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Original Research

Mycelial Growth and Enzymatic Activities of Fungi Isolated from Recycled Paper Wastes Grown on Di (2-ethylhexyl) phthalate

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Abstract

Phthalate esters are often discharged into the ecosystem by paper and plastic industries during manufacturing processes, contributing to environmental pollution. Di (2-ethylhexyl) phthalate (DEHP) is a plasticizer widely used in the manufacture of plastics that imparts flexibility to polyvinyl chloride resins. This is an endocrine-disrupting compound that could lead to cancer. Filamentous fungi were isolated from mixed pulp waste in a paper recycling facility. Ribosomal DNA internal transcribed spacers sequencing were performed, which allowed for the identification of the fungal species of the isolates as *Neurospora sitophila*, *Fusarium culmorum*, *Trichoderma atroviride*, *Hypocrea lixii*, and *Trichoderma harzianum*. Radial growth rate (u_r), mycelial biomass, and laccase and esterase activities of the fungal isolates in media containing different concentrations of DEHP (0, 500, 750, 1,000, 1,200, and 1,500 mg/l) were carried out. The highest u_r was shown by *N. sitophila* in the medium added with 1,500 mg of DEHP/l. The largest mycelial biomass was produced by *F. culmorum*, *T. atroviride*, *H. lixii*, and *T. harzianum* in the medium containing 1,500 mg of DEHP/l. *F. culmorum* and *T. harzianum* had higher esterase activity than laccase activity in all the media tested. This study demonstrates that fungi isolated from the recycled paper wastes in a paper industry were capable of utilizing DEHP as the sole carbon source. These fungal isolates can be used for the bioremediation of DEHP-contaminated sites.

Keywords: di (2-etylhexyl) phthalate, esterase, filamentous fungi, laccase, molecular identification

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Introduction

Phthalate esters are often discharged by the paper and plastic industries during the manufacturing processes into the ecosystem, contributing to environmental pollution [1]. These compounds are aromatic esters widely used as plasticizers in the manufacture of plastics, which impart flexibility to polyvinyl chloride resins [2]. Phthalates are not chemically bonded to the plastics polymer and might eventually migrate from the polyvinyl chloride resins or plastics into the environment. Di (2-etylhexyl) phthalate (DEHP) is one of the most used plasticizers worldwide [3]. DEHP is an endocrine-disrupting compound that causes adverse reproductive, developmental, and immune effects in humans and wildlife, and could also lead to cancer [3, 4]. Microorganisms have been studied as an alternative pathway for phthalate biodegradation. It has been reported that esterases are enzymes involved in microbial degradation of phthalate esters [5-7]. Phthalates can be degraded by microorganisms such as *Gordonia* sp. [8], *Pseudomona alcaligenes*, *P. tutzeri*, *Bacillus subtils*, *Micrococcus* sp., *Rhodococcus* [9], *Bacillus stearothermophilus* [10], *Fusarium* sp., *Trichosporon* sp. [7], *Pleurotus ostreatus*, *Irpex lacteus*, *Polyporus brumalis*, *Merulius tremellosus*, *Trametes versicolor*, and *T. versicolor* TVMR12 [11, 12]. Microbial consortiums also have the capacity to degrade these compounds [2, 3]. In the present research, *N. sitophila*, *F. culmorum*, *T. atroviride*, *H. lixii*, and *T. harzianum* were isolated from the recycling of mixed paper wastes in a paper recycling facility and were identified using an internal transcribed spacer sequence of nuclear ribosomal DNA (ITS). Radial growth rate (u_r) mycelial biomass, and laccase and esterase activities of these filamentous fungi were evaluated in media containing 0, 500, 750, 1,000, 1,200, and 1,500 mg of DEHP/l.

Experimental Procedures

Sampling and Growth of the Fungal Isolates

Samples were taken from three different stages (pulping, addition of biocide, and pretreatment of pulping wastewater) from a recycled paper facility that converts paper wastes into cardboard rolls. The process can be described as follows: paper wastes are placed in a pulp mixer using a forklift truck (called pulping), and then biocide is added to the pulp in order to reduce the presence of microorganisms. Soaked paper (pulping material containing biocide) is transformed into a long sheet of paper using heated metal rollers that compress and further dry the paper through evaporation. The long sheets of paper are converted into cardboard rolls using a bobbin winding machine. Water from the draining stage is stored in tanks for pretreatment of pulping wastewater and activated sludge is obtained by precipitation from the wastewater treatment plant. Treated pulping wastewater is stored in a large tank (Fig. 1). Samples were inoculated in the agar surface of plates containing malt extract agar using a sterile loop. The plates were incubated up to 7 d at 25ºC. Colonies were separated on the basis of morphological parameters, isolated onto individual plates, and purified by transfer where necessary.

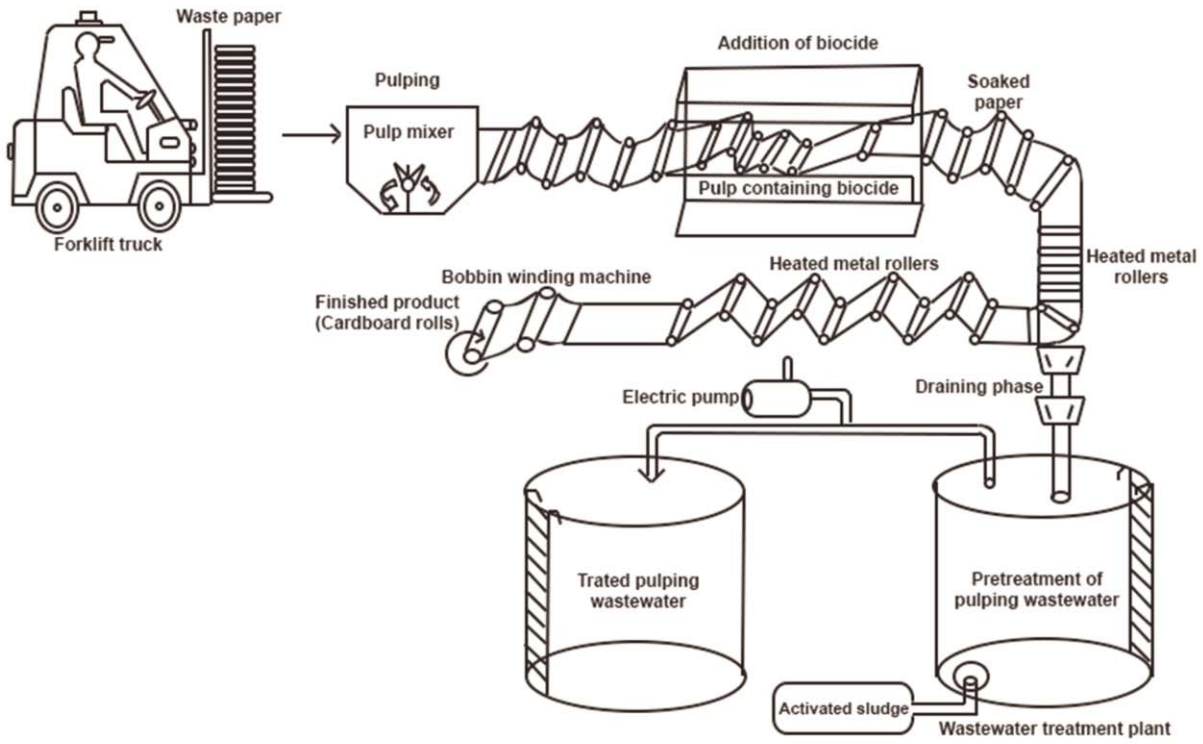

Fig. 1. Schematic representation of a paper recycling facility showing the production of cardboard rolls as final product.

Table 1. Identification of fungal isolates from three different stages of the recycling of mixed paper wastes in a paper recycling facility.

Stage of recycling	Closest match	% homology	GenBank accession number
Pulping	*Neurospora sitophila* (Shear and B.O. Dodge)	99%	HF947519
Pulping	*Fusarium culmorum* (W.G. Sm. Sacc.)	91%	HF947520
Addition of biocide	*Trichoderma atroviride* (P. Karst)	100%	HF947518
Preteatment of pulping wastewater	*Hypocrea lixii* (Pat)	97%	HF947522
Preteatment of pulping wastewater	*Trichoderma harzianu* (Rifai)	100%	HF947517

DNA Extraction and Identification of Fungi

Individual fungal strains were grown in potato dextrose agar plates for 7 d at 25ºC. The mycelia were removed from the agar and the DNA extraction was carried out as previously reported [13]. To identify isolates, DNA was amplified by PCR using the primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) [14]. The PCR conditions were as follows: initial activation of the Taq-DNA-Polymerase for 1 min at 95ºC, followed by 25 cycles of 30 s denaturation at 94ºC, annealing for 45 s at 50ºC, and extension for 5 min at 72ºC. Each PCR reaction was carried out using AmpliTaq Gold PCR kit (Applied Biosystems, E.U.A.), consisting of 25 µl of master mix, 5 µl of 10 mM of each primer, 2 µl DNA template (ca 50 ng), and 13 µl of water (final volume of 50 µl). PCR products were then sequenced by Seq Wright DNA Technology Services (U.S.A.) and used to interrogate the European Bioinformatic Institute (EBI, Cambridge, England; www.ebi.ac.uk/ena).

Culture Media

Six culture media were used:

1) mineral medium (MM)
2) MM + 500 mg of DEHP/l
3) MM + 750 mg of DEHP/l
4) MM + 1,000 mg of DEHP/l
5) MM + 1,200 mg of DEHP/l
6) MM + 1,500 mg of DEHP/l.

The MM contained (in g/l): KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $(NH_4)_2SO_4$, 1.0; $CaH_4(PO_4) \cdot H_2O$, 0.3; $FeSO_4 \cdot 7H_2O$, 0.2; $ZnSO_4 \cdot 7H_2O$, 0.2; and $MnSO_4 \cdot 7H_2O$, 0.2. The pH was adjusted to 6.0 using 1 M NaOH. DEHP was added to the sterile MM and then the culture media were sonicated in aseptic conditions for approximately three minutes using an ultrasonic processor (GEX 130) until the DEHP was fully dispersed. Petri plates were made with MM supplemented with different concentrations of DEHP as mentioned above.

Radial Growth Rate and Mycelial Biomass

Petri dishes were inoculated in the center of the plate using inoculum (4 cm of diam) from the periphery of each fungal strain grown on potato dextrose agar. The radius of the mycelial growth was measured every 4 h in *N. sitophyla* colonies; every 8 h in *T. harzianum*, *T. atroviride*, and *H. lixii* colonies; and every 12 h in *F. culmorum* colonies using a digital Vernier (Mitutoyo) until full invasion of the plates. The radial growth rate (u_r) was calculated as the slope of the radius of mycelial growth (measured as mentioned above) versus time plots (times specified above), analyzed by linear regression [15]. The mycelial biomass was evaluated in each plate after full invasion of the culture media. The mycelia were separated from the culture media using a boiling-water bath in a pre-weighed watch glass. This was weighed, and then oven-dried at 60ºC for 24 h, then weighed again [15].

Extracellular Laccase and Esterase Activities

Mycelia were removed and discarded using a scalpel from the surface of well-developed colonies of the fungal isolates grown on Petri dishes containing the media tested. The extracellular enzymatic extract (EE) was obtained from the surfaces of these Petri dishes that were flooded with sterile deionized water (25 ml) for 24 h using a glass Pasteur pipette. Laccase activity was determined in the EE by changes in the absorbance at 468 nm (using a Jenway 6405UV/Vis spectrophotometer), using 2, 6-dimethoxyphenol (DMP, SIGMA) as substrate. The assay mixture contained 900 µL of 2 mM DMP in 0.1 M acetate buffer pH 4.5 and 100 µl of either enzymatic extract, which were incubated at 40ºC for 1 min. Esterases activity was determined by changes in the absorbance at 405 nm (using a Jenway 6405UV/Vis spectrophotometer), using p-nitrophenyl butyrate (*p*NPB) as substrate. The reaction mixture contained 10 µl of *p*NPB solution (1.76% (v/v) of *p*NPB in acetonitrile), 790 µl of 50 mM phosphate buffer pH 7.0, 0.04% Triton X-100, and 100 µl of EE, which were incubated at 37ºC for 5 min [16, 17]. One enzymatic unit of laccase activity or esterase activity (U) is defined as the amount of enzyme that gives an increase of 1 unit of absorbance per min in the reaction mixture. The enzymatic activities were expressed in U/l of enzymatic extract.

Statistical Analysis

All the experiments were carried out in triplicate. Data were evaluated using one-way ANOVA and Tukey post-test using the Graph Pad Prism program.

Table 2. Radial growth rate (mm/h) of filamentous fungi isolated from the recycling of mixed paper grown on different concentrations of DEHP.

Strain	Concentration of DEHP (mg/l)					
	0	500	750	1,000	1,200	1,500
	10^{-3}					
Neurospora sitophila	460^{c} (0.1)	340^{d} (0.05)	480^{c} (0.006)	1,005^{b} (0.02)	1,006^{b} (0.5)	1190^{a} (0.03)
Fusarium culmorum	300^{d} (0.01)	300^{d} (0.007)	310^{d} (0.005)	550^{c} (0.009)	890^{a} (0.02)	750^{b} (0.005)
Trichoderma atroviride	500^{c} (0.04)	640^{b} (0.02)	560^{b} (0.01)	690^{a} (0.004)	560^{b} (0.009)	520^{d} (0.006)
Hipocrea lixii	600^{c} (0.006)	660^{b} (0.001)	510^{d} (0.01)	780^{a} (0.02)	490^{e} (0.01)	460^{f} (0.02)
Trichoderma harzianum	440^{d} (0.07)	530^{c} (0.004)	490^{d} (0.05)	970^{a} (0.002)	740^{b} (0.02)	540^{c} (0.06)

Means with the same letter (a, b, c, or d) within a row are not significantly different.
Numbers in parentheses correspond to standard deviation of three separate experiments.

Table 3. Mycelial biomass (g/cm^2) of filamentous fungi isolated from the recycling of mixed paper grown on different concentrations of DEHP.

Strain	Concentration of DEHP (mg/l)					
	0	500	750	1,000	1,200	1,500
	10^{-3}					
Neurospora sitophila	40^{d} (0.0006)	40^{d} (0.001)	50^{d} (0.001)	65^{c} (0.001)	120^{a} (0)	80^{b} (0.001)
Fusarium culmorum	45^{e} (0.001)	50^{e} (0.001)	70^{d} (0.001)	110^{c} (0.002)	180^{b} (0.003)	250^{a} (0.004)
Trichoderma atroviride	20^{e} (0.0006)	40^{d} (0.0006)	50^{c} (0.001)	75^{b} (0.002)	75^{b} (0.001)	105^{a} (0.004)
Hipocrea lixii	30^{c} (0.0006)	30^{c} (0.001)	66^{b} (0.001)	66^{b} (0.006)	89^{a} (0.001)	89^{a} (0.003)
Trichoderma harzianum	45^{d} (0.0004)	60^{c} (0.001)	80^{b} (0.006)	80^{b} (0.003)	80^{b} (0.002)	100^{a} (0.004)

Means with the same letter (a, b, c, d, or e) within a row are not significantly different.
Numbers in parentheses correspond to standard deviation of three separate experiments.

Results

Fungal Isolates

N. sitophila and *F. culmorum* were isolated from the pulping, *T. atroviride* was isolated from the pulp containing biocide, and *H. lixii* and *T. harzianum* were isolated from the pretreatment of pulping wastewater of the recycling of mixed paper wastes in a recycled paper industry (Table 1).

Radial Growth Rate

T. atroviride, *H. lixii*, and *T. harzianum* showed the largest u_r in media containing 1,000 mg of DEHP/l. *F. culmorum* had the greatest u_r in medium added with 1,200 mg of DEHP/l. The highest u_r was shown by *N. sitophila* in the medium added with 1,500 mg of DEHP/l. *F. culmorum*, *T. atroviride*, and *T. harzianum* showed the lowest u_r in the medium lacking DEHP (Table 2).

Mycelial Biomass

The largest mycelial biomass was produced by *F. culmorum*, *T. atroviride*, *H. lixii*, and *T. harzianum* in the medium containing 1,500 mg of DEHP/l. *N. sitophila* had the greatest mycelial biomass in the medium added with 1,200 mg of DEHP/l. The lowest mycelial biomass was observed in the medium lacking DEHP (Table 3).

Extracellular Laccase and Esterase Assays

N. sitophila had the highest extracellular laccase activity in medium added with 500 mg of DEHP/l, followed by *Thrichoderma* species and *H. lixxi* in media containing 1,500 and 1,200 mg of DEHP/l, respectively (Table 4). *T. harzianum* showed the major extracellular esterase activity in medium containing 500 mg of DEHP/l (Table 5). *F. culmorun* and *T. harzianum* had higher esterase activity than laccase activity in all the media tested (Table 5). The extracellular laccase and esterase activities were lower in the medium lacking DEHP than in the medium added with 1,500 mg of DEHP/l for all fungal isolates (Tables 4 and 5).

Discussion

In this work, two strains of *Trichoderma* were isolated from the recycling of mixed paper wastes in a paper recycling facility. Khokhar et al. [18] isolated *Arpergillus* sp.

Table 4. Extracellular laccase activity (U/l) of filamentous fungi isolated from the recycling of mixed paper grown on different concentrations of DEHP.

Strain	Concentration of DEHP (mg/l)					
	0	500	750	1,000	1,200	1,500
Neurospora sitophila	8.3^{e} (0.03)	33.9^{a} (0.16)	12.3^{d} (0.009)	9.3^{e} (0.05)	18.6^{c} (0.06)	23.1^{b} (0.03)
Fusarium culmorum	7.8^{e} (0.001)	11.6^{d} (0.03)	12.4^{c} (0.03)	15.3^{b} (0.02)	8.8^{e} (0.01)	17^{a} (0.02)
Trichoderma atroviride	4.4^{d} (0.05)	24.4^{b} (0.05)	5.5^{c} (0.01)	5.1^{c} (0.01)	5.1^{c} (0.01)	27.1^{a} (0.001)
Hipocrea lixii	7.1^{d} (0.02)	11.1^{c} (0.02)	5^{e} (0.01)	7.2^{d} (0.01)	21.4^{a} (0.05)	16.1^{b} (0.03)
Trichoderma harzianum	8.7^{c} (0.02)	8.04^{c} (0.03)	2^{e} (0.01)	7.1^{d} (0.04)	18.1^{b} (0.06)	22.3^{a} (0.01)

Means with the same letter (a, b, c, d, or e) within a row are not significantly different.
Numbers in parentheses correspond to standard deviation of three separate experiments.

Table 5. Extracellular esterase activity (U/l) of filamentous fungi isolated from the recycling of mixed paper grown on different concentrations of DEHP.

Strain	Concentration of DEHP (mg/l)					
	0	500	750	1,000	1,200	1,500
Neurospora sitophila	11.3^{d} (0.05)	22.7^{c} (0.05)	6.5^{e} (0.01)	22.1^{c} (0.07)	38.3^{a} (0.16)	34.1^{b} (0.12)
Fusarium culmorum	14.8^{e} (0.03)	69.4^{b} (0.08)	63.3^{c} (0.16)	40.3^{b} (0.009)	20.5^{d} (0.02)	55.3^{a} (0.04)
Trichoderma atroviride	5.7^{c} (0.04)	3.6^{d} (0.02)	19.5^{b} (0.12)	3.11^{d} (0.009)	21^{b} (0.12)	27.1^{a} (0.18)
Hipocrea lixii	7.1^{d} (0.02)	12^{c} (0.01)	22.3^{a} (0.13)	7.4^{d} (0.003)	18.4^{b} (0.02)	18^{b} (0.006)
Trichoderma harzianum	39.7^{f} (0.11)	225.7^{a} (0.14)	79.4^{e} (0.09)	95.5^{d} (0.15)	122.1^{b} (0.31)	111.2^{c} (0.30)

Means with the same letter (a, d, c, d, e, or f) within a row are not significantly different.
Numbers in parentheses correspond to standard deviation of three separate experiments.

Penicillum sp. and *Trichoderma* sp. from soil, from the wastewater of a paper recycling facility, and from a textile facility, respectively. *T. harzianum*, *T. virile*, and *A. nidulans* showed the highest cellulolytic enzymatic activity. Raju et al. [19] also isolated *Trichoderma* sp. from contaminated soil samples of south Indian paper mills. In the present study, fungal strains capable of degrading DEHP were isolated from the recycling of mixed paper wastes in a recycled paper industry, which could be due to the presence of phthalates in such paper wastes. It has been reported that phthalate esters are often discharged by the paper and plastic industries during the manufacturing processes into the ecosystem [1]. It has been reported that *Pleurotus pulmonarius* and *F. oxysporum* had higher u_r in medium containing 1,000 mg of DEHP/l than in medium added with glucose [20]. Suárez-Segundo et al. [20] studied the u_r of *Pleurotus* sp., *Mortierella alpine*, and *F. oxysporum* in media containing 500 and 1,000 mg of either DEHP or dibutyl phthalate (DBP) per liter and in medium added with glucose, and reported that *F. oxysporum* had the highest u_r in media containing DEHP. *F. oxysporum* also showed the largest mycelial biomass on media added with DEHP. In this research, the growth of the fungal isolates in the medium lacking DEHP could be due to the use by the fungi of a certain amount of nutrients that unavoidably remain in the inoculum and/or the use of nutrients produced by hyphal lysis from the inoculum as it was previously reported [20]. Kim et al. [5] reported that cutinases are enzymes able to degrade phthalates. The capability of degrading phthalates by *F. oxysporum* is due to its extracellular cutinase production [21, 22]. Fungi have a unique enzymatic system that makes these organisms useful in bioremediation processes [23]. Hwang et al. [12] studied the degradation of butyl benzyl phthalate (BBP) (100 mg/l) by *P. ostreatus*. It was found that the degradation of this compound was higher when BBP was added to an optimum liquid medium (yeast-malt extract-glucose) than when it was added to a minimal medium, and that esterases were more important than laccases in the degradation of this phthalate. Córdoba-Sosa et al. [24] reported that the esterase activity of *Pleurotus ostreatus* was much higher than the laccase activity at the beginning of the stationary phase in medium containing 1,500 mg of DEHP/l in submerged fermentation.

Conclusions

In the present work, *F. culmorum* and *T. harzianum* showed higher esterase activity than laccase activity in all the media tested. This study demonstrates that fungi isolated from recycled paper waste were capable of utilizing DEHP as the sole carbon source.

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