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Biodegradation of 4-*n*-nonylphenol by the non-ligninolytic filamentous fungus *Gliocephalotrichum simplex*: A proposal of a metabolic pathway

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ABSTRACT

4-Nonylphenols (NPs) are endocrine disrupting compounds (EDCs) which are known to interfere with the endocrine system of humans and animals. The aim of this study was to test the ability of non-ligninolytic filamentous fungus *Gliocephalotrichum simplex* to biodegrade 4-*n*-NP. The results revealed that during the first 24 h of incubation, 4-*n*-NP at the concentration of 50 mg L^{-1} was eliminated from the culture medium by 88%, whereas at the concentration of 100 mg L^{-1} by 50%. In this paper, glucose utilization as a co-substrate during toxic compound degradation was also shown. It was found that the presence of 4-*n*-NP caused sugar metabolism retardation and this inhibition was dependent on NP concentration. The qualitative GC–MS analysis showed the presence of products of *G. simplex* 4-*n*-NP biodegradation. We proposed the metabolic pathway of 4-*n*-NP biodegradation, which is based on subsequent C1 removals from the alkyl chain followed by the aromatic ring cleavage. In further experiments with 4-*n*-NP [ring-^{14}C(U)] we proved aromatic ring cleavage occurrence. After 72 h of incubation the evolution of $^{14}CO_2$ was observed and the mineralization efficiency was on the level of 29%. The results suggest the existence of a novel mechanism of 4-*n*-NP degradation in fungi.

1. Introduction

Endocrine disrupting compounds (EDCs) are toxic, anthropogenic compounds which are known to interfere with the endocrine system and, in consequence, cause serious health problems in wildlife. It was found that these compounds have adverse effects on the development and reproduction of several species of fish, mollusks and other animals [1,2]. EDCs possess various chemical structures and physical properties, but there are two main mechanisms of their action on eukaryotic organisms—one is mimicking the occurrence of natural hormones and the other is blocking normal hormonal functions [3,4]. The most common EDCs in the environment are nonylphenol etoxylates (NPEOs), which are widely used as surfactants, antistatic agents, detergents and solubilisers [3,5]. The common wastewater treatments of sewage containing NPEOs usually lead to their incomplete degradation and occurrence of nonylphenols (NPs), which are more resistant to degradation. There are a lot of data concerning NP effect on wildlife [6–10]. Also, there are some data indicating that these compounds are able to induce hormone dependent breast tumor cells proliferation [11], by imitating the natural feminine estrogen—17β-oestradiol while competing with the receptor for the binding site for the natural hormone [4,12].

NPs in the natural environment have an influence on miscellaneous ecosystems due to their bioaccumulation and remaining in the ecosystems for many years. The removal of NPs during wastewater biodegradation treatments is considered as an important issue, thus the investigations of their biodegradation mechanisms are of fundamental interest for understanding the fate of NPs [13,14]. NPs are present in the environment mostly as forms with branched side chains, but NP with a linear alkyl chain (4-*n*-NP) is also a possible decisive feature of the catabolic pathways [15]. It was found that some microbial strains possess the ability to degrade 4-*n*-NP [16,17].

Bacterial biodegradation of NPs has been studied extensively, but there are only some data concerning the ability of fungal cultures to utilize NPs. Additionally, the investigated fungal strains mostly belong to the ligninolytic or mitosporic fungi with the ability to produce laccase [18–20].

The aim of this study was to check the usability of *Gliocephalotrichum simplex* for NP elimination with a 4-*n*-NP as an investigation model. The attention was paid to metabolite identification, capacity of the fungus for 4-*n*-NP mineralization and also simultaneous glucose utilization during biodegradation.

2. Materials and methods

2.1. Chemicals

4-*n*-NP (Fluka) was used as a substrate. For radioactive experiments 4-*n*-NP [ring-^{14}C(U)] (ARC, USA) with specific activity

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52 mCi mM^{-1} and radiochemical purity 99%, dissolved in ethanol, was applied. All chromatographic solvents were purchased from J.T. Baker (Holland). Other chemicals (pure or high purity) were purchased from Sigma–Aldrich, Germany.

2.2. Microorganism and growth conditions

Filamentous fungus *G. simplex* (synonym of *Cylindrocladium simplex*) strain number IM 2358 from the collection of the Department of Industrial Microbiology and Biotechnology, University of Łódź, Poland was used in this work. Spores from 14 d old cultures on ZT slants [21] were used to inoculate 20 mL of mineral medium with 2% of glucose, pH = 6.8 as described earlier [22] in 100 mL Erlenmayer flasks. After 24 h of incubation precultures were transferred to fresh medium in the ratio 1:9. 4-*n*-NP dissolved in ethanol (stock solution 20 mg mL^{-1}) was aseptically added to the cultures to give the final concentrations of 50 or 100 mg L^{-1}. The control cultures were supplemented with the same amounts of ethanol. Additionally abiotic (uninoculated) controls were prepared. All samples were incubated at 28 °C on rotary shaker (150 rpm). At appropriate times culture samples were withdrawn for analyses.

2.3. Estimation of biomass

Dry weight biomass was quantified by filtering the whole culture through a predried and preweighed Sartorius filter membranes (0.25 μm). The samples were dried to a constant weight at 100 °C and the results were expressed as g L^{-1}. All samples were analyzed in triplicates, averages and standard deviations are quoted in the figures. Normality of distribution was verified with Shapiro–Wilk's *W*-test. To estimate significances, one way ANOVA (with NIR test) was used.

2.4. 4-n-NP extraction and sample preparation

Samples were prepared according to the method described by Ref. [23] with some modifications. The samples were acidified to pH = 2 and homogenized (MISONIX, England) at 4 °C and with 120 W power input with 20 mL ethyl acetate (a first extraction step). After homogenization, the samples were extracted by methylene chloride. The extracts were mixed together, dried with anhydrous sodium sulfate and evaporated under reduced pressure at 40 °C.

2.5. Derivatization, GC–MS analysis and metabolites identification

1 mL of ethyl acetate was added to the evaporated samples and 200 μL was transferred to chromatography vials and subjected to GC–MS in order to determine the amount of 4-*n*-NP. For 4-*n*-NP metabolite identification, 50 μL ethyl acetate solution was evaporated to dryness by a N_2 gas stream and derivatization was performed as follows. 50 μL of BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) was added and heated to 60 °C for 1 h. Afterwards, the samples were supplemented with 200 μL ethyl acetate and analyzed with GC–MS.

GC analyses of the extracts were performed on a Hewlett-Packard HP 6890 series GC equipped with a mass selective detector HP 5973, using a Restek RTX-5MS capillary column (60 m × 0.25 mm × 0.25 μm). The injection volume was 2 μL. The inlet was set to split mode with split ratio 10:1 (split flow 10 mL min^{-1}) and the temperature was maintained at 275 °C. Helium was used as a carrier gas. Temperature parameters of the column were as follows: 60 °C maintained for 2 min, 20 °C min^{-1} to 300 °C and maintained for 8 min. Mass selective detector parameters were as follows: ms source 250 °C, ms quad 200 °C, scan mode with the mass range set from 45.0 to 550.0 amu. All compounds and their byproducts were identified on the basis of the retention time and mass spectra analysis done with MS Calc Pro (ChemSW), Isotope Calculator (NIST) and AMDIS software and confirmed by NIST08 MS library when possible, as it was described previously by us [24–27]. Culture samples without the addition of 4-*n*-NP served as a point of reference for the identification of unknown compounds. Quantitative analyses were done on the basis of standard equation, which showed linearity in the ranges from 0 to 100 μg mL^{-1} of 4-*n*-NP.

2.6. Glucose determination with LC–MS/MS

For glucose content determination, 200 μL samples were withdrawn aseptically and placed in Eppendorf tubes. The samples were centrifuged 2600 × *g* (Sigma) for 10 min. After centrifugation the supernatants were transferred to new Eppendorf tubes and the amount of glucose was determined using HPLC MS/MS Agilent 1200 coupled with the mass detector QTRAP 3200 (Applied Biosystems). The tested samples were injected directly to MS/MS with the flow rate 1 mL min^{-1} of methanol:water—40:60 under the temperature of 35 °C. The detection of glucose was made on MS/MS detector working in MRM negative ionization mode. The monitored MRM pairs were 179–59 (CE = −25) and 179–89 (CE = −19). The other parameters of the detector were: CUR: 10.00; TEM: 450.00; GS1: 50.00; GS2: 50.00; ihe: ON; IS: −4500.00; CAD: Medium; DP: −25.00; EP: −2.00: CEP: −11.00; CXP: −4.00; CEM: 2700. Quantitative analyses were done on the basis of standard equation, which showed linearity in the ranges from 0 to 10 mg mL^{-1} of glucose.

2.7. Radioactive experiments

Radioactive experiments were done according to the modified method described previously [28]. Briefly, 1 μCi of 4-*n*-NP [ring-^{14}C(U)] and non-labelled NP were applied to provide the final concentration 50 mg L^{-1}. Radioactive experiments were performed in 100 mL Erlenmayer flasks sealed with silicone caps. In order to trap $^{14}CO_2$, each cap was equipped with two glass capillaries. The inlet capillary, sterile air was supplied continuously (with the flow rate 10 mL min^{-1}) and the outlet capillary was joined by silicone and glass capillaries with glass tubes filled with 10 mL CarboSorbE (PerkinElmer). The biodegradation experiments were conducted at 28 °C on a rotary shaker (150 rpm). For each experiment control flasks without fungus were prepared. Whole samples were withdrawn for analyses at appropriate times in the following way: the cultures were filtered through glass filter membranes (pore size 0.45 μm) and whole masses of the fungus were transferred to the scintillation vials and supplemented with 2 mL Ultima Gold liquid scintillation cocktail (PerkinElmer). The volumes of 0.25 mL of the filtrates were collected in the scintillation vials and filled up to 2 mL with Ultima Gold cocktail. In order to determine the amount of $^{14}CO_2$, every 24 h the CarboSorbE was replaced with a new portion. The measurements of adsorbed $^{14}CO_2$ were done by mixing CarboSorbE with LSC cocktail Permafluor E+ in a ratio 1:1. The obtained mixture was transferred to the scintillation vials. All radioactivity determinations were performed with 1450 MicroBeta Liquid Scintillation Counter Wallace LKB (Turku, Finland).

3. Results and discussion

3.1. The growth of G. simplex in the presence of 4-n-NP

In the preliminary experiments the growth intensity of *G. simplex* in the presence of two different concentrations of 4-*n*-NP was tested (Fig. 1). The most significant growth retardations in the samples incubated with 50 and 100 mg L^{-1} of 4-*n*-NP were noticed in

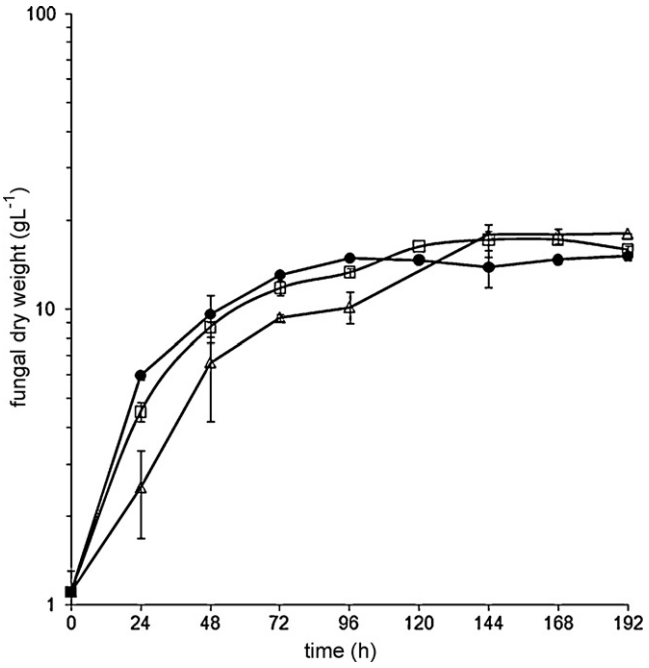

Fig. 1. Time courses of growth of *G. simplex* in the presence of 4-*n*-NP in the concentrations 50 mg L^{-1} (white squares) and 100 mg L^{-1} (white triangles) compared to the control without toxic substrate (black circles).

24 h of incubation when the dry masses of the fungus were equal to 4.51 ± 0.82 and 2.51 ± 0.33 g L^{-1}, respectively, in comparison to the controls 5.98 ± 0.21 g L^{-1} ($p < 0.05$ and $p < 0.002$). After 72 h of incubation the difference between the samples with 50 mg L^{-1} of 4-*n*-NP and controls did not reach statistical significance, while the growth in the presence of a doubled concentration of the 4-*n*-NP was repressed significantly until 96 h of incubation. The growth inhibition of fungal cultures was also observed for some other fungal species e.g. *Neurospora crassa* growth was reduced in the presence of 20 mg L^{-1} 4-*n*-NP [29]. It has been shown that ligninolytic fungal strains exhibit even higher 4-*n*-NP sensitivity. The biodegradation experiments for these fungi were carried out in the concentration as low as 2.5 mg L^{-1} [18]. The results obtained in this paper suggest that *G. simplex* possesses an ability to grow in the presence of high concentrations of 4-*n*-NP.

3.2. Removal of 4-n-NP by G. simplex

In the current study we performed the experiments on 4-*n*-NP elimination (Fig. 2). The obtained results showed that 4-*n*-NP in the concentration 50 mg L^{-1} was eliminated by 88% from the culture medium during the first 24 h and only trace amounts of this compound in the cultures in 48 h of incubation were detected. It was also noticed that the removal of 4-*n*-NP in the concentration 100 mg L^{-1} had a different course. It was found that in 24 h of incubation about 50% of the substrate was still present in the culture and complete utilization of the substrate took place in 144 h of incubation.

In other 4-*n*-NP biodegradation studies, ligninolytic fungal strains with the ability to produce extracellular enzymes with low substrate specificity were frequently applied. Cajthaml et al. [18] found that none of eight species of ligninolytic filamentous fungi possesses the ability for 4-*n*-NP degradation (in the concentration 2.5 mg L^{-1}) during 7 d of incubation, however, after 14 d of incubation, a complete removal of this substrate was observed. In other studies the utilization of 4-*n*-NP as a sole carbon and energy source by *Candida aquaetextoris* was described. It was demonstrated that

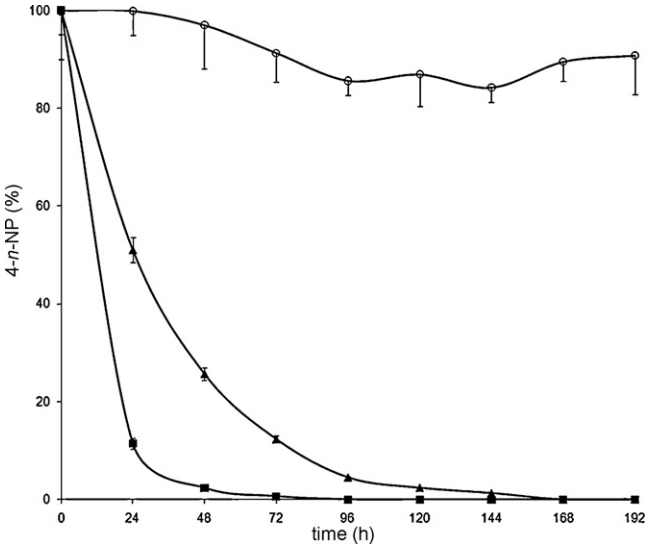

Fig. 2. 4-*n*-NP elimination by *G. simplex* IM2358 in mineral medium in the concentration 50 mg L^{-1} (black squares) and 100 mg L^{-1} (black triangles) in comparison to the control without fungus (white circles).

after 14 d of yeast incubation with this xenobiotic in the concentration 100 mg L^{-1} only trace amounts of 4-*n*-NP were left in the medium [17]. *G. simplex* had the ability to degrade 4-*n*-NP present at relatively high concentrations (50 or 100 mg L^{-1}) and the degradation rate was similar for both concentrations. Moreover, the degradation took place in a shorter period of time than in other experiments with fungi [17,19].

3.3. Glucose utilization by G. simplex in the presence and absence of 4-n-NP

In the present study, *G. simplex* was found to be a convenient model for NP utilization applied at relatively high concentrations. 4-*n*-NP was not a sole carbon and energy source, but glucose in the concentration of 2% was present in the culture medium. Data showed in Fig. 3 suggest that in the cultures without the xenobiotic, glucose utilization was very efficient up to 48 h of incubation, while in the samples supplemented with 4-*n*-NP, glucose removal was retarded. It was noticed that in the samples supplemented with 50 mg L^{-1} of 4-*n*-NP the total glucose utilization was observed in 120 h of incubation, whereas in the samples with

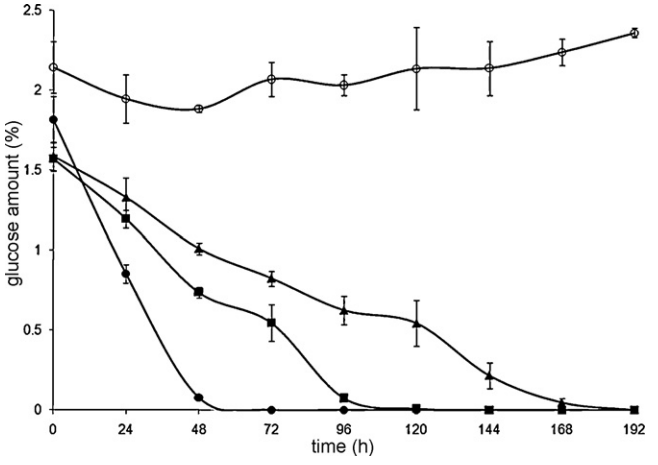

Fig. 3. *G. simplex* glucose utilization in the presence of 4-*n*-NP in the concentration 50 mg L^{-1} (black squares) and 100 mg L^{-1} (black triangles) compared to the controls without toxic substrate (black circles) and without fungus (white circles).

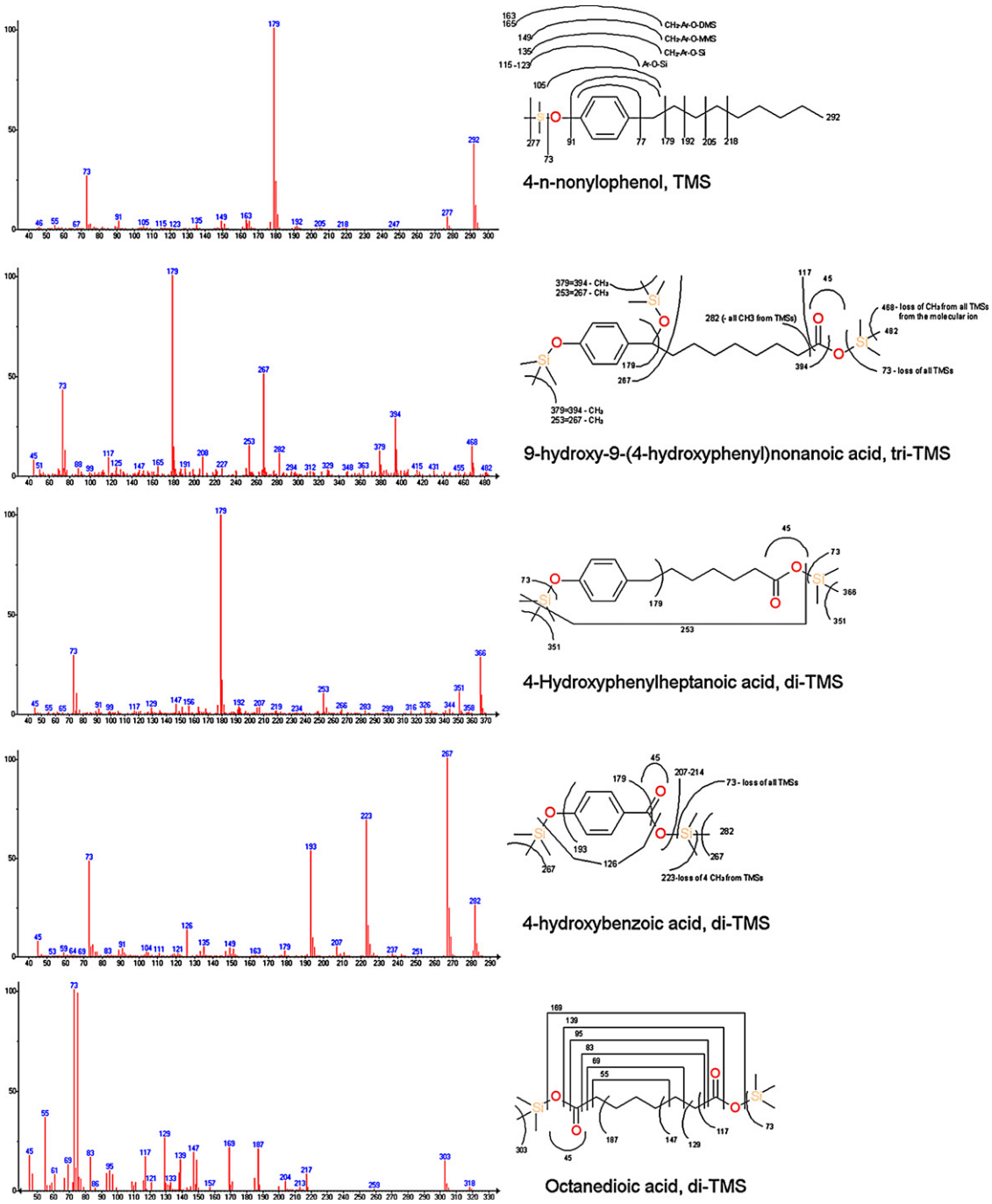

Fig. 4. Examples of mass spectra analysis of the selected compounds.

100 mg L^{-1} it was noticed in 192 h of incubation. As it was shown in Fig. 2, for 4-*n*-NP in the concentration of 50 mg L^{-1}, only 0.63% of the initial amount of this toxic compound was still present in the cultures in 72 h of incubation and the data obtained for glucose consumption (Fig. 3) revealed that the most efficient glucose utilization took place between 72 and 96 h of incubation, while 4-*n*-NP in was completely degraded. It seems that the observed phenomenon is associated with inhibition of the glucose utilization by the traces of 4-*n*-NP in the culture medium. The obtained results may also suggest that the presence of 4-*n*-NP, in the concentration dependent manner, caused the retardation of *G. simplex* glucose metabolism. Although the 4-*n*-NP was metabolized with a constant rate (as it was mentioned above), it inhibited *G. simplex* metabolism, as was evidenced by the lower biomass content and the inhibition of co-substrate utilization. There is a lack of literature data concerning glucose utilization in the presence of 4-*n*-NP.

3.4. Qualitative analysis of 4-n-NP metabolites and mineralization

Qualitative analysis of 4-*n*-NP (50 mg L^{-1}) degradation metabolites during *G. simplex* culturing was performed after extracts derivatization with BSTFA and GC–MS analysis done in 6 h incubation periods up to 72 h of the experiment. Most of the degradation metabolites were detected between 6 and 24 h of incubation. After 72 h of incubation, biodegradation products were not found. Because the majority of the compounds were not commercially

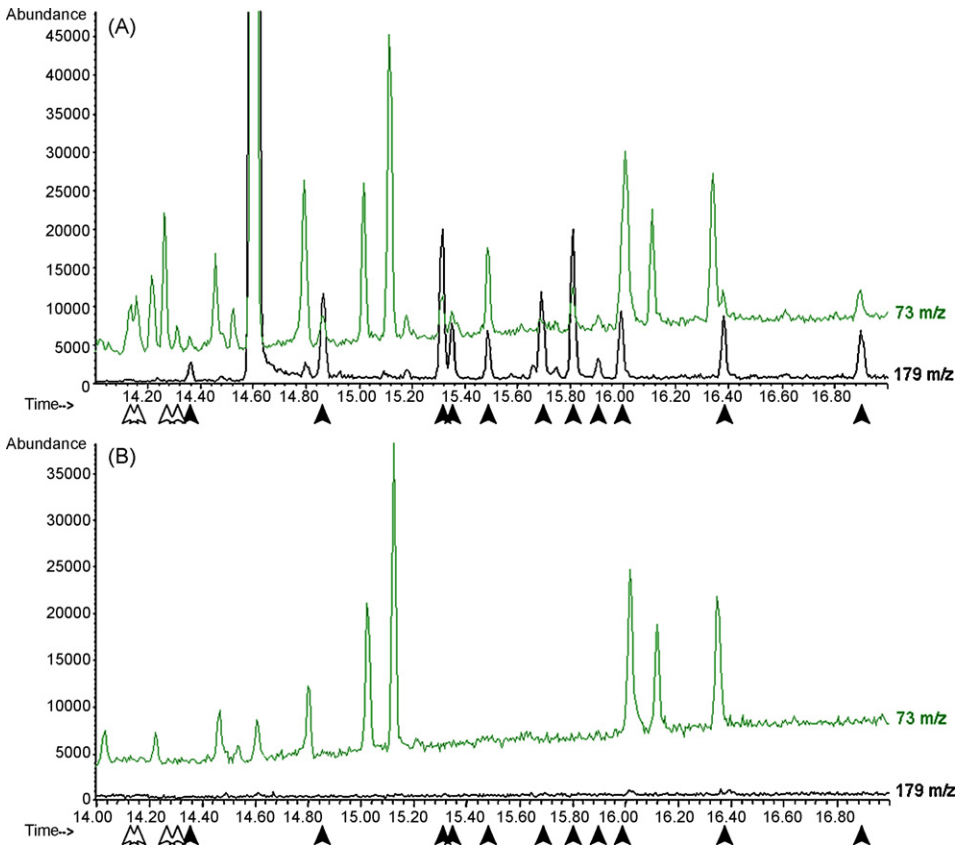

Fig. 5. An example of extracted ion chromatogram (73 and 179 *m*/*z*) in the tested sample (A) and biotic control (B) after 6 h of incubation. Black arrows marks differences in 179 *m*/*z* abundance, white arrows marks differences in 73 *m*/*z* abundance.

available, chemical structures were assigned from the mass spectra data.

In the first step of identification, we checked the way how trimethylsilyl (TMS) derivative of 4-*n*-NP was fragmenting. It was found that the compound followed a typical fragmentation and produced three major ions (Fig. 4): 292 *m*/*z*—molecular ion (M), 179 *m*/*z*—base peak (CH_2–Ar(aromatic ring)–O–TMS) and 73 *m*/*z* (TMS). The additional fragments important to structure investigation also were: 91 *m*/*z* (Ar), 105 *m*/*z* (Ar–OH) and 277 *m*/*z* coming from the loss of CH_3 from TMS from the molecular ion (M-15). The other fragments are also shown and interpreted in Fig. 4.

The number of TMSs reflecting the number of hydroxyl groups was estimated upon the isotopic pattern of the molecular ion and high mass fragments. Based on the obtained fragmentation pattern of 4-*n*-NP ions 73 and 179 *m*/*z* were chosen as main markers in the search for its metabolites. All samples were subsequently investigated in the extracted ion mode (73 and 179 *m*/*z*) and compared to the appropriate biotic controls which were serving as reference. The peaks exhibiting any differences in the tested mass signals, were chosen for further analysis as possible metabolites of 4-*n*-NP biodegradation (Fig. 5).

Not all compounds have been identified especially in cases of very low concentrations where the mass spectra were contaminated, as a result of a high noise-to-signal ratio and therefore difficult to interpret. Compounds containing a hydroxylated aromatic ring and a carboxyl group on the distal point of the alkane chain followed a similar fragmentation to 4-*n*-NP, giving three major ions: molecular ion, 179 and 73 *m*/*z*. Additional fragments confirming the presence of substructure were ions: 91 *m*/*z* (Ar), 105 *m*/*z* (Ar–OH), loss of CH_3 from TMS from the molecular ion (M-15) and ion 45 coming from the carboxyl group. A fragmentation of 4-hydroxyphenylheptanoic acid, di-TMS is presented as an example in Fig. 4. Another kind of metabolites contained a hydroxylated aromatic ring as well as hydroxylated and carboxylated linear chain. The fragmentation was similar to the one described above with one exception—strong ion 267 *m*/*z* coming from the substructure built of TMS–O–Ar–C–O–TMS confirming the presence of the hydroxyl group in the aliphatic chain was located next to the aromatic ring. Short chain compounds (1–2 carbons in the aliphatic substructure) showed a slightly different fragmentation. Ion 179, although present, was weak in the compounds containing double bonded oxygen in the aliphatic chain situated next to the aromatic ring. The same situation happened in case of double bonded carbon. The example of such behavior was exhibited by 4-hydroxybenzoic acid, di-TMS (Fig. 4). The compound fragmented giving the main ions: 282 *m*/*z* (M), 267 *m*/*z* as a result of a loss of CH_3 from the molecular ion (M-15), 223 *m*/*z* as a result of a loss of four methyl groups from TMS from the molecular ion, 193 *m*/*z* coming from Ar–COO–TMS (as a result of the detachment of O-TMS from the aromatic ring) and 73 *m*/*z* from TMS. Other ions that confirmed the structure were: 91 *m*/*z* (Ar), 105 *m*/*z* (Ar–OH), 126 *m*/*z* (O–Ar–CO), 45 (COO), prominent ion 121 *m*/*z* with one prominent ion (59) in the cluster 53–61 *m*/*z* confirming the presence of OH group in *para* position of the aromatic ring.

The qualitative analysis revealed the presence of products of *G. simplex* 4-*n*-NP biodegradation. Mass spectra of identified compounds are listed in Table 1. The literature data concerning 4-*n*-NP biodegradation by different organisms were based usually on the detection of only 4 or 5 metabolites [17,30,31]. In the light of the data presented in this study, we propose the biodegradation pathway (with the metabolites mentioned in Table 1) in Fig. 6.

An analysis of the metabolic pathway proposed by us in this study revealed, that there are two possible routes of 4-*n*-NP degradation by *G. simplex*. One of them starts with carbons

Table 1
The results of GC–MS qualitative analysis of 4-*n*-NP biodegradation.

Compound number	Compound name (trimethylsiloxy derivatives)	Retention time	Chemical formula	Molecular weight	Mass spectrum *m*/*z* (10 largest ions relative intensity)
1	1-(4-Hydroxyphenyl)ethanone, TMS	12.15	$C_{11}H_{16}O_2Si$	208.33	193(99.9) 208(25.7) 194(15.7) 73(14.1) 151(11.1) 89(8) 75(5) 135(4.9) 91(4.6) 195(4.5)
2	4-(1-Hydroxyvinyl)phenol, di-TMS	12.80	$C_{14}H_{24}O_2Si_2$	280.52	73(99.9) 279(76.6) 265(44.1) 280(36.8) 147(33.6) 45(24) 281(17.3) 77(16.3) 315(14.9) 75(14.8)
3	4-Hydroxybenzoic acid, di-TMS	13.01	$C_{13}H_{22}O_3Si_2$	282.49	267(99.9) 223(65.2) 193(49.8) 268(26.9) 73(24.6) 282(22.5) 224(15.2) 126(10.2) 269(10) 194(9.3)
4	2-(4-Hydroxyphenyl)acetic acid, di-TMS	13.07	$C_{14}H_{24}O_3Si_2$	296.52	73(99.9) 179(25.3) 75(23.5) 281(15.9) 296(15.4) 257(15.1) 164(13.9) 117(13.6) 252(12.6) 45(11.2)
5	3-(4-Hydroxyphenyl)propanoic acid, di-TMS	13.76	$C_{15}H_{26}O_3Si_2$	310.54	179(99.9) 192(65.7) 73(52.3) 310(21.7) 75(17.3) 177(16.3) 180(15.7) 193(14.3) 55(8.6) 295(7)
6	2-Hydroxy-2-(4-hydroxyphenyl)acetic acid, tri-TMS	14.05	$C_{17}H_{32}O_4Si_3$	384.70	193(99.9) 73 (77.8)311(20.5) 281(17.3) 194(15.1) 45(13) 223(10.5) 267(9.6) 165(8.4) 74(7.2)
7	3,4-Dihydroxybenzoic acid, tri-TMS	14.07	$C_{16}H_{30}O_4Si_3$	370.67	193(99.9) 370(52.2) 371(22.3) 355(20.4) 73(19.5) 311(19.5) 372(14.5) 177(13.5) 223(12.9) 356(11.7)
8	4-Hydroxyphenylbutyric acid, di-TMS	14.30	$C_{16}H_{28}O_3Si_2$	324.57	192(99.9) 73(37.7) 75(21.8) 179(19) 193(16.3) 309(15) 324(13.9) 177(13.2) 147(5.7) 45(5.6)
9	4-*n*-Nonylophenol, TMS	14.60	$C_{18}H_{32}OSi$	292.54	179(99.9) 292(39) 180(21.8) 73(16.6) 293(10.6) 181(6.6) 277(5.9) 163(4) 165(3.7) 149(3.3)
10	4-Hydroxyphenylpentanoic acid, di-TMS	14.81	$C_{17}H_{30}O_3Si_2$	338.60	179(99.9) 73(45.2) 338(28.8) 192(23.1) 75(22) 323(21.2) 180(15.4) 205(12.4) 45(12) 206(9.3)
11	3,4-Dihydroxybenzaldehyde, di-TMS	14.99	$C_{13}H_{22}O_3Si_2$	282.11	267(99.9) 268(23.5) 73(13.9) 269(10.1) 207(5.8) 282(5.3) 179(4.1) 266(3.0) 281(3.0) 270(2.8)
12	4-Hydroxyphenylhexanoic acid, di-TMS	15.33	$C_{18}H_{32}O_3Si_2$	352.63	179(99.9) 73(26.6) 352(24.2) 180(15.1) 75(14.2) 337(10.2) 353(7.6) 253(7.1) 207(5.7) 181(5.2)
13	8-Hydroxy-8-(4-hydroxyphenyl)octanal, di-TMS	15.51	$C_{19}H_{32}O_4Si_2$	380.22	380(99.9) 267(77.7) 73(56.2) 381(32.7) 179(32.5) 268(23) 382(14) 156(9.1) 269(8.9) 365(7.8)
14	4-Hydroxyphenylheptanoic acid, di-TMS	15.84	$C_{19}H_{34}O_3Si_2$	366.65	179(99.9) 366(31.3) 73(28.8) 180(18.3) 351(11.9) 367(11.5) 75(10.2) 253(7.4) 181(5.4) 192(5.3)
15	8-Hydroxy-8-(4-hydroxyphenyl)octanoic acid, tri-TMS	16.39	$C_{23}H_{44}O_4Si_3$	468.25	179(99.9) 73(61.1) 267(44.4) 380(42.2) 117(21.3) 97(21.2) 45(17.4) 180(16.1) 357(14.4) 381(13.5)
16	9-Hydroxy-9-(4-hydroxyphenyl)nonanoic acid, tri-TMS	16.91	$C_{24}H_{46}O_4Si_3$	482.27	179(99.9) 267(50.8) 73(42.7) 394(28.7) 253(15.1) 468(15) 180(14.5) 395(13.1) 75(12.8) 379(12.4)

detachment to yield 4-hydroxyphenylheptanoic acid (**14**) in 6 h of incubation. In the next hours, subsequent detachments of single carbons from the aliphatic chain lead to formation a of 3-(4-hydroxyphenyl)propanoic acid (**5**) in 12 h of cultivation, which is then converted to 4-(1-hydroxyvinyl)phenol (**2**), on a side route and to 2-(4-hydroxyphenyl)acetic acid (**4**) and 4-hydroxybenzoic acid (**3**) on the main route. Hydroxylation of the aromatic ring in a *meta* position occurred for compounds (**3**) and (**1**), but the aromatic ring fission degradation products were not detected with the applied method.

It seems that the second route starts with hydroxylation in the ninth (next to aromatic ring) position and carboxylation in the first (distal carbon) position of the nonyl-moiety resulting in a formation of 9-hydroxy-9-(4-hydroxyphenyl)nonanoic acid (**16**). In 6 h of incubation 4-hydroxybenzoic acid (**3**) was found as a one of the main metabolite. We also detect octanedioic acid with retention time 13.16 and fragmentation pattern interpreted in Fig. 4, but the presence of this compound as a metabolite of 4-*n*-NP degradation product needs further confirmations. It is probable that fatty acids can occur in fungal cultures under toxic environmental conditions. The presence of hydroxybenzoic acid (**3**) in 6 h of incubation strongly supports the hypothesis, that the linear chain was detached, because such compounds as (**5**) were not detected in the cultures before 12 h of incubation. It was documented that 9-hydroxy-9-(4-hydroxyphenyl)nonanoic acid (**16**) was also further transformed to 8-hydroxy-8-(4-hydroxyphenyl)octanoic acid (**15**) probably by the same mechanism as in the first route.

Biodegradation of 4-NPs by different species with metabolites formation has been widely discussed in the literature [3,17,32,33]. In the yeast cultures of *C. aquaetextoris* the major metabolites of the 4-*n*-NP breakdown were identified as 4-hydroxy-acetophenon and *trans*-4-hydroxycinnamic acid, indicating an oxidative attack at the alkyl chain. Both compounds tend to accumulate [17]. In our experiments with *G. simplex*, the presence of metabolites up to 72 h of incubation in samples supplemented with 50 mg L^{-1} of 4-*n*-NP were confirmed. There are no literature data suggesting toxic effect of detected metabolites. The qualitative analysis performed after 72 h of incubation did not reveal any biodegradation products. The obtained data suggest that in cultures with *G. simplex* there was no accumulation of biodegradation products. Two major metabolites

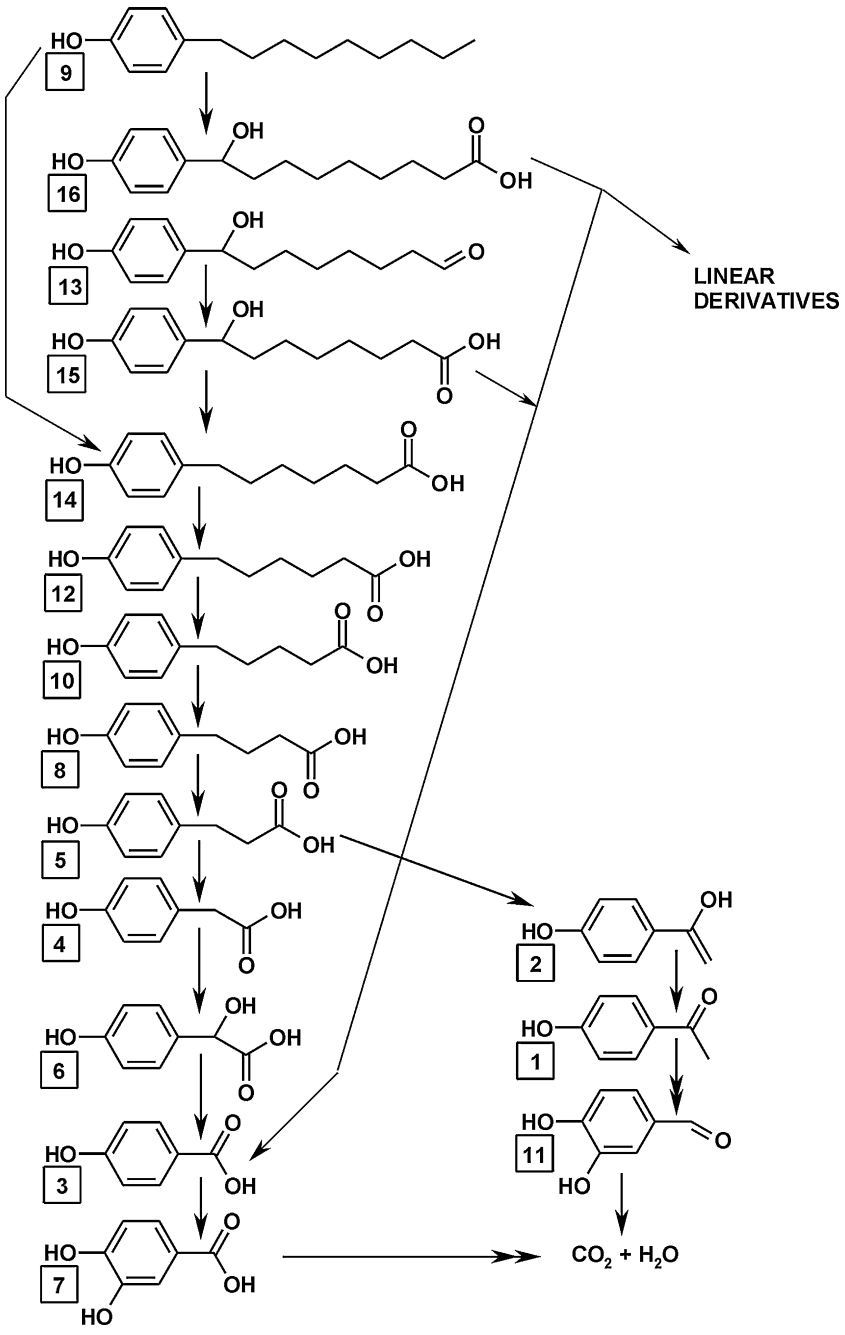

Fig. 6. A pathway for *G. simplex* biodegradation of 4-*n*-NP proposed in this study based on the data presented in Table 1.

of 4-*n*-NP degradation by *G. simplex* 3-(4-hydroxyphenyl)propanoic acid (**5**) and 4-hydroxybenzoic acid (**3**) were also found during the metabolism of this compound in rats and fish [30,31,34]. Moreover, *G. simplex* was found to remove C1 moieties in contrast to data suggesting the removal of C2 moieties as the β-oxidation process [15]. In our previous work, the fungus tested in this work was found to possess the ability of 11α-hydroxylation of steroids [21]. It is possible, that the monooxygenase present in the fungus is involved in the hydroxylation and subsequent degradation of a nonyl chain. In our studies linear chain detachment probably also occurred. A similar process was observed for *Sphingomonas* TTNP3 where hydroxylated alkyl chain was detected as a product of NP degradation [15].

Table 2
Balancing of the applied radioactivity with 4-*n*-NP [ring-^{14}C(U)]*.

Time of incubation (h)	Abiotic control		*G. simplex* IM2358		
	Medium	CO_2	Filtrate	Biomass	CO_2
0	98 ± 2	0	100	0	0
72	97 ± 2	0	66 ± 13	1 ± 0	29 ± 13

* Values of radioactivity are expressed in % of the initial applied radioactivity.

Corvini et al. [15] pointed out that in filamentous aquatic fungi and in the yeast *C. aquaetextoris*, the hydroxylation of NP aromatic ring was not detected. In our studies with *G. simplex* used as a 4-*n*-NP degrader, two metabolites 3,4-dihydroxybenzoic acid (**7**) and 3,4-dihydroxybenzaldehyde (**11**) with hydroxylation in a *meta*, *para* position were found. Unfortunately, the cleavage of aromatic ring products was not detected with the applied method. In order to prove the aromatic ring degradation, we performed the experiments with 4-*n*-NP [ring-^{14}C(U)] (Table 2). It was demonstrated that during the incubation period, a total recovery of the applied radioactivity was not lower than 97%, and in abiotic controls (without the fungus) no $^{14}CO_2$ evolution was found. The cleavage of the aromatic ring of 4-*n*-NP was confirmed by the data concerning the mineralization of the tested compound with the evolution of $^{14}CO_2$. Low amount of ^{14}C associated with biomass of *G. simplex* suggests that aromatic ring cleavage products are incorporated into $^{14}CO_2$ and into degradation intermediates.

The degradation of ^{14}C-labelled 4-*n*-NP by the fungus *Trametes versicolor* with the evolution of $^{14}CO_2$ was also described in the literature. The authors found that after 12 d of incubation only 6% of the initially applied radioactivity was detected in $^{14}CO_2$ [35]. The other studies with *Sphingomonas* TTNP3 revealed that one of the isomers *of p*-NP was also mineralized, and after 3 d 28% of the applied radioactivity was transferred to $^{14}CO_2$, but in those studies NP was added as a single carbon and energy source [15].

4. Conclusions

In the presented paper we report that non-ligninolytic fungus *G. simplex* effectively degraded 4-*n*-NP during 72 h of incubation. The data obtained in our study also revealed several 4-*n*-NP degradation products. An analysis of the pathway proposed by us suggest the existence of a novel mechanism of 4-*n*-NP degradation in fungi with the biodegradation of the aromatic ring of 4-*n*-NP confirmed by the radioactive experiments and the evolution of $^{14}CO_2$. The obtained results indicate that *G. simplex* is a valuable research tool for the study of NP degradation by fungi, because of the fast pace of the process and a novel mechanism. Moreover, complete degradation with no accumulation of biodegradation products, described in this paper, suggests the potential application of the tested strain in the environment.

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