Isolation of phylogenetically diverse nonylphenol ethoxylate-degrading bacteria and characterization of their corresponding biotransformation pathways

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ABSTRACT

Most nonylphenol ethoxylate (NPEO)-degrading isolates have been assigned to *γ-Proteobacteria*, which is different from the results acquired by using molecular ecological techniques. To better understand the environmental fate of NPEOs, bacterial isolation strategy characterized by the use of gellan gum as a gelling reagent and a low concentration of target carbon source were used to isolate phylogenetically diverse NPEO-degrading bacteria from activated sludge, and the biotransformation pathways of the isolates were investigated. Eight NPEO-degrading isolates with high diversity were acquired, which were distributed among seven different genera: *Pseudomonas*, *Sphingomonas*, *Sphingobium*, *Cupriavidus*, *Ralstonia*, *Achromobacter* and *Staphylococcus*. The latter five genera have never been reported to be able to degrade NPEOs. Three biotransformation pathways of NPEOs were observed in the eight strains. Six strains belonging to α , β and γ classes of *Proteobacteria* and *Firmicutes* phylum degraded NPEOs by initially shortening the EO chain and then oxidizing the terminal alcohol of the shortened NPEOs to the corresponding nonylphenoxy carboxylates (NPECs), which could explain most of the reported observations for the degradation of NPEOs in environment. An isolate (NP42a) belonging to the genus *Sphingomonas* degraded NPEOs through a non-oxidative pathway, with nonylphenol monoethoxylate (NP₁EO) as the dominant product. Another isolate (NP47a) belonging to the genus *Ralstonia* degraded NPEOs by oxidizing the EO chain directly without the formation of short chain products.

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1. Introduction

Nonylphenol ethoxylates (NPEOs) are highly cost effective surfactants that are widely used in industrial, agricultural, commercial and household applications (Thiele et al., 1997). They reach wastewater treatment plants (WWTPs), in which they are incompletely degraded to short-chain NPEOs, nonylphenoxy carboxylates (NPECs), carboxyalkylphenol polyethoxycarboxylates (CAPECs), and nonylphenol (NP) (Ahel et al., 1994; Di Corcia et al., 1998a; Maguire, 1999; Ying et al., 2002; Montgomery-Brown and Reinhard, 2003; Gonzalez et al., 2007; Koh et al., 2008; Nagarnaik et al., 2010). Some metabolites such as NP and nonylphenol mono- and diethoxylate (NP₁EO and NP₂EO) have long been focused because they act as estrogenic hormones in wildlife at low concentrations (Jobling et al., 1996; Routledge and Sumpter, 1996). Therefore, it is important to understand the fates of NPEOs from the viewpoint of environmental risk management.

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The biodegradation of NPEOs is generally believed to start with the stepwise shortening of the ethoxy (EO) chain (Thiele et al., 1997; Montgomery-Brown and Reinhard, 2003). To date, two EO chain-shortening pathways have been elucidated using pure cultures: (i) non-oxidative EO chain (hydroxyl shifts) exo-cleavage with short-chain NPEOs as the main metabolites ($n = 2$ or 3), which occurs through the release of one molecule of acetaldehyde at each step (Fig. 1A) (John and White, 1998; Lu et al., 2008) and (ii) EO chain oxidation and exo-cleavage with short chains of NPEOs and NPECs as the main metabolites ($n = 2$ or 3), which occurs through release of one molecule of glyoxylic acid at each step (Fig. 1B) (Sato et al., 2001, 2003; Liu et al., 2006). However, many laboratory and field studies have shown that NP₁EO or CAPECs are the main metabolites (Ahel et al., 1994; Di Corcia et al., 1998a; Jonkers et al., 2001), which cannot be explained well by the above pathways. On the other hand, based on an evaluation of NPEO metabolites in WWTPs, it was suggested that NPEOs were gradually shortened, after which the short-chain NPEOs were directly oxidized to their corresponding NPECs without exo-cleavage (Ahel et al., 1994; Di Corcia et al., 1998a; Montgomery-Brown and Reinhard, 2003). So it is possible that some other pathways might exist, which requires further studies using pure cultures.

Fig. 1. Generally proposed aerobic biodegradation pathway of NPEOs (John and White, 1998; Sato et al., 2001). (A) Hydroxyl shift cleavage model, and (B) terminal oxidation cleavage model. The exact structure of the alkyl chain is not specified.

At the same time, bacteria capable of degrading NPEOs or octylphenol ethoxylates (OPEOs) have been isolated from a wide variety of environments, including activated sludge (Maki et al., 1994; John and White, 1998; Barberio et al., 2001; Liu et al., 2006), sea water (Nguyen and Sigoillot, 1997), contaminated soil (Sato et al., 2001) and sediment (Chen et al., 2005). Except for several OPEO-degrading bacteria belonging to β - and α -*Proteobacteria*, such as *Burkholderia cepacia* and *Sphingomonas* sp., most NPEO- or OPEO-degrading isolates have been assigned to γ -*Proteobacteria* (mainly *Pseudomonas* spp.) (Maki et al., 1994; Nguyen and Sigoillot, 1997; John and White, 1998; Chen et al., 2005). However, community structure analysis of NPEO-enriched activated sludge using multiple molecular ecological techniques has demonstrated that bacteria affiliated with α - and β -*Proteobacteria* are dominant in NPEO-amended reactors, suggesting that these bacteria might be involved in the degradation of NPEOs (Lozada et al., 2004, 2006). So it is possible that pure cultures acquired through conventional isolation methods may not represent the actual distribution of NPEO-degrading bacteria in the environment, which might have affected the results of pathway researches.

Recently, strategies such as adjusting the gelling agent used in the solid medium and avoiding the use of nutrient-rich growth media have successfully narrowed the gaps in the results between molecular and cultivation-based analyses (Janssen et al., 2002; Tamaki et al., 2005). Gellan gum can effectively increase the cultivability of organisms and overcome some of the toxic effects that agar has on various groups of microorganisms. In addition, the clarity of the gum gels makes it possible to detect micro-colonies in plates that could not be seen on agar. For example, the percentage of β -*Proteobacteria* isolates recovered from lake sediment by gellan gum-based cultivation (40%) was much higher than that recovered by agar-based cultivation (3.3%) (Tamaki et al., 2005). At the same time,

high concentrations of target organic compounds normally used for enrichment tend to favor fast-growing species (Ferrari et al., 2005).

The purpose of this study was to isolate phylogenetically diverse NPEO-degrading bacteria using the above cultivation strategy characterized by the use of gellan gum as a gelling reagent and a low concentration of target carbon source. The degradation pathways of NPEOs for the acquired isolates were then characterized. This study will improve the understanding of the fates of NPEOs in the environment.

2. Materials and methods

2.1. Reagents and chemicals

Technical grade NPEO surfactant mixtures with an ethoxylate range of 4–19 and an average EO chain length of 9 (NP_{av9}EO) and 4-n-NP (used as an internal standard for GC–MS analysis) were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). NH₂ column (250 × 4.6 mm, 5 μ L, Agilent-Zorbax, USA) with gradient elution coupled to UV detection (277 nm) to detect the distribution of EO chain (Ahel and Giger, 1985). Since the molar percentage of short chain NPEO oligomers (NP1–3EO) was less than 2% of the total mixtures as shown in Fig. S1 (Supplementary Material), its contribution to technical surfactant mixtures was omitted in this study. Mixed NPEO standards, NPECs standards with EO chain lengths ranging from 0 to 9 corresponding to NP₁EC to NP₁₀EC, and NP were obtained from Hayashi Pure Chemicals (Osaka, Japan). N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethyl-chlorosilane (TMCS), which was used for trimethylsilylation, as well as gellan gum and agar used as gelling agents, were obtained from Supelco (Bellefonte, PA, USA). Pure linear alcohol ethoxylates (AE0₈) and 2,4-dinitrophenylhydrazine (DNPH) were obtained from Fluka AG (Buchs, Switzerland). All organic solvents (HPLC grade) were obtained from Fisher Company (Muskegon, MI, USA). All water (18.2 M Ω) used in this study was purified using a PURIC-MXII apparatus (Organo, Japan).

2.2. Screening and identification of NPEO-degrading bacteria

A sludge sample was collected from an activated sludge reactor used to treat NPEO-containing wastewater (Zhang et al., 2008a) and inoculated into modified PE03 medium containing NP_{av9}EO (50 or 500 mg L^{−1}) as the only carbon source. One liter of the modified PE03 medium contained: 0.38 g of KH₂PO₄, 0.39 g of K₂HPO₄, 0.5 g of (NH₄)₂SO₄, 5 mL of basal salts solution and 1 mL of vitamin solution (Hanada et al., 1995). The cultures were then incubated aerobically at 30 °C with shaking at 140 rpm. The enrichment cycles were repeated at 10-day intervals for 6 months. Finally, 100 μ L of culture broth diluted appropriately was spread on PE03-NP_{av9}EO (50 or 500 mg L^{−1}) gellan gum or agar plates and then incubated at 30 °C in the dark for 3 days. Next, 16 morphologically distinct colonies were selected, purified and tested for their ability to degrade NP_{av9}EO. NPEO-degrading strains were further identified by amplification of their 16S rRNA genes using universal bacterial primers 27F and 1492R (Lane, 1991). The nucleotide sequences of the 16S rRNA genes obtained were then submitted to GenBank and assigned the following accession numbers: NP31a (EU580701), NP213b (EU580710), NP47a (EU580702), NP15b (EU580707), NP22b (EU580697), NP41b (EU580708), NP42a (EU580703), and NP426a (EU580698).

2.3. Biodegradation of NPav9EO using pure cultures

All isolates were previously grown to the log phase in Luria–Bertani liquid medium supplemented with NP_{av9}EO (500 mg L^{−1}).

A 100- μ L aliquot of the culture medium, which had an optical density at 600 nm that was adjusted to 0.5, was then used to inoculate 250-mL Erlenmeyer flasks containing 100 mL of PE03-NP_{av9}EO (500 mg L⁻¹) medium. The samples were then subjected to constant shaking at 140 rpm and 30 °C for 20 days and the growth was evaluated by measuring the optical density at 600 nm using a spectrophotometer (Hitachi, UV3010). All assays were conducted in duplicate and test blanks were added to measure the abiotic losses.

After a designated time, the culture medium (2 mL) was collected from the test tubes, stabilized with 20 μ L of formaldehyde and acidified to pH 2 with HCl. The medium was then extracted using an equal volume of ethyl acetate/methanol (7:3, v/v) twice by shaking the mixture vigorously. The collected organic phases were then blown dry under a gentle flow of nitrogen. For LC–MS analysis, dried samples were redissolved in 1 mL of methanol/Nanopure water 1:1 (v/v). The recoveries of the NP, NPEOs, and NPECs ranged from 72.5% to 96.4%.

2.4. Biodegradation of NP₁₁EO using resting cells

Cells grown in PE03-NP_{av9}EO (500 mg L⁻¹) medium up to the late log phase were harvested by centrifugation (8500g, 10 min), washed twice with sterile distilled water and then used as resting cells. The biodegradation tests using resting cells were similar to the NP_{av9}EO degradation tests, except that NP₁₁EO (35 mg L⁻¹) was used instead of NP_{av9}EO, and 50-mL Erlenmeyer flasks that each contained 20 mL of PE03 medium were used for the reactions.

Individual NPEO oligomer (NP₁₁EO) was prepared from the NP_{av9}EO mixture by normal-phase HPLC using a previously described method (Hayashi et al., 2005). Gradient separation was performed on a Agilent-Zorbax NH₂ column (4.6 mm $\times$ 250 mm, 5 μ m) using *n*-hexane/2-propanol (93:7, v/v) and 2-propanol/water as the mobile phases (95:5, v/v) (27). The NP₁₁EO fraction was obtained by collecting the eluate over the corresponding period and then evaporating the sample to dryness under a gentle nitrogen stream.

2.5. Preparation and use of cell extracts to detect the intermediates

Strains were incubated in 500-mL Erlenmeyer flasks containing 200 mL of PE03-NP_{av9}EO (500 mg L⁻¹) medium. Cells were grown to the late log phase and then pelleted by centrifugation (8000g, 20 min), washed once with 10 mM potassium phosphate buffer (pH 7.0), washed twice with 20 mM Tris–HCl buffer (pH 8.0), suspended in the same buffer, disrupted by sonication at 4 °C (eight times for 15 s each with a 10 s interval) and centrifuged (10,000g, 15 min). The resultant supernatants were then used as cell extracts after filtration through 0.2 μ m pore filters. Aliquots (1 mL) of the cell extracts were mixed with 1 mL of PE03 medium supplemented with NP_{av9}EO or AEO₈ (2000 mg L⁻¹) and then incubated at 28 °C for 4 h.

Formation of aldehydic compounds from NPEOs in the cell extracts was detected by derivatization with DNPH combined with LC/MS (Richardson et al., 2000). The reagent solution was prepared by dissolving 20 mg of DNPH in 15 mL of a solution that contained HCl (~12 mol L⁻¹), water, and acetonitrile at a ratio of 2.5:1 (v/v). Derivatization was conducted by adding 500 μ L of the reagent solution to the sample. After 12 h of reaction (40 °C), the solution was injected directly into the LC/MS system using a mobile phase consisting of acetonitrile and 5 mmol L⁻¹ ammonium acetate solution. Changes in the percentage of acetonitrile were as follows: 30% at time zero, 95% at 25 min, and 100% at 30 min. The flow rate of the mobile phase was 0.2 mL min⁻¹. Aldehydic compounds were detected by selected ion recording (SIR) of the corresponding (M–H)⁺ ions in negative ion mode.

2.6. Analysis of NPEOs and related compounds

Simultaneous analysis of the NPEOs with long chains and NPECs was conducted using a Waters LC/MS system (Alliance 2690 HPLC system linked to a Micromass ZQ 4000) equipped with a Waters Symmetry ShieldTM RP18 column (150 $\times$ 2.1 mm, 3.5 μ m) as previously (Zhang et al., 2007). The mobile phase was a mixture of 5 mmol L⁻¹ ammonium acetate solution (A) and pure methanol (B). All analyses were performed in SIR mode using the deprotonated molecular ions of NPECs during negative ionization and the ammonium adducts of NPEOs in positive mode. The method recoveries ranged from 75% to 98% and the relative standard deviation (RSD) was below 12%. Instrumental limits of detection and linear range were 25 pg and 2.5–500 μ g L⁻¹ for NP_{1–10}EC, respectively. The concentration (0.5–39.3 g L⁻¹), SIR peak S/N (38–486), detection limit (0.4–4.6 pg) of individual NPEO (*n* > 3) oligomer in 300 μ g L⁻¹ of NP_{av9}EO were calculated based on the assumption of the same molar absorption coefficient (Table S1, Supplementary Material).

Short-chain NPEOs (NP₂EO and NP₁EO) and NP were analyzed using a gas chromatograph (Agilent Technologies, 6890 GC) interfaced with a DB-5MS capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m) and a mass spectrometer (Agilent Technologies, 5975i MSD) (Isobe et al., 2001). Due to the coelution problems (Crescenzi et al., 1995), the signal intensity of short-chain NPEOs (*n* = 1–3) was much lower than that of long chain NPEOs oligomers. So, GC–MS, which could provide reliable identification and high sensitivity, was used for analyzing short-chain NPEOs (Thiele et al., 1997). Briefly, the dried samples were redissolved in *n*-hexane, derivatized with BSTFA, and then subjected to GC–MS analysis. The GC column temperature was programmed to increase from 50 °C (initial equilibrium time 2 min) to 200 °C via a ramp of 20 °C min⁻¹, from 200 to 260 °C via a ramp of 5 °C min⁻¹, and from 260 to 280 °C via a ramp of 20 °C min⁻¹, after which it was maintained at 280 °C for 2 min. Helium carrier gas was maintained at a constant flow rate of 1 mL min⁻¹. Compounds were identified by comparison with previously published results (Isobe et al., 2001) and quantified in selective ion monitoring mode (SIM).

3. Results and discussion

3.1. Taxonomic characteristics of NPEO-degrading isolates

Eight NPEO-degrading bacterial strains were successfully isolated from activated sludge acclimated with NP_{av9}EO. The 16S rRNA sequences of strains NP42a, NP31a, NP213b, NP15b and NP426a were >99% homologous with *Sphingomonas* sp. LB126, *Alcaligenes* sp. NyZ215, *Cupriavidus* sp. KU-26, *Pseudomonas putida* KT2440, and *Staphylococcus haemolyticus*, respectively. The sequences of NP41b, NP22b, and NP47a were 96–98% homologous with *Sphingomonas* sp. JS5, *Sphingobium* sp. TP340-2, and *Ralstonia* sp. BPC3, respectively.

These isolates, which were distributed widely in the α , β and γ classes of *Proteobacteria* and the *Firmicutes* phylum, could be assigned to seven different genera: *Pseudomonas* (NP15b), *Sphingomonas* (NP41b, NP42a), *Sphingobium* (NP22b), *Cupriavidus* (NP213b), *Ralstonia* (NP47a), *Achromobacter* (NP31a) and *Staphylococcus* (NP426a) (Fig. 2). Among the eight isolates, only NP15b (*Pseudomonas*) was found to be closely related to previously reported NPEO-degrading bacteria (Maki et al., 1994; Nguyen and Sigoillot, 1997; John and White, 1998; Barberio et al., 2001; Liu et al., 2006). No members of the latter five genera have previously been identified as NPEO degraders. Among the eight NPEO-degrading isolates, three isolates belonged to β -*Proteobacteria* (the order *Burkholderiales*) and three to α -*Proteobacteria* (*Sphingomonas* and

Fig. 2. Phylogenetic distribution of NPEO-degrading bacteria based on the nucleotide sequence of the 16S rRNA. The phylogenetic tree was constructed using a neighbor-joining algorithm with the Jukes-Cantor distance in the PHYLIP software (<http://evolution.genetics.washington.edu/phylip.html>). The 16S rRNA gene sequence of *Geotoga petraea* (belonging to the Phylum Thermotogae) was selected as the outgroup. Reference sequences were obtained from the Ribosomal Database Project database (<http://rdp.cme.msu.edu/>). The Genbank accession numbers are shown in parentheses.

Sphingobium). This was in accordance with the culture independent (molecular) studies showing that α - and β -Proteobacteria are dominant in NPEO-amended reactors (especially the order *Burkholderiales* and the genus *Sphingomonas*) (Lozada et al., 2004, 2006; Zhang et al., 2008b).

It is clear that the strategy used to isolate NPEO-degrading bacteria was successful in the present study. While eight strains were identified using the gellan gum based media, only three (NP22b, NP213b, NP15b) were obtained on the agar based media (data not shown), showing that the use of gellan gum might have resulted in the isolation of phylogenetically diverse NPEO-degrading species. On the other hand, only NP15b (isolated by both agar and gellan gum) and NP31a (isolated by gellan gum) were acquired when using 500 mg L⁻¹ of NPEOs, suggesting that keeping a relatively low concentration of NPEOs might also be important for acquiring phylogenetically diverse isolates.

3.2. NPEO biotransformation pathway studies

3.2.1. Pathway 1: hydroxyl shift cleavage of NPEOs with NP₁EO as the major degradation product

Strain NP42a, which belongs to the *Sphingomonas* genus (α -Proteobacteria), was capable of degrading >95% (molar percentage) of the NP_{av9}EO within 2 days, and its growth was correlated with the disappearance of NP_{av9}EO (Fig. 3A). NP₁EO and NP₂EO were the primary degradation products at the beginning, but NP₁EO soon became the dominant products (Fig. 3B). NPECs were not formed throughout the whole period, which suggests that NPEOs should be biodegraded through a non-oxidative pathway. A mass balance at different sampling times for strain NP42a was summarized in Table S2 (Supplementary Material). The main metabolites detected (NP_{1–3}EO) accounted for about 4.3–84% molar percentage of NP_{av9}EO transformed during 15 days, which is in accordance with other studies (Jonkers et al., 2001). A complete mass balance was difficult to achieve in present study, which often occurs in similar biodegradation studies (Jonkers et al., 2001). The incomplete mass balance was possibly due to the poor extraction efficiency of NP_{av9}EO adsorbed on cells and the existence of some undetected metabolites. According to John and White (1998), in the non-oxidative pathway, the first step in the EO chain removal by strain

Fig. 3. Utilization of NP_{av9}EO as a growth substrate for strain NP42a. (A) Growth was assessed based on the optical density at 600 nm (OD₆₀₀). Residual amounts of NPEOs (EO chain length $n > 3$) were determined by LC-MS analysis of organic extracts of the entire culture medium. (B) Changes in the concentration of NP₁EO and NP₂EO in the culture media during the incubation of strain NP42a. Values represent the means of duplicate experiments, the standard deviations were too small to illustrate in some cases.

Pseudomonas putida is a hydroxyl shift from the terminal to the penultimate carbon to produce a labile hemiacetal, which results in the stepwise release of C₂ units as acetaldehyde to form the corresponding NPEOs with shorter EO chains. As shown in Table 1, acetaldehyde was identified as the major aldehydic compound formed in the reaction of NP_{av9}EO with the NP42a cell extract (Table 1), which further demonstrates that the EO chain was shortened via a non-oxidative hydroxyl shift cleavage pathway (Fig. 1A).

Table 1

The formation of acetaldehyde (mM) from the reaction of cell extracts with NP_{av9}EO by NPEO-degrading isolates^a.

Bacterial strain	Substrate	
	NP _{av9} EO	AEO ₈
NP42a	0.86	0.24
NP15b	0.13	0.03
NP47a	ND	ND

^a Other aldehydic compounds such as glyoxylic acid were not detected in the reaction of cell extracts with NP_{av9}EO and AEO₈ by any NPEO-degrading isolates. ND = not detected.

As shown in Table 1, aldehyde was also formed during the reaction of AEO₈ with the NP42a cell extract, which suggests that NP42a can degrade a wide spectrum of compounds with similar structures via the non-oxidative pathway (White et al., 1996; Di Corcia et al., 1998b; Lu et al., 2008).

Fig. 4. Changes in the distribution of individual NPEO metabolites (NPEOs and NPECs) during NP₁₁EO (50 μmol L⁻¹) biodegradation by strain NP15b for (A) 6, (B) 12, (C) 24 and (D) 96 h.

On the other hand, NP_{2,3}EO have been found to be the dominant degradation products through the hydroxyl shift cleavage pathway in most of the studies (John and White, 1998, as shown in Fig. 1A). Few pure strains that are capable of converting NPEOs to NP₁EO, which has been regarded as the most toxic and estrogenic NPEO homologue (Servos, 1999; Metcalfe et al., 2001) and is widely found in many environments as the main metabolites of NPEOs (Ahel et al., 1994; Ferguson et al., 2001; Kolpin et al., 2002), have been isolated to date. Nishio et al. (2005) isolated a bacterial strain belonging to the genus *Sphingomonas* that could produce OP₁EO through the degradation of OPEOs. However, they found that a high ratio of OP₂EO existed in the degradation products (the ratio of NP₁EO:NP₂EO was 2.2:1 on day 7), which is different from the results of the present study. Liu et al. (2006) also isolated two strains (*Ensifer* sp. strain AS08 and *Pseudomonas* sp. strain AS90) capable of transforming NP₂EO to NP₁EO. Overall, this is the first study to report the transformation of long chain NPEOs by a pure culture with NP₁EO as the major metabolite. The suggested NPEO-degradation pathway is shown in Fig. 5A.

3.2.2. Pathway 2: hydroxyl shift cleavage of NPEOs followed by terminal oxidation of the EO chain

The biodegradation of NP₁₁EO by resting strain NP15b is shown in Fig. 4. NP₁₁EO was transformed into NP_{2–10}EO and a small amount of NP_{2–10}EC (about 13% molar percentage) within 6 h of culture. NPEOs with an EO chain length greater than four almost disappeared, while NP_{4–10}EC remained almost unchanged around 5.6–9.2% after incubation for 12 h. At the same time, NP_{2–3}EO (about 39–53%) and NP_{2–3}EC (about 35–49%) became the predominant products. After incubation for 96 h, a small amount of NP₁EO (about 5.1%) and NP₁EC (4.4%) appeared, and the concentration of NP_{2–3}EC accounted for approximately 54% of the total intermediates. No NP₁₁EC was detected throughout the degradation period. A mass balance at different sampling times for strain NP15b was summarized in Table S3 (Supplementary Material). The main metabolites detected (NP_{1–10}EO and NP_{1–10}EC) accounted for

Fig. 5. Biodegradation pathways of NPEOs proposed based on the results of this study. (A) hydroxyl shift cleavage-oxidation model, and (B) terminal oxidation model (non shortening). The exact structure of the alkyl chain is not specified.

about 71–96% molar percentage of NP₁₁EO transformed during 96 h. Strains NP41b, NP22b, NP213b, NP426a, and NP31a showed NPEO-degradation behavior similar to NP15b.

As shown in Table 1, acetaldehyde was also the major aldehydic compound produced in the reaction of cell extract with NP_{av9}EO by strain NP15b. So it is speculated that the degradation of NP₁₁EO was divided into two main steps: shortening of the EO chain via a hydroxyl shift during the first step and terminal oxidation of the short-chain NPEOs to short chain NPECs without the liberation of glyoxylic acid. The suggested pathway is shown in Fig. 5A. Because six of the eight isolated strains with high phylogenetically diversity showed similar NPEO-degrading patterns, it is reasonable to assume that Pathway 2 might be a major mechanism involved in the degradation of NPEOs in the environment, which is also consistent with the observations of aerobic biodegradation of NPEOs in mixed cultures and environments (Ahel et al., 1994; Di Corcia et al., 1998a; Montgomery-Brown and Reinhard, 2003).

3.2.3. Pathway 3: direct oxidation of NPEOs without the cleavage of EO chain

It is interesting to note that strain NP47a exhibited the ability to transform NP₁₁EO to its corresponding carboxylated products (NP₁₁EC) without the cleavage of the EO chains (Fig. 6). On day 7, the concentration of NP₁₁EC and NP₁₁EO reached 19.6 and 23.4 $\mu\text{mol L}^{-1}$, accounting for 40% and 50% of the initial NP₁₁EO concentration respectively. The remaining 10% might be an error margin or due to the existence of some undetected metabolites, such as CAPECs. The degradation rate and the bacterial growth rate were both relatively low, perhaps because the energy gained from oxidation of the terminal alcohol group was much lower than the stepwise ether cleavage. As shown in Table 1, none of the aldehydic compounds were produced in the reaction of NP47a cell extract with NP_{av9}EO or AEO₈. These findings indicate that the biodegradation of the NPEOs by strain NP47a exhibited a novel oxidation pathway as shown in Fig. 5B.

To date, all studies conducted to evaluate the biodegradation of NPEOs by pure cultures have shown that degradation proceeds via the successive exo-cleavage of the EO chain. Although NPECs with long EO chains have not been widespread in the environment (Ying et al., 2002; Montgomery-Brown and Reinhard, 2003; Komori et al., 2006), the formation of NPECs without ether cleavage has also been observed in some studies (Di Corcia et al., 1994; Jonkers et al., 2001; Hayashi et al., 2005). For example, Jonkers et al. (2001) found that mid-chain NPECs with the same distribution profile as the added NPEOs occurred as the most abundant species at the beginning of their biodegradation experiment in river water. Hayashi et al. (2005) found that NPEOs were oxidized to the corresponding NPECs instead of shortened in the presence of organic materials such as methanol, glucose, and yeast extract. Di Corcia et al. (1994) found that the concentration of NPECs with long EO

chains in the secondary effluents of a major WWTP in Italy remained high over 1 year of monitoring. Therefore, NP47a may represent a small group of bacteria capable of transforming long chain NPEOs directly to the corresponding NPECs under special environmental conditions.

At the same time, strain NP47a was also found to be capable of degrading short chain of NPEOs (NP_{av2}EO, $n = 1, 2$, and 3) to CAPEC₅ in another study (data not shown), further showing that this isolate possesses a high oxidation ability.

4. Conclusions

Phylogenetically diverse NPEO-degrading strains were isolated from activated sludge by using a cultivation strategy characterized by the use of gellan gum as a gelling reagent and a low concentration of target carbon source. Among the eight NPEO-degrading isolates, six isolates with high phylogenetic diversity were found to degrade NPEOs via hydroxyl shift cleavage of NPEOs followed by terminal oxidation of the EO chain, suggesting that this pathway is a major mechanism for the degradation of NPEOs in diverse environments. At the same time, there exist two other pathways: a non-oxidative pathway with nonylphenol monoethoxylate (NP₁EO) as the dominant product, and an oxidative one by oxidizing NPEOs directly without the cleavage of EO chains.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemosphere.2010.04.044.

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Fig. 6. Time course of NP₁₁EO biodegradation and the formation of metabolites by strain NP47a.

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