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Enrichment, isolation, and biodegradation potential of long-branched chain alkylphenol degrading non-ligninolytic fungi from wastewater

Ranjith Kumar Rajendran[a], Chu-Ching Lin[a], Shir-Ly Huang[b], Roland Kirschner[c,*]

[a] Graduate Institute of Environmental Engineering, National Central University, No. 300, Zhongda Rd., Zhongli District, Taoyuan City 32001, Taiwan
[b] Institute of Microbiology and Immunology, National Yang Ming University, Taipei, Taiwan
[c] Department of Biomedical Sciences and Engineering, No. 300, Zhongda Rd., Zhongli District, Taoyuan City 32001, Taiwan

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ABSTRACT

4-*t*-Octylphenol (4-*t*-OP) has become a serious environmental concern due to the endocrine disruption in animals and humans. The biodegradation of 4-*t*-OP by pure cultures has been extensively investigated only in bacteria and wood-decaying fungi. In this study we

isolated and identified 14 filamentous fungal strains from wastewater samples in Taiwan using 4-*t*-OP as a sole carbon and energy source. The isolates were identified based on sequences from different DNA regions. Of 14 fungal isolates, 10 strains grew effectively on solid medium with a wide variety of endocrine disrupting chemicals as the sole carbon and

energy source. As revealed by high-performance liquid chromatography analysis, the most effective 4-*t*-OP degradation (> 70%) in liquid medium was observed in *Fusarium falciforme* after 15 days. To our knowledge, this is the first report on the degradation of 4-*t*-OP as a sole carbon and energy source by non-ligninolytic fungi.

1. Introduction

Long chain alkylphenols such as branched chain octylphenol (4-*t*-OP) are ubiquitous, persistent endocrine disrupting compounds (EDCs), and are known to possess estrogenic activity. 4-*t*-OP mainly originates from the incomplete biological and abiotic transformation of octylphenol polyethoxylate (OPEO*n*); OPEO*n* is extensively used in industrial sectors for various purposes such as detergents, emulsifiers, dispersing agents, and solubilizers (Ahel et al., 1996; Thiele et al., 1997; Ying et al., 2002). The increasing demand and the use of OPEO*n* have led to the continuous discharge of 4-*t*-OP into the environment from the wastewater treatment process. An earlier study reported that the influent and effluent concentration of OP was ranged from 38 to 159 ng L^{-1} and 10 to 95 ng L^{-1}, indicating that the percentage of OP removal from a wastewater treatment plant was less than 40% (Zhou et al., 2010). Consequently, 4-*t*-OP is released to and found in various environmental matrices including soil, sea, and fresh water, and sediments. The predicted environmental concentration of 4-*t*-OP was found to be 44 ± 174 ng g^{-1} dw^{-1} in surface sediments, 9.2–12.1 μg g^{-1} in sewage sludge, and 0.12–2.5 μg L^{-1} in the effluent (Dong et al., 2015; Jin et al., 2008). OP is also leached out from plastics into the sea water (Staniszewska et al., 2016) and accumulates in marine fishes (Errico et al., 2017). The exposure to very low concentrations of 4-*t*-OP can cause serious adverse effects in aquatic animals and humans with respect to spermatogenesis, reduced male sexual behavior, reduced testes weight and induced vitellogenin production (Bayley et al., 1999; Boockfor and Blake, 1997; Gray et al., 1999). Moreover, 4-*t*-OP has higher estrogenic activity than the other long-chain alkyphenols such as nonylphenol (NP) and their parental compounds including nonylphenol polyethoxylates (NPEO*n*) and OPEO*n* (White et al., 1994; Jobling et al., 1994). Due to the widespread occurrence of 4-*t*-OP in the environment as well as its toxicity and estrogenic activity, the development of highly efficient remediation methods to overcome these problems are needed.

Microorganism-based bioremediation is considered a promising, effective approach for removal of recalcitrant organic pollutants compared to the conventional physicochemical treatments such as absorption by activated carbon, membrane filtration, photodecomposition, and chemical oxidation, which are known for their relatively high costs and low removal efficiency (Liu et al., 2009; Lobo et al., 2013; Lv et al., 2016). Most research to date has mainly been focused on the degradation of branched long-chain alkylphenols and their degradation mechanism by bacteria and wood-decaying white-rot (ligninolytic) fungi. Several of these bacterial strains, especially those belonging to the *Sphingomonas* group, have been reported to use 4-*t*-OP as the sole carbon and energy source (Corvini et al., 2006; Toyama et al., 2011). As for fungal strains, the majority of the studies on 4-*t*-OP degradation by

* Corresponding author.
E-mail address: kirschner@ncu.edu.tw (R. Kirschner).

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fungi have primarily involved white-rot fungi (WRF) because of their production of extracellular lignin-modifying enzymes, which may also play a crucial role in the biotransformation of aromatic pollutants (Cajthaml et al., 2009). For example, *Trametes versicolor* and *Phanerochaete sordida* could degrade *t*-NP and 4-*t*-OP by producing laccase and lignin-peroxidases (Catapane et al., 2013; Tamagawa et al., 2007; Wang et al., 2012). However, WRF in nature mainly grow in lignocellulosic substrates, require high oxygen concentrations, and favor acidic conditions. In addition, WRF are sensitive under shear stress conditions, which may affect the extracellular enzyme production (Marco-Urrea et al., 2015). Therefore, in order to overcome the disadvantages of WRF, non-ligninolytic fungal groups such as species of the highly diverse *Ascomycota*, should be tested for 4-*t*-OP degradation, particularly those being common in polluted environments and resisting environmental stress (Aranda, 2016; Harms et al., 2011).

So far, only a few ascomycetous non-ligninolytic fungi, including *Aspergillus versicolor* (Krupiński et al., 2014), *Clavariopsis aquatica* (Junghanns et al., 2005), *Gliocephalotrichum simplex* (Różalska et al., 2010) and *Penicillium expansum* (Kuzikova et al., 2017) have been demonstrated for the degradation capacity of NP. Chang et al. (2016) reported that *Penicillium* sp. isolated from antarctic soil could degrade medium- and long-chain alkylphenols such as 4-*t*-butylphenol, 4-*t*-OP and 4-*t*-NP at 4 °C in the presence of dextrose. Another study has shown that a non-ligninolytic fungus *Umbelopsis isabellina* (Zygomycota) was capable of metabolizing 4-*t*-OP through beta-oxidation in a rich medium (Janicki et al., 2016). Notably, in our recent study we reported for the first time that ascomycetous yeast strains isolated from a wastewater treatment plant were capable of utilizing 4-*t*-OP as the sole carbon source (Rajendran et al., 2016, 2017).

Despite several studies showing the capability of non-ligninolytic fungi to degrade medium- and long-chain APs, to date no study has reported on the degradation of 4-*t*-OP as the sole carbon source by filamentous ascomycetous non-ligninolytic fungi. Thus, the present study has the following aims: (i) to isolate and identify non-ligninolytic fungi that can utilize 4-*t*-OP as the sole carbon and energy source from wastewater samples; (ii) to assess the ability of isolated fungi to utilize a wide range of structurally relevant toxic pollutants including alkylphenols, alkylphenol polyethoxylates, as well as natural and synthetic estrogens; (iii) to quantitatively investigate the 4-*t*-OP degradation capacity of selected fungi. To our knowledge, this is the first report on the degradation of 4-*t*-OP as the sole carbon and energy source by non-ligninolytic fungi isolated from environmental sources.

2. Materials and methods

2.1. Chemicals and reagents

4-*t*-Octylphenol (4-*t*-OP, purity ≥ 97%), 4-*t*-nonylphenol (4-*t*-NP; ring and chain isomer mixture), octylphenol polyethoxylate ($OPEO_n$, purity ≥ 97%), nonylphenol polyethoxylate ($NPEO_n$, purity ≥ 97%), estrone (E1, purity ≥ 99%), 17*β*-estradiol (E2, purity ≥ 98%), and 17*α*-ethynylestradiol (EE2, purity ≥ 98%) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Ethyl acetate was purchased from Merck Co., USA. Acetonitrile and other organic solvents of HPLC grade were obtained from Sigma-Aldrich Co. (St. Louis, MO). All other chemicals used in this study were of analytical grade purchased from Showa Chemicals Co., Japan and Sigma-Aldrich Co., USA.

2.2. Enrichment and isolation of 4-t-OP degrading fungi

Samples were collected from different sources including sewage water (SW), sewage sludge (SS) from Dihua sewage treatment plant, Taipei, and soil and wastewater from plastic industry, Taoyuan, in Taiwan. All the collected samples, i.e., SS, SW, and wastewater (5 mL) and soil (5 g) were enriched and inoculated individually into 250 mL Erlenmeyer flask containing 50 mL sterilized modified minimal salt broth, pH 6.0, as described by Rajendran et al. (2016), and contained 50 mg L^{-1} 4-*t*-OP as the sole carbon source. All the flasks were aerobically incubated at 25 °C on a rotary shaker at 150 rpm in the dark. After 10 days incubation, an aliquot of 5 mL of each enrichment culture was transferred into 45 mL freshly prepared pre-sterilized MMSB amended with 4-*t*-OP (50 mg L^{-1}). The above steps were repeated for three times to attain a 4-*t*-OP degrading fungal consortium. To prevent the growth of bacteria, during the first two enrichments, tetracycline was sterilized through sterile 0.2 μm polypropylene membrane syringe filter (Acrodisc® syringe filter, USA) and added aseptically to a final concentration of 100 mg L^{-1}. The final enrichment cultures were serially diluted and plated on MMSB with 4-*t*-OP (50 mg L^{-1}) and agar (1.5%) plates. All the plates were incubated at 25 °C for 10 days; colonies were picked and purified by restreaking on Malt Extract Agar (MEA, Scharlau). All the isolated fungal strains were maintained at 4 °C for a month. Pure cultures of the fungal strains were deposited at the Bioresource and Research Center (BCRC), Hsinchu, Taiwan.

2.3. Fungal DNA extraction, polymerase chain reaction (PCR) amplification, and DNA sequencing

The isolated fourteen fungal strains were pre-grown on MEA with chloramphenicol (100 mg L^{-1}) at 25 °C for 5–7 days. After incubation, a single agar plug (~10–12 mm diameter) was aseptically transferred and complete genomic DNA was extracted using Genomic DNA spin kit (Bioman Scientific Co. Ltd., Taiwan) according to the manufacturer's protocol with modification. PCR amplification was performed using different primers: ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990) for the internal transcribed spacer region of the ribosomal RNA gene (ITS rDNA), H3F1 and H3R1 (Bills et al., 2009) for the partial histone H3 (HisH3) gene, Tub2fd and Tub4rd (Groenewald et al., 2012) for the partial beta-tubulin (*β*-tub) gene, and ef1 (Geiser et al., 2004) and ef2 (O'Donnell et al., 1998) for the partial fragment of the translation elongation factor 1-alpha gene (*tef*1α). The detailed information of the primers used in this study is listed in Table 1. Each PCR reaction was comprised of 5 μL 10 × reaction buffer, 3 μL 25 mM $MgCl_2$, 4 μL dNTP-mix, 1 μL of the forward and reverse primers, 0.25 μL of Taq-DNA polymerase, 5 μL of genomic DNA, and 30.75 μL nuclease free water for a total volume of 50 μL. For negative control, genomic DNA was replaced with an equal volume of nuclease free water. The thermocycler programs for each DNA region were given in Table S1. The amplified PCR product size was fractionated using gel electrophoresis (2%) with GelRed™ (Biotium, Hayward, California, USA) and then visualized with a UV transilluminator. PCR products were purified using Gel Band purification kit according to the manufacturer's instruction (GE healthcare, UK) and then stored at − 20 °C. The purified PCR products were sequenced in both directions with the same primers as for the PCR and the sequencing was done by Mission Biotech (Nangang, Taipei). DNA sequences were edited using Codon Code Aligner version 4.0.1 (Codon Code Corporation, USA) and the edited sequences were deposited in GenBank. The GenBank accession and BCRC numbers of the isolated fungal strains were given in Table 4.

2.4. Phylogenetic analysis

In order to assess the relationship between own and the other strains of the *Fusarium solani* species complex (FSSC), newly generated *tef*1α sequences were aligned with sequences of various other closely related sequences downloaded from GenBank using BLAST search (http://www.ncbi.nih.gov/BLAST/) and from relevant publications about FSSC (O'Donnell et al., 2008; Lombard et al., 2015). Accession numbers of the edited sequences are given in the phylogenetic tree (Fig. 1). All the sequences were edited and aligned without manual editing except trimming off the uneven ends of the alignment block using the program MEGA 6.0 (Molecular Evolutionary Genetic Analysis Software, ver. 6.0) with the implemented MUSCLE algorithm (Tamura et al., 2013). A

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