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Research Article

High density polyethylene (HDPE) biodegradation by the fungus *Cladosporium halotolerans*

Michela Di Napoli[1], Brigida Silvestri[2], Giusy Castagliuolo[1], Andrea Carpentieri[3], Giuseppina Luciani[2], Antimo Di Maro[4], Sergio Sorbo[5], Alessandro Pezzella[6], Anna Zanfardino[1,*,†], Mario Varcamonti[1,*,†]

[1]Department of Biology, University of Naples Federico II, Via Cintia, 80126 Naples, Italy
[2]Department of Chemical, University of Naples Federico II, Materials and Production Engineering, Via Cintia, 80126 Naples, Italy
[3]Department of Chemical Sciences, University of Naples Federico II, Via Cintia, 80126 Naples, Italy
[4]Department of Environmental, University of Campania 'Luigi Vanvitelli', Biological and Pharmaceutical Sciences and Technologies (DiSTABiF), Via Vivaldi, 81100 Caserta, Italy
[5]University of Naples Federico II, CeSMA, Via Cintia, 80126 Naples, Italy
[6]Department of Physics "Ettore Pancini", University of Naples Federico II, Via Cintia, 80126 Naples, Italy
*__Corresponding author:__ Department of Biology, University of Naples Federico II, Via Cintia, 80126 Naples, Italy, Tel.: +39-081-679039-133; Fax: +081-679233; E-mail: anna.zanfardino@unina.it & varcamon@unina.it
[†]These authors contributed equally to this work and they are co-corresponding authors.
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Abstract

Polyethylene (PE) is high molecular weight synthetic polymer, very hydrofobic and hardly biodegradable. To increase polyethylene bio-degradability it is very important to find microorganisms that improve the PE hydrophilic level and/or reduce the length of its polymeric chain by oxidation. In this study, we isolated *Cladosporium halotolerans,* a fungal species, from the gastric system of *Galleria mellonella* larvae. Here, we show that *C. halotolerans* grows in the presence of PE polymer, it is able to interact with plastic material through its hyphae and secretes enzymes involved in PE degradation.

Keywords: bioremediation, *Cladosporium halotolerans*, enzymatic assay, Fourier transform infrared spectroscopy, high density polyethylene degradation, oxidoreductase

Introduction

Polyethylene is composed of ethylene monomers ($CH_2 = CH_2$) and it is the most common polymer used in the plastic industry. In the 1950s, Karl Ziegler polymerized ethylene in the presence of various metals forming different organized structures. For instance, HDPE is mainly composed of linear polyethylene polymers producing a narrow, dense and organized structure. Since Polyethylene is a hydrophobic and high molecular weight synthetic polymer, it is very difficult to biodegrade (Montazer et al. 2020). Its use in disposal production or packaging materials causes deleterious environmental accumulation (Krupp and Jewell 1992). For example it can cause, like other types of plastics, intestinal blockage of marine birds and mammals (Spear et al. 1995, Seechi and Zarur 1999). To make it biodegradable, PE requires a modification of its crystalline structure, molecular weight and of the mechanical properties responsible for PE resistance to degradation (Albertsson et al. 1994). This can be achieved by improving the hydrophilic level of PE and/or reducing the length of its polymeric chain by oxidation such that it becomes accessible for microbial degradation (Bikiaris et al. 1999). This degradative process starts by the secretion of enzymes which can either directly convert PE in oligomeric/monomeric units or add oxygen moieties to the C-backbone. Catabolism of the oligomeric units leads to the progressive enzymatic dissimilation of the macromolecules from the ends of the chain. Finally, those oligomers will be short enough to be used by microorganisms (Lau et al. 2009) to produce final compounds, such as CO_2 and H_2O (Lucas et al. 2008). Several studies reported PE biodegradation using fungal isolates, such as *Phanerochaete chrysosporium*, *Aspergillus niger* (Orhan and Buyukgungor 2000, Manzur et al. 2004), other *Aspergillus* species including *A. terreus, A. fumigatus* (Zahra et al. 2010), and *A. flavus* (El-Shafei et al.1998) and *Acremonium, Fusarium, Penicillium, Phanerochaete* (Restrepo-Flórez et al. 2014, Zhang et al. 2020). In more recent studies, it has been shown that many fungal strains isolated from lakes have the ability to degrade polyurethane (Brunner et al. 2018), while others isolated from the ocean, such as *Zalerion maritimum,* have the ability to degrade polyethylene as well (Zeghal et al. 2021). Thus, fungi play a very important role in plastic biodegradation: they secrete not only degrading enzymes (i.e. cutinases, lipases, and proteases, lignocellulolytic enzymes) but also pro-oxidant ions to improve the degradation process. This enzymatic oxidation and/or hydrolysis creates functional groups that improve the hydrophilicity of plastic polymers, and consequently allow their degradation low molecular weight oligomers (Srikanth et al. 2022). Moreover, several larvae of insect species, most notably coleopterans and lepidopterans, were found to possess the remarkable ability to consume and degrade different polymers (Yang et al. 2014, Chalup et al. 2018, Kundungal et al. 2019). For example, *Plodia interpunctella* (Indian mealworms) can consume polyethylene and harbours several polyethylene- degrading intestinal bacteria (Yang et al. 2014). Similarly, the larvae of *Tenebrio molitor* (yellow mealworm) can ingest and metabo-

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lize polyethylene and polystyrene (Yang et al. 2015, Brandon et al. 2018). Interestingly, polyethylene biodegradation seems to be dramatically accelerated *in vivo*, inside the insect host, as opposed to when microbial species are isolated and grown *in vitro* (Yang et al. 2015). Therefore, it is likely that the accelerated breakdown of plastics in insect larvae is a complex process that is interdependent on both the microbiome and its host. Recently, Bombelli et al. (2017) described the extraordinary capacity of caterpillar larvae of the greater wax moth, *Galleria mellonella*, to ingest and degrade polyethylene at unprecedented rates. Although *G. mellonella* does not actively plump on plastics in its natural environment, it is a ubiquitous pest of apiaries (Kwadha et al. 2017). Similar to polyethylene, the structure of honeycomb is rich in long aliphatic chains (Dadd 1966, Kong et al. 2019). Therefore, it is possible that symbiotic gut biome provides to caterpillar set of physiological adaptations to derive energy from such a complex diet (Bombelli et al. 2017, Kong et al. 2019). In the beeswax the most frequent hydrocarbon bond is C-C and C-H, as in PE. Further investigation is required to elucidate the details of wax biodegradation and it seems likely that single C-C bonds of these aliphatic compounds are targets for digestion. The aim of the present study was to isolate new PE-degrading microorganisms from the *Galleria mellonella* larvae gut content and identify their potential PE-degrading mechanism. Here, we describe a new strain of *Cladosporium halotolerans*, isolated from larvae intestine and capable of degrading HDPE microplastic particles.

Materials and methods

Isolation, screening and identification of intestinal microorganisms from galleria mellonella larvae

Small, medium and large size larvae were taken from a contaminated beeswax frame and surface sterilized by 70% ethanol and aseptically dissected to extract their guts. The guts were transferred into 1.5 ml conical microcentrifuge tubes with 0.2 ml sterile distilled water (SDW) with 0.05% Tween 80 and homogenized with a tight hand homogenizer for 1 min, then 0.8 ml SDW with 0.05% Tween 80 was added to each tube. Serial dilutions of the homogenate were made and streaked onto nutrient agar Luria Bertani (LB) to estimate the number of bacteria or fungi cells in the samples. Plates were incubated at 30°C for 48 h. The formed colonies were streaked on agar medium in the presence of beeswax (Sigma-Aldrich- 243248) used as single carbon source and on agar medium in the presence of polyethylene with density of 0.955 g cm^{-3} (5502LW, China Petrochemical Corporation, Maoming). Plates were incubated at 30°C for 7–10 days. One specific colony, able to grow on both plates, was isolated. Morphological features were determined using macroscopic and microscopic examinations (Watanabe 2002). Molecular identification was performed at the BMR genomics s.r.l. by PCR amplification and sequencing of the rRNA operon including the rRNA 5.8S gene and the flanking internal transcribed spacers (ITS). *Cladosporium halotolerans* was identified using BLAST N software, since the rRNA sequence showed about 99% identity with this fungal strain (MK762599.1).

Polyethylene degradation analysis

The isolated fungus was inoculated in Luria Bertani (LB) 0.1X medium with or without 1% polyethylene powder with density of 0.955 g cm^{-3} mixed in 2% agar. The agar plate assay was a semi-quantitative method to follow microbial polymer degradation. After inoculation the fungus onto the medium containing polyethylene powder, the presence of a clear halo around the colony indicated the presence of HDPE degradation (Nishida and Tokiwa 1993). The same experiment was also performed by inoculating fungus in LB 0.1X without agar.

Table 1. Weight determination of samples at initial time (T_0) and after 15 days of incubation (T_{15}).

SAMPLES	DRY WEIGHT (mg) T_0	DRY WEIGHT (mg) T_{15}
LB + HDPE	25 ± 0.6	25 ± 0.8
LB + *Cladosporium halotolerans* + HDPE	25 ± 0.7	18 ± 0.5

HDPE weight loss and scanning electron microscopy (SEM)

In order to evaluate the polyethylene utilization, *Cladosporium halotolerans* was used at an initial OD_{600} nm of 0.1OD and grown with or without 20 mg of HDPE polymer. In 2 ml of a LB 10-times diluted medium (2 mg of tryptone, 2 mg of NaCl and 1 mg of yeast extract). After 15 days samples were dried and their weight was recorded. The amount of polyethylene consumed by *Cladosporium halotolerans* was calculated by making the difference between the sample containing only HDPE and the sample containing HDPE and fungus. The experiment was performed in triplicate and the reported result in Table 1 was an average of three independent experiments.

Scanning electron microscopy was used to observe interaction of fungus with HDPE. For scanning electron microscopy (SEM), samples of fungi cultured both in absence and in presence of HDPE powder were cut from subapical parts using a sharp razor blade, fixed with 3% glutaraldehyde in phosphate buffer (pH 7.2–7.4) for 2 h at room temperature, post-fixed with 1% osmium tetroxide in the same phosphate buffer for 1.5 h at room temperature, and completely dehydrated with ethanol and critical point drying. Both the biological samples prepared as before and samples of unprepared HDPE micro particles alone were then mounted on aluminum stubs, coated with a thin gold film using an EdwardE306 Evaporator, and observed under a FEI (Hillsboro, OR, USA) Quanta 200 ESEM in high vacuum mode (P 70 Pa, HV 30 kV, WD10 mm, spot 3.0).

Fourier transform infrared spectroscopy (FTIR)

Polyethylene degradation was confirmed by using Fourier Transform Infrared Spectroscopy (FTIR). Suspensions of Polyethylene with or without *C. halotolerans* were prepared by dispersing equal amount of HDPE (1% W/V) in LB 0.1X cell culture medium. After 15 days all samples before frozen at −80°C and they were lyophilized and homogenized. The samples were indicated in the followings as HDPE and HDPE + *C. halotolerans*, respectively. Fourier transform infrared transmittance analysis were carried out by using a Nexus FTIR spectrometer connected to DTGS KBr detector. For each sample, three pellets were prepared by dispersing 2 mg of lyophilized powders in 200 mg of KBr. All spectra were recorded in the 4000–400 cm^{-1} range, with 2cm^{-1} value of resolution and KBr as blank was used.

Screening of oxidoreductase

Earlier studies revealed involvement of laccase in polyethylene degradation (Viswanath et al. 2008). Hence, screening, of this en-

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zyme in *C. halotolerans* was carried out. Laccase activity assay was performed on plates containing the following composition (g/l): 3.0 peptone, 10.0 glucose, 0.6 KH_2PO_4, 0.001 $ZnSO_4$, 0.4 K_2HPO_4 0.0005 $FeSO_4$, 0.05 $MnSO_4$, 0.5 $MgSO_4$, 20.0 agar (pH 6.0) supplemented with 0.02% guaiacol. *Cladosporium halotolerans* and *Candida albicans*, used as negative control, were plated and plates were incubated at 30°C for 7 days. Laccase activity was visualized on plates containing 0.02% guaiacol since laccase catalyzes the oxidative polymerization of guaiacol to form reddish brown zones in the medium (Coll et al. 1993). It has previously been shown that the production of laccase by fungi depends greatly on the composition of the growth medium (Rogalski et al. 1991), for this reason the effects of copper, known to affect the production of this enzyme, were evaluated. Copper ($CuSO_4$), at 1, 5, and 10 mM were used. The concentration of copper that did not cause mortality was chosen to perform Fourier transform infrared spectroscopy (FTIR).

Proteins from fungal supernatant and SDS-PAGE

The isolated fungus was sub-cultured in LB 0.1X agar plates and incubated at 30°C for three days. A loopful of the fungal culture was inoculated into LB 0.1X broth in presence or absence of HDPE 1% and incubated at 30°C for 15 days (static light conditions). After the incubation the culture was filtered with filter paper to remove the biomass. The supernatant was filter-sterilized with a 0.22-μm filter (Millipore, Bedford, MA, USA). Supernatant protein concentration was determined by the Bradford protein assay (Bio-Rad) (Zanfardino et al. 2017). Subsequently, to remove the excess of salts due to the culture broth, a precipitation in trichloroacetic acid (TCA) was carried out, 1 volume of TCA (100%) was added to 4 volumes of protein sample and incubated for 10 min at 4°C. The sample was centrifuged at maximum speed for 5 min, the supernatant was removed, leaving the whitish protein pellet intact. The pellet was washed and allowed to dry by placing the tube in a thermal block at 95°C for 5–10 min to eliminate the acetone. The 4X Laemmli buffer (SIGMA) has been added to samples, boiled at 100°C for 5 min and resolved by Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (10%) an analytical technique to separate proteins based on their molecular weight (Zanfardino et al. 2010). Subsequently the gel was colored with the Comassie blue (0.1% Comassie Brilliant blue in acetic acid 10%).

Supernatant activity on solid medium

The supernatant obtained after incubation of *Cladosporium halotolerans* for 15 days, in the presence and absence of high density polyethylene, was concentrated 100X. Subsequently, to evaluate the presence of oxidoreductase in the supernatants, 20 microlitres of each concentrated sample (+ HDPE,—HDPE) were disposed on LB agar plate containing 0.02% guaiacol, a chromogenic substrate used for the screening of enzymes with oxidoreductase activity. The positive control of the experiment was represented by commercial purified laccase from *Trametes versicolor* (Sigma Aldrich) used at the same concentration as for the liquid assays (Papinutti and Martinez 2006).

In-situ hydrolysis, LC-MS/MS analysis and protein identification

Mono-dimensional SDS-PAGE gel was stained with Coomassie Brilliant Blue, the band approximately at 50 KDa, was excised and de-stained with 100μL of 0.1 M ammonium bicarbonate (AMBIC) and 130 μL of acetonitrile (ACN) and subsequently subjected to *in-situ* hydrolysis with 0.1 μg μL trypsin mM in AMBIC for 18 hrs at 37°C. The hydrolysis was stopped by adding acetonitrile and 0.1% formic acid. The sample was then filtered and dried in a vacuum centrifuge The peptideS mixture thus obtained were directly analyzed by LTQ Orbitrap XL™ Hybrid Ion Trap-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany). C-18 reverse phase capillary column 75 μm 10 cm (Thermo Fisher Scientific), was performed using a flow rate of 300 nl/min, with a gradient from eluent A (0.2% formic acid in 2% acetonitrile) to eluent B (0.2% formic acid in 95% acetonitrile). The following gradient conditions were used: t = 0 min, 5% solvent B; t = 10 min, 5% solvent B; t = 90 MIN, 50% solvent B; t = 100 min, 80% solvent B; t = 105 min, 100% solvent B; t = 115 min, 100% solvent B; t = 120 min; 5% solvent B. Peptides analysis was performed using data-dependent acquisition of one MS scan followed by CID fragmentation of the five most abundant ions. For the MS scans, the scan range was set to 400–1800 m/z at a resolution of 60 000, and the automatic gain control (AGC) target was set to $1 \cdot 10^6$. For the MS/MS scans, the resolution was set to 15 000, the AGC target was set to $1 \cdot 10^5$, the precursor isolation width was 2 Da, and the maximum injection time was set to 500 ms. The CID normalized collision energy was 35%. Data were acquired by Xcalibur™ software (Thermo Fisher Scientific). In-house Mascot software (version 2.4.0) was used as a search engine to identify proteins. The software returns a list of proteins associated with a probability index (score), calculated as–10 Log P, where P was the probability that the observed event was a random one. Proteins were considered as identified if a minimum number of 2 peptides reach the calculated score. Since the proteome of *Cladosporium halotolerans* did not yet available, the database used for protein identification was the one of *Rachicladosporium antarticum* (18 598 sequences; 9 390 076 residues), an organism phylogenetically related.

Results

Isolation of cladosporium halotolerans from wax larvae

The gastric content of several *Galleria mellonella* larvae were plated on LB agar medium. The microorganism obtained were screened for their ability to grow in Luria Bertani medium containing beeswax and on agar plates containing high density polyethylene (HDPE). Fifteen colonies were isolated and among them one fungal microorganism was selected for its ability to grow on solid medium containing HDPE as principal carbon source. The 5.8S rRNA gene sequence allowed us to classify the isolate as *Cladosporium halotolerans* (99% identity).

Cladosporium halotolerans ability to degrade HDPE

Recent studies have reported that several types of microorganism, living within the guts of insect larvae, are capable to degrade plastic material (Sedacca 2017, Zhang et al. 2020). Since 1961, when Fuhs reported the ability of some microorganisms to consume paraffin as a carbon source (Fuhs 1961), the biodegradation potential for PE and other groups of plastics have been studied and different microorganisms (bacteria and fungi) have been studied. Generally, bacteria-mediated biodegradation of film-type or micro particles plastics require a minimum of four weeks to be observed (Yang et al. 2014, 2015, Zhang et al. 2020). In this study, we used high density polyethylene powder. In liquid medium HDPE powder, as shown in Fig. 1 (panel A) is highly hydrophobic and binds to the walls of the flask remaining separated from the liquid medium. In Fig. 1B and C, it is shown the growth of *C. halotolerans*

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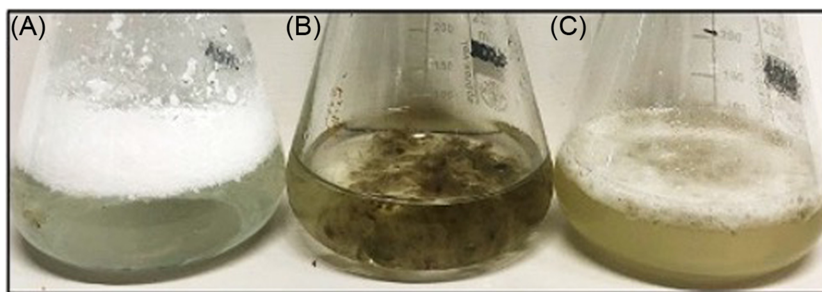

Figure 1. PE macroscopic observation in presence and the absence of *Cladosporium halotolerans*. Panel A: polyethylene in absence of the microorganism is highly hydrophobic and remains separated from the culture medium. Panel B shows *Cladosporium halotolerans* culture. Panel C: HDPE solubilization induced by *Cladosporium halotolerans*.

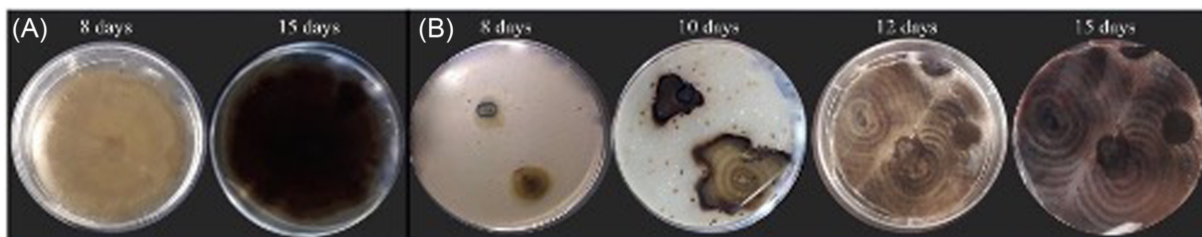

Figure 2. Fifteen-day monitoring of *Cladosporium halotolerans* growth. Panel A fungal growth on plates without HD-polyethylene; panel B fungal growth on HD-polyethylene agar plate.

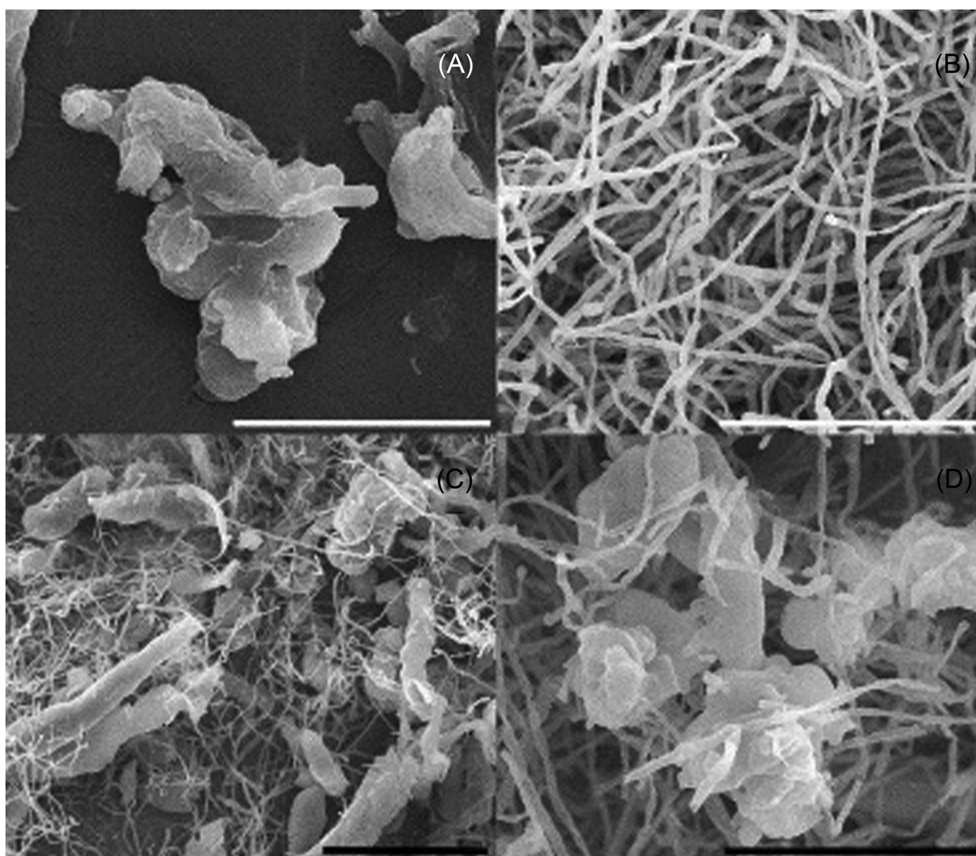

Figure 3. Scanning Electron Microscopy (SEM). **(A)** Typical HDPE particles; **(B)** hyphae of *Cladosporium halotolerans*; **(C)** fungus and HDPE particles at low magnification; **(D)** fungus and HDPE particles at high magnification. Scale bars: 50 μ (A, B, D); 100 μ (C).

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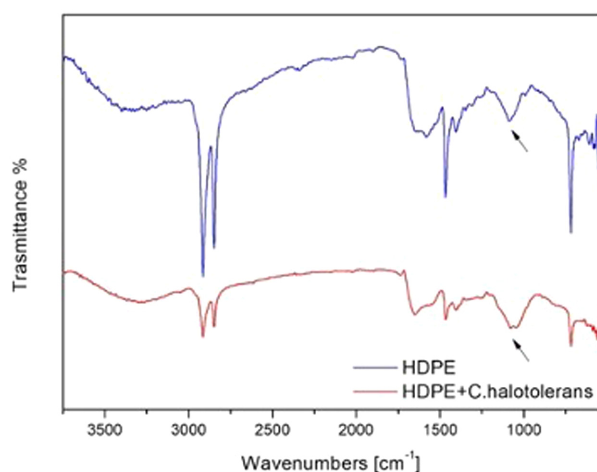

Figure 4. FTIR analysis of HDPE degradation. Spectra of polyethylene (blue line) and polyethylene with *Cladosporium halotolerans* (red line). The arrow indicates the main peak at 1080 cm^{-1} chosen as the internal standard.

with (C) or without (B) HDPE powder. Interestingly *C. halotolerans* growth with HDPE resulted in an increased turbidity of the culture and solubilization of HDPE. In particular, *Cladosporium halotolerans* mediates the solubilization of HDPE particles due to its degradation activity and/or producing surfactant molecules already after 15 days.

HDPE degradation was observed also after a test carried out on plates containing 1% polyethylene powder mixed in the growth medium. After 15 days of incubation, as shown in Fig. 2A, the absence of high density polyethylene allows the fungus to grow on the entire surface of the plate. In presence of HD-polyethylene, the fungus grows forming clearer concentric circles indicative of polyethylene biodegradation (Fig. 2B).

The time necessary for the fungus to degrade HDPE is of fundamental interest, after only 12 days the microorganism forms concentric and transparent circles on solid medium containing HDPE, indicative of the strong fungal degradative capacity.

Recently, several bacteria and fungi with similar catabolic activity have been isolated. However, they showed much slower degradation capacity under different conditions from our setting. For instance, *Penicillium simplicissimum* YK degrades the plastic polymer in 3 months (Yamada-Onodera et al. 2001), *Aspergillus flavus* in 30 days (Zhang et al. 2020), and *Aspergillus tubingensis* VRKPT1 in 30 days too (Sangeetha Devi et al. 2015).

HDPE weight loss

To evaluate the fungal induced polyethylene weight loss, *Cladosporium halotolerans* cultures were grown with and without HDPE micro particles for 15 days. As reported in Table 1, the dry weight of the sample containing *C. halotolerans* with HDPE was reduced compared to that with only HDPE. To explain this result, and in agreement with previous studies, (Shah *et al.* 2008) we speculate that HDPE degradation is associated to oxidative fragmentation of the carbon backbone of the polymeric chain with consequential production of volatile or gaseous species that cause the observed weight reduction. Using a growth medium with a very low amount of carbon source, we cannot exclude that the observed weight loss could be in part the result of the mineralization of carbon sources other than plastic. To support the hypothesis that *C. halotolerans* interacts with HDPE powder, scanning electron microscopy has been performed. SEM observations showed that polyethylene powder is composed of a mixture of small particles (from 50 to 100 μm) (Fig. 3A). *Cladosporium halotolerans* develops a thick twine of hyphae (Fig. 3b), which, when cultured in presence of HDPE, tightly envelop the plastic particles (Fig. 3C and D). This observation is in agreement with the mechanical degradation by the fungal hyphae reported in several studies, which contributes to PE fragmentation (Khan et al. 2017).

HDPE degradation analyzed by FTIR

To validate the hypothesis that the fungus degrades polyethylene powder, FTIR analysis of HDPE and HDPE + *C. halotolerans* was performed. Spectra of each sample are reported in Fig. 4. Both samples show the typical absorption band of polyethylene. The peaks at 2920 cm^{-1} and 2852 cm^{-1}were assigned to C–H asymmetric and C–H symmetric stretching vibrations in –CH_2–, respectively. The peak at 1466 cm^{-1} was attributed to C–H deformation vibrations in –$(CH_2)_n$–, whereas the peak at 723 cm^{-1} to C–C rocking vibrations in –$(CH_2)_n$– (Gulmine et al. 2002). Furthermore, some bands related to LB ten-times diluted culture medium were also visible at about 3500 and 1640 cm^{-1} attributed to-OH stretching vibration and H_2O bending. The band at 1370 cm^{-1} was related to COO- symmetric stretching and CH_3 bending vibration of lipids and proteins, while the band at 1080 cm^{-1} to hydrogen phosphate ions stretching groups (Aksoy and Severcan 2012). To evaluate possible variations in the amount of HDPE in the presence of *C. haloterans* the main peak at 1080 cm^{-1} (arrow in Fig. 4) was chosen as internal standard. In particular, the ratio between the area of the peak in the range 2920 and 2852 cm^{-1} (main peaks of HDPE) and the area of the peak at 1080 cm^{-1} (main peak of the culture medium) was chosen as a parameter to evaluate the evolution of the amount of HDPE in the culture medium. By keeping the peak area constant at 1080 cm^{-1}, a reduction in the intensity of the main HDPE bands at 2920 and 2852 cm^{-1} was clearly visible (red spectrum of Fig. 4). Comparing the spectra, the main HDPE absorption bands in the presence of *C. halotolerans* looks strongly reduced. Although biofilm might form on HDPE surface, this phenomenon is bound not to influence HDPE main bands (Wang et al. 2020), thus this decrease could be strictly related to plastic consumption by fungus.

Oxidoreductase screening

In some species of fungi oxidoreductase enzymes are involved in the degradation process of several plastic materials (Sowmya et al. 2015). Thus, to evaluate whether *Cladosporium* is an oxidoreductase producer, a colorimetric screening method was followed using the oxidation of guaiacol, a chromogenic substrate (Ostojić et al. 2017). As shown in Fig. 5A, *Candida albicans* does not react with the substrate, indicating the absence of oxidoreductase activity, and for this reason it was used as a negative control. In panels B *Cladosporium halotolerans* was grown without (B1) or with guaiacol (B2). In B2, after 7 days of dark incubation, the appearance of reddish color indicated the oxidation of the guaiacol by fungal oxidative enzyme(s).

Cladosporium halotolerans protein supernatants analysis

To verify whether the 15-days polyethylene treatment causes changes in the expression of the fungus-secreted proteins in the supernatant, an electrophoretic gel preparation was performed under denaturing conditions. To prevent salts alteration of protein migration, a precipitation was carried out in trichloroacetic acid (TCA). Fig. 6A shows a protein band (indicated by a red arrow), corresponding to a molecular weight of 80-90 KDa, with higher in-

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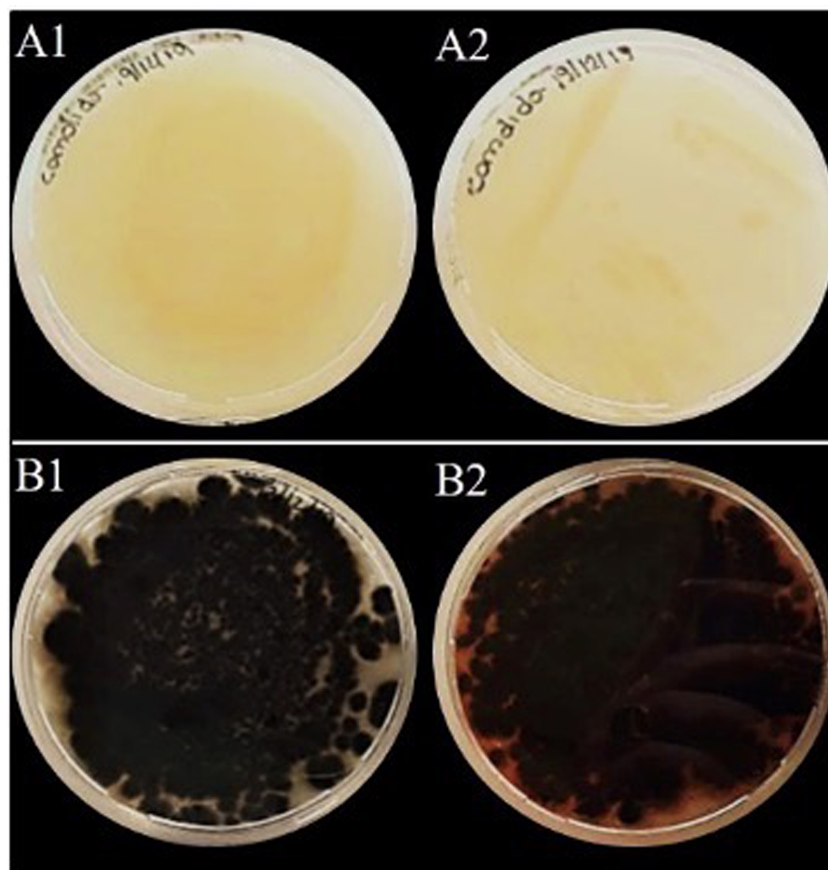

Figure 5. Guaiacol oxidoreductase test. Panels A: *Candida albicans* plates used as negative control of the experiment. Panels B: *Cladosporium halotolerans* plates. Fungi were grown in absence (A1 and B1) or in presence (A2 and B2) of guaiacol substrate.

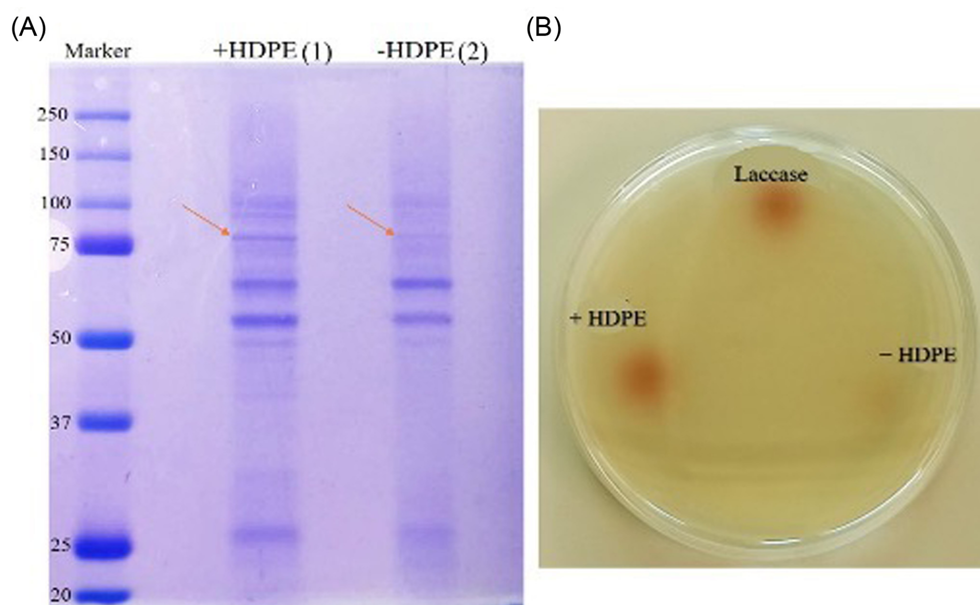

Figure 6. *C. halotolerans* supernatant analysis by SDS-PAGE and enzymatic screening. Panel A: SDS-PAGE of supernatants obtained from *Cladosporium halotolerans* in presence (lane 1) or absence (lane 2) of HDPE. Panel B: Guaiacol oxidoreductase test of fungal supernatants (+ HDPE,—HDPE). As positive control of the experiment was used commercial purified laccase from *Trametes versicolor*. These experiments were riproduced at least three times.

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Table 2. Identified protein from PE+ *C. halotolerant*; B. Identified protein from *C. halotolerans* (control sample).

prot_acc	prot_desc	prot_score	Number of peptides
A.			
|A0A1V8SKK9	Aminopeptidase	182	8
A0A1V8T3G2	Uncharacterized protein	62	6
A0A1V8T2V2	Amidase domain-containing protein	59	6
A0A1V8SD42	Beta-hexosaminidase	37	3
A0A1V8SF32	FAD_binding_3 domain-containing	35	2
A0A1V8SP57	Fungal_trans domain-containing protein	34	2
A0A1V8S928	Peptide hydrolase	26	2
B.			
A0A1V8TED2	Uncharacterized protein	76	2
A0A1V8T2V2	Amidase domain-containing protein	74	2
|A0A1V8SKK9	Aminopeptidase	52	2
A0A1V8T380	SWR1-complex protein	37	2
A0A1V8S928	Peptide hydrolase	33	2
A0A1V8T8Y1	Kinesin-like protein	33	2
A0A1V8SD42	Beta-hexosaminidase	32	2

tensity when the fungus was grown in presence of HDPE (lanes 1 and 2). In panel B of the same figure is shown a plate enzymatic assay of culture supernatants grown in the presence or absence of HDPE. The intensity of red spots is very different, indicating that the corresponding enzyme is secreted and the presence of HDPE increases its activity. A similar phenomenon is reported in *Trametes hirsuta* for cellulose: in presence of this substrate an increase of laccase enzymatic activity is observed (Rodríguez Couto et al. 2006).

Then, the identification of the bands indicated in Fig. 6 as PE + *C. halotolerans* secreted proteins and untreated *C. halotolerans* secreted proteins was performed. Identified proteins are summarized in Table 2. However, the proteic profile resulted very similar, FAD_binding_3 domain protein (GO: 0016491 GONUTS page) resulted to be very interesting among HDPE+ identified proteins, since it was uniquely identified in the sample obtained after HDPE addition.

Discussion

This study propose *Cladosporium halotolerans,* a fungal strain isolated for the first time from *Galleria mellonella* larvae gastrointestinal system, as new microorganism able to degrade HDPE. In particular, this fungus determines a change in the HDPE surface chemical structure, which passes from insoluble to soluble within 15 days. The observed weight loss and degradation of a plastic substance are observed in a relatively short time, which is of considerable importance for a possible application in bioremediation processes. *C. halotolerans* can probably combine a mechanism of mechanical (as confirmed by SEM images) and enzymatic degradation, due to structural components like hyphae, and to the presence of secreted enzymes having oxidoreductase activity. The substantial decrease of HDPE was also confirmed by FTIR analysis, where all the HDPE peaks 2920 and 2852 cm^{-1} appear strongly reduced in the samples with *Cladosporium*. Moreover, the analysis of the supernatant proteic pattern of the fungus revealed some differences associated with polyethylene presence. The most significative one is the presence of FAD_binding_3 domain containing enzyme, a protein that binds FAD as prosthetic group used by various oxidoreductase enzymes. FAD is an electron carrier molecule that function as a hydrogen acceptor. This protein catalyzes an oxidation-reduction (redox) reaction in which a CH_2 or CH-CH group acts as a hydrogen or electron donor and reduces a hydrogen or electron acceptor (GO:0016725 GONUTS page; GO:0016627 GONUTS page). This enzyme could be responsible of the guaiacol oxidation assay used in this study. It is known, in fact, that the FAD_binding_3 domain protein has an oxidoreductase activity even on phenolic substrates such as pyrogallol (GO:0018706; GO:0016679).

The fungal production of specific enzymes such as oxidoreductase, at high concentrations and for a long period of time, triggers the breaking of the C-C bonds of the plastic polymers into oligomers, dimers and monomers (Pathak and Navneet, 2017). Following the bio-fragmentation process, the monomers produced are absorbed by microorganisms, and oxidized to produce energy, allowing the microbial cell to grow and reproduce.

Our experiments show that the HDPE degradation mechanism performed by *Cladosporium halotolerans* is very complex and it is most likely a combination of mechanical and enzymatic processes. The mechanical process is exerted by the fungal hyphae, which are in close contact with the HDPE particles, while fungal secreted enzymes are responsible for the enzymatic process.

Authorship contribution statement

Michela Di Napoli, Giusy Castagliuolo, Antimo Di Maro, Alessandro Pezzella: Methodology, Writing—original draft, Investigation. Andrea Carpentieri: LC-MS/MS analysis. Brigida Silvestri and Giuseppina Luciani: IFTR analysis and writing—original draft. Sergio Sorbo: Microscopy analysis. Anna Zanfardino and Mario Varcamonti: Conceptualization, Methodology, Writing—original draft, Investigation, Visualization, supervision.

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