EXPLORING THE POTENTIAL OF FUNGAL ENZYMES FROM THE GENUS CYCLOCYBE CYLINDRACEA FOR EFFICIENT BIODEGRADATION OF SYNTHETIC POLYMERS

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ABSTRACT

The global concern over synthetic plastic waste has intensified, with 22 million metric tons entering the environment annually, contributing to a total accumulation of over 6 gigatons since the widespread plastic production began in 1950. Current strategies to alleviate this environmental burden are inadequate, necessitating alternative and sustainable approaches for tackling plastic pollution[1,2]. Microbial enzymes, particularly those from fungal strains, are emerging as biotechnological tools for waste circularity. The broad enzymic machinery of fungi, capable of degrading not only recalcitrant organic compounds such as lignin, cutin, and waxes but also xenobiotics with similar structures, demonstrates the potential for plastic materials degradation[3]. This study aimed to evaluate various strains of the genus *Cyclocybe* (Basidiomycota) for their ability to depolymerize plastics and, one step further, to identify the possible enzymatic mechanism. Screening of 29 fungal strains for polymer-degrading activity was conducted on agar plates containing synthetic polymers, including polyester and polyether polyurethane. Strain identification at the species level was achieved through the study of morphoanatomic characteristics and DNA sequencing. Positive biodegradation results led to the selection of strains for evaluation in submerged fermentations using the mentioned polymers as sole carbon sources. Throughout the fermentation process, culture supernatants were assayed for turbidity, protein content, while selected oxidative and hydrolytic enzymatic activities were quantified through spectrophotometric assays. Substrate degradation was assessed for potential chemical modifications via ATR-FTIR spectroscopy. The established screening foundation could predict enzymic activity on synthetic polymers, providing a crucial tool for discovering novel enzymes, supporting the potential of fungi and their enzymic machinery as powerful tools for degrading plastic waste.

KEYWORDS

Plastic, biodegradation, fungi, screening, biocatalysis

INTRODUCTION

The widespread use of plastic materials can be attributed to their advantageous properties, such as lightweight design, durability, and ease of low-cost production[4]. While certain characteristics facilitate the convenient disposal of plastic objects, they also play a role in the significant annual discarding of approximately 150 million tons of solid plastics worldwide, which accounts for nearly half of the total annual global production[5]. Especially with the emergence of the global COVID-19 pandemic, there has been a surge in the mass production of plastic items such as masks, protective suits, and gloves, leading to an increase in plastic waste[6]. It is estimated that without improvements

in waste management practices, approximately 12000 million kilograms of plastic waste will accumulate in landfills or the environment by 2050[7].

To tackle the global challenge of plastic waste, conventional approaches have been utilized: the process of incineration can result in the release of harmful and toxic emissions, including dioxins, furans, heavy metals, and sulphides, which further contributes to environmental pollution[8].While recycling presents a more favorable option for managing plastic waste, it alone does not offer a definitive solution to the plastic problem. Mechanical recycling results in a deterioration of polymer quality, hindering the plastic's potential for indefinite reuse (downcycling)[9,10], while chemical recycling involves the use of elevated temperatures and harmful solvents[11].

Biodegradation plays a crucial role in mitigating the adverse effects of plastics. Microorganisms, including fungi, are believed to be the inherent decomposers of not only natural polymers, but also artificial compounds like plastics[12]. Fungi and their enzymic machinery remain an unexplored source of biological tools that could be uncovered and utilized to address emerging challenges, such as the bioconversion of plastics.

This study aims to evaluate fungal strains of the genus *Cyclocybe* (Basidiomycota) for their ability to depolymerize plastic materials, more specifically an anionic aliphatic polyester-polyurethane dispersion (Impranil® DLN-SD), an anionic polycarbonate polyurethane dispersion (Impranil® DL 2077) and polycaprolactone (PCL). This study also highlights the intraspecific variation observed in the degradation capacity of each strain. The biodegradation potential of fungi is being explored with the objective of contributing to the development of sustainable solutions for plastic waste management.

METHODOLOGY

Fungal strains

29 strains of the genus *Cyclocybe* (Basidiomycota), isolated from diverse habitats of Greek territory and preserved in the culture collection of the Laboratory of General and Agricultural Microbiology, AUA were screened for their ability to grow on agar plates and liquid cultures containing synthetic polymers. Identification of strains at the species level was previously accomplished by examining morphoanatomic features and conducting DNA sequencing analysis. The fungal strains were cultivated on PDA plates for 8 days at 25 °C, and one piece from the edge of the culture was cut with a loop of specific dimensions and used as inoculum for the screening tests.

Agar plate screening assays

In case of Impranil® DLN-SD and PCL, the screening tests were conducted in agar plates containing Czapek-Dox medium supplemented with 0.4% (v/v) Impranil® DLN-SD or 0.5% (w/v) PCL in place of glucose as sole carbon source. The evaluation of the strains was carried out by measuring the growth rate of the mycelium and the formation of clear zones around the inoculum.

Liquid screening assays

To perform liquid screening assays, the fungal strains were cultivated in 10 mL liquid cultures containing Czapek-Dox medium along with 0.4% (v/v) Impranil® DLN-SD or 0.6% (v/v) Impranil® DL 2077 as the sole carbon source, at 25°C under agitation (80 rpm) for 18 days. The evaluation of the strain growth and the polymer degradation were conducted with both measuring the decrease in the turbidity of the samples (every 3 day-measurement of the optical density at 600 nm, OD_{600}) and weighing the final dry fungal biomass.

Determination of PU properties

Impranil® DLN-SD degradation was assessed by Fourier-Transform Infrared (FT-IR) spectroscopy, aiming to detect any changes in PU chemical signature.

Evaluation of enzymatic activities in the culture supernatants

Determination of enzymatic activities in culture supernatants when 0.4% (v/v) Impranil® DLN-SD was used as carbon source took place. Esterase activity was determined spectrophotometrically by hydrolysis of p-nitrophenyl acetate (p-NPA) at λ_{405} nm. Protease activity was determined spectrophotometrically by casein hydrolysis at λ_{280} nm. Laccase activity was determined spectrophotometrically by oxidation of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) at λ_{420} nm.

RESULTS AND DISCUSSION

A. Impranil® DLN-SD (PU)

i. *Agar plate and liquid culture screening*. Polyester- polyurethane degradation by the 4 most efficient strains was demonstrated in both solid and liquid cultures with Impranil® DLN-SD. The competent strains formed zones of clearing around the inoculum in the plates (Figure 1) while decreasing the turbidity of Impranil® DLN-SD (Figure 2), as indicated by measurements of the optical density at 600 nm in the liquid cultures (Figure 3), a fact which implies the polymer substrate breakdown in both cases.

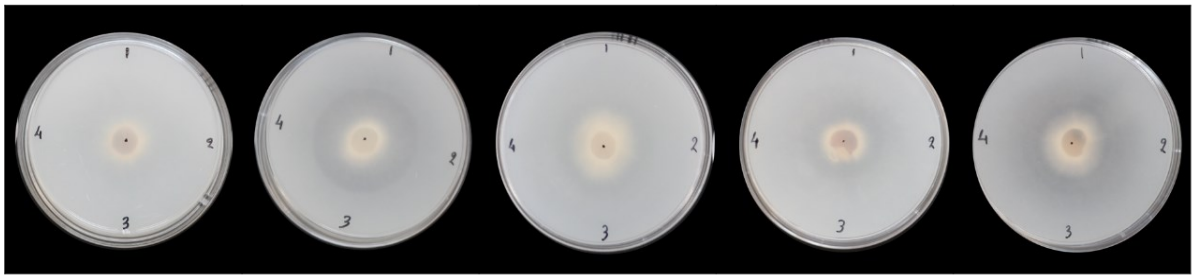

Figure 1. *Czapek-Dox medium with 0.4% (v/v) Impranil® DLN-SD inoculated with the strains #964, 0834, 965, 970, 960 (from left to right) after 18 days of incubation in agar plates at 25°C. Strain #964 (left) shows no polymer degradation ability compared to the other strains.*

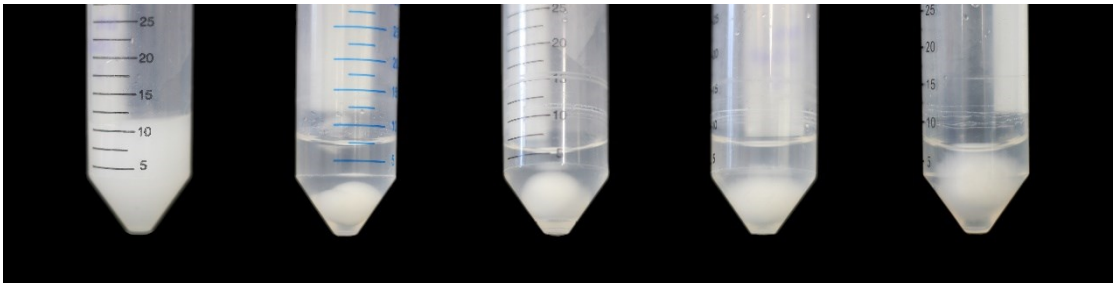

Figure 2. *Czapek-Dox medium with 0.4% (v/v) Impranil® DLN-SD inoculated with the strains #964, 0834, 965, 970, 960 (from left to right) after 18 days of incubation in liquid cultures at 25°C, under agitation (80 rpm). Strain #964 (left) shows neither growth nor polymer degradation ability compared to the other strains.*

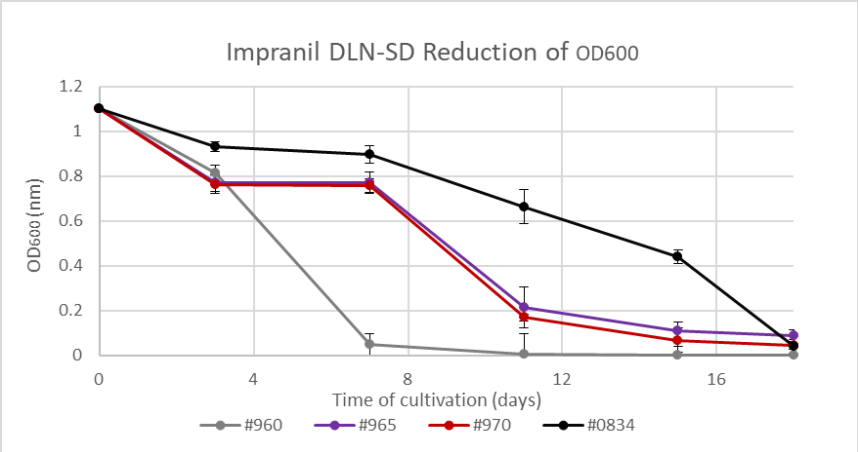

Figure 3. *The reduction of the turbidity of Impranil® DLN-SD by the strains #960,965, 970 and 0834.*

ii. *Evaluation of enzymatic activities in the culture supernatants*. Esterase activity was determined in low levels in the culture supernatants at the 8th day of cultivation, while it decreased later. Neither protease nor laccase activity were detected.

iii. *FT-IR spectroscopy of residual Impranil® DLN-SD.* The breakdown of the substrate is primarily evidenced by a significant decrease in the intensity of the peak at 1735 cm^{-1}, indicating the stretching of C=O bonds in esters and/or N-aryl urethanes. Following treatment with *C. cylindracea* 960, a shift to 1710 cm^{-1} is observed, indicating the formation of aryl-carboxylic acids. Additionally, the peaks at 1547 cm^{-1} and 1246 cm^{-1} increase, indicating bending and stretching of urethane C-N-H bonds, respectively, suggesting that degradation by *C. cylindracea* 960 occurs through hydrolysis of ester and urethane bonds. The rise in the 1380 cm-1 peak is attributed to C-H bending from symmetrical deformation of -CH_3, likely due to the generation of alkanes resulting from the depolymerization of the Impranil® DLN-SD substrate.

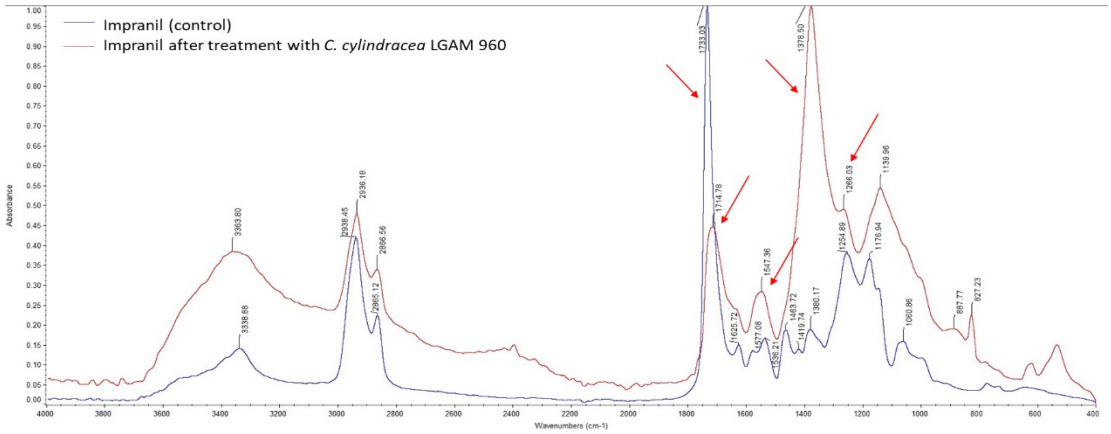

Figure 4. *FTIR spectroscopy analysis of Impranil® DLN-SD degradation by C. cylindracea 960. Spectra of Impranil® DLN-SD at the beginning of cultivation (control, blue) and after 18 days of incubation with C. cylindracea 960 (red).*

B. Impranil® DL 2077 (PU)

i. *Liquid cultures screening*. Polycarbonate polyurethane degradation was assessed by the decrease in the turbidity of Impranil® DLN-SD (Figure 4), as indicated by measurements of the optical density at 600 nm in the liquid cultures (Figure 5).

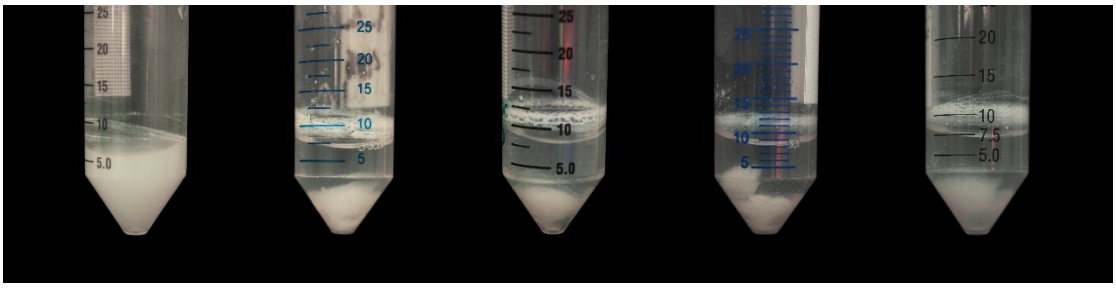

Figure 5. *Czapek-Dox medium with 0.6% (v/v) Impranil® DL 2077 inoculated with the strains # 965, 967, 964, 960 and 961 (from left to right) after 18 days in liquid cultures at 25°C under agitation (80 rpm). Strain #965 (left) shows neither growth nor polymer degradation ability compared to the other strains.*

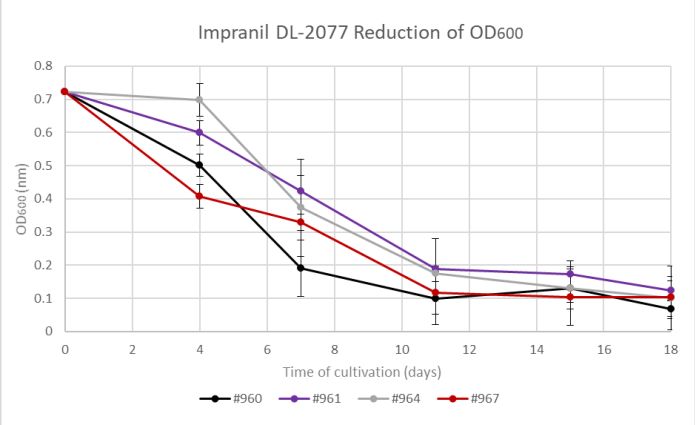

Figure 6. *The reduction of the turbidity of Impranil® DLN-SD by the strains #960, 961, 964 and 967.*

C. Polycaprolactone (PCL)

i. *Agar plate screening*. In the PCL degradation assay, halo zones were visible around the colonies of many fungal strains, after 7 days of incubation. Indicatively, as shown in Figure 6, strains #965, 964, 960, 961 formed a clear zone around colonies on PCL agar plates indicative of their ability to degrade this polymer. The diameter of the zones increased depending on the incubation time.

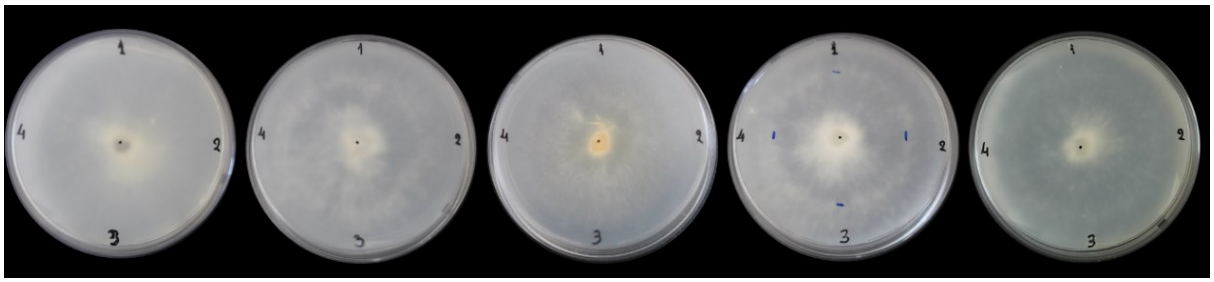

Figure 7. *Czapek-Dox medium with 0.5% (w/v) PCL inoculated with the strains # 967, AA, 965, 960 and 0834 (from left to right) after 18 days of incubation in agar plates at 25°C. Strain #967 (left) shows no polymer degradation ability compared to the other strains.*

Table 1. *Summary table of some of the most competent strains in each substrate. The degree of biodegradation is represented using the symbols '+,-': (-): no degradation, (+): moderate degree of degradation, (++): high degree of degradation.*

Strain/substrate	Impranil® DLN-SD (PU)	Impranil® DL 2077 (PU)	Polycaprolactone (PCL)
960	**++**	**++**	**++**
961	+	++	-
964	-	++	+
965	++	-	++
967	+	++	-
970	++	+	-
0834	++	-	+
AA	+	-	++

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