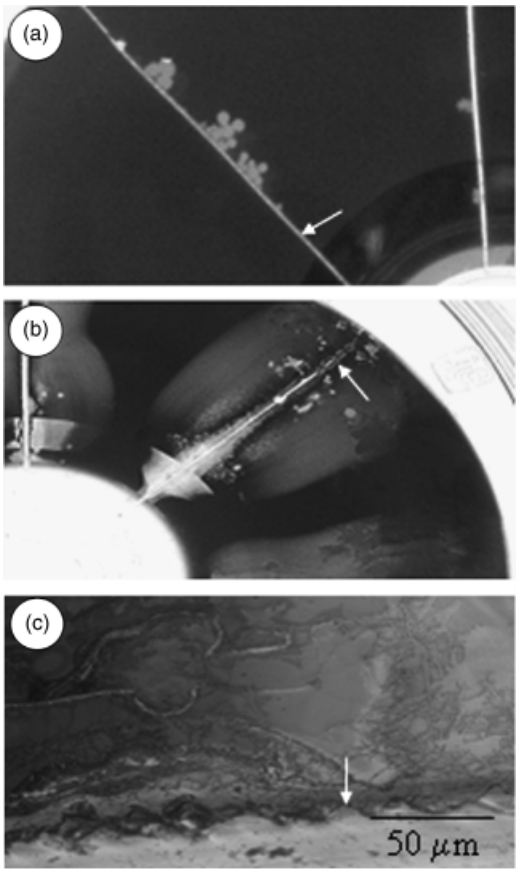

Fig. 1. Different types of CDs showing degradation paths after incubation on malt agar plates colonized by the *Geotrichum*-like fungus. (a) CD-A after 6 months of incubation. (b) CD-R after 3 months of incubation. (c) Detail of the colonized area in a CD-R analysed by phase-contrast microscopy (× 100) showing fungal hyphae. Arrows indicate scratches made to accelerate the infection process.

and activated oxygen species involved in these degradation processes (Schoemaker *et al.*, 1991; Reddy, 1995).

Similar degradation ways have been found in CDs from other tropical countries although the responsible organisms have not been studied (García-Guinea *et al.*, 2001). High humidity and temperatures could be the key factors in the biodeterioration process and good conservation of these CDs could avoid these damages.

Polycarbonate degradation

An increasing volume of plastic waste is generated by industry and households. Therefore, there is a growing interest in the study of the biodegradation of these polymers to overcome their environmental problems (Shimao, 2001; Kim & Rhee, 2003). Here, the biodegradability of the synthetic polyester poly(bisphenol A carbonate) was examined, using the *Geotrichum*-like fungus. For this, the fungus was grown in liquid medium supplemented with fragments of CDs obtained in the first stage of production (grooved polycarbonate fragments), to examine the changes in the pits and lands structures.

SEM analysis of the fragments surface at different incubation times revealed fungal growth and progressive degradation of the polycarbonate layer. Figure 2 shows the time course of the process using CD-A in the first stage of manufacture (grooved polycarbonate). After 1 month of incubation, the pits moulded into the top of the polycarbonate layer appeared damaged, and completely disappeared after 2 months of fungal treatment. No changes were observed in the fragments incubated in the uninoculated control flasks. These results show clear evidence on polycarbonate degradation by the fungus, and open new perspectives for the biodegradation of this material. Although more studies are necessary to know the degradation mechanisms, the results obtained could contribute to the development of new strategies for the biological treatment of polycarbonate wastes.

Aluminium solubilization

The capacity of the isolated fungus to mobilize aluminium from the CDs in both solid and liquid culture was studied. In this case, CD fragments were used in the second and third stages of production (i.e. grooved polycarbonate covered with aluminium, and either with or without a protective lacquer layer). Figure 3 shows the progressive disappearance of the reflective metal with the incubation time in solid medium. The first signs appear after 15 incubation days from the border, the metal practically disappearing after 2 months of incubation. The deterioration process was similar in the fragments with the lacquer, although in this case the process was delayed because the lacquer protected the metal against deterioration.

In the liquid cultures small fragments of the same material were added after 10 incubation days. At this time the pH of the culture was around 7 and it was slightly modified by the fungus during the subsequent incubation (pH 7–8). After 7 days of incubation the aluminium started to disappear from the fragment surface, especially in the fragments without lacquer. After 20 days of incubation, the presence of the metal in the fragments, fungal mycelium, and liquid culture was analysed by ICP-OES. Less than 10% of the metal remained in the fragments, the rest being detected in the culture liquid and mycelia (around 35% and 55%, respectively). In the case of fragments with lacquer, it was observed that the aluminium disappeared more slowly (69% remained in the fragments, and 12% and 19% were in the mycelia and liquid, respectively). No metal solubilization was detected in the controls when the pH was maintained among 7–8 (the pH of the fungal culture). Different organic acids, such as acetic, citric, and oxalic,

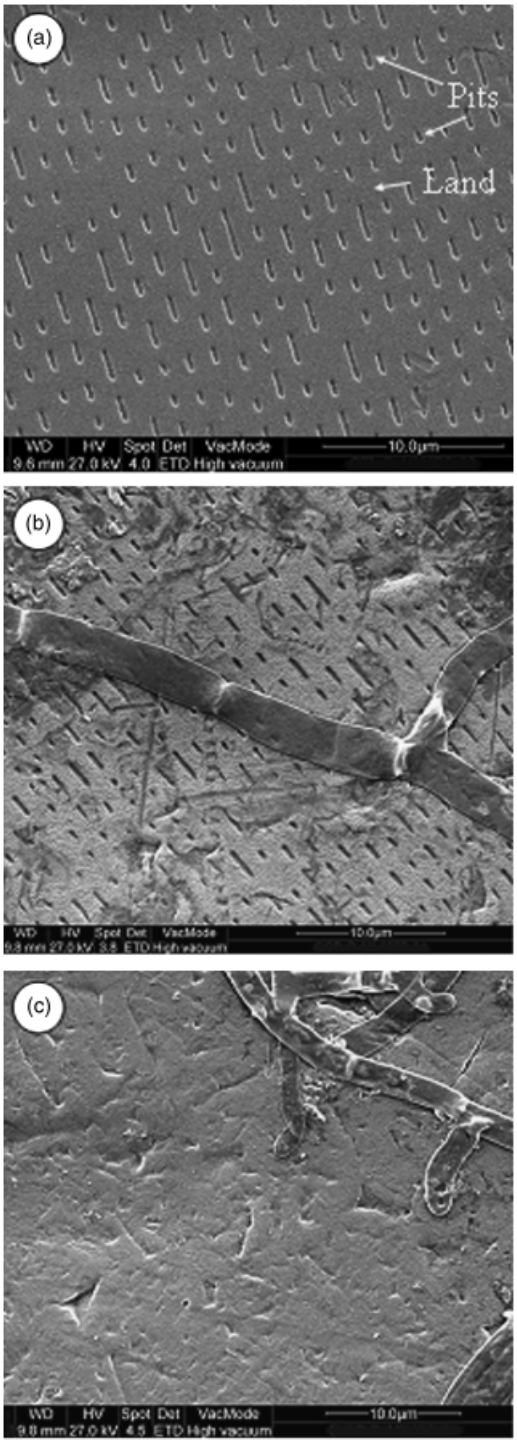

Fig. 2. SEM images of the *Geotrichum*-like fungus growing on grooved polycarbonate CD fragments and progressive deterioration of pits and lands structures. (a) Control. (b) Fungal hyphae and pits and lands after 1 month of incubation. (c) Disappearance of these structures after 2 months of incubation.

were detected by HPLC in 30-day-old cultures, in the presence or absence of CD fragments. These compounds are also secreted by other basidiomycetes (Dutton *et al.*, 1993) and it has been reported that they are involved in the tolerance of these organisms to metals (Bellion *et al.*, 2006). The presence of aluminium in the mycelium can be explained because fungi present detoxification mechanisms such as metal sequestration at the cell wall or intracellular chelation (Bellion *et al.*, 2006).

Aluminium recovery from CDs is economically unattractive and energy consuming, because the amount of this metal is very small (a few grams per tonne of discs). However, recycling of poly(bisphenol A carbonate) is economically valuable and this process includes removal of the coatings from this polymer. There is a variety of chemical and mechanical methods for CD recycling (e.g. chemical recovery, melt filtration, and mechanical abrasion) but each technique has some disadvantages in terms of environment or energy (Zevenhoven & Saeed, 2002). The ability of this fungus to remove aluminium, lacquer, and printing could provide an environmental-friendly alternative for its application in the stage of separation of coatings from the polycarbonate polymer.

Enzymatic activities

Extracellular laccase, peroxidase, and AAO activities were determined in glucose–peptone–yeast extract fungal cultures. Reducing sugars reached basal levels after 7 days of incubation (Fig. 4) and total proteins attained the highest level at the same time (data not shown). Figure 4 summarizes the time course of the enzymes during 14-day incubation time. Under these conditions, the fungus only produced appreciable amounts of AAO (up to 400 mU mL^{-1}). The Mn^{2+}-oxidizing peroxidase activity (up to 140 mU mL^{-1}) only was detected in the cultures supplemented with $MnSO_4$, but this cation did not affect AAO activity. The fungus did not produce either lignin peroxidase or laccase in any of the conditions assayed. All the above enzymes are involved in the degradation of lignin and other recalcitrant aromatic compounds (Schoemaker *et al.*, 1991). It has been reported that Mn^{2+}-oxidizing peroxidases play an important role in the degradation of dyes and aromatic pollutants (Heinfling *et al.*, 1998a; Rodríguez *et al.*, 2004). AAO is an enzyme involved in the production of the extracellular H_2O_2 required as cosubstrate by peroxidases (Guillén *et al.*, 1994). Mn^{2+}-oxidizing peroxidases have been reported in most ligninolytic basidiomycetes (Hatakka, 1994; Peláez *et al.*, 1995) but extracellular AAO has been only detected in species of *Pleurotus* (Fr.) P. Kumm. and *Bjerkandera* (Varela *et al.*, 2000).

In addition, low levels of esterase activity were detected in the *Geotrichum*-like cultures using *p*NPB as substrate (Fig. 4).

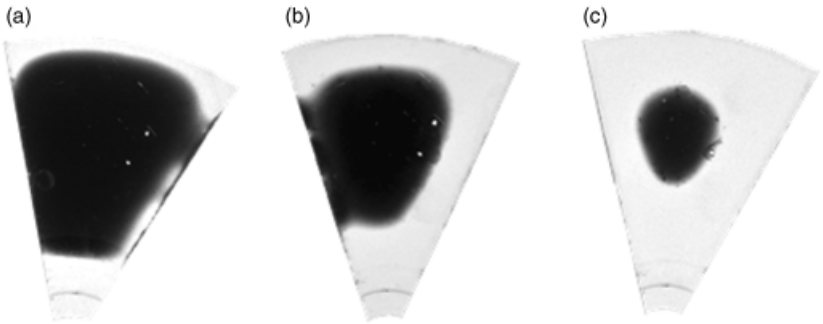

Fig. 3. Progressive solubilization of the aluminium in the CD-A fragments without the protective layer after 15 (a), 30 (b), and 60 (c) days of incubation on an agar plate.

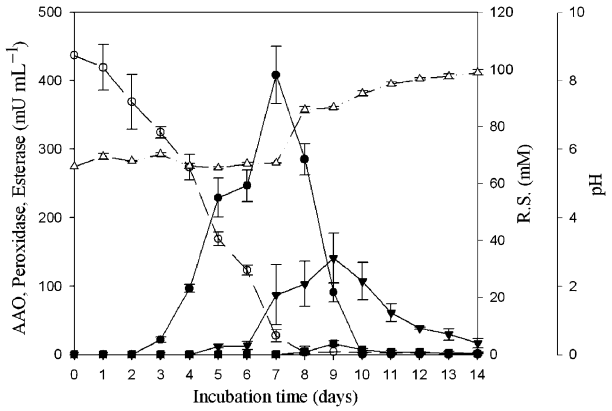

Fig. 4. Time courses of AAO (●), peroxidase (▼), and esterase (■) activities, reducing sugars (RS) (○), and pH (△) in the extracellular fluid of cultures of the *Geotrichum*-like fungus in glucose–peptone medium containing manganese (means and 95% confidence limits).

Recently, the role of different lipases in the degradation of poly(bisphenol A carbonate) has been reported (Sivalingam & Madras, 2004). These enzymes are induced by the presence of olive oil (Calero-Rueda *et al.*, 2002) and further studies using several inducers will contribute to characterize the enzyme produced by the *Geotrichum*-like fungus and its role in the degradation of poly(bisphenol A carbonate).

Identification of the fungus

In a previous report, the strain isolated from the deteriorated CD was identified as a *Geotrichum*-like fungus on the basis of morphological characteristics such as fast growing, white dry colonies, hyaline hyphae without clamp connections, and cylindrical conidia, together with cell wall polysaccharide composition, and lack of sexual forms (García-Guinea *et al.*, 2001).

To complete the identification of this fungus, the sequences of the ribosomal DNA including ITS1, 5.8S, and ITS2 regions were used, because this area has been identified as a suitable target for analysis of fungal phylogeny (White *et al.*, 1990). A 604-bp fragment was amplified by PCR using the ITS1/ITS4 primers. This sequence, deposited in GenBank with the accession number EF441742, showed 99% identity with the ITS region of several isolates deposited as *Thanatephorus cucumeris* (A.B. Frank) Donk (accession numbers AF455463, AF455459, AF455445, AF455438), two unclassified basidiomycetes (accession numbers AF455454, AJ279471), and several strains of *Bjerkandera* sp. and *B. adusta* (Willd.) P. Karst (accession numbers DQ060096, DQ060095, AY633927, AY089741). *T. cucumeris*, the teleomorph of *Rhizoctonia solani* J.G. Kühn, is a soilborne plant pathogen characterized by brownish hyphae, absence of conidia, and formation of basidiospores and sclerotia (Sneh *et al.*, 1991). According to these features the isolate from the CD cannot be included in this species. However, the morphology of the isolated fungi is similar to the anamorphic state of *Polyporus adustus* (Willd.) Fr. (= *B. adusta*), which shows a *Geotrichum*-like conidial state (Barnett & Hunter, 1998). In the same way, other authors identified isolates of *Bjerkandera* sp. as a *Geotrichum*-like fungi (Wirsel *et al.*, 2001; Kornillowicz-Kowalska *et al.*, 2006). On the other hand, the taxonomic position of the above *T. cucumeris* isolates should be verified.

Another aspect that confirms the identification of the fungus isolated from the Belize biodeteriorated CD is the enzymes secreted in liquid medium. AAO and Mn-oxidizing peroxidase have previously been detected in different *Bjerkandera* species (Muheim *et al.*, 1990; Heinfling *et al.*, 1998b; Palma *et al.*, 2000). Furthermore, laccase, which was not detected in the fungus isolated from the CD, is present in most white-rot basidiomycetes but it has never been reported in *Bjerkandera* species.

Conclusion

It can be concluded that the *Geotrichum*-like fungus isolated from the Belize-deteriorated CD is an anamorph of *B. adusta*. This strain can colonize different CD types

and destroys the information kept in these optical supports since it is able to solubilize the aluminium present in the reflective layers and also degrades the polycarbonate support where the data are grooved as well as the phthalocyanine dye layer. The purification and characterization of the enzymes secreted by this fungus, currently underway, will contribute to clarify their role in the degradation of different CD components.

To avoid CD and DVD biodeterioration, it is necessary to protect these informatics supports. Humidity and temperature are important factors to be taken into account to extend their half-life together with a high quality control during the manufacture to remove the defective products. Finally, the use of this fungus in removing coatings from old compact discs to recover the polycarbonate should be considered in the future although the process conditions must be established to minimize loss of this valuable polymer.

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