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Characterization of TCE-Degrading Bacteria and Their Application to Wastewater Treatment

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Abstract Two bacterial strains capable of degrading trichloroethylene (TCE), isolated from soils contaminated with various chlorinated alkenes, were identified as *Alcaligenes odorans* N6 and *Nocardia* sp. H17. In addition, four KCTC strains, including three strains of *Pseudomonas putida* and one strain of *Sphingomonas chlorophenolica*, exhibited an ability to degrade toluene. *A. odorans* N6 and *Nocardia* sp. H17 degraded 84% of the initial amount of TCE in a basal salts medium (BSM), containing 0.2 mM TCE as the sole source of carbon and energy, in a day. The optimal pH for growth was within a range of 7.0–8.0. A mixed culture of the four toluene-degrading isolates degraded 95% of 0.2 mM TCE with 1.5 mM toluene as an inducer, whereas no TCE was degraded by the same mixture without an inducer. When a mixed culture of all 6 isolates was used, the degradation efficiency of 0.2 mM TCE was 72% without an inducer, in a day, and 82% with toluene as an inducer. In a continuous treatment, 1,000 mg/l of TCE in an artificial wastewater was completely removed within 18 h when an activated sludge was used along with the microbial mixture, which was 27 h faster than when only an activated sludge was used. Accordingly, it would appear that such a microbial mixture could be effectively applied to the biological treatment of wastewater containing TCE with or without an inducer.

Key words: *Alcaligenes odorans*, microbial mixture, *Nocardia* sp., trichloroethylene, wastewater treatment

Volatile chlorinated aliphatic hydrocarbons are a major concern as a potential health hazard in drinking water [18]. A broad cross-section of compounds are increasingly finding their way into the groundwater, contaminating aquifers and subsurface environments because of their common usage in various industries [12]. One of the most prevalent among these compounds is TCE, which is known to be one of the US EPA priority pollutants and a suspected mutagen [1].

TCE biodegradation has already been studied at various contaminated sites [6, 16]. Recently, it was reported that aerobic bacteria, such as *Nitrosomonas*, *Methylosinus*, and *Bukhholderia*, can actually degrade TCE [3, 13, 20]. These microorganisms catalyze the oxidation of carbon monooxide, methane, methanol, ethylene, propylene, and bromoethene. These compounds are substrates for other broad-specificity monooxygenases such as the methane monooxygenase. In these pathways, ammonia and methane function as electron donors [3, 12, 13]. Most TCE-degrading bacteria require an inducer, such as toluene or phenol, to degrade TCE. TCE degradation with toluene as the inducer is caused by two kinds of toluene-oxygenases [9, 13, 23]. *Burkholderia cepacia* G4 employs a novel toluene pathway using a toluene *ortho*-monooxygenase [13]. Toluene dioxygenase of *Pseudomonas putida* F1, as observed by Wackett and Gibson [22], can also degrade TCE in the presence of toluene. Recently, it was found that *Pseudomonas stutzeri* OX1 and the white rot fungus *Phanerochaete chrysosporium* can degrade PCE and TCE, respectively, without an inducer [15, 24]. However, despite the extensive research done on the biodegradation of TCE, much more information is still needed for the effective biological treatment of wastewater and groundwater containing TCE.

Accordingly, in the current study, we isolated aerobic bacteria with the capability of degrading TCE without an inducer and investigated their characteristics. Thereafter, a microbial mixture was created and tested with artificial wastewater containing TCE.

Materials and Methods

Chemicals and Media

TCE was obtained from Aldrich Chemical (Milwaukee, U.S.A.). For the degradation of TCE, the isolates were

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cultured on a Luria-Bertani (LB) medium or a basal salt medium [BSM: K_2HPO_4 (4.35 g), KH_2PO_4 (1.7 g), NH_4Cl (2.1 g), $MgSO_4$ (0.2 g), $MnSO_4 \cdot 7H_2O$ (0.05 g), $FeSO_4 \cdot 7H_2O$ (0.01 g), $CaCl_2 \cdot 2H_2O$ (0.03 g), distilled water (1,000 ml)] containing 0.2 mM TCE. The pH of the medium was adjusted to 7.0.

Isolation and Culture Conditions

Samples from a variety of soils and wastewaters in various areas of Taegu and Kumi that had a history of contamination with chlorinated alkenes were collected and screened for possible TCE degradation. Ten strains were initially isolated, and two strains that exhibited the best degradation of TCE were then selected. These two isolates, strain N6 and strain H17, were enriched and developed by adding soils and wastewaters to BSM containing TCE. The liquid portion of the enrichment was transferred to a fresh medium to obtain an aquifer material-free enrichment. After 1 week of incubation, the aquifer material-free enrichment was subcultured in a fresh BSM and subsequently diluted with the same medium. Further isolation and confirmation of purity were performed using BSM along with 2% agar. The well-isolated colonies on the agar plate were selected and restreaked to attain pure cultures of strains N6 and H17.

The isolates were incubated in the LB medium for 24 h. The culture was then centrifuged at 14,500 ×g and washed twice with a phosphate buffer. The diluted isolates were mixed with BSM to create a medium and isolate mixture. Aliquots (5 ml) of the mixture were then dispensed into 60-ml headspace vials. The vials were sealed with teflon-faced neoprene rubber septa (Wheaton, Millville, NJ, U.S.A.) secured with aluminum hole caps to allow access by a syringe. Using a syringe through the septum of each vial, TCE was added as an aqueous stock to give a final concentration of 0.2 mM. The vials were placed on a shaker (120 rpm) for 3 days at 30°C.

Identification

Various physiological and biological tests on isolates N6 and H17 were performed using the API 20E system (BioMerieux), based on Bergey's Manual of Systematic Bacteriology [8] and Manual for the Identification of Medical Bacteria [2]. The color of the strains changed by their metabolites and reagent kit for incubation. The result was discriminated by the color based on APILAB Plus (BioMérieux, France), a software for identification. The cell morphology was confirmed using scanning electron microscope (Philips 515, Eindhoven, The Netherlands).

Analytical Methods

The TCE degradation was quantitatively investigated based on cell growth and the reduction of TCE using a spectrophotometer (Shimadzu UV-160, Kyoto, Japan) at A_{600} and gas chromatography (Varian 3400 CX, Wallnut creek, CA, U.S.A.). The samples were injected with a 250-μl gas-tight syringe into a gas chromatograph equipped with a flame ionization detector and Supelco (Supelco Dark, Bellefonte, P.A., U.S.A.) SPB-5 on a 30 m×250 μm capillary column (column temperature, 70°C; injector temperature, 150°C; detector temperature, 250°C; 30 ml/min; N_2 as the carrier gas). The soluble total organic compound (TOC) was analyzed using a TOC analyzer (Shimadzu 5000A, Kyoto, Japan). All measurements were carried out in triplicate.

Manufacture of Microbial Mixture

The microbial mixture was manufactured using the culture solution and a carrier, defatted rice bran in this case. A microbial culture was prepared for strain N6, strain H17, *Sphingomonas chlorophenolica*, and three strains of *Pseudomonas putida*. The microbial cultures were sprayed onto and adsorbed by the rice bran, and then mixed using a ribbon impeller (d, 100 mm). The mixtures were then

Table 1. Characterization of *Alcaligenes odorans* N6 and *Nocardia* sp. H17.

Test	*Alcaligenes odorans* N6	*Nocardia* sp. H17
Gram/Shape	-/R	+/R
Spores	-	-
Motility	+	+
Growth in air	+	+
Growth anaerobically	-	-
Catalase	+	+
Oxidase	+	-
Glucose (acid)	-	-
O/F/-	-	-
Pigmentation	-	
KCN (growth on)	+	-
Citrate as C source	+	+
Carbohydrates (in peptone media), acid		
Glucose	-	-
Lactose	-	-
Sucrose	-	-
Xylose	-	-
Carbohydrates, acid from:		
Glucose	-	-
Lactose	-	-
Maltose	-	-
Xylose	-	-
Aesculin hydrolysis	-	+
Nitrate hydrolysis	+	+
Indole	d	-
Gelatin liquefactin	-	-
Urease	-	+

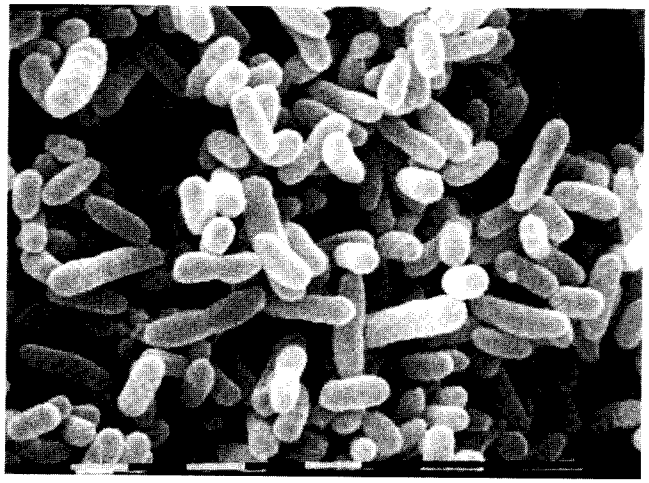
R, Rod shape; d, different reaction in different strains.

incubated for 2 days and dried at room temperature. The cell number in the microbial mixtures was expressed as the colony forming units (CFU) per g dry weight.

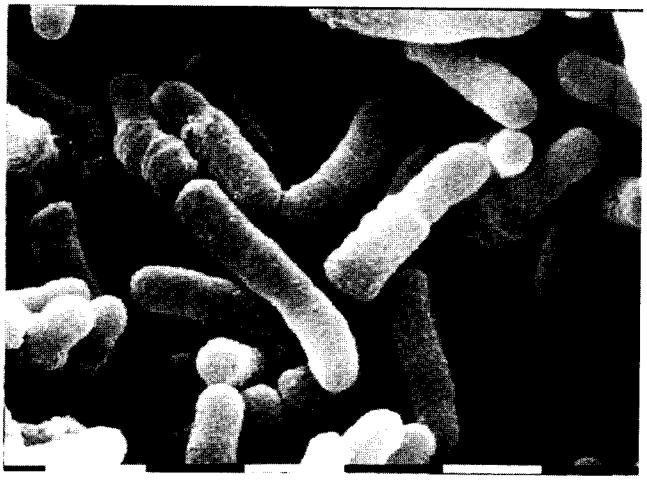
RESULTS AND DISCUSSION

Characterization of TCE-Degrading Bacteria

Ten bacterial strains were isolated from soils contaminated with chlorinated alkenes and wastewaters containing TCE. Two isolates, the strains N6 and H17, were finally selected based on their ability to degrade TCE. Their physiological and biological characteristics were then investigated (Table 1). The strain N6 was found to be a Gram-negative aerobic, rod-shaped bacterium (Fig. 1a) with motility. It was also catalase- and oxidase-positive, but urease-negative. In addition,



(a)



(b)

Fig. 1. Scanning electron micrographs of *Alcaligenes odorans* N6 (a) and *Nocardia* sp. H17 (b) after 48 h of incubation in Luria-Bertani broth.
The lengths of the white and black bars are 1 μm.

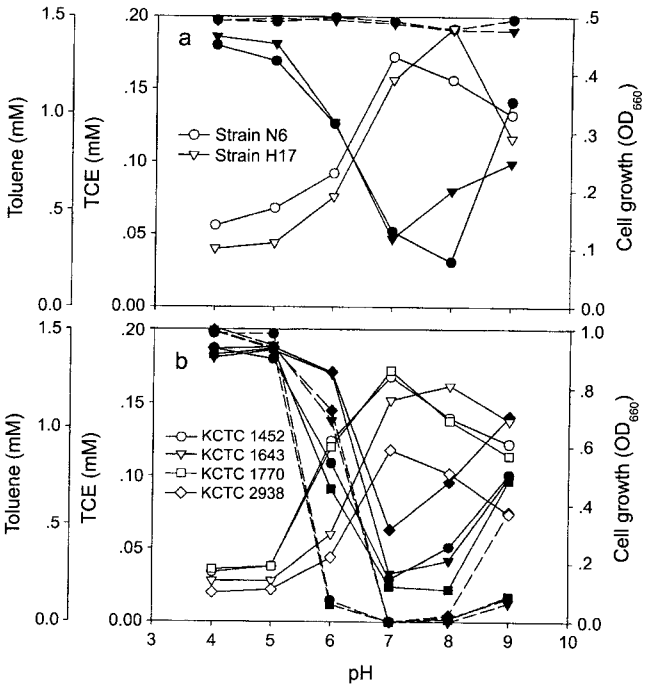


Fig. 2. Effect of initial pH on the degradation of TCE and toluene by the isolates (a) and toluene-degrading bacteria (b). Closed symbols are TCE (solid line) and toluene (dotted line). Open symbols represent cell growth.

it did not utilize glucose, sucrose, or lactose as carbon sources. The characteristics of the strain N6 were found to be very similar to those of the standard species, *Alcaligenes odorans*. As such, the strain N6 was designated as *Alcaligenes odorans* N6. The strain H17 was found to be a Gram-positive aerobic, rod-shaped bacterium (Fig. 1b) with motility. It was also catalase- and urease-positive, but did not utilize glucose, sucrose, or lactose as carbon sources. Since these characteristics were similar to those of the *Nocardia* sp., the strain H17 was named *Nocardia* sp. H17.

The optimal pH for the degradation of TCE and toluene was investigated after incubation with the bacterial strains in a medium containing 0.2 mM TCE. The degradation of TCE by *A. odorans* N6 was the highest at pH 8, which also exhibited the highest cell growth (Fig. 2a). In the case of *Nocardia* sp. H17, the optimum pH for TCE degradation and cell growth was also 8. The cell growth of *A. odorans* N6 and *Nocardia* sp. H17 was significantly reduced under acidic conditions below pH 5. These two strains were also unable to degrade toluene, which was added as an inducer. The three strains of *Pseudomonas putida* (KCTC 1452, 1643, and 1770) and one strain of *Sphingomonas chlorophenolica* (KCTC 2938) were able to degrade both TCE and toluene (Fig. 2b). The maximum pH for the degradation and cell growth of these strains was 7 or 8. In regards to the cell growth of the inoculated strains, there was a significant decrease of both TCE and toluene.

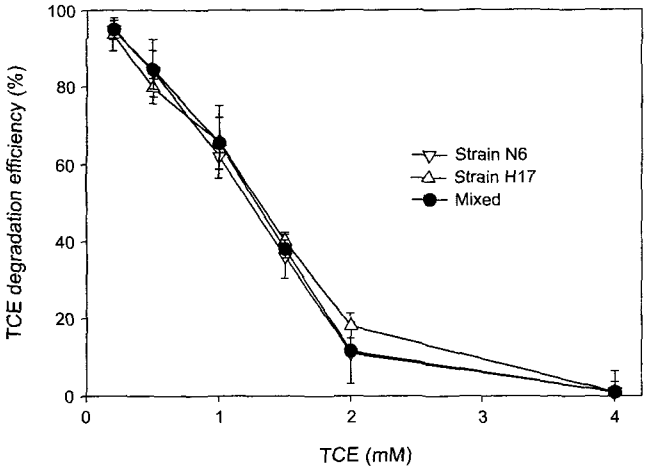


Fig. 3. TCE degradation efficiency with various concentrations of strain N6, strain H17, and mixed culture.

Under batch culture conditions, *A. odorans* N6 and *Nocardia* sp. H17 were cultured separately and also as a mixture, along with various concentrations of TCE, such as 0.2, 0.5, 1.0, 1.5, 2.0, and 4.0 mM (Fig. 3). There was no difference in the TCE degradation efficiency among the various treatments. The degradation efficiency was about 93% with 0.2 mM TCE and then dramatically decreased when the TCE concentration was increased up to 2.0 mM. No degradation was observed with a 4.0 mM TCE concentration. According to a previous report [17], decreased degradation efficiencies at high concentrations was due to the toxicity of TCE.

Numerous research efforts have been focused on the biodegradation of TCE. For example, *Alcaligenes* sp. strain JMP134 [5] can grow in a TCE concentration of 3 mg/l (equivalent to 0.023 mM TCE). Miura and Dalton [11] investigated the degradation of alkene by *Nocardia corallina* B-276. *Pseudomonas cepacia* G4 [4] and *Xanthobacter* sp. Py2 [10] can degrade TCE at a concentration of up to 0.17 mM and 0.1 mM, respectively. However, in the current study, we found that *A. odorans* N6 and *Nocardia* sp. H17 could degrade TCE at concentrations of up to 2.0 mM, which is much higher than any other previous reports.

Degradation of TCE by Microbial Mixture

The two isolates (*A. odorans* N6 and *Nocardia* sp. H17) and four toluene-degrading strains (KCTC 1452, 1643, 1770, and 2938) were mixed, and the mixture was incubated with TCE. Mixture A was composed of *A. odorans* N6 and *Nocardia* sp. H17, while Mixture B was composed of the four toluene-degrading strains. The degradation of 0.2 mM TCE by each strain individually or by Mixture A and Mixture B was analyzed every 3 h over a 24-h period. Mixture A was found to be very effective in degrading TCE without the use of toluene (Fig. 4a), whereas mixture B showed a rapid degradation of TCE when 1.5 mM toluene was included (Fig. 4b). A mixture of A+B exhibited a stable degradation capability with or without toluene as an inducer. Toluene was degraded concomitantly with TCE by the microbial mixture including mixture B, as it was capable of degrading toluene (Fig. 4c).

Mixture A was unable to degrade any toluene, but mixture B and a mixture of A+B both degraded all toluene within 24 h (Table 2). Mixture A degraded 72% of the TCE without toluene and 67% of the TCE with toluene, thus indicating that addition of toluene as an inducer did not affect the degradation efficiency of TCE by mixture A. In contrast, mixture B exhibited the highest degradation efficiency of TCE (95%) with toluene, yet was unable to degrade any TCE without toluene. As such, the use of toluene as an inducer was indispensable for the degradation of TCE by mixture B. The mixture of A+B degraded 84% of the TCE with toluene and 61% without toluene. Therefore, the use of mixture A+B exhibited a stable degradation of

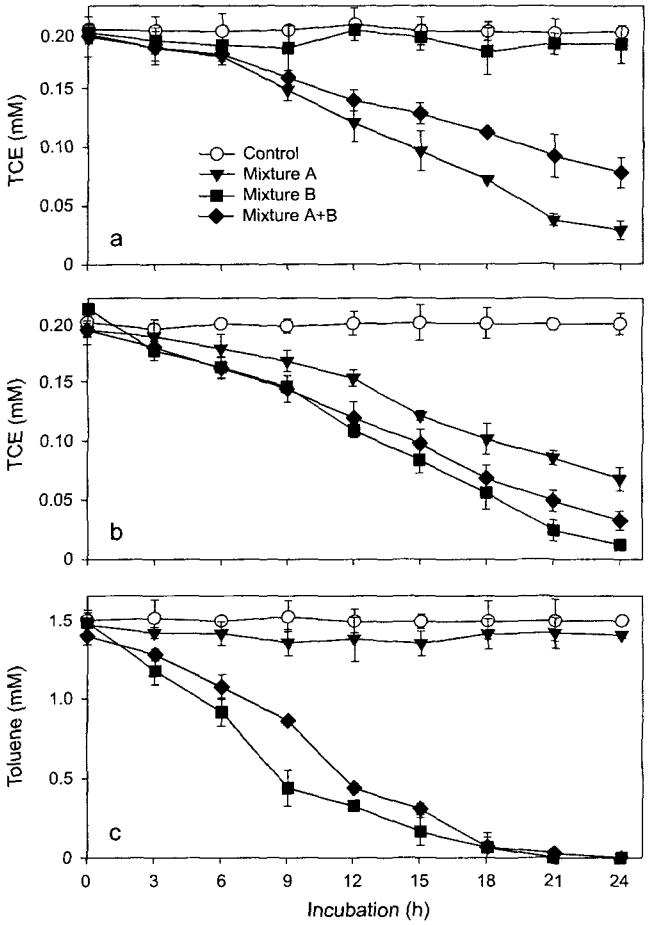


Fig. 4. Degradation of TCE by mixed culture without (a) or with (b) toluene and degradation of toluene (c) over 24 h.
Control, no added microorganism; Mixture A, strains N6 and H17; Mixture B, toluene-degrading bacteria (KCTC 1452, 1643, 1770, and 2938).

Table 2. Degradation efficiency of TCE and toluene by microbial mixtures, with or without inducer, after 1-day incubation.

Culture	Degradation efficiency (%)		
	Without inducer	With inducer	
	TCE	TCE	Toluene
Control	0	1	2
Mixture A*	72	67	6
Mixture B**	6	95	100
Mixture A+B	61	84	100

*Mixture A is composed of strains N6 and H17.
**Mixture B is composed of KCTC 1452, 1643, 1770, and 2938.

TCE with or without toluene as an inducer, and was also capable of degrading toluene. Consequently, it would seem that the microbial mixture of A+B could be effectively applied to the biological treatment of TCE-containing wastewater with or without the use of an inducer.

According to previous studies [18, 19], *P. putida* and *S. chlorophenolica* can degrade TCE through toluene 2-monooxygenase, which transforms TCE into TCE epoxide, or using toluene dioxygenase, which transforms TCE into glyoxylate and formate [23]. However, *A. odorans* N6 and *Nocardia* sp. H17 did not degrade through toluene 2-monooxygenase, therefore, TCE degradation by *A. odorans* N6 and *Nocardia* sp. H17 did not require the toluene as an inducer. Recently, Ryoo *et al.* [15] reported that certain waste sites may be remediated by adding a bacterium, since chlorinated alkene induces its own degradation. Therefore, it appears that *A. odorans* N6 and *Nocardia* sp. H17 contain an enzyme that either has a different specificity or requires other chemicals rather than toluene as an inducer for the degradation of TCE.

Treatment of Wastewater Containing TCE

Six kinds of microbial cultures were created using strains capable of degrading TCE. The cell numbers of the microbial cultures were within a range of 2.3×10^7–1.6×10^9 CFU/g (Table 3). The mixture, composed of *A. odorans* N6, *Nocardia* sp. H17, and four toluene-degrading bacteria, had a cell number of 6.2×10^8 CFU/g. The bulk density, water content, and particle size of the mixture were 0.301, 27%, and 50–100 mesh, respectively, which were not much different from those of each microbial culture. This mixture was then used as the inoculum in a pilot-scale experiment for the treatment of TCE.

Table 3. Characteristics of individual and mixed microbial cultures.

Strains	Total viable count (CFU/g)	Bulk density	Water content (%)	Particle size (mesh)
Strain N6	2.6×10^8	0.306	29	50–100
Strain H17	4.1×10^8	0.310	27	50–100
KCTC 1452*	1.6×10^9	0.302	27	50–100
KCTC 1643*	3.1×10^8	0.298	29	50–100
KCTC 1770*	1.5×10^9	0.303	29	50–100
KCTC 2938**	2.3×10^7	0.300	27	50–100
Mixture	6.2×10^8	0.301	27	50–100

*KCTC 1452, 1643, and 1770 are *Pseudomonas putida.*
**KCTC 2938 is *Sphingomonas chlorophenolica.*

The pilot-scale experiment was carried out with an artificial wastewater containing TCE, using a continuous culture in a reactor, as previously reported by Oh *et al.* [14]. The artificial wastewater included several organic compounds, as indicated in JSWA [7], and dry cleaning wastewater taken from a local laundry. The TOC in the wastewater was about 10,000 mg/l. The operating conditions of the reactor were as follows: DO controlled by an air blower, 2–3 mg/l; hydraulic retention time (HRT), 24 h; temperature, 25±2°C. Activated sludge from a pharmaceutical company was added to the reactor and the concentration of the mixed liquor suspended solids (MLSS) was maintained at 2,000–3,000 mg/l by recycling the sludge in a settling tank.

The control did not include any activated sludge or the microbial mixture. After 18 h of incubation, the TCE concentrations in the control, sludge, and sludge+isolates

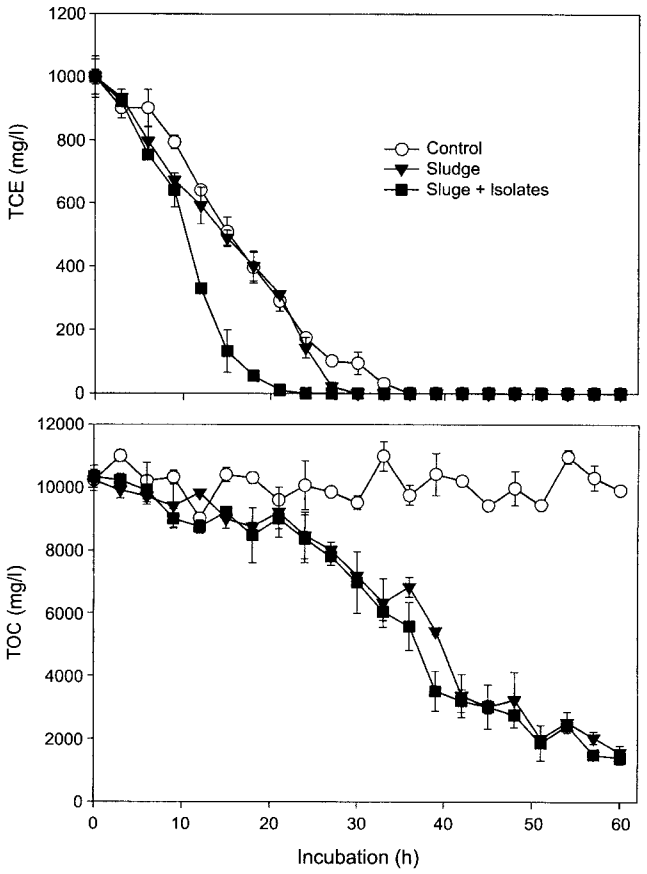


Fig. 5. Removal of TCE and TOC by activated sludge and microbial mixture in a continuous culture for 60 h.

Table 4. Removal efficiencies of TCE and TOC by microbial mixture in wastewater.

Incubation (h)	Removal efficiency of TCE (%)			Removal efficiency of TOC (%)		
	C*	S**	S+M***	C	S	S+M
9	30	33	36	0	8	13
18	60	60	95	0	14	18
27	90	98	100	4	22	25
36	100	100	100	5	33	46
45	100	100	100	8	71	71

*C, influent.
**S, only activated sludge added to influent, as control.
***S+M, activated sludge and microbial mixture added to influent.

were 396, 401, and 54 mg/l, respectively. The TOC concentrations in the control, sludge, and sludge+isolates were 9,924, 1,569, and 1,402 mg/l, respectively, after 60 h of incubation (Fig. 5), thus, indicating that the TCE concentration was not dependent on the addition of the activated sludge, but rather on the addition of the isolates. In contrast, the TOC concentration was found to be dependent on the addition of the activated sludge, and not on the addition of the isolates.

Due to its volatility, TCE was 100% removed by aeration after 36 h. When the isolates were added to the activated sludge, the removal efficiency of TCE was as high as 95% after 18 h of incubation, whereas the removal efficiency of TCE by the activated sludge without the isolates was only 60%. However, the addition of the isolate consortia did not affect the removal efficiency of TOC (Table 4).

In a continuous culture, the degradation of TCE by the microbial mixture was investigated using a pilot reactor designed on the basis of an activated sludge system. Again, due to its volatility, most of the TCE was removed very rapidly. The addition of the microbial mixture to the activated sludge also shortened the TCE removal time, yet did not affect the TOC removal.

According to previous studies, *Xanthobacter* sp. Strain Py2 cells are known to degrade TCE in a continuous culture [10]. At the highest TCE concentration tested (390 μM, equivalent to 51 mg/l TCE), 30% of TCE was converted into epoxide in a continuous culture, when incubated in a medium containing TCE in a 1.5-liter fermentor. Brar and Gupta [1] reported that 97% of 30 mg/l TCE was removed in a RBC (rotating biological contactor) containing *Thiosphaera pantotropha*, while Sponza [21] reported that TCE was degraded with TCE-degrading granules when a UASB (upflow anaerobic sludge blanket) was used. However, the TCE-degrading granules in this case required 230 days of continuous operation to remove the TCE. In contrast, the microbial mixture used in the current study containing *A. odorans* N6 and *Nocardia* sp. H17 rapidly removed a high concentration of TCE (1,000 mg/l). In addition, the current test was the first time that both a microbial mixture and activated sludge system were used to degrade TCE in wastewater.

In conclusion, *A. odorans* N6 and *Nocardia* sp. H17 were shown to degrade a high concentration of TCE even without the use of toluene as an inducer. Therefore, such a microbial mixture could be effectively applied to the removal of TCE in wastewater.

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