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TETRACHLOROETHYLENE-DECREASING BY BIOLOGICAL SUBSTANCE(S) FROM ACTIVATED SLUDGE

ROZKŁAD TETRACHLOROETYLENU SUBSTANCJAMI BIOLOGICZNYMI OSADU CZYNNEGO

Tetrachloroethylene (PCE) is a volatile organic carbon and is used in various industries such as dry-cleaning or electronics. Around the world, PCE leaked from factories has been found in subsurface (soil and groundwater) close to industries. We collected activated sludge (MLVSS was adjusted to 10k mg/L) from wastewater treatment of an electronic manufacturer where aliphatic chlorinated compounds are treated daily and acclimatized to 1000 – 2000 mg/L of PCE every week by direct addition. PCE in liquid phase was 0.7 mg in acclimatized activated sludge and 0.9 mg in autoclaved activated sludge. 0.2 mg of PCE in liquid phase was decreased by acclimatized activated sludge within 24 hours. Interestingly, 0.45 - 0.61 mg of PCE was decreased by the filtrate of activated sludge within 24 hours. The filtrate used to remove flocks or bacteria was glass fiber filter GS-25 and membrane filter (1 μm and 0.2 μm pore size, respectively). The cell-free filtrate was molecular weight cut-offs (MWCO) by centrifuge type of ultrafiltration membrane. PCE was decreased by less than 10,000, 5,000 and 3,000 MWCO fractions. In addition, the biological substance(s) was measured by Qubit system and bicinchoninic acid (BCA) method as proteinous substance(s). These results suggested low (less than 3,000) molecular weight like peptide was related to PCE-decreasing. A part of PCE-decreasing mechanisms was elucidated by X-ray absorption fine structure (XAFS) analysis with synchrotron focusing state of bond of chloride-element and other elements. We found that the covalent bonds of chloride and carbon (C-Cl) in PCE were craved and chloride separated from carbon was stayed as ionic binding after reaction of PCE and biological subsctance(s). This result indicated that PCE was degraded by biological substance(s) from activated sludge.

Chlorinated ethylene like PCE has been used in various industrial processes such as dry-cleaning and cleaning of E-Infrastructure. Recently, many contaminated sites are being found in Japan every year because of enactment of the soil environmental quality standards and Soil Contamination Countermeasures Act. One of the treatment of pollutant is through bioremediation, and has a low environmental burden. Biostimulation is a general method of remediation in Japan. Bioaugmentation has been hardly applied but, bioaug-

mentation is a very popular remediation approach in Germany and the US. Most methods of bioaugmentation for PCE rehabilitation have been used in anaerobic reaction, and aerobic reaction has not yet been used. The report of aerobic degradation of PCE by Pseudomonas sturzeri OX1 is the only one to date [1, 2, 3]. Pseudomonas sturzeri OX1 degrades PCE by toluene o-xylene monooxygenase as cometabolism. Microorganisms capable of using PCE as energy and sole carbon source have not been found yet. The purpose of this research is to degrade high concentration of PCE in aerobic condition and elucidate the mechanisms.

1. Materials and Methods

1.1. Acclimatizing to PCE

Activated sludge were collected from a wastewater treatment plant receiving electronic components and put it into a round-bottom flask attached to a Liebig condenser above and aerated by pump to keep the aerobic condition. PCE (Wako, special grade, as a carbon source), ethanol (solubilized agent of PCE), and ammonium phosphate dibasic [$(NH_4)_2HPO_4$], as P and N source were added to the flask. PCE solution was added to a final concentration of 2,000 - 10,000 mg/L every one week or two weeks interval. Also, ammonium phosphate dibasic was supplied to the same flask to a final concentration of 5 mg/L once a week. Furthermore, we added a little amount of yeast extract (Difco, for cell culture) as sources of minerals or vitamins. After long term acclimatization to PCE, we determined the degradation of PCE.

1.2. PCE-decreasing assay

PCE degradation was examined using 100 ml sealed vials. 20ml of activated sludge was added to the vials, followed by supplementation of PCE solution to a final concentration of 200 mg/L. Autoclaved (121 ˚C, 30 min) activated sludge was used as a control. The vials were tightly closed with Teflon-coated butyl rubber and aluminum cap, and incubated at 20 ˚C, 120 rpm for 24 hr. PCE concentration in each vial was analyzed by GC-MS (SHIMADU, GCMS-QP2010 plus, Perkin Elmer, Headspace Sampler Turbo Matrix 40).

1.3. Filtration

Filtration of the acclimatized activated sludge was performed using a GS-25 glass fiber filter (ADVANTEC, Tokyo, Japan) followed by a mixed-cellulose-ester membrane filter of 0.2 μm-pore size (ADVANTEC, Tokyo, Japan) to remove flocks and microorganisms. These filtrates (20 ml) were used to test for the PCE-decreasing activity as described above.

1.4. Protein quantification in membrane filtrate

Protein concentration of the filtrates was measured with Quant-iT Protein assay kit and Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. Non contaminated water (Milli-Q; Mil-lipore GmbH, Schwalbach, Germany) was used as a negative control.

1.5. Molecular weight cut-offs (MWCO)

The filtrates obtained from the membranes were ultrafiltered by using Vivaspin 20 filters (GE healthcare, Little Chalfont, Buckinghamshire, UK) with different sizes of MWCO; 10,000 cut-off was initially applied followed by 5,000 and 3,000 cut-offs. Respective fractions of each MWCO steps were mixed with PCE to examine the PCE-decreasing activity.

1.6. Mechanisms of the PCE-decreasing activity

To elucidate the mechanisms involved in the decrease of PCE, we focused on the C-Cl bonds. Atomic chlorides in PCE were analyzed by X-ray absorption fine structure (XAFS) with synchrotron radiation (Aichi Synchrotron Radiation Center, Aichi Japan). Spectrum of chloride was changed depending on neighbor elements.

2. Results and Discussion

2.1. Acclimatization to PCE and PCE-decreasing assay

Activated sludge collected from waste water treatment plant (PCE-free waste water) were acclimatized with PCE in aerobic condition and maintained for four years. Concentration of PCE in the culture was monitored periodically to confirm the decrease of PCE. PCE was added into activated sludge at a final concentration of 1000 mg/l. We added 800 mg of PCE to 800 ml of activated sludge after acclimatization and PCE concentration in liquid phase was analyzed by head-space GCMS continually for 7 days. PCE was apparently decreased to below 1 mg/l at 24 hr and below 0.03 mg/l at 72 hr (data not shown). To exclude the occurrence of partial evaporation of PCE since activated sludge was constantly aerated in the acclimatization system with Liebig condenser installed, a closed system (batch style) using Teflon-coated septum, aluminum cap and glass vial was used. An aliquot of acclimatized culture was taken from the flask and assayed for PCE-decreasing.

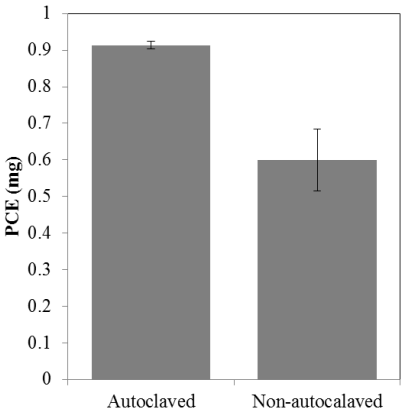

Fig. 1. PCE-decreasing activity in activated sludge

With activated sludge (non-autoclaved), remaining PCE was 0.65 mg whereas autoclaved activated sludge was 0.92 mg. The decrease of PCE observed was 0.27 mg (FIGURE 1), indicating biotic reaction might have been involved in the decrease of PCE.

2.2. PCE-decreasing by filtration of activated sludge

First step of the filtration was performed by using GS-25 glass-fiber-filter (pore size=1μm) to remove flocks, and second step was carried out by using membrane filter (pore size=0.2 μm) to remove bacteria. TABLE 1 shows PCE-decreasing activity by the two filtrates of activated sludge. For the negative control, remaining PCE was 0.88 mg. On the other hand, remaining PCE in the filtrate from GS-25 filtrate was 0.43 mg, whereas for the membrane filtrate was 0.27 mg. The difference between negative control and GS-25 filtrate was 0.45 mg and 0.61 mg for membrane filtrate. PCE-decreasing ratios were 54% and 74%, respectively. These results implied that some sort of biological substances(s) below 0.2 μm size have PCE-decreasing activity.

Tab. 1. PCE-decreasing activity in each filtrates

Filter	PCE-decreasing[a] mg	Decreasing ratio[b] %
GS-25	0.45±0.10	54
Membrane	0.61±0.03	74

2.3. Influence of protein in membrane filtrate on PCE-decreasing

We attempt to elucidate the relationship between amount of protein and the change in amount of PCE as shown in TABLE 2. Protein amount was 5.9 mg for non-concentrated filtrate, and 23.7 mg in concentrated filtrate. PCE amount for non-concentrated filtrate was 0.15 mg, and 0.68 mg for concentrated filtrate. The amount of protein was 4 times and PCE-decreasing activity was increased 4.5 times after concentrated. This result indicates that protein from membrane filtrate was responsible for PCE-decreasing activity because the amount of PCE decreased was in proportion to the amount of protein.

Tab. 2. Relationship between proteinous in membrane and PCE-decreasing activity

	PCE concn. (mg/l)	Protein concn.(mg/l)	PCE-decreasing (mg)-A	Protein (mg)-B	Specific activity (A/B)
Non-concentrated	36.1 ± 2.8	295	0.15	5.9	0.02
1% NaCl in water	44.8 ± 2.2	-	-	-	-
Concentrated	18.1 ± 2.2	1185	0.68	23.7	0.02
5% NaCl in water	43.6 ± 2.9	-	-	-	-

2.4. PCE decreased by molecular weight cut-off fractions

TABLE 3 showed the decrease in PCE concentration by each MWCO fractions. PCE-decreasing activity was found for each MWCO fractions and all of activities of MWCO fractions were higher than that of membrane filtrate. Less than 3,000 MWCO fraction decreased PCE concentration to 1.6 times as much as ≥10,000 MWCO fraction did. These results indicate that organic matter(s) with a lower MW (<3,000) was involved in the decrease of PCE concentration. We confirmed that PCE-decreasing activity also correlated with the protein. The specific activity of less than 3,000 MWCO fraction was 3.25 times as much as that of membrane filtrate. We assumed that either organic matter(s) unrelated to the decrease of PCE were removed by ultrafiltration or inhibitory substances against PCE treatment were excluded. These results indicated that peptide-like substance(s) might be involved in the decrease of PCE, and it was also supported by UV spectrum analysis. The ultrafiltrate (<3,000 MWCO) was found to have absorbance at 200 to 230 nm, which is general absorbance of peptide (data not shown).

Tab. 3. PCE-decreasing activity in each MWCO fractions

Fraction (MWCO)	PCE-decreasing (mg) – A	Protein (mg) – B	Specific activity (A/B)	Specific activity yield
Membrane filtrate	0.22±0.06	5.08±0.40	0.04	1.00
≥10,000	0.09±0.03	1.11±0.11	0.08	2.00
<10,000	0.20±0.01	2.76±0.25	0.07	1.75
5,000 – 10,000	0.09±0.03	0.66±0.12	0.14	3.50
<5,000	0.38±0.02	3.12±0.23	0.12	3.00
3,000 – 5,000	0.10±0.02	0.72±0.05	0.14	3.50
<3,000	0.37±0.04	2.86±0.10	0.13	3.25

[a] Each MWCO was fractionated by ultrafiltration

2.5. Mechanism of PCE degradation

FIGURE 2 showed the spectrum of atomic chloride from PCE as analyzed by XAFS with synchrotron radiation. When the Spectrums (A) PCE only and (B) mixture of PCE and membrane filtrate were compared, the spectrum was shifted to high energy. Spectrum mixture of PCE and membrane filtrate was not similar to (C) covalent bond, while (D) ionic bond matched. These results suggested that C-Cl bonds were calved and chloride from PCE existed in ionic state.

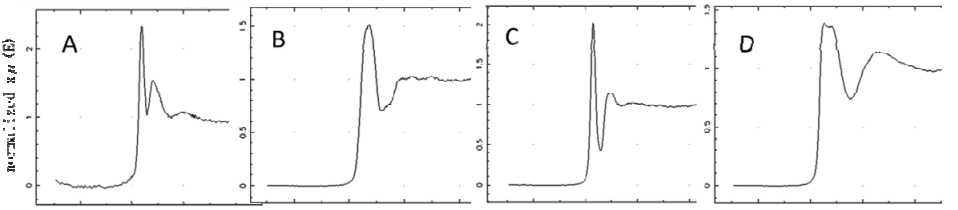

Fig 2. Spectrum of each chloride bond by EXAFS analysis after normalization A: PCE only, B: PCE and membrane filtrate, C: trichloroacetic acid (covalent bond), D: sodium chloride (ionic bond)

3. Conclusion

PCE-decreasing activity was successfully observed and the mechanisms of PCE-decreasing phenomenon from activated sludge after acclimatization to PCE, was also successfully elucidated. C-Cl bonds in PCE were cleaved and chlorides existed in ionic state. PCE degradation was caused by bacterial product(s) from activated sludge and the product(s) was less than 3,000 molecular weight and peptide-like compound. In the near future, we hope to determine the composition and structure of the products.

Reference

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