Degradation behaviors of nonylphenol ethoxylates by isolated bacteria using improved isolation method

GU Xin¹, ZHANG Yu^{1,*}, ZHANG Jing¹, YANG Min^{1,*}, Hideyuki Tamaki², Yoichi Kamagata².

1. State Key Laboratory of Environmental Aquatic Chemistry, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China. E-mail: bpguxin@gmail.com

2. Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology, Tsukuba, Ibaraki 305-8566, Japan

Received 21 May 2008; revised 2 July 2008; accepted 23 July 2008

Abstract

Nonylphenol ethoxylate (NPEO)-degrading bacteria were isolated from activated sludge using an improved isolation method, and the corresponding degradation behaviours were investigated. Eight NPEO-degrading strains distributed in genera *Pseudomonas*, *Sphingomonas*, *Sphingobium*, *Cupriavidus*, *Ralstonia*, *Achromobacter*, and *Staphylococcus* were acquired. The latter five genera have never been reported for the degradation of NPEOs. Four degradation patterns were observed for the eight pure strains. In pattern A, NPEOs were converted to short-chain NPEOs and carboxylated products, while in pattern B, lower ethoxylated oligomers appeared. Nonylphenol monoethoxylate was the main product in pattern C, while in pattern D ethoxylated units was oxidized but not shortened. Pattern C and D have not yet been reported.

Key words: nonylphenol ethoxylate (NPEO); biodegradation; isolation method

Nonylphenol ethoxylates (NPEOs) are a major class of nonionic surfactants used in large amounts in industrial and agriculture applications (Thiele *et al.*, 1997). The environmental fate of NPEOs has received wide attention because some of their biodegradation intermediates in different aquatic environments, such as nonylphenol (NP), nonylphenol monoethoxylate (NP₁EO), nonylphenol diethoxylate (NP₂EO) and low ethoxylated nonylphenoxy carboxylates (NPECs) act as estrogenic hormone to aquatic organisms, mammals and birds at low concentration (Jobling and Sumpter, 1993; Jobling *et al.*, 1996; White *et al.*, 1994).

NPEOs enter the environment primarily via industrial and municipal wastewater treatment plants (WWTPs) (Ying *et al.*, 2002), where higher ethoxylated NPEOs are efficiently bioconverted into low ethoxylated products. Thus, the biodegradation process converts the relatively harmless surfactants into more endocrine-disruptive components. An understanding of this bioconversion mechanism by isolated strains will be important to assess environmental risk for this group of products. Until now, most of the isolated bacteria showing the ability to assimilate NPEOs belong to the class γ -Proteobacteria (mainly the genus *Pseudomonas*, *Acinetobacter*, *Aeromonas*). However, culture independent (molecular) studies on NPEO-degrading activated sludge reactors (Lozada *et*

al., 2004, 2006) and natural water microcosms (Zhang *et al.*, 2008a) indicated that the α -Proteobacteria and β -Proteobacteria play an important role in NPEO degradation. It has been reported that some improved cultivation-based technique permitted successful isolation of unknown or novel bacteria (Tamaki *et al.*, 2005). Therefore, the object of this work was to isolate novel NPEO-degrading bacteria with high genetic diversity from activated sludge by using improved cultivation-based technique and thereafter explore the corresponding degradation behaviors.

The bacterial strains in this study were isolated from activated sludge acclimated with NPEOs surfactant mixtures (technical grade, with an ethoxylate units (EOs) range of 4–19 and average EO chain length of 9, NP_{av9}EO) in a simulated NPEO-degrading bioreactor (Zhang *et al.*, 2008). Eight NPEO-degrading bacteria were isolated using an improved isolation method (Tamaki *et al.*, 2005) with a PE03 medium containing 50 mg/L NP_{av9}EO as the sole added carbon source. The isolated NPEO-degrading bacteria belong to α -, β -, γ -Proteobacteria and Firmicutes Phylum (Fig.1), and the members of β - and α -Proteobacteria were dominated, which is consistent with the results obtained by culture independent methods from an NP₁₀EO-amended sludge reactor (Lozada *et al.* 2004, 2006).

The 8 isolates were assigned to seven different genera: *Pseudomonas* (NP15b), *Sphingomonas* (NP41b, NP42a), *Sphingobium* (NP22b), *Cupriavidus* (NP213b), *Ralstonia*

* Corresponding author. E-mail: yangmin@rcees.ac.cn (Yang Min); zhangyu@rcees.ac.cn.

Fig. 1 Phylogenetic distribution of NPEOs-utilizing cultures isolated from enrichment cultures. Calculation of the phylogenetic tree was based on the neighbor-joining method with bootstrapping. The tree was rooted with the 16S rRNA gene sequence of *Escherichia coli* as the outgroup.

(NP47a), *Achromobacter* (NP31a), and *Staphylococcus* (NP426a) (Fig.1). To our knowledge, the latter five genera were the first time to be reported for the degradation of NPEOs.

The growth and degradation tests were performed for each isolates in liquid PE03 medium with 500 mg of NP_{av9}EO per liter as the sole carbon source. Seven isolates could degrade NP_{av9}EO within 2 d, while strain *Ralstonia* NP47a showed a poor but visible growth and a partial degradation. No abiotic NPEO removal was observed (data not shown).

NP_{av9}EO biodegradation intermediates were analyzed by liquid chromatography/mass spectrometry (LC/MS) and gas chromatography/mass spectrometry (GC/MS). NPEO degradation could be classified into four patterns (patterns A-D) based on different degradation products.

In pattern A, long homologs ($n > 4$) disappeared within 2 d, while more persistent intermediate homologs such as NP₂EO and NP₃EO and short-chain NPECs accumulated as the major end products. Four strains (*Sphingobium* NP22b, *Sphingomonas* NP41b, *Cupriavidus* NP213b, *Staphylococcus* NP426a) showed this degradation pattern, and typical changes of the four short-chain NPEOs and NPECs by NP22b are shown in Fig.2. These compounds are the most frequently reported intermediates formed during the aerobic biodegradation of NPEOs (Jonkers *et al.*, 2001; Hayashi *et al.*, 2005).

The concentrations of the carboxylated products were relatively low in the first two days, and the concentrations of NP_nEC ($n > 4$) remained low during the tested period. After the formation of short chain oligomers reached a plateau, the concentrations NP₂EC and NP₃EC increased rapidly, with NP₂EC reaching a maximum concentration of 102 $\mu\text{mol/L}$ (47% of the total intermediates molar concentration).

Most NPEO-degrading strains reported in previous publications can be classified into pattern A, and most of reported strains belong to genera *Pseudomonas* (Maki *et al.*, 1994; Sato *et al.*, 2001) of γ -Proteobacteria. Our study indicated that the pattern A NPEO-degrading microbes existed diversely, belonging to α -Proteobacteria, β -Proteobacteria, and Firmicuts.

Fig. 2 Changes in the concentrations of typical metabolites during the biodegradation period by strain NP22b (Pattern A).

In pattern B (*Achromobacter* NP31a and *Pseudomonas* NP15b), except for NP₂EO and NP₂EC as the main degradation products, two new metabolites, NP₁EO and its carboxylated product NP₁EC were produced within 14 d and accumulated gradually (data not shown). The concentration of NP₁EO increased to around 25 $\mu\text{mol/L}$ in 25 d, which is nearly 30% of the concentration of NP₂EO. Growth reached the maximum in 1 d, and complete transformation of NP_{av9}EO occurred within 1 d together with the maximum growth of microbes. It has been reported that *Sphingomonas macrogoltabidus* G-3 isolated from topsoil of golf courses is capable of bioconverting octylphenol ethoxylates (OPEOs) to small amount of octylphenol monoethoxylate (OP₁EO) together with large amount of OP₂EO (Nishio *et al.*, 2005).

In pattern C (*Sphingomonas* NP42a), NP₁EO was the main degradation products (Fig.3), and the concentrations of the carboxylated products was very low ($< 4\%$). NP₁EO is known as the most toxic and estrogenic in NPEOs homologue. NP₁EO was identified as a main metabolite from aerobic and anaerobic degradation of NPEOs in many of the bench-scale studies as well as in field studies, nevertheless there is little information about the bioconversion mechanisms and the pure strains which are capable to

Fig. 3 Changes in the concentrations of NP₁EO and NP₂EO during the biodegradation period by Strain NP42a (Pattern C).

convert NPEOs to NP₁EO. This is the first report about NP₁EO producing pure culture, which will improve the understanding of bioconversion mechanism of NPEOs.

In pattern D (*Ralstonia* NP47a), NPEOs were converted to their carboxylated products without the formation of lower ethoxylated oligomers (Fig.4). Until now almost all studies on NPEO biodegradation by pure culture indicated that the degradation proceeds via the successive exocission of the ethylene oxide chain (Maki *et al.*, 1994; Jones *et al.*, 1998; Sato *et al.*, 2001). It is very interesting that strain NP47a has the ability to oxidize the EO chain, accordingly lacks the ability to shorten the EO chain. The only NPEOs biodegradation mechanism that has been validated by axenic strains is the EO chains exocission pathways (oxidative and non-oxidative). However, pattern D exhibits many differences from the reported pathways, and the further mechanism is still under study.

Fig. 4 Time course of NP_{av9}EO degradation by strain NP47a (Pattern D).

As a conclusion, eight NPEO-degrading bacteria belong to α -, β -, γ -*Proteobacteria* and *Firmicutes* Phylum and 7 different genera were successfully isolated from the NPEO degrading bioreactor by an improved isolation method. Five of the 7 genera have not yet been reported previously for the biodegradation of NPEOs. These 8 isolates demonstrated four different degradation patterns, and two of them have not yet been reported. This study improved the understanding of bioconversion process of NPEOs in the environment.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 50578153, 50525824) and the Ministry of Science and Technology, China (No. 2006DFA91870).

References

- Hayashi S, Saito S, Kim J H, Nishimura O, Sudo R, 2005. Aerobic biodegradation behavior of nonylphenol polyethoxylates and their metabolites in the presence of organic matter. *Environ Sci Technol*, 39(15): 5626–5633.
- Jobling S, Sheahan D, Osborne J A, Matthiessen P, Sumpter J P, 1996. Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. *Environ Toxicol Chem*, 15(2): 194–202.
- Jobling S, Sumpter J P, 1993. Detergent components in sewage effluent are weakly oestrogenic to fish: An in vitro study using rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Aqua Toxicol*, 27(3-4): 361–372.
- Jones F W, Westmoreland D J, 1998. Degradation of nonylphenol ethoxylates during the composting of sludges from wool scour effluents. *Environ Sci Technol*, 32(17): 2623–2627.
- Jonkers N, Knepper T P, De Voogt P, 2001. Aerobic biodegradation studies of nonylphenol ethoxylates in river water using liquid chromatography-electrospray tandem mass spectrometry. *Environ Sci Technol*, 35(2): 335–340.
- Lozada M, Figuerola E L M, Itria R F, Erijman L, 2006. Replicability of dominant bacterial populations after long-term surfactant-enrichment in lab-scale activated sludge. *Environ Microbiol*, 8(4): 625–638.
- Lozada M, Itria R F, Figuerola E L M, Babay P A, Gettar R T, de Tullio L A, Erijman L, 2004. Bacterial community shifts in nonylphenol polyethoxylates-enriched activated sludge. *Water Res*, 38(8): 2077–2086.
- Maki H, Masuda N, Fujiwara Y, Ike M, Fujita M, 1994. Degradation of alkylphenol ethoxylates by *Pseudomonas* sp. strain tr01. *Appl Environ Microbiol*, 60(7): 2265–2271.
- Nishio E, Yoshikawa H, Wakayama M, Tamura H, Morita S, Tomita Y, 2005. Isolation and identification of *Sphingomonas* sp. that yields tert-octylphenol monoethoxylate under aerobic conditions. *Biosci, Biotechnol, Biochem*, 69(7): 1226–1231.
- Sato H, Shibata A, Wang Y, Yoshikawa H, Tamura H, 2001. Characterization of biodegradation intermediates of non-ionic surfactants by matrix-assisted laser desorption/ionization-mass spectrometry 1. bacterial biodegradation of octylphenol polyethoxylate under aerobic conditions. *Polym Degrad Stab*, 74(1): 69–75.
- Tamaki H, Sekiguchi Y, Hanada S, Nakamura K, Nomura N, Matsumura M, Kamagata Y, 2005. Comparative analysis of bacterial diversity in freshwater sediment of a shallow eutrophic lake by molecular and improved cultivation-based techniques. *Appl Environ Microbiol*, 71(4): 2162–2169.
- Thiele B, Günther K, Schwuger M J, 1997. Alkylphenol ethoxylates: Trace analysis and environmental behavior. *Chem Rev*, 97(8): 3247–3272.
- White R, Jobling S, Hoare S, Sumpter J, Parker M, 1994. Environmentally persistent alkylphenolic compounds are estrogenic. *Endocrinology*, 135(1): 175–182.
- Ying G, Williams B, Kookana R, 2002. Environmental fate of alkylphenols and alkylphenol ethoxylates: a review. *Environment International*, 28(3): 215–226.
- Zhang J, Yang M, Zhang Y, Chen M X, 2008. Biotransformation of nonylphenol ethoxylates during sewage treatment under anaerobic and aerobic conditions. *Journal of Environmental Sciences*, 20(2): 135–141.
- Zhang Y, Sei K, Toyama T, Ike M, Zhang J, Yang M, Kamagata Y, 2008a. Changes of catabolic genes and microbial community structures during biodegradation of nonylphenol ethoxylates and nonylphenol in natural water microcosms. *Biochem Eng J*, 39(2): 288–296.