

Growth factors, kinetics and biodegradation mechanism associated with *Pseudomonas nitroreducens* TX1 grown on octylphenol polyethoxylates

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Abstract

The growth properties and biodegradation mechanism of a Gram-negative bacterium, *Pseudomonas nitroreducens* TX1 that was able to grow on branched octylphenol polyethoxylates (OPEO_n, average $n = 9.5$) as the sole carbon source over a wide concentration range (1–100,000 mg l⁻¹) were studied. Analysis of growth factors indicated the highest specific growth rate (μ) of 0.53 h⁻¹ was obtained at an initial concentration of 5000 mg l⁻¹ OPEO_n. An optimal C/N ratio of 12 was obtained for (NH₄)₂SO₄ as the nitrogen source in a cultivated medium at pH 7. The kinetic analysis demonstrated that bacterial growth and OPEO_n degradation followed the Monod equation and were based on a substrate concentration inhibition model and pseudo-first-order reaction, respectively. The substrate inhibition coefficient was over 18,000 mg l⁻¹ and this indicates that the strain has an ability to sustain growth at high concentrations of OPEO_n and use it as the sole carbon source under such a stress condition. Furthermore, LC-MS analysis showed that the biodegradation mechanism of dodecyl octaethoxylate (AEO₈) by *P. nitroreducens* TX1 was the sequential cleavage of the ethoxylate chain.

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1. Introduction

Octylphenol polyethoxylates (OPEO_n) belong to the non-ionic surfactant family, the alkylphenol polyethoxylates (APEO_n), and these are used in numerous commercial and industrial products such as detergents, emulsifiers, wetting agents, solubilizers and dispersants. These surfactants have a relatively low toxicity for mammals but a higher toxicity for aquatic organisms (Osburn and Benedict, 1996; Planas et al., 2002). However, in the environment, the ultimate metabolites of APEO_n are more toxic and resistant to biological degradation than are the parent compounds (Tanenbaum et al., 1998; Servos, 1999; La Guardia et al., 2001). The biodegradation metabolites of

APEO_n are thought to imitate natural hormones and the levels present in the environment may be sufficient to disrupt endocrine activity in wildlife and even humans (White et al., 1994; Ferguson et al., 2000; Ying et al., 2002). Thus, the use of these surfactants has been prohibited for domestic activities in the UK, Germany and Switzerland (Manzano et al., 1999).

The biodegradability of APEO_n has been studied mainly in aquatic environments including fresh water, wastewater and seawater (Staples et al., 1999, 2001; Tanghe et al., 2000). Bacterial isolates such as *Pseudomonas* sp. TR01, marine *Pseudomonas*, *Pseudomonas putida*, *Burkholderia cepacia*, and *Sphingomonas* sp. TTNP3 have been reported as being able to biodegrade APEO_n (Tanghe et al., 1998, 2000; Hideaki et al., 1994; Nguyen and Sigoillot, 1997; John and White, 1998; Nishio et al., 2002). However, the growth factors and growth kinetics of these APEO_n-degrading isolates have been seldom investigated in the previous studies. Additionally, most of the biodegradation studies deal with low concentrations of APEO_n in the range

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5–1000 mg l⁻¹ (Planas et al., 2002; Manzano et al., 1999; Tanghe et al., 1999, 2000; John and White, 1998; Franska et al., 2003). It is well documented that most commercial surfactants in the low concentration range show rapid and extensive biodegradation in the aerobic environment. However, the growth factors and biodegradation mechanism for high concentrations remain unknown. Surfactant concentrations may be increased dramatically during remediation processes when there is exogenous addition of surfactant, as well as when the surfactants are used as additives to pesticides for agriculture activities (Iglesias-Jimenez et al., 1996; Harwell et al., 1999). A novel bacterial strain, *Pseudomonas nitroreducens* TX1, is used in this study to investigate catabolism of OPEO_n up to a maximum of 100,000 mg l⁻¹ OPEO_n in MSB medium. This strain has the possibility of being applied to the removal of high concentrations of OPEO_n from aquatic and soil environments.

Bacterial growth factors such as substrate concentration, pH, and nutrient level can have an impact on the utilization of substrate, affect protein synthesis, change the synthesis of the cytoplasm and modify the release of the intermediates. Thus, growth factors are critical for the understanding of bacterial physiology, the evaluation of the persistence of organic pollutants, and the design of the facilities for bioremediation. This study investigates factors including nutrients and pH, etc., for the growth kinetics of *P. nitroreducens* TX1 at relatively high concentrations of the surfactant. This study provides helpful information on the substrate degradation rate and microbial growth rate for the development of *P. nitroreducens* TX1 as a tool in bioremediation of APEO_n contamination in the field.

2. Materials and methods

2.1. Chemicals

Commercially available OPEO_n surfactant (commercial name, Triton X-100) was purchased from Merck Chemical Co. (Darmstadt, Germany) and used for this bacterial growth and biodegradation study. The average number of ethoxylate (EO) units for Triton X-100 is 9.5 according to the manufacturer's information, which corresponds to an average molecular weight of ca. 625. The molecular structure of OPEO_n and properties are shown in the manufacturer's information webpage (<http://www.chemistrystore.com/triton-x100.htm>). The dodecyl octaethoxylate (AEO₈) used in this study was purchased from the Fluka Chemical Co., Switzerland. An AEO₈ molecule consists of eight EO units, which corresponds to a molecular weight of ca. 538. The chemical structures of OPEO_n and AEO₈ are shown in Fig. 1.

2.2. Bacterial strain

The strain, *P. nitroreducens* TX1, was isolated from the recycle-drainage of a rice field in the northern part of

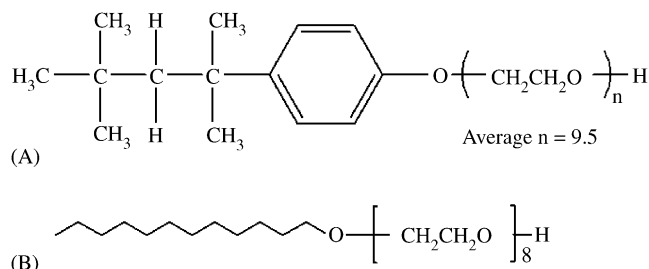


Fig. 1. The chemical structures of: (A) OPEO_n and (B) AEO₈.

Taiwan by enrichment culture using OPEO_n as the sole carbon source. A sterilized test tube was filled with 10 ml of MSB, 0.5 g of environmental sediment sample and 0.05% OPEO_n. The test tubes were incubated at 30 °C in a shaker operated at 200 rpm. When the optimal density at 600 nm (OD₆₀₀) of the culture was higher than 1, one-tenth of the culture was sub-cultured to a newly prepared MSB medium with the concentration of OPEO_n doubled. This procedure was repeated ten times. The isolates were plated out on an OPEO_n-MSB plate to isolate single colonies. The isolate was then identified by the Biolog method (Bochner, 1989), 16S rDNA sequence analysis (Ausubel et al., 1990) and fatty acid methyl ester analysis (Sasser, 1991). The Gram-negative strain was identified as *P. nitroreducens* by the Biolog method with a similarity index (SIM) of 0.90, and as a *Pseudomonas* species with 98% similarity by 16S rDNA sequence analysis and as *Pseudomonas aeruginosa* by fatty acid analysis. The bacterial strain is negative for gelatin hydrolysis.

2.3. Media

The strain was grown in MSB containing OPEO_n as the sole carbon source and 0.02% (NH₄)₂SO₄ as the nitrogen source. The composition of the MSB included 5.5 g Na₂HPO₄·7H₂O l⁻¹, 2.7 g KH₂PO₄ l⁻¹, 0.5 g (NH₄)₂SO₄ l⁻¹, 0.15 g KOH l⁻¹, 0.6 g MgSO₄·7H₂O l⁻¹, 0.2 g nitrilotriacetic acid l⁻¹, 67 mg CaCl₂ l⁻¹, 2 mg FeSO₄·7H₂O l⁻¹, 0.815 mg (NH₄)₂Mo₇O₂₄·4H₂O l⁻¹, and 1 ml of trace metal solution. The trace metal solution consisted of 2.5 g Na₂EDTA·2H₂O l⁻¹, 11 g ZnSO₄·7H₂O l⁻¹, 5 g FeSO₄·7H₂O l⁻¹, 1.54 g MnSO₄·H₂O l⁻¹, 0.4 g CuSO₄·5H₂O l⁻¹, 0.25 g Co(NO₃)₂·6H₂O l⁻¹, and 0.18 g Na₂B₄O₇·10H₂O l⁻¹ (Stanier et al., 1966). All the chemicals were purchased from Merck Chemical Co. (Darmstadt, Germany).

2.4. The growth factors and kinetics analysis

Various concentrations of OPEO_n (1.0 × 10⁵, 7.5 × 10⁴, 5.0 × 10⁴, 2.5 × 10⁴, 1.0 × 10⁴, 5.0 × 10³, 2.5 × 10³, 1.0 × 10³, 1.0 × 10², 10.0, 1.0 and 0.0 mg l⁻¹) were added to a series of 250-ml flasks containing 50 ml of MSB. The culture was inoculated from a single colony on an MSB plate containing 0.5% OPEO_n. The cell growth and OPEO_n degradation were measured every 2 h. Two types of

nitrogen source, $(\text{NH}_4)_2\text{SO}_4$, and KNO_3 , were included in this evaluation. In the presence of 5000 mg l^{-1} of OPEO_n , various concentrations of $(\text{NH}_4)_2\text{SO}_4$ and KNO_3 were used to achieve a C/N ratio from 8 to 35. The effects of the nitrogen source on the bacterial growth were measured by OD_{600} , specific growth rate, and surfactant biodegradation rate. Different pH values (5–9) were evaluated for MSB containing 0.5% OPEO_n as the sole carbon source and 0.16% $(\text{NH}_4)_2\text{SO}_4$ and 0.18% KNO_3 as the nitrogen source. The flasks were incubated in a reciprocal shaker at 200 rpm, at 30°C .

2.5. OPEO_n extraction and measurement

OPEO_n was extracted from a 2-ml bacterial culture by adding 25 ml MgSO_4 solution (72.4% of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) and 0.5 ml of 5 N H_2SO_4 (Nguyen and Sigoillot, 1997). The total mixture was extracted three times with 25 ml CHCl_3 each time. The combined organic phase was dried by anhydrous Na_2SO_4 and then concentrated to 1 ml residue. The residue was then dissolved in 5 ml of acetonitrile for HPLC analysis (Nguyen and Sigoillot, 1997). The amount of OPEO_n in the extracts was determined by HPLC using a C_{18} column (HPLC BT 7900, BIOTRONIK, Oregon, USA) equipped with a UV detector (LINEAR UVIS 200, BIOTRONIK, Oregon, USA) at 275 nm (Marcomini and Giger, 1987). The mobile phase was acetonitrile/ DDH_2O (7/3). Calibration was performed with standards over the range of $50\text{--}275 \mu\text{g l}^{-1}$ of OPEO_n in acetonitrile. The OPEO_n concentrations and bacterial numbers in the soil were obtained from three samples and an average with standard deviation was obtained. The data shown in all the figures and tables are the means calculated each time from three samples except the LC-MS chromatogram. All statistical analysis were conducted using STATISTICA 6.1 software.

2.6. The degradation mechanism analysis

The biotransformation of 0.5% AEO_8 was performed by the resting cells of *P. nitroreducens* TX1 to investigate the biodegradation mechanism of OPEO_n . The solution was taken from the culture medium and sequentially extracted by adding 1:1 CHCl_3 . The mixture was then sonicated for 30 min. The extracted organic phase was immediately filtered using a $0.22 \mu\text{m}$ filter for analysis. The AEO_8 and its intermediates were analyzed by an LC-MS (Waters Alliance 2690, Massachusetts, USA) equipped with an electrospray ionization-mass spectrometer (Platform LC, Micromass, UK). The injection volume was $10 \mu\text{l}$ and the flow rate was 0.5 ml min^{-1} . A C_{18} column (Waters $\mu\text{Bondapak}^{\text{TM}}$, $3.9 \times 300 \text{ mm}$, Massachusetts, USA) was used for the analysis. A gradient mobile phase was developed made up of 0.1% aqueous formic acid and acetonitrile, which started at 30% of acetonitrile and reached 80% and 90% acetonitrile at 15 and 60 min, respectively. The potentials of the ionization source were

3.5 V for the capillary and 35 V for the cone voltage. The source temperature was 100°C and the flow rate of nitrogen gas was 300 l h^{-1} . In addition, the relative intensity of AEO_n and the transformed metabolites were obtained by comparing the corresponding peak intensities in the total ion chromatogram. This is a semi-quantitative method to display the kinetics of degradation of AEO_8 and its metabolites by *P. nitroreducens* TX1. In addition, the intermediates of the OPEO_n biodegradation by *P. nitroreducens* TX1 were also detected by the same methods by using the electrospray ionization-mass spectrometer.

3. Results and discussion

3.1. Initial concentration

As presented in Fig. 2, when the initial concentration of OPEO_n is below 5000 mg l^{-1} , the specific growth rate of *P. nitroreducens* TX1 gradually increases with an increase in OPEO_n . The greatest specific growth rate was observed at an initial concentration of 5000 mg l^{-1} . However, the specific growth rate apparently declined as the OPEO_n concentration increased above 5000 mg l^{-1} up to $100,000 \text{ mg l}^{-1}$. The observed specific growth rate of *P. nitroreducens* TX1 was estimated to be in the range of $0.36\text{--}0.53 \text{ h}^{-1}$ when the initial OPEO_n concentration was in the range $2500\text{--}50,000 \text{ mg l}^{-1}$. Furthermore, 0.11 h^{-1} of specific growth rate was still obtained even at an initial concentration of OPEO_n as high as $100,000 \text{ mg l}^{-1}$.

It is interesting to note that the *P. nitroreducens* TX1 still showed an apparent growth rate at high concentrations of OPEO_n with this chemical as the sole carbon source. This indicates that the isolate has a novel ability to resist the high OPEO_n concentration. The growth demonstrated at this level of surfactant by *P. nitroreducens* TX1 is higher than the other APEO_n degrading microorganisms reported previously, such as microorganisms from an industrial

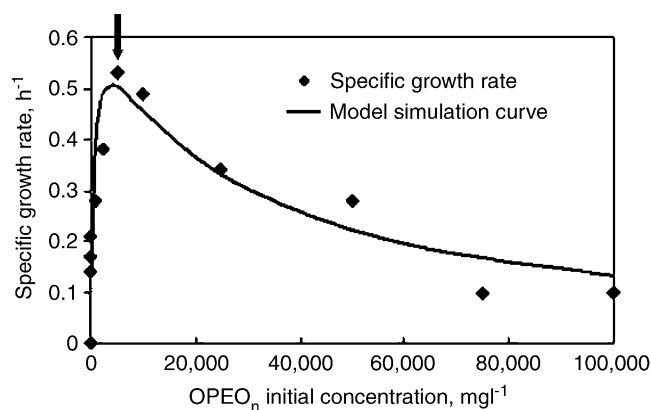


Fig. 2. The initial concentration of OPEO_n corresponds to the specific growth rate by *P. nitroreducens* TX1. The specific growth rate (μ) was derived from the cell density of OD_{600} , and the exponential cells growth period (t) which was measured by every 2 h. The arrow indicates the OPEO_n in the concentration of 5000 mg l^{-1} .

sewage treatment process (Turkovskava et al., 1996), river water in Japan (Maki et al., 1996), the sieved sludge from a New Bedford wastewater treatment plant (MA, USA) (Hideaki et al., 1994), and the sludge from a continuous flow activated sludge chamber (Franska et al., 2003).

3.2. Nitrogen sources

Both the specific growth rate and OPEO_n degradation rate were used as the parameters to measure the response of *P. nitroreducens* TX1 to changes in the C/N ratio. As illustrated in Fig. 3, the results show that a variation of C/N in the range 8–35 produced a prominent effect on these two parameters. A C/N ratio of 12 with (NH₄)₂SO₄ and 22 with KNO₃ was found to be optimal for both specific growth rate and OPEO_n degradation rate, respectively. Since the C/N ratio of heterotrophic bacteria is generally in the range of 12–20, the optimal value of the C/N ratio for *P. nitroreducens* TX1 was in such a range. However, the variation in the C/N ratio on the either side of the optimal value showed an adverse effect on these two parameters. The specific growth rate declined by 40–45% with (NH₄)₂SO₄ and the OPEO_n degradation rate decreased by 35–40% with KNO₃ when the C/N ratio was above 22. Similarly, at a C/N ratio of 8, the value of these two parameters decreased by 30% with (NH₄)₂SO₄ and 40% with KNO₃. This indicates that the specific growth rate and OPEO_n degradation rate are susceptible to changes in nitrogen source and C/N ratio.

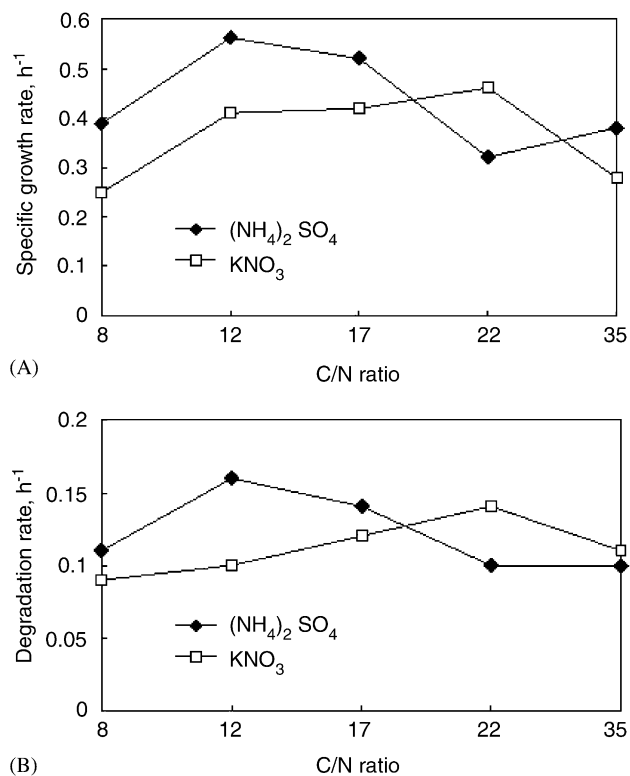


Fig. 3. The effect of nitrogen sources and C/N ratio on *P. nitroreducens* TX1: (A) specific growth rate and (B) OPEO_n degradation rate.

In theory, the synthesis of one mole cell requires the transportation of 20 and 28 electrons to the terminal electron acceptor (oxygen) when NH₄⁺ and NO₃⁻ are used as the nitrogen source, respectively (Rittmann and McCarty, 2001). This indicates that less energy is required when using NH₄⁺ instead of NO₃⁻ as the nitrogen source. The results of this study are consistent with this. It is also interesting to note that the optimal C/N ratio using (NH₄)₂SO₄ was almost two-fold that of KNO₃. However, with the optimal C/N ratio for these two nitrogen sources, an 18% enhancement of the specific growth rate was obtained when using (NH₄)₂SO₄ instead of KNO₃ as the nitrogen source. Since the protein and DNA contents of cells are reported to be affected by C/N ratio (Bhattacharya and Bubey, 1997), this indicates that metabolism and synthesis may be quite different when *P. nitroreducens* TX1 is grown on (NH₄)₂SO₄ and KNO₃ as sole nitrogen sources.

3.3. Optimal growth pH

Since the availability of macronutrients and the enzyme activity are highly pH-dependent (Skladany and Baker, 1994), specific growth rate should be optimized with respect to the cultivation pH. As presented in Fig. 4, the specific growth rate of *P. nitroreducens* TX1 was estimated over a pH range of 5–9 with either (NH₄)₂SO₄ or KNO₃ as the nitrogen source. The results indicated that the specific growth rate increased 100–150% in the pH range from 5 to 7. Moreover, growth was lower at a pH above 7. The specific growth rate decreased significantly by over 80% and a specific growth rate of only 0.1 h⁻¹ was obtained at pH 9. Consequently, the effect of pH on growth of *P. nitroreducens* TX1 is in agreement with most microorganisms, which favor growth at pH levels ranging from 6.0 to 8.0 (Skladany and Baker, 1994). Moreover, Fig. 4 also shows that the use of different nitrogen sources did not obviously affect the cell growth at various pH.

It should be mentioned that the pH of the cultivation medium was found to decline gradually from an initial pH

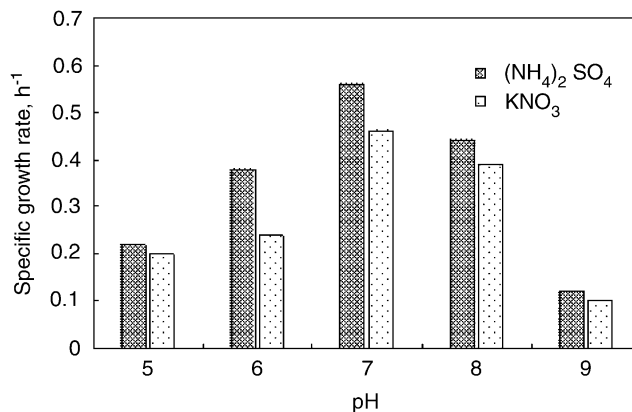


Fig. 4. The effect of pH on the specific growth rate of *P. nitroreducens* TX1.

of 7 to about pH 6 when *P. nitroreducens* TX1 was cultivated in 5000 mg l⁻¹ OPEO_n-MSB medium. The increase in acidity suggests the production of acidic metabolites, such as octylphenol ethoxy acetic acid (OPEC_n), from the metabolism of OPEO_n (Staples et al., 1999; Marcomini and Giger, 1987; DiCorcia et al., 2000). The acidic pH may also be associated with the formation of carbon dioxide as OPEO_n is biodegraded (Swisher, 1987).

3.4. The kinetics analysis

Since models are widely accepted for the prediction of the transition between zero- and first-order kinetics and related kinetic parameters, kinetics analysis following the Monod equation and the Haldane model (Simkins and Alexander, 1984) were performed as part of this study.

The bacterial growth model was proposed as follows:

$$\mu = \frac{\mu_{\max} S}{K_s + S + (S^2/K_i)} \quad (1)$$

$$N = N_0 e^{\mu t} \quad (2)$$

The model of the substrate degradation is

$$q = \frac{q_{\max} S}{K_s + S + (S^2/K_i)} N \quad (3)$$

where μ is the specific growth rate (h⁻¹), μ_{\max} is the maximum specific growth rate (h⁻¹), S is the substrate concentration (mg l⁻¹), K_s is the half-velocity constant (mg l⁻¹), K_i is the substrate inhibition coefficient (mg l⁻¹), N_0 is the initial amount of cells in OD₆₀₀, N is the amount of cells in the late log phase of the cells growth in OD₆₀₀, t is the incubation time (h), q is rate of substrate utilization (h⁻¹), and q_{\max} is the maximum specific rate of substrate utilization (h⁻¹).

Fig. 2 shows the substrate inhibition model which is able to describe the relationship between the specific growth rate and initial OPEO_n concentration. The model simulation curve in Fig. 2 is derived by Eq. (1). The time course of the growth curve and the corresponding OPEO_n disappearance are presented in Fig. 5. The model simulation curve for the bacterial growth and OPEO_n biodegradation in Fig. 5 are derived by Eqs. (2) and (3), respectively. This result indicates that a significant growth rate of *P. nitroreducens* TX1 can be obtained with OPEO_n as the sole carbon source at initial concentrations of 1000 and 10,000 mg l⁻¹ and the maximum OD₆₀₀ of the culture is achieved at 24 h with an OPEO_n removal of 70–90%. When the initial concentration of OPEO_n is below 100 mg l⁻¹, the OD₆₀₀ of the culture gradually decreases after 24 h due to insufficient carbon source. It is interesting to note that the *P. nitroreducens* TX1 is able to grow on OPEO_n even at concentrations as high as 100,000 mg l⁻¹.

In fact, it is difficult to compare the kinetic parameters of surfactant-degrading bacteria to other reported isolates due to the variety of structures of the surfactants used and

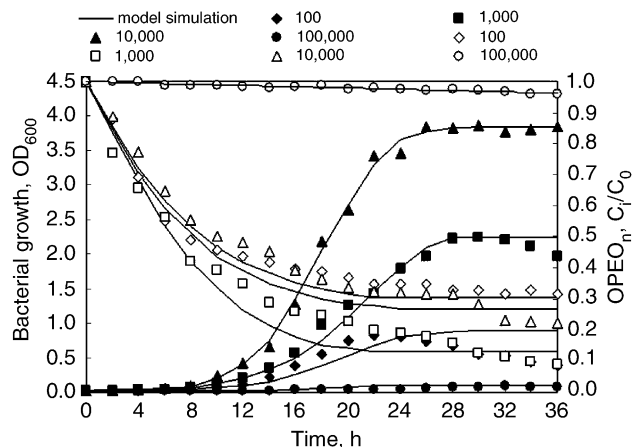


Fig. 5. The bacterial growth curves and the corresponding OPEO_n disappearance by *P. nitroreducens* TX1. The OPEO_n concentration is in mg l⁻¹; The filled symbols: the bacterial growth at the four tested concentration; The open symbols: the OPEO_n degradation profile.

the variation in laboratory conditions. However, since the kinetic analysis of bacteria using APEO_n as the sole carbon source has not been reported in previous studies, the result of the kinetic analysis of *P. nitroreducens* TX1 is compared to bacterial strains using other surfactants as the sole carbon source. As shown in Table 1, *P. nitroreducens* TX1 shows a higher specific growth rate (μ and μ_{\max}) with 5000 mg l⁻¹ of OPEO_n as the sole carbon source than the strain *Pseudomonas* C12B does with sodium dodecyl sulphate (SDS), activated sludge with SDS, linear primary alcohol ethoxylates (LPAE), and linear secondary alcohol ethoxylates (LSAE) in the range of 500–2500 mg l⁻¹. The K_s obtained with *P. nitroreducens* TX1 also indicates that there may be a higher affinity between the *P. nitroreducens* TX1 and OPEO_n molecules than between the activated sludge and LPAE as presented in Table 1. However, the degradation rate (shown by the coefficient, k) for almost all surfactants is in the same order of magnitude except for LSAE. Furthermore, this result also shows that the K_i of OPEO_n biodegradation is over 18,000 mg l⁻¹ by *P. nitroreducens* TX1.

Basically, OPEO_n is one kind of detergent and generally shows toxic inhibition of microorganisms. Since the previous results have demonstrated that the *P. nitroreducens* TX1 are able to grow on 100,000 mg l⁻¹ of OPEO_n, *P. nitroreducens* TX1 may have a novel mechanism to resist such a high concentration of OPEO_n and use it as the sole carbon source.

In addition, it should be noted that the μ of 0.53 h⁻¹ (12.72 d⁻¹) was obtained at 5000 mg l⁻¹ of OPEO_n with *P. nitroreducens* TX1. Since aerobic heterotrophic bacteria generally have a μ_{\max} in the range of 8.4–13.2 d⁻¹ (Rittmann and McCarty, 2001), this indicates that the specific growth rate of *P. nitroreducens* TX1 using OPEO_n as the sole carbon source is higher than for most aerobic heterotrophic bacteria.

Table 1
The comparison of kinetics parameters of surfactant-degrading bacteria on various surfactants

Strain	Surfactant	Conc. (mg l ⁻¹)	μ (h ⁻¹)	μ_{\max} (h ⁻¹)	K_s (mg l ⁻¹)	k_b^a	K_i	K^b (h ⁻¹)	Reference
<i>Pseudomonas nitroreducens</i> TX1	OPEO _n	5000	0.53	0.67	659	0.001	18,656	0.17	This study
<i>Pseudomonas</i> C12B	SDS ^c	20				0.022		0.19	Marchesi et al. (1997)
Activated sludge ^d	SDS	500	0.038						Zhang et al. (1999)
	SDS	2500	0.056	0.12	438	0.0002		0.18	
	LPAE ^e	500	0.08	0.10	2450	0.00004		0.24	
	LSAE ^f	500	0.07					1.03	

^a k_b : interaction coefficient, it is derived where $k_b = \mu_{\max}/K_s$.

^b k : OPEO_n degradation rate.

^cSDS = sodium dodecyl sulphate.

^dThe activated sludge obtained from central municipal wastewater treatment plant in Baton Rouge, LA, USA.

^eLPAE = linear primary alcohol ethoxylates.

^fLSAE = linear secondary alcohol ethoxylates.

3.5. The biodegradation mechanism of OPEO_n

The chemical structure of AEO₈ contains both an alkyl chain and a polyethoxylate chain and is similar to OPEO_n except for the lack of an aromatic ring (Fig. 1). *P. nitroreducens* TX1 showed an oxygen uptake rate (OUR) of 245 nmole min⁻¹ g⁻¹ using AEO₈ as a substrate compared to 528 nmole min⁻¹ g⁻¹ using OPEO_n, indicating that the bacterial enzyme system induced by OPEO_n can also work on AEO₈. Consequently, the mechanism of degradation of OPEO_n by *P. nitroreducens* TX1 as studied using AEO₈ due to its similar side chain structure and because AEO₈ is a single compound (John and White, 1998). Fig. 6 shows mass spectra of the transformation of AEO₈ by *P. nitroreducens* TX1 in a resting cell experiment. The relative intensity of AEO₈ apparently decreased as transformation time increased (12–48 h). At the same time, the distributions of more intense ion peaks gradually shifted to AEO₄, AEO₅ and AEO₆. In addition, the carboxylated metabolite, such as dodecyl octaethoxy acetic acid (AEC₈), was apparent at 48 h. AEC_n ($n = 5-8$) are the biodegradation metabolites from AEO_n. The time course of bacterial growth and the quantity of AEO₈ and its corresponding metabolites after transformation are shown in Fig. 7. The results indicate that the rate of bacterial growth apparently increased within the first 12 h (Fig. 7(A)). Moreover, the relative intensity of AEO₇, AEO₆ and AEO₅ also increased along with a corresponding decrease in AEO₈ (Fig. 7(B)). These metabolites gradually declined when AEO₈ had almost disappeared. Moreover, it is interesting to note that the carboxylated metabolites, AEC₈, AEC₇, AEC₆ and AEC₅, were not observed until 12 h, and then accumulated after this time (Fig. 7(C)).

John and White (1998) have also investigated the mechanism of biotransformation of 0.1% AEO₈ under aerobic conditions by the bacterium *P. putida*. They found that the metabolites AEO₇, AEO₆, and AEO₅ were generated after 90 min of transformation (John and White, 1998). This was similar to our study. Both bacterial strains demonstrated transformation of AEO₈ by sequential

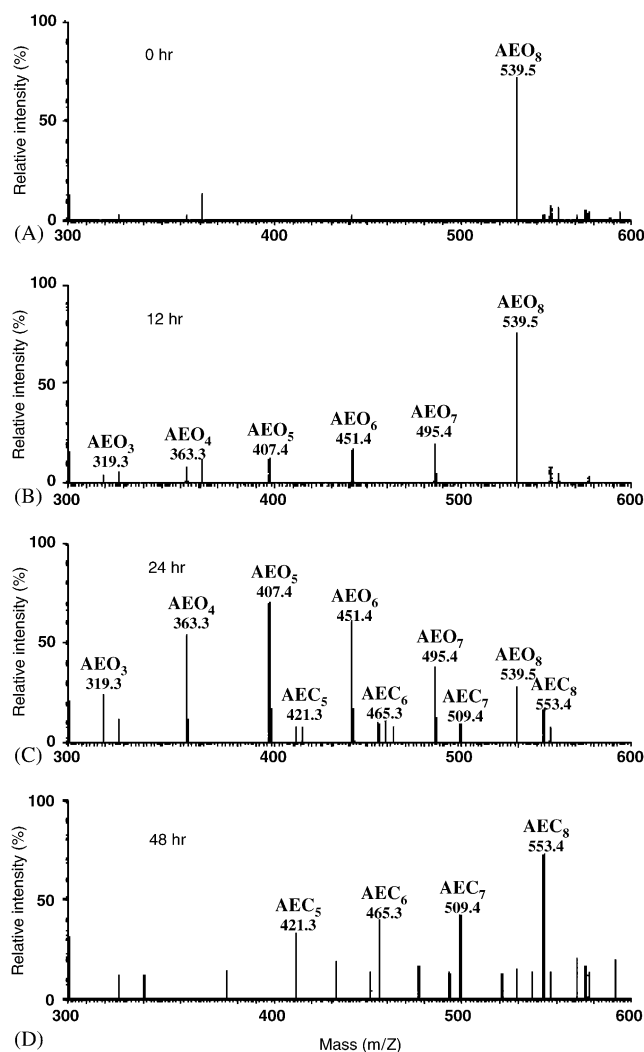


Fig. 6. The mass spectra of initial AEO₈ and its metabolites after transformation for 12, 24 and 48 h by *P. nitroreducens* TX1. AEC_n is the AEO_n with a terminal EO units carboxylated ($n = 5-8$).

cleavage of EO units, suggesting that in both cases the degradation of OPEO_n may use the same mechanism. In addition, several reports have shown that the carboxylated

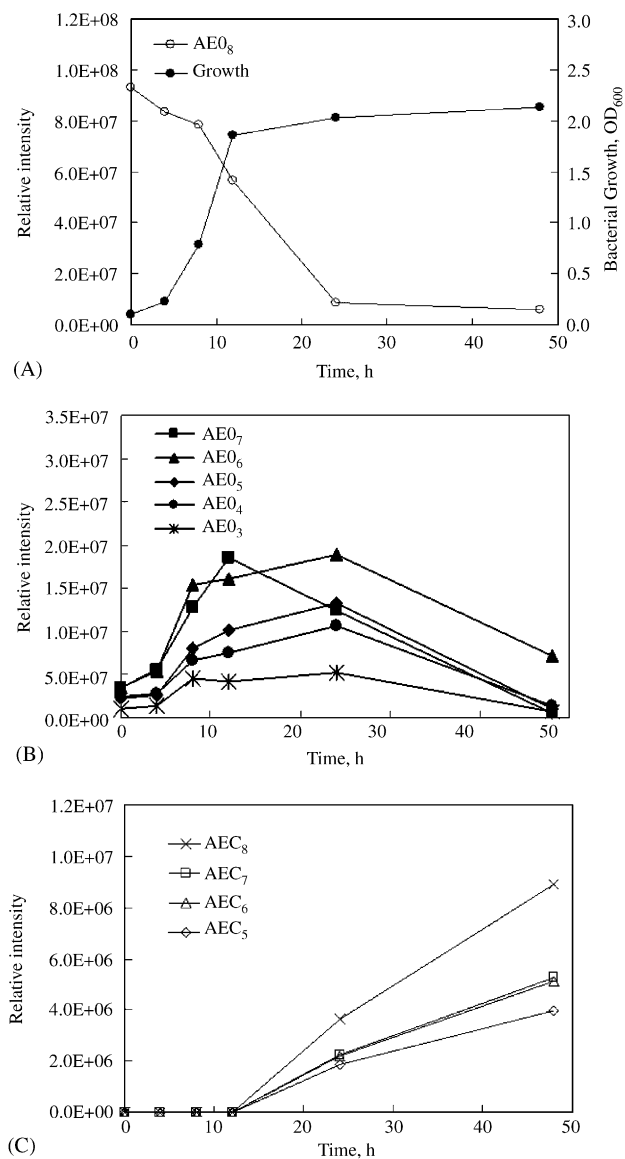


Fig. 7. The kinetics of relative intensity of AEO₈ and its biotransformation intermediates by LC-MS analysis: (A) AEO₈ and bacterial growth; (B) the metabolites after the cleavage of EO units; and (C) carboxylated metabolites.

intermediates, such as short-chain APEC_n, were usually observed to be accumulated in the process of biodegradation (Staples et al., 1999; John and White, 1998; Marcomini and Giger, 1987; DiCorcia et al., 2000). Thus, Sato et al. (2003) proposed a terminal oxidative model for the cleavage of the polyethoxylate chain under aerobic conditions. The terminal EO unit is first oxidized to generate a carboxylated OPEO_n. The carboxylated EO unit in the polyethoxylate chain is then cleaved along with the release of glyoxylic acid (Sato et al., 2003). Since carboxylated metabolites of AEO₈ were observed in our study, the biodegradation of OPEO_n by *P. nitroreducens* TX1 may also follow the terminal oxidative model. Furthermore, AEC_n ($n = 5-8$) were observed during the stationary phase, suggesting the accumulation of these carboxylated

metabolites resulting from low cleavage activity of the EO units in an aged culture. Additionally, the intermediates of OPEO_n biodegradation were also detected; the results show that the intermediates of OPEO₂ and OPEO₃ were apparently accumulated in the culture medium. However, the ultimate metabolites of OPEO_n biodegradation still need to be determined in a future study.

4. Conclusions

The isolate *P. nitroreducens* TX1 was demonstrated to be able to degrade OPEO_n in an MSB medium using OPEO_n as the sole carbon source (1–100,000 mg l⁻¹). The optimal specific growth rate was estimated to be 0.53 h⁻¹ and was observed at an initial concentration of 5000 mg l⁻¹ of OPEO_n. The C/N ratio is a significant factor for bacterial growth when using OPEO_n as the sole carbon source. *P. nitroreducens* TX1 showed a higher specific growth rate using (NH₄)₂SO₄ than KNO₃, while both nitrogen sources were able to give an optimal C/N ratio. Moreover, the optimal growth rate was observed at pH 7 over a range of pH 5–9. The kinetic analysis showed that *P. nitroreducens* TX1 has a substrate inhibition coefficient over 18,000 mg l⁻¹. This indicates that the *P. nitroreducens* TX1 has the ability to resist high concentrations of OPEO_n and has a high specific growth rate in comparison to most aerobic heterotrophic bacteria. The mechanism of biodegradation of OPEO_n in a resting cell experiment was demonstrated to be the cleavage of the polyethoxylate chain using AEO₈. The carboxylated metabolites of AEO₈, AEC₅₋₈, were observed to accumulate in the stationary phase. Consequently, the use of *P. nitroreducens* TX1 as an exogenous microorganism is able to potentially enhance the bioremediation of OPEO_n-contaminated soils.

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