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# Triggering and identifying the polyurethane and polyethylene-degrading machinery of filamentous fungi secretomes

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**Abstract**

The uncontrollable disposal of plastic waste has raised the concern of the multidisciplinary community, which tries to face this environmental burden by discovering and applying new techniques. Regarding the biotechnology field, several important microorganisms possessing the necessary enzymatic arsenal to utilize recalcitrant synthetic polymers as an energy source have been discovered. In the present study, we screened various fungi for their ability to degrade intact polymers, such as ether-based polyurethane (PU) and low-density polyethylene (LDPE). For this Impranil and a mixture of long-chain alkanes was used, not only as sole carbon sources but also as enzyme indicators and inducers for polymer degradation. An agar plate screening revealed three fungal strains belonging to *Fusarium* and *Aspergillus* genera, whose secretome was further studied for its ability to degrade the respective polymers. Specifically for ether-based PU, the secretome of a *Fusarium* species reduced the sample mass and the average molecular weight of the polymer by 24.5 and 20.4 %, respectively, while the secretome of an *Aspergillus* species caused changes in the molecular structure of LDPE, as evidenced by FTIR. At the same time, the proteomic analysis revealed that the enzymatic activities induced in presence of Impranil can be associated with urethane bond cleavage, a fact which was also supported by ether-based PU degradation. Meanwhile, the mechanism of LDPE degradation was not completely elucidated, although the presence of oxidative enzymes could be the main factor contributing to polymer modification.

*Keywords*: microbial degradation; fungus; enzyme; plastics; polyurethane; polyethylene

## 1. Introduction

Synthetic polymers have become one of the most massively produced materials, having a great spectrum of properties that are directly combined to their composition and chemical structure. More specifically, plastics are inexpensive, durable, and lightweight; properties that make them suitable for a great variety of applications throughout the global economy, including food packaging, building construction, health, and agriculture industry (Agarwal & Gupta, 2017; Chia et al., 2020; L. Liu et al., 2022). Especially, during the last decade, the annual global production of plastics exceeded 360 million metric tons (Geyer et al., 2017) with polyethylene (PE) and polyurethane (PU) being among the most demanded polymers throughout Europe (Wei & Zimmermann, 2017); their demand accounts for 30.3 and 7.8% of the total plastic demand, respectively (PlascticsEurope, 2021).

Despite plastics' massive production, countries around the globe have not yet developed an effective strategy to deal with their uncontrolled disposal (Chen & Yan, 2020). Regarding PU and PE waste, almost 50 % of the post-consumed and post-produced PU is landfilled (Kemona & Piotrowska, 2020), while high-density PE (HDPE) and low-density PE (LDPE) recovery rates do not exceed 10 and 5 %, respectively (Rahimi & Garciá, 2017). The plastic waste that is not reused or incinerated is buried in landfills or the natural environment (Geyer et al., 2017), increasing ecotoxicity, due to the release of toxic and harmful compounds such as plasticizers, additives, and copolymers (L. Liu et al., 2022).

When plastics are exposed to the environment, abiotic and/or biotic factors result in plastic breakdown, whose rate is highly affected by their hydrophobicity, structure complexity, and composition (Lambert et al., 2014). Considering these factors,

simulation studies predict that polymer half-lives' can vary from a few months up to millions of years (Chamas et al., 2020). This long-term process starts with polymer bio-fragmentation, according to which smaller units are released and subsequently utilized by the microorganisms as an energy or nitrogen source (Lambert et al., 2014; Mouafo Tamnou et al., 2021; A. et al., 2020).

Among the reported microorganisms that utilize synthetic polymers belong several bacteria, microalgae, but also numerous plant biomass-degrading fungi, whose enzymatic machinery is considered a promising tool in the field of biodegradation (Mahajan & Gupta, 2015; Zerva et al., 2021; Nikolaivits et al., 2021; Sangale et al., 2019; K. Zhang et al., 2022).

Microorganisms able to produce enzymes with high catalytic activity against synthetic polymers are of high interest, although there is a strong need to screen efficiently organisms developing new, time-, and source-saving methodologies. Regarding screening for putative PU-degrading bacterial and fungal isolates, a common and simple strategy used often includes a commercial polyester-polyurethane dispersion, namely Impranil DLN-SD (Álvarez-Barragán et al., 2016; Howard et al., 2001; Khruengsai et al., 2022; Molitor et al., 2020). Ester-based PU is more susceptible to degradation (Danso et al., 2019; Howard, 2002), so screening tests using Impranil can be considered a time-saving and successful technique for the detection of microorganisms with PU depolymerizing potential (Biffinger et al., 2015). However, Impranil hydrolysis does not guarantee the degradation of the urethane bond, because according to reports, the hydrolysis of ester-based PU is mainly a result of the ester rather than the urethane bond cleavage (Álvarez-Barragán et al., 2016; Shah et al., 2013). As a consequence, it is clear, that hydrolases recognizing only the ester bond of ester-based

PU cannot be used for breaking down mixtures of PU waste, so the discovery of enzymes selectively cleaving the urethane bond is very important, as only this bond is ubiquitous among the different PU structures.

Concerning screening for putative PE fungal degraders, a regular, but a time-consuming technique which can last weeks or even months, includes growth assays in agar plates using PE film or powder suspension (Bonhomme et al., 2003; Dominguiano et al., 2011; Yamada-Onodera et al., 2001; Zahra et al., 2010), sometimes followed by PE degradation in liquid cultures (Yamada-Onodera et al., 2001). Among the various fungal species, *Aspergillus*, *Cladosporium*, *Penicillium,* and *Fusarium* have been identified as PE degraders (Kumar Sen & Raut, 2015), which secrete oxidoreductive ligninolytic enzymes and therefore utilize polymer as carbon source (Srikanth et al., 2022). Even though bacterial strains have been most commonly associated with PE degradation, in the environmental systems the degradation of organic matter is predominantly caused by fungal species. For this reason, fungi are considered to be the main contributors not only to lignocellulose but also to synthetic polymer breaking down (Chen et al., 2020).

Taking into consideration all the above-mentioned points, this study, firstly, aimed to devise and implement a protocol for a fast and reliable preliminary microorganism screening utilizing compounds resembling oligomers or break-down products of plastics. After the preliminary screening, instead of whole-cell biocatalysis, the positive fungal strains were grown in liquid cultures supplemented with the same compounds, and the induced extracellular enzymes were tested for virgin LDPE and PU degradation. This procedure renders the determination of polymer properties easier after treatment since in other cases, the microbial colonization on the polymer surface should be carefully

removed prior to analysis. Moreover, according to reports studying natural polymer deconstruction, the insoluble materials themselves cannot directly trigger the induction of depolymerizing enzymes, therefore elicitors or inducers are necessary to activate the microbial enzyme machinery (Liao et al., 2014; Ogunmolu et al., 2018; Reina et al., 2014).

For this reason, in our case, the role of Impranil and lcAlk as inducers for depolymerizing enzymes was investigated, proving that the enzymes secreted are able to degrade the ether-based PU and functionalize LDPE. Apart from material analysis, the enzyme activities triggered were also identified through proteomic analysis, providing more information about the enzyme groups that should be further studied, in respect of recalcitrant polymer breakdown. To the best of our knowledge, the idea of using the secretomes as screening tools for synthetic polymer degradation has not yet been reported, not to mention that lcAlk are used for the first time in screening/induction for potential PE degraders, providing a reliable indication for PE degradation potential.

## 2. Materials and Methods

### 2.1 Chemicals and polymeric Materials

Ether-based thermoplastic PU pellets (Laripur 7560) were purchased from Coim group (Milano, Italy), while the anionic aliphatic polyester-polyurethane dispersion Impranil® DLN-SD, hereinafter referred to as Impranil, was received from Covestro Solution Center (Germany). PE pellets were received from Exon Mobil (U.S.A) and lcAlk ($C_{24}$, $C_{28}$, $C_{36}$) were purchased from Sigma-Aldrich (U.S.A). PU and PE pellets were ground into powder using the cryogenic grinding apparatus Pulverisette 14 (Fritsch Corp., Idar-Oberstein, Germany), as described previously (Nikolaivits et al., 2022). After

grinding, powder of particle size <500 μm was dried under vacuum (300 mbar) at 90 °C for 3 h.

## 2.2 Sample collection, isolation, and identification of fungal strains

Soil samples were collected from different sites (Table S1): Ruma (Serbia, 44.985348, 19.815829), Hrtkovci (Serbia, 44.873975, 19.789936), Belgrade (Serbia, 44.792250, 20.537033), and Athlone (Ireland, 53.428411, -7.875629). Additionally, material from decaying oak log (Kosutnjak, Belgrade, Serbia, 44.764715, 20.439966), plastic-contaminated river sediment (Brzece, Serbia, 43.297260, 20.882913), deteriorating plastic bag (Belgrade, Serbia, 44.790537, 20.536422), and dead isopods were also used to isolate fungi. One gram of soil of different samples was suspended in 9 mL of sterile phosphate-buffered saline (PBS; pH 7.4) and vortexed for 20 min. After vortexing, the samples were left to settle, and serial dilutions were prepared. The dilutions were used to inoculate agar plates containing 1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) dextrose, and 2 % (w/v) agar (YPDA) and were incubated for 3 days at 30 °C. Single colonies were transferred onto fresh plates to obtain pure cultures.

For molecular identification, the powdered biomass of each fungal strain was used for the isolation of genomic DNA. The entire ITS region was PCR amplified, using ITS1 and ITS4 PCR primers, following the protocol described previously (Gardes & Bruns, 1993; White et al., 1990) (see Electronic supplementary material). The sequenced ITS regions were used for fungi identification and were deposited to GenBank with the following accessions: OQ155213, OQ155214, OQ155221, OQ155226.

## 2.3 Preparation of Impranil and lcAlk agar plate screening assays

The isolated fungal strains were routinely cultivated on YPDA plates for 3 days at 27 ºC. One piece (0.5 cm x 0.5 cm) from the edge of these was used as inoculum for the following tests. All the screening tests were conducted in mineral medium (MM) agar plates (15 g $L^{-1}$) supplemented with a different carbon source. MM was composed of 19 mM $NaH_2PO_4$, 33.5 mM $K_2HPO_4$, 7.6 mM $(NH_4)_2SO_4$, 250 µM $MgSO_4$, 147 µM $FeCl_3$, 14 µM $ZnCl_2$, 12 µM $CoCl_2$, 12 µM $Na_2MoO_4$, 10 µM $CaCl_2$, 11 µM $CuCl_2$, 12 µM $MnCl_2$, 12 µM $H_3BO_3$ and 1.8 mM HCl. MM was sterilized in an autoclave at 121 ºC for 20 min.

For the detection of PU degrading strains, MM was supplemented with 0.4 % (v/v) Impranil as sole carbon source, which was added after the sterilization of MM, as the extensive heating can have an adverse effect on product properties, whereas plates with no carbon source were used as control.

Regarding the detection of potential PE-degraders, tests were also conducted on agar plates containing an emulsified growth medium composed of MM containing 5 % (v/v) a mixture of lcAlk dissolved in hexadecane ($C_{16}$). The lcAlk mixture was composed of 80 g $C_{24}$/L, 80 g $C_{28}$/L, and 40 g $C_{36}$/L. The MM/lcAlk solution was emulsified after 5 min of ultrasonication using the ultrasonic processor VC 600 (Sonics and Materials, USA), at 40 % amplitude, before being poured onto plates. Agar plates using an emulsified growth medium composed of MM (95 % v/v) – hexadecane (5 % v/v) were used as control plates.

In both PU and PE plate assays, agar plates were incubated at 27 ºC for 5 days.

*2.4* Induction of depolymerizing enzymes using Impranil and lcAlk

The strains able to grow on agar plates with Impranil forming zones of clearing around the colonies were further tested for their ability to degrade virgin ether-based PU powder. Specifically, a preculture was prepared by the inoculation of the positive fungal strains grown on YPD (one piece of 0.5 cm x 0.5 cm dimensions) in 10 mL sterilized MM supplemented with 0.4 % (v/v) Impranil. Fungal precultures were incubated at 27 °C, under continuous stirring at 120 rpm, for 4 days and were subsequently used for the inoculation (10 % v/v) of 100 ml of the same sterilized MM/Impranil medium. The cultures were incubated under the same growth conditions for 5 days, and the culture medium was removed every 24 h, centrifuged at 4,000 x *g* for 15 min at 4 °C, and sterilized using a 0.22 μm filter. For choosing the most competent strain for PU degradation, the culture supernatants of fungi grown on MM/Impranil medium were concentrated using an Amicon ultrafiltration apparatus (exclusion size, 10 kDa; Amicon chamber 8400 with membrane Diaflo PM10, Millipore, Billerica, MA) until the different culture supernatants of each day get similar protein content.

Regarding the strains, whose growth was greater on plates with lcAlk, these were also grown in liquid cultures using MM/lcAlk solution, while cultures with MM/hexadecane were used as a control. More specifically, the emulsified growth media were prepared as mentioned in the case of agar plates, but the addition of 0.1 % (v/v) Tween 20 in the culture medium was necessary to prevent phase separation, increasing this way emulsion stability. Similar to MM/Impranil preculture preparation, one piece of the fungal strain grown in YPD plates was used for the inoculation of 10 mL emulsified MM/hexadecane and MM/lcAlk solution. These fungal precultures were incubated under the same conditions as those mentioned above and were subsequently

used for the inoculation of the corresponding 100 ml MM/hexadecane and MM/lcAlk media. The cultures were again incubated at 27 °C, under continuous stirring at 120 rpm, for 4 days. After that, they were centrifuged, sterilized using a 0.22 μm filter, and used as biocatalysts for LDPE degradation.

## 2.5 Enzymatic degradation of ether-based PU and PE powder

The culture supernatants obtained after inducing putative depolymerizing enzymes using Impranil or lcAlk were tested for their ability to degrade ether-based PU or LDPE powder, respectively. In more detail, 50 mg of PU or LDPE powder were incubated with 50 mL of the culture supernatant at 30 °C under continuous stirring at 160 rpm, for 4 days. Regarding control samples, PU powder was incubated with an equal amount of culture supernatant, which had been previously inactivated by boiling for 15 min, while in the case of LDPE, MM/hexadecane supernatant was used as control.

After incubation, plastic powders were isolated by centrifugation at 20,000 x *g* for 10 min, resuspended in 10 mL of sodium dodecyl sulfate solution (SDS) (2 % w/v), and kept under stirring for 10 minutes to remove potentially attached proteins. After discarding the SDS solution, polymeric powders were rinsed twice with 40 mL ultrapure water and isolated by centrifugation. Finally, each plastic powder was dried before its properties were determined.

## 2.6 Determination of polymer properties after treatment with secretomes

After the enzymatic treatment, PU powder was completely dissolved in tetrahydrofuran (THF >=99.9 % purity, Macron Fine Chemicals, Poland), at a concentration of 2 mg/mL. After dissolution, the samples were filtered through a polytetrafluoroethylene (PTFE) filter (0.22 μm), and the filtrate was analyzed with a

guard column (PLgel 5 µm) and two PLgel MIXED-D 5 µm columns (300 ×7.5 mm) using the Agilent 1260 Infinity II equipped with refractive index detector (RID), as described previously (Nikolaivits et al., 2022).

PE degradation was assessed by Fourier-Transform Infrared (FT-IR) spectroscopy aiming to detect any changes in PE molecular fingerprint. The FT-IR spectrum was recorded by the Bruker Alpha II FTIR spectrometer using the ATR method with a diamond crystal, while Software OPUS 8.5 was used to analyze the recorded spectrum, which was received as 16 scans in the 400–4000 $cm^{-1}$ range. Identification of PE characteristic peaks was performed based on vibrational bands and comparing IR spectra against standards in the software's spectral library.

## 2.7 Esterase activity assay in secretomes

The esterase activity in each sample was measured using *p*-nitrophenyl butyrate (*p*-NPB) as a substrate in 0.1 M phosphate-citrate buffer pH 6, as previously described (Nikolaivits et al., 2022). The activity was calculated in units (U), where 1 unit is defined as the amount of enzyme required to release 1 µmol of *p*-nitrophenol per minute under the given assay conditions. Protein concentration in cell-free extracts was estimated according to the Lowry assay (Lowry et al., 1951), while quantification was performed using Bovine Serum Albumin (BSA) solution as a standard.

## 2.8 Proteomic analysis

### 2.8.1 Sample preparation and LC-MS/MS analysis

After selecting the most promising fungal strains for PU and PE degradation, the secreted proteins in presence of Impranil, hexadecane, and lcAlk solution were identified by proteomic analysis. Briefly, the extracellular fraction of each strain grown

in MM/Impranil, MM/hexadecane and MM/lcAlk was filter-sterilized, concentrated and dialyzed, before it was analyzed by LC-MS/MS (see Electronic supplementary material).

### 2.8.2 Data analysis and peptide identification

LC-MS/MS samples were searched with MaxQuant v2.0.3.0 (Tyanova et al., 2016) against their corresponding proteome and the MaxQuant common contaminant database with default search settings, including, a false discovery rate (FDR) set at 1 % on peptide spectrum match (PSM), peptide and protein level. Reversed sequences of all protein entries were concatenated to the database for estimation of the FDR.

For the sample of BPOP18 and the sample of IA2 grown on Impranil proteins were quantified with the iBAQ (intensity Based Absolute Quantification) algorithm (Schwanhäusser et al., 2013). The 2 LC-MS/MS samples of MM36 grown in the presence of hexadecane and MM/lcAlk solution were searched together, matching between runs was enabled and proteins were quantified with the iBAQ and the MaxLFQ algorithm (Schwanhäusser et al., 2013) for relative quantification using a minimum ratio count of 2 unique and razor peptides.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2022) partner repository (https://www.ebi.ac.uk/pride/) with the dataset identifier PXD038835 and 10.6019/PXD038835.

### 2.8.3 Functional annotation of proteins

Available functional annotations were downloaded from UniProt. Proteins were classified as extracellular based on their subcellular location annotation when available or the presence of signal peptide and the absence of transmembrane helices in the mature protein. Additionally, Interproscan v5.56-89.0 (Jones et al., 2014) was used to identify protein motifs and domains in the proteins. CAZymes were identified with BlastP against the CAZy database (release 08062022) and HMMER3 against the library of run_dbcan v3.0.7 (Zhang et al., 2018). BlastP alignments were filtered with an e-value 1e-50 and 95 % coverage of both the query and subject sequence.

## 3. Results and Discussion

### 3.1 Agar plate screening and fungi identification

Bacterial and fungal strains capable of PU degradation have usually been isolated from the surface of soil-buried polyester PU and tested in flasks or in composting conditions (Barratt et al., 2003; Nakajima-Kambe et al., 1995). In this study, a variety of environmental samples was collected, including dead isopods and decaying oak log not necessarily containing PU and PE materials. The microorganisms isolated from the different samples were inoculated in agar plates (Figure S1), and especially the fungal strains (Table S1) were further tested for their plastic degradation capability.

Screening for potential PU degrading strains was performed in agar plates supplemented with Impranil (**Figure 1**). The competent strains formed zones of clearing around the inoculum, a fact which implies PU dispersion breakdown. Strains #7, #8, and #10 (Table S1) were proved to be the most promising strains, so their potential for ether-based PU degradation was investigated. On the other hand, strain #2 showed limited Impranil degradation, therefore it was not selected for further experiments, but it was identified as a potential PU degrader possibly after optimization

of the reaction conditions. Plates with MM were used as control, in which no sign of growth was observed.

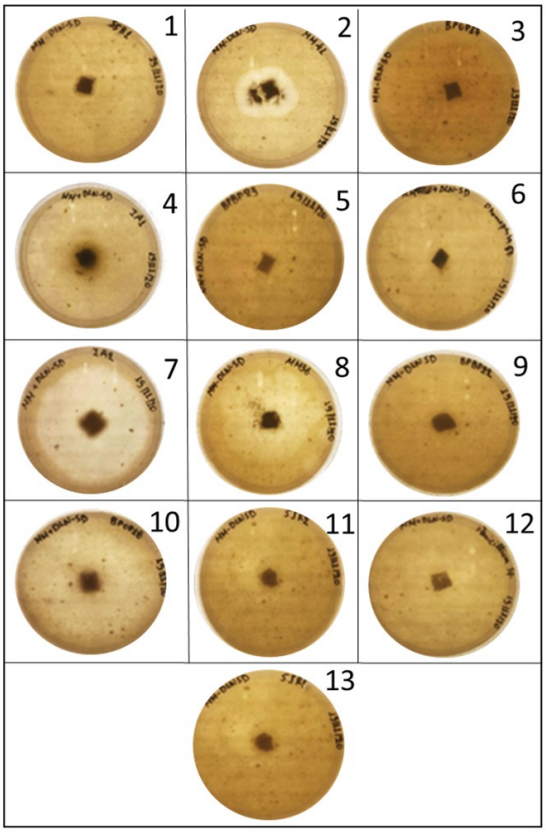


**Figure 1** Growth of 13 fungal strains on mineral medium using Impranil as carbon source, after 5 days of incubation.

Agar plates used for PE screening consisted of MM supplemented with hexadecane or a mixture of lcAlk prepared in hexadecane. Since little is known about alkane degradation by fungi, a report on different bacterial strains by Wang & Shao, 2013, clearly mentions that the alkane degradation pathway is regulated only in the presence of the appropriate hydrocarbons, whose carbon chain mainly ranges from $C_{14}$ to > $C_{22}$. Additionally, other reports mention that the enzymes able to oxidize medium-chain alkanes ($C_5$ to $C_{17}$) cannot oxidize the longer ones, because of the size limit imposed

by the enzyme binding pocket, concluding that the enzymes oxidizing lcAlk differ from those acting on the shorter ones (Rojo, 2009; Wang & Shao, 2013). For these reasons, even though hexadecane is the longest liquid alkane allowing the creation of the lcAlk solution (Griesbaum et al., 2000), its assimilation cannot be considered as a sign of PE degradation potential. Consequently, the plates with MM/hexadecane were used as control, whereas plates with MM/lcAlk solution were used to screen the different fungal strains as putative PE degraders.

The strains whose growth on lcAlk (Figure 2B) was greater than those on hexadecane (Figure 2A), were selected as potential PE degraders, and their ability on PE breakdown was further studied. The first indications derived from the plate screening, showed strains #2 and #8 (Table S1) can sufficiently grow on agar plates supplemented with lcAlk. Specifically, strain #8 was selected as the most promising candidate for PE degradation due to comparatively rapid growth, and its potential was further evaluated by using its enzymes as biocatalysts for virgin PE degradation.

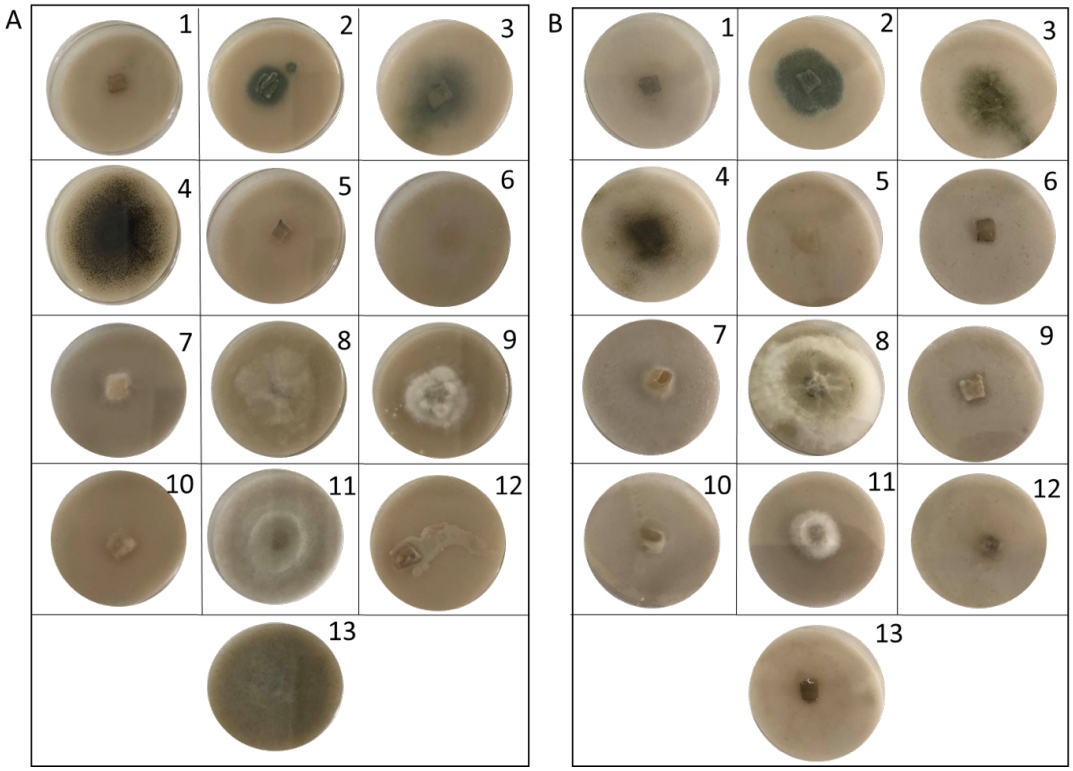


**Figure 2** Growth of 13 fungal strains on mineral medium supplemented with hexadecane (A) and Hexadecane-lcAlk (B) after 5 days of incubation

Regarding the identification of the most promising fungal strains, the ITS sequences of strains MM41 (#2), IA2 (#7), MM36 (#8), and BPOP18 (#10) were aligned to the UNITE database (https://unite.ut.ee/). The ITS sequence of MM36 was identical to the ITS sequences of *Aspergillus transmontanensis* and *Aspergillus parasiticus* and was thus classified as *Aspergillus* sp. MM36. The ITS sequence of MM41 was identical to the ITS of *Penicillium citrinum* and was classified as *Penicillium citrinum* MM41. Strains IA2 and BPOP18 were identified as members of the *Fusarium* genus and were further searched against the *Fusarium* MLST database (https://fusarium.mycobank.org/) with an identity threshold of 99.6 % to assign these two strains at the species level, as suggested for filamentous fungi (Vu et al., 2019). This search classified BPOP18 as Fusarium oxysporum, however IA2 could not be assigned to the species level. Interestingly, *Fusarium* and *Aspergillus* species have been

repeatedly referred in ester-based PU degradation (Khan et al., 2020; Mathur & Prasad, 2012; Ren et al., 2021; Zafar et al., 2013), with the latter to be also mentioned as a promising candidate for LDPE breakdown (DSouza et al., 2021; El-Sayed et al., 2021; J. Zhang et al., 2020), implying that these genera possess enzymes which are worth being studied and utilized against recalcitrant polymer breakdown.

3.2 Fungal secretome as biocatalyst for ether-based PU degradation

Even though *Fusarium* sp. IA2, *Aspergillus* sp. MM36, and *F. oxysporum* BPOP18 were identified as putative ester-based PU degraders, Impranil degradation does not confirm urethane bond hydrolysis, as ester-based PU is more vulnerable to degradation, due to the ester rather than the urethane bond cleavage (Akutsu et al., 1998; Schmidt et al., 2017). For this reason, these strains were subsequently grown in liquid cultures using Impranil as the sole carbon source and the extracellular fraction was used as a biocatalyst for ether-based PU hydrolysis. As shown in **Figure 3**, the maximum weight loss and number-average molecular mass ($\overline{M_n}$) decrease observed by *Aspergillus* sp. MM36 secretome was 5.2 and 4.8 %, respectively. On the other hand, even better results were achieved when PU powder was treated with the extracellular fraction from *Fusarium* strains. More specifically, the enzymes secreted by *Fusarium* sp. IA2 and *F. oxysporum* BPOP18, reduced PU weight by 13.5 and 9.0 %, while $\overline{M_n}$ decreased by 7.2 and 5.5 %, respectively (**Figure 3**).

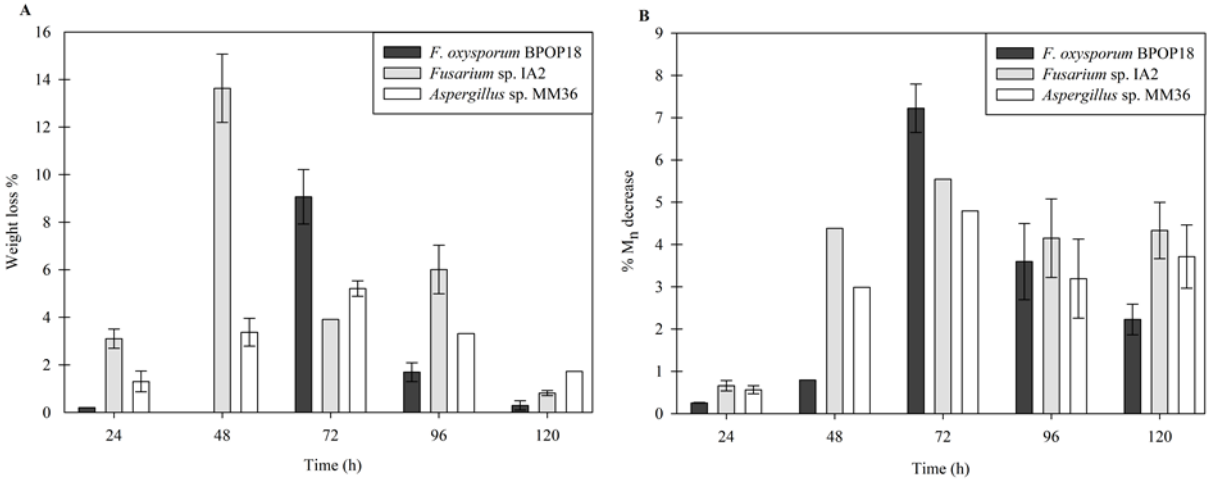


**Figure 3** Measured weight loss (%) (A) and $\overline{M_n}$ decrease (B) of PU powder after treatment with the fungal secretomes during the time course experiment. The dark grey, light grey and white bars correspond to *F. oxysporum* BPOP18, *Fusarium* sp. IA2, and *Aspergillus* sp. MM36, respectively.

To appropriately interpret these results, PU degradation was correlated with the enzymatic activity in the culture supernatant, providing some evidence about the enzymes induced in presence of Impranil. In this study, the esterase activity was monitored during cultivation time, as esterases are predominant in ester-based PU degradation (J. Liu et al., 2021), hydrolyzing ester and in some cases also urethane linkage (Santerre et al., 1994; Smith et al., 1987), releasing an amine, an alcohol and a molecule of carbon dioxide (Magnin et al., 2020).

As shown in **Figure 4**, the esterase activity in *F. oxysporum* BPOP18 and *Aspergillus* sp. MM36 culture supernatant was peaked after 72 h of induction, almost reaching 4 and 2.4 U/mg protein, respectively, a fact which can be correlated with the maximum degradation yields recorded. Concerning *Fusarium* sp. IA2, the esterase activity was also peaked at the same time point exceeding 1.4 U/mg protein, whereas

the maximum weight loss was measured after treating PU powder with the culture supernatant obtained after 48 h growth (**Figure 4**). That might indicate that the enzymatic activity having the most effect on the material is not necessarily an ester-cleaving one.

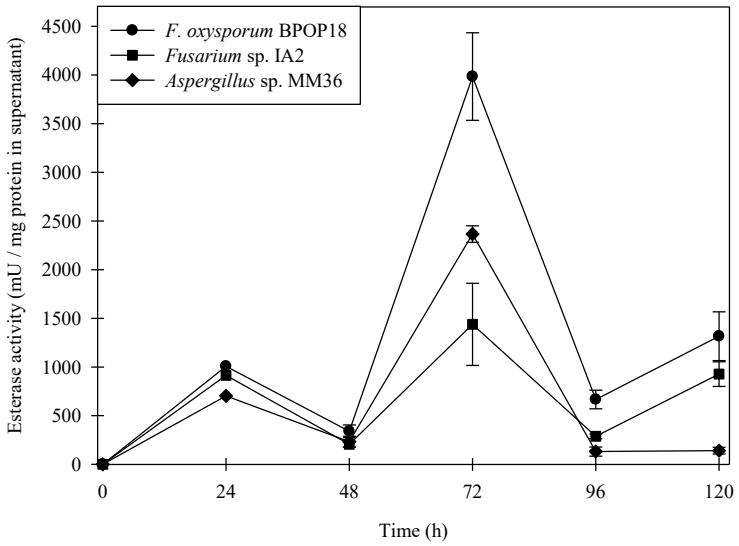


**Figure 4** Esterase activity (mU/mg protein) in supernatant of *F. oxysporum* BPOP18 (black circles), *Fusarium* sp. IA2 (black squares), and *Aspergillus* sp. MM36 (black diamonds) induced by Impranil during 5 days of incubation.

Based on the degradation yields mentioned above, both *Fusarium* strains were proved to have higher depolymerizing potential in PU degradation, therefore *Aspergillus* sp. MM36 was not studied further. However, considering that weight loss measurement and $\overline{M_n}$ decrease were similar in *Fusarium* strains, the culture supernatants were concentrated, so they get higher, but also similar protein content (**Table S2**), increasing this way the chances of succeeding higher degradation yields and better comparing the two secretomes' potential. The results presented in **Figure 5**, prove that the esterolytic activity of *Fusarium* sp. IA2 accompanied by weight loss and

$\overline{M_n}$ decrease fluctuates at the same levels, as previously observed, although the protein content is higher. More specifically, the maximum esterase activity did not exceed 1.3 U/mg protein and it was detected after 48 h of incubation when at the same time the weight loss of PU powder decreased by 9.1 %. Regarding the $\overline{M_n}$, the maximum recorded reduction was 8.2 %, and it was observed after using the secretome of 72 h (**Table S3**). On the contrary, the esterase activity in the extracellular fraction of the *F. oxysporum* BPOP18 increased by 1.4-fold after concentration, almost reaching 5.6 U/mg protein after 72 h. At the same time point, polymer weight loss and molecular weight decrease were maximized, as they were reduced by 24.5 (**Figure 5B**) and 20.4 % (**Table S3**), respectively.

Interestingly, the degradation results accompanied by the enzyme assay data provide an assumption about the enzymes induced in presence of Impranil, which probably differ between the *Fusarium* strains. After concentrating the culture supernatant of *F. oxysporum* BPOP18, the esterolytic activity was increased and also coincided in time with maximum PU degradation yield. On the contrary, in the case of *Fusarium* sp. IA2, the highest esterolytic activity measured in the concentrated culture supernatant is 4-fold lower when compared to those of *F. oxysporum* BPOP18, so it can be probably inferred that PU breakdown is not only a result of esterase activity, but more enzymes could synergistically act towards PU degradation. However, the low esterase activity assay alone does not provide enough evidence for underestimating esterases' action, as *p*-nitrophenyl butyrate is used to detect mostly cutinases, while lipases preferentially hydrolyze acyl esters with >10 carbon atoms (Liu et al., 2019). In any case, these diversity signs between the *Fusarium* strains can be of high interest, so the secreted enzymes of both strains in presence of Impranil were identified by proteomic analysis.

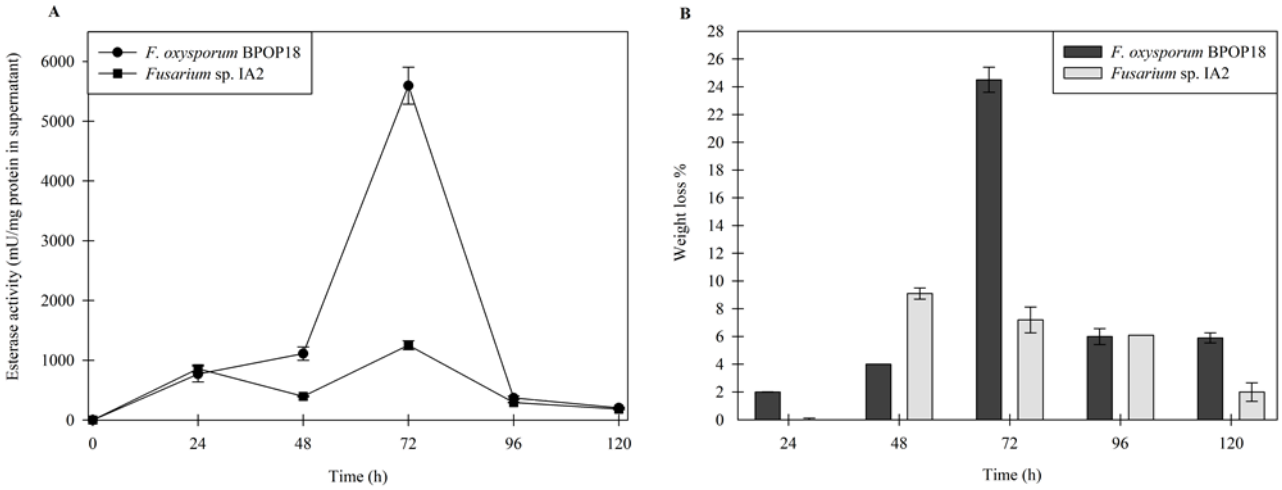


**Figure 5 A.** Esterase activity (mU/mg protein in supernatant) of *F. oxysporum* BPOP18 (black circles), *Fusarium* sp. IA2 (black squares) after culture supernatant concentration **B.** Weight loss of PU powder after treatment with the fungal secretomes during the time course experiment with the concentrated culture supernatants. The dark grey and light grey bars correspond to *F. oxysporum* BPOP18, *Fusarium* sp. IA2, respectively.

One more key benefit that should be highlighted is that Impranil could induce the rapid secretion of enzymes, which can recognize and cleave the urethane bond of ether-based PU. This fact can be critical if we consider all the research reports that have tested only the ester-based PU breakdown, after screening on Impranil. To the best of our knowledge, after the preliminary screening of different fungal strains on Impranil, only Álvarez-Barragán et al. have studied their degradation potential on ether-based PUs, which are known to remain intact to microbial attack (Álvarez-Barragán et al., 2016). As a consequence, this can justify why the discovery of novel enzymes able to degrade ether-based PU has almost no progress over the last years, with the majority of the published research to have been focused on microbes, and, by extension, on their secreted enzymes

capable of hydrolyzing polyesters such as PET or ester-based PU (Danso et al., 2019; Mohanan et al., 2020).

3.3 Secretome analysis of *F. oxysporum strains*

Apart from the development of a novel screening method and the confirmation of the depolymerizing potential of fungal secretome, the secreted proteins in presence of the inducers were also identified. Secreted protein identification can lead to the discovery of important enzymes, making incremental progress toward plastic waste valorization, following effective green technologies.

The secreted proteins revealed in presence of Impranil by the proteomic analysis were analyzed against known *Fusarium* proteomes. To choose a suitable proteome for *Fusarium* sp. IA2 all non-redundant *Fusarium* proteomes were downloaded from UniProt and the LC-MS/MS data of IA2 were searched against them with MaxQuant. The proteome of *Fusarium oxysporum* f. sp. *narcissi* (UP000290540, 20595 sequences) resulted in the highest identification rate on the PSM and protein level. UP000290540 was also among the best proteomes for the sample of *Fusarium oxysporum* BPOP18. Both samples of IA2 and BPOP18 grown on Impranil were thus searched against UP000290540.

Specifically, in the secretome of *F. oxysporum* BPOP18 292 proteins were detected, from which 176 (60 %) were classified as extracellular and 17 (6 %) as membrane-bound. As Impranil degradation takes place extracellularly, the analysis mainly focused on these proteins. According to the proteomic analysis, several hydrolases such as lipases, cutinases, acetylesterases, carboxypeptidases, peptide hydrolases, and oxidoreductases were induced **(Supplementary_ excel file)**, whose role has been already associated with PU degradation (Nikolaivits et al., 2021). Among the

10 most abundant proteins belong an esterase, a protease, a hydrolase, and 6 oxidoreductases. As mentioned in a similar study conducted by Zhang et al. (2022), the transcriptomic analysis in a *Cladosporium halotolerans* strain, revealed that the upregulated expressed genes in presence of Impranil also encode cutinases, peroxidases, lipases, and hydrophobic surface binding proteins, with the latter possibly absorbed to hydrophobic Impranil forming complexes with the other hydrolases mentioned. Among the 25 oxidoreductive enzymes, which were identified in our case study, several peroxidases and tyrosinases are included **(Supplementary_excel file)**, nevertheless, especially for tyrosinases, we assumed that their role in Impranil degradation can be supplementary since this enzyme mainly oxidizes phenolic compounds. However, concerning the virgin PU, these enzymes can modify its structure, as a previous report describes a laccase-mediated system from *Trametes versicolor*, which can degrade different PU materials by forming carbonyl groups (Magnin et al., 2021).

Regarding the secretome of *Fusarium* sp. IA2, the analysis detected 70 proteins in total, from which 52 (74 %) were predicted to be extracellular or membrane-bound. Interestingly, among the 10 most abundant detected enzymes belong 3 cutinases, a lipase, and a peptide hydrolase **(Supplementary_excel file)**. At this point, it is worth mentioning that proteases form the largest group of the secreted enzymes, which is consisted of serine-type or metallo-proteases with *endo*- and *exo*- activity (Ozsagiroglu et al., 2012; Skleničková et al., 2020). Regarding the enzymes with esterolytic activity, only 7 were detected in the fungal secretome, which belong to cutinases, lipases, and carboxyl ester hydrolases.

Surprisingly, in both *Fusarium* secretomes, proteases are dominant compared to the other enzymes identified **(Figure 6)**. This discovery is of high significance since the detection of protease activity in culture mediums supplemented with Impranil is not so

frequently reported (Álvarez-Barragán et al., 2016; Khruengsai et al., 2022; K. Zhang et al., 2022), while proteases are referred to be more effective than esterases in PU degradation (Skleničková et al., 2020). In any case, the large number of the detected proteases can be justified, if we consider that the main product of the urethane bond hydrolysis after esterase or protease action is an amine (Magnin et al., 2020), so the secretion of enzymes with proteolytic activity is expected to be induced.

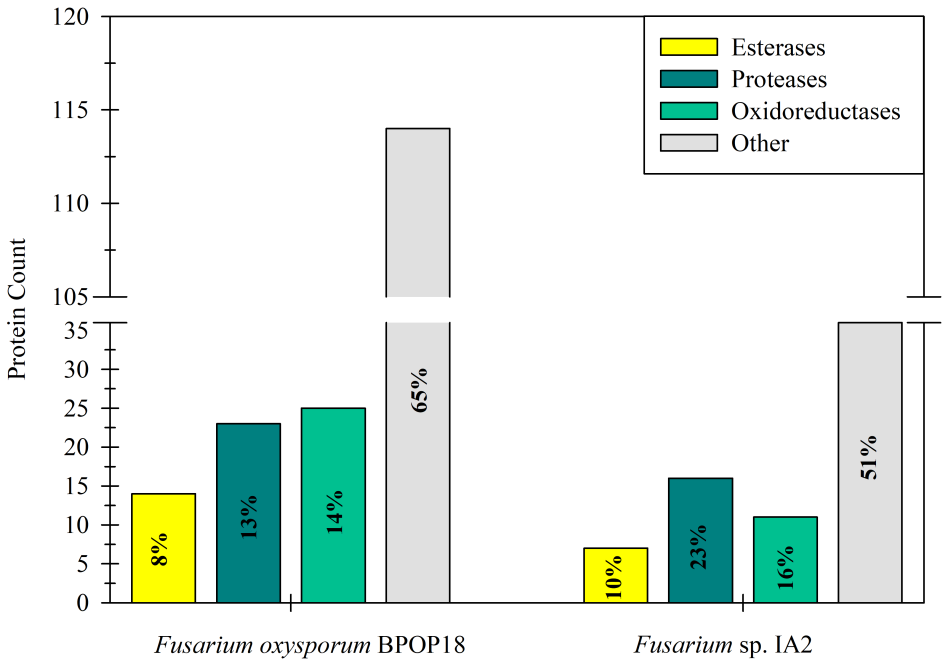


**Figure 6** Comparative analysis of the secreted enzymes in presence of Impranil in *F. oxysporum* BPOP18 and *Fusarium* sp. IA2. In each bar, each enzyme group is presented as a percentage of the secreted proteins.

On the whole, based on the enzymatic activities induced in presence of Impranil, it can be assumed that ether-based PU degradation, was caused after urethane bond cleavage. Simultaneously, the same enzymatic activities can degrade ester-based PU and the intermediate products can be subsequently utilized, providing a carbon source

necessary for fungal growth. The esterase activity in *Fusarium sp.* IA2 culture supernatant was not as high as in the case of *F. oxysporum* BPOP18, a fact which is also justified by the proteomic analysis, since proteases form the largest group in *Fusarium* sp. IA2 secretome. However, this does not necessarily mean that the PU degradation mechanism between these two *Fusarium* species substantially differs, as it seems that in any case, the complete degradation demands an enzymatic mixture, consisting of both esterases and proteases.

3.4 Enzymatic treatment of LDPE using the secretome of *Aspergillus* sp. MM36

The secretome of *Aspergillus* sp. MM36 induced by MM/lcAlk growth medium was used for LDPE breakdown. After 4 days of incubation, the LDPE powder was analyzed using FT-IR spectroscopy to detect any modifications in the molecular structure (**Figure 7**).

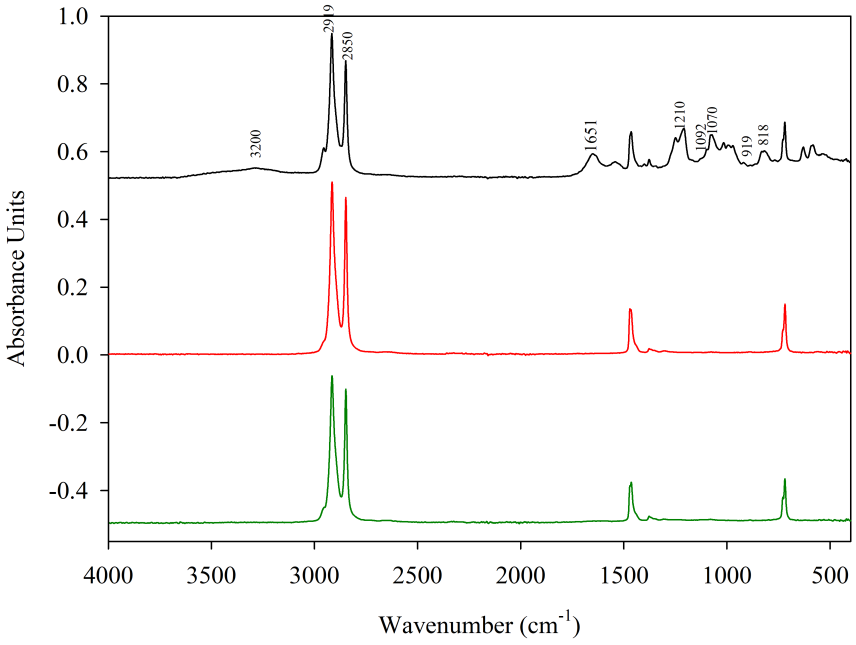

**Figure 7** FT-IR spectrum of virgin LDPE powder (green line), LDPE powder after treatment with supernatant of *Aspergillus* sp. MM36 grown in MM/hexadecane (red line), and MM/lcAlk solution (black line)

The spectra from the virgin PE in comparison with PE powder treated with the supernatant of *Aspergillus* sp. MM36 grown in MM/hexadecane (green and red line, respectively) showed only four peaks, which belong to the characteristic LDPE peaks (**Figure 7**). More specifically, absorptions at 2919 and 2850 $cm^{-1}$ correspond to $CH_3$ and $CH_2$ stretching vibrations, respectively, and their presence is typical in alkanes (Raghavan & Torma, 1992).

The spectrum of PE powder, when treated with the supernatant of MM/lcAlk cultures, is significantly different compared to those of virgin PE powder and the treated PE powder using the extracellular fraction of MM/hexadecane cultures. Firstly, the monitored absorption at ca.3200 $cm^{-1}$ (3090-3650 $cm^{-1}$) confirms the presence of oxidized moieties containing -OH groups (Georgiopoulou et al., 2021), which are typical degradation products for polyolefins. The C-O stretching frequencies ranging from 1072-1300 $cm^{-1}$ indicate biodeterioration (Ghatge et al., 2020; Satlewal et al., 2008), which is the first step of the PE degradation mechanism with the following steps of bio-fragmentation, bio-assimilation, and mineralization (Dey et al., 2020; Mohanan et al., 2020). More precisely, the peak at 1210 $cm^{-1}$ corresponds to the C-O stretching due to the formation of acyl groups, while the peaks at 1092 and 1070 $cm^{-1}$ are attributed to the C-O in the formed alkoxy groups. The formation of the alkoxy groups can be related to hydroxylation thus providing evidence for PE oxidative degradation. Besides, The C-O formation could also be attributed to the stabilization of peroxy radicals; although hydroperoxides, which are products of the oxidative degradation of

PE, decompose leading to the formation of hydroxyl groups and carbonyl groups or radicals (Peixoto et al., 2017). However, during PE biodeterioration, the carbonyl groups (1715-1740 $cm^{-1}$) are also expected to be formed during biodeterioration mainly due to oxidation by abiotic factors (e.g., physical and chemical treatment) (Balasubramanian et al., 2014). The strong absorbance intensity of the C=C double bond at 1651 and 818 $cm^{-1}$, (Ghatge et al., 2020) monitored in the MM/lcAlk spectrum, is also related to PE biodeterioration (Esmaeili et al., 2014) and indicates chain scission (Peixoto et al., 2017). Chain scission through vinyl end formation ($H_2C$=C-) is also monitored for the MM/lcAlk treated PE powder at the absorption 919 $cm^{-1}$ (Dey et al., 2020; Peixoto et al., 2017). The significantly different spectra recorded by FT-IR analysis provide a strong indication of the LDPE molecular structure modifications after treatment with *Aspergillus* sp. MM36 secretome, attributed to biodeterioration.

3.5 Proteomic analysis of the secreted proteins in presence of lcAlk

The 2 LC-MS/MS samples of *Aspergillus* sp. MM36 grown in the presence of hexadecane (control) and lcAlk solution were searched together against the proteome of *Aspergillus transmontanensis* (UP000325433, 14,200 sequences) with MaxQuant. The analysis identified 63 proteins in presence of lcAlk, from which 39 (62 %) were classified as extracellular and 2 as membrane-bound. The different enzymes detected in *Aspergillus* sp. secretome can be classified into esterases, proteases, oxidoreductases, and glycoside hydrolases (GH) **(Figure 8)**. Considering the enzymes whose activity can be attributed to lcAlk degradation, the proteomic analysis revealed an increased production of a flavin adenine dinucleotide (FAD) dependent-oxidoreductase, which belongs to the AA7 family of CAZy database **(Supplementary_excel file)**. The role of this enzyme is supposed to be predominant in LDPE degradation, since flavoenzymes

take part in a wide range of hydroxylation and oxidation reactions, such as oxygen activation and electron transfer (Fraaije & Mattevi, 2000).

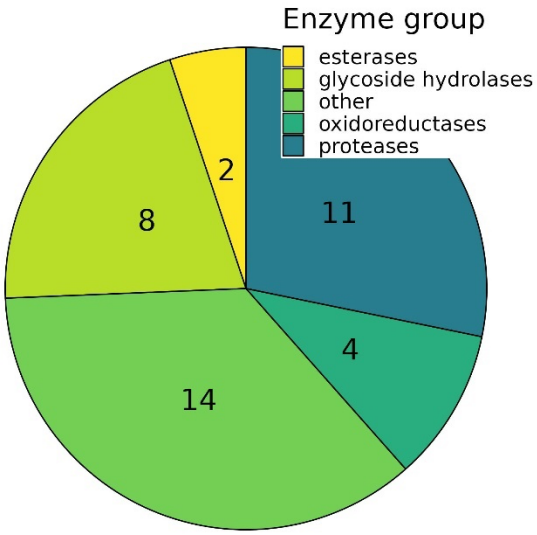


**Figure 8** The different enzyme groups detected in *Aspergillus* sp. MM36 secretome after growth in MM/lcAlk solution

Another possible explanation for the LDPE modification after treatment with culture supernatants can be also attributed to reactive oxygen species (ROS). Interestingly, a similar mechanism of LDPE degradation was also described in the case of the marine bacterium *Alcanivorax* sp., suggesting that several secreted oxidoreductases can generate extracellular ROS, although no homology with already characterized superoxide-generating enzymes was found (Zadjelovic et al., 2022). The proteome UP000325433 of *A. transmontanensis* contains a transmembrane NADPH homologous to an oxidase of *Aspergillus nidulans* able to generate ROS (Lara-Ortíz et al., 2003). As observed not only by Zadjelovic et al. but also in our case study, the hypothesis of LDPE oxidation by ROS is also strengthened after detecting a superoxide

dismutase and a catalase in the presence of lcAlk; enzymes which are probably expressed as a response to high levels of superoxide radicals (Zadjelovic et al., 2022).

## 4 Conclusions

In this work, we proved that the utilization of Impranil and lcAlk, can be a successful techique for screening putative PU and PE degraders. Among the fungal species screened, the fungal strains belonging to *Fusarium* and *Aspergillus* sp. were proved to be of high interest, as their enzyme machinery can successfully downgrade recalcitrant polymers. At the same time, synthetic polymer degradation using fungal secretomes in presence of inducers, such as Impranil and lcAlk, is referred for the first time, proposing a new, time-saving methodology for evaluating the microbe's degradation potential.

Especially for the secreted enzymes in presence of inducers, the proteomic analysis, revealed that cutinases, proteases, and oxidases expressed in presence of Impranil by *Fusarium* strains are probably responsible for ether-based PU breakdown. Concerning LDPE modification by *Aspergillus* sp. MM36, this may occur by the detected oxidoreductase, belonging to the AA7 family, and probably be assisted by generated reactive oxygen species, as proteomic analysis revealed an upregulated secretion of dismutases. Regarding lcAlk, their dual role not only as a carbon source but also as an inducer that can directly trigger the enzymatic machinery toward the assimilation of polyolefins can be considered a notable discovery, as these polymers belong to the most recalcitrant ones and to the best of our knowledge their complete degradation has not yet been reported.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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