Journal of Hazardous Materials 359 (2018) 203–212

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Different pathways for 4-n-nonylphenol biodegradation by two *Aspergillus* strains derived from estuary sediment: Evidence from metabolites determination and key-gene identification

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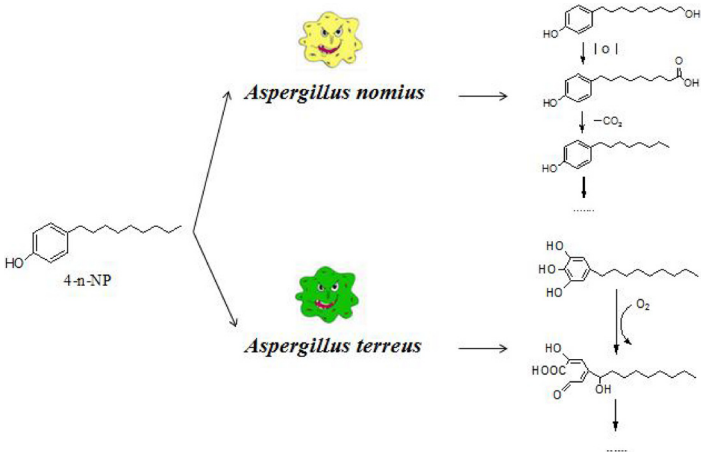
GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
NPs biodegradation
Degradation mechanism
Key gene
Aspergillus

ABSTRACT

Nonylphenols (NPs) are known as Endocrine Disputing Chemicals (ECDs) and Persistent Organic Pollutants (POPs) and have attracted continuous attention. Biodegradation is one of the effective ways for pollutant removal in aquatic, sedimentary and soil environments. In this study, two estuarine derived fungi strains, NPF2 and NPF3, were screened from Moshui river estuarine sediment and identified as genus *Aspergillus*. The growth curves of the two strains as well as the removal and degradation rates for 4-*n*-NP in Potato Dextrose(PD)medium were used to evaluate their degradation ability. Both strains showed high efficiency for 4-*n*-NP degradation with 86.03% and 98.76% removal rates in 3 days for NPF2 and NPF3, respectively. Determination of degradation intermediates by LC–MS suggested that the mechanisms for 4-*n*-NP biodegradation by NPF2 and NPF3 are quite different. Some key functional genes for the two strains also provided supplementary evidences for the different biodegradation mechanism. On strain NPF2, with participation of *Cox1*, *2* and *3*, 4-*n*-NP degradation starts from reaction at the terminal of the long alkyl chain. The chain reduces one carbon atom once within a cycle of hydroxylation, subsequent oxidation at *α*-C position and decarboxylation. However, on NPF3, with involvement

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https://doi.org/10.1016/j.jhazmat.2018.07.058
Received 5 January 2018; Received in revised form 11 July 2018; Accepted 12 July 2018

of *sMO*, *Cel7A*, *Cel7B* and *ATEG-00639*, 4-*n*-NP degradation starts from benzene ring, converting into fatty acids. The latter bio-pathway was the first time reported for NPs degradation on fungi.

1. Introduction

Nonylphenols (NPs), known as Endocrine Disputing Chemicals (EDCs), are the degradation products of Nonylphenol Polyethoxylates (NPEOs) in anaerobic environment. NPEOs have been widely used in detergent, printing and dyeing, and chemical industries. NPs are more toxic, hydrophobic and difficult to degrade than the parent NPEOs [1], and had been identified as Persistent Organic Pollutants (POPs) in the 1980s by the United Nation Environment Programme (UNEP) [2–4].

With the extensive usage in industries and accumulation in general environments, NPs were detected not only in terrestrial and atmospheric environments but also in aquatic and marine environments [5–7]. Surface runoff, water discharge and atmospheric dry/wet deposition are the major pathways for transportation of NPs and NPEOs [8]. While entered into aquatic and marine environments, NPs and NPEOs are preferentially adsorbed on particles and finally buried in sediments [9]. Sediments are then generally regarded as one of the major sinks of NPs because of their relatively low solubility in water (4.9 mg L^{-1}) [10] and high hydrophobicity with a log K_{ow} value of 4.48 [11]. Therefore, NPs were more frequently detected in sediments with higher concentrations, rather than dissolved in aqueous phase [12].

Biological removal of NPs has attracted great attention for the past decades because of its advantages of effectiveness, eco-friendliness, economy and expedience [13]. However, relatively less attention had been paid to the biodegradation of NPs in natural environments, despite that it is one of the major pathways for elimination of NPs, especially in sedimentary environment.

Some fungi such as *Gliocephalotrichum simplex* [14], *Aspergillus versicolor* [15] and *Metarhizium* [16] have attracted more attention because of their efficiency and universality in different environments. Based on a few metabolites detected, Rózalska et al. proposed, for the first time, that the degradation starts from the alkyl chain of NPs [14]. Krupiński et al. [17] and Różalska et al. [18] reported some proteome participating in NPs degradation, including reduction-oxidation system (ROS) of nitrate reductase, superoxide reductase and superoxide dismutase system, tricarboxylic acid cycle (TCA cycle) and energy system. Chang [19] also reported *Penicillium* sp. derived from Antarctic soil could effectively degrade 4-NP at 4 °C (low temperature) within three weeks and at 15 °C (medium temperature) within one week. Some bacteria were also found to be effective for NPs degradation. Most of them are belonging to *Sphingomonas*, *Stenotrophomonas*, *Pseudomonas*, and *Bacillus* [20]. Watanabe et al. [21] isolated *Acidovorax* from river water. The bacteria could increase the degradation rate with increasing NP concentration. Study by Wang et al. [22] indicated that ammonia oxidizing archaea (AOA) were more responsive to NP amendment, compared to ammonia-oxidizing bacteria (AOB). *Gammaproteobacteria*, *Alphaproteobacteria* and *Bacteroidetes* were the largest bacterial groups derived from marine sediment with NP-degrading ability, while bacterial genera of *Brevundimonas*, *Flavobacterium*, *Lysobacter* and *Rhodobacter* might be involved in NP degradation in river sediment [22].

As stated above, NPs are preferentially accumulated in sediments. Therefore, sediments are certainly excellent matrices for screening microorganisms that can use high concentration NPs as the carbon and energy sources [23]. To the best of our knowledge, there were very limited reports on 4-*n*-NP biodegradation mechanism, especially by fungi derived from marine or estuary sediments; and there was no reasonable interpretation for the reported mechanism from a genic point of view.

Therefore, the objectives of the present work are: to screen and identify high performance 4-*n*-NP degrading fungi from estuarysediments, and to elucidate and confirm the biodegradation mechanism. We found two completely different pathways for 4-*n*-NP degradation on two *Aspergillus* strains derived from estuary sediments, one of which is the first report for NPs degradation by fungi.

2. Materials and methods

2.1. Chemicals

4-*n*-NP (purity 99.3%), tert-butyl-phenol (purity 99.5%) and (1, 2, 3, 4, 5, 6-hexamethyl) benzene (purity 99.5%) was purchased from Dr. Ehrenstorfer GmbH. Solvents, including methanol, ethyl acetate and n-hexane, were all of HPLC grade from J&K Technology Co. Ltd. (Beijing, China). Agar and glucose for culture media were of BR grade and purchased from Haibo Biotechnology Co. Ltd. (Qingdao, China). Milli-Q water (l8.2 MΩ cm) were supplied in house with a Millipore Gradient A10 system (Bedford, USA).

2.2. Fungi screening and identification

The target strains were derived from Moshui river estuarine sediment of Jiaozhou bay, Qingdao, China. The sediment was collected in August 2015, and the detailed fungi separation and purification process was previously described [24]. A laser scanning confocal microscope (Olympus, MIU-IBL) was used to observe the morphological characteristics of strains. ITS rDNA molecular biology identification and PCR amplification were used to identify the strains species and genes, respectively. Detailed operational processes can be found in Purnomo's essay [25].

The sequencing was performed at the "Basic Local Alignment Search Tool" (BLAST) for sequence alignment (http://blast.ncbi.nlm.gov/Blsast.cgi), and the compiled sequence was uploaded to the GENBANK (gbsub@ncbi.nlm.nih.gov) to obtain accession numbers MH421850 and MH421849 for NPF2 and NPF3, respectively.

2.3. Degradation of 4-n-NP

Potato dextrose (PD) were used as the media for strains growth and for 4-*n*-NP degradation assessment [26]. The removal and degradation rates, defined in the following formula, were used to indicate the efficiency for removing and degrading 4-*n*-NP. They are expressed as mean value ± standard deviation with triple independent measurements.

A stock solution (10 mg mL^{-1}) was prepared by dissolving 4-*n*-NP in methanol. Incubation was conducted in 150-mL Erlenmeyer flasks with 50 mL of PD medium and 100 μL of the stock solution (to obtain an initial concentration of 20 mg L^{-1}) in each flask. After sterilizing at 121 °C for 30 min and cooling to the room temperature, the purified fungi were inoculated into the media and cultured for 0, 1, 2, 3, 4, 5, 6 and 8 days with rotary shakers at 29 °C, 120 rpm. At each time point, samples in triplicate were withdrawn along with a control, filtrated to separate fermentation broth and mycelium. For intermediates detection in Section 2.4, samples were taken every 6 h in the first two days, and every 1 or 2 days thereafter.

After separation, 100 μL 20 mg L^{-1} tert-butyl-phenol (in methanol) was added into the broth as a surrogate. Then the broth with surrogate was extracted twice with 50 mL ethyl acetate each time. The extract was evaporated with a vacuum rotary evaporator to about 0.5 mL, transferred to a 4 mL GC sample vial and dried under a gentle nitrogen stream. The extract was then re-dissolved with 100 μL methanol, and the residual 4-*n*-NP was analyzed with a gas chromatograph (GC). For