BIOREMEDIATION OF GROUNDWATER POLLUTED WITH CHLORINATED ETHYLENES

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ABSTRACT

Chlorinated solvent is one the most significant categories of hazardous groundwater contaminants in Japan. Bioremediation is considered as a most promising clean-up technique for the solvent, although this technology is still in a developing stage. Microbial degradation of a most important chlorinated solvent, trichloroethylene (TCE), by phenol-dgerading bacteria was studied for evaluating the feasibility of the groundwater bioremediation. Enumeration and physiological characterization of phenol-degrading bacteria in soil samples suggested the common and abundant presence of various types of phenol-degrading bacteria in the natural environment. Among 14 different phenol-degrading bacteria tested, all with only one exception showed TCE-degrading capability, when grown on phenol. Although the general trend was observed that bacterial strains with higher phenol-degrading activity showed higher initial TCE degradation rate, the extent of TCE degradation (ultinate removal) had no relationship with phenol-degrading activity. These results suggested that the biostimulation of indigenous phenol-degrading bacteria is possible, however, that their TCE-degrading properties, i.e. activity and stability, should be taken into consideration for achieving effective bioremediation.

KEY WORDS

Bioremediation, biostimulation, bioaugmentation, chlorinated solvents, phenol-degrading bacteria, trichloroethylene

INTRODUCTION

Chlorinated solvent is one of the most significant categories of hazardous soil/groundwater contaminants which is known to persist long in natural environments and to be toxic or carcinogenic for living things. Typical ones of this category include chlorinated ethylenes like trichloroethylene (TCE) and tetrachloroethylene (PCE). Local government of Japan surveyed on the groundwater quality from fiscal 1984 to 1988, resulting in the finding that the quality of 7.7 and 4.1 % of the samples collected from approximately 27,000 wells exceeded the standard values concerning TCE (0.03 mg/l) and PCE (0.01 mg/l), respectively (Environmental Agency Japan, 1996). Thus, it is of urgent necessity to remove such chlorinated ethylenes from polluted groundwater in order to protect our water supplies from the contamination.

Generally chlorinated ethylenes in aquifers are not fully mobilized by flowing water, physico-chemical treatment such as pump-and-treat strategy is not efficient. Even when the solvent can be pumped out, air stripping or sorption treatment can merely transfer it from water phase to atmosphere or sorbents. The catalytic oxidation treatment has a problem of high costs. Another physico-chemical alternative, digging up of the contaminated soils and their disposal, is practically impossible to be applied to large area contaminations (Rittmann et al., 1994).

On the other hand, biological degradation of the chlorinated ethylenes at the targeted sites seems to be a best way for complete and cost-effective removal, and this approach is called *in situ* bioremediation. Bioremediation includes two approaches, namely "biostimulation" and "bioaugmentation". The former refers to activation of indigenous degrading microbes, and the latter to amendment of effective exogenous degrading microbes. In biostimulation, nutrients and inducers required for the microbial growth and enzyme production are injected into the contaminated aquifer with air or hydrogen peroxide as an electron acceptor for aerobic degradation. In bioaugmentation, effective TCE-degrading microorganisms which

was isolated and cultivated outside should be introduced into the contaminated aquifer in addition to nutrients, inducers and electron acceptors. A schematic of *in situ* bioremediation is described in Fig. 1.

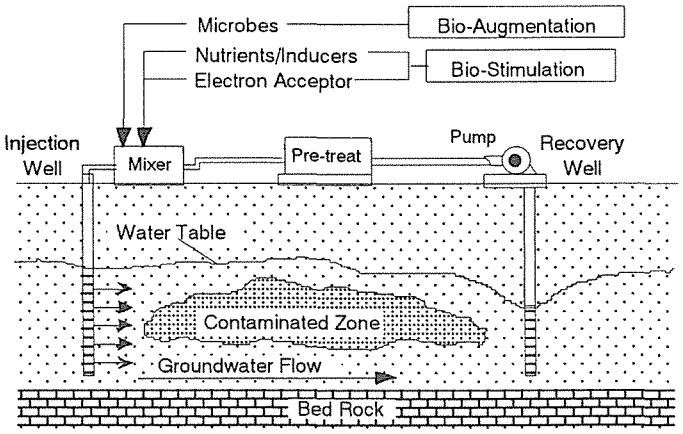

Fig. 1 A schematic of *in situ* bioremediation

Since soil/groundwater environment is generally under anaerobic or micro-aerobic conditions, microbial degradation of chlorinated ethylenes can proceed via anaerobic pathway(s). In the anaerobic metabolism, TCE (or PCE) is reductively degraded with successive substitution of chlorine for hydrogen (Chaudhry and Chapalamadugu, 1991). In this process, TCE is converted into 1,1-dichloroethylene (1,1-DCE), *cis*-1,2-dichloroethylene (cis-DCE) or *trans*-1,2-dichloroethylene (trans-DCE), and finally transformed into vinyl chloride (VC) or ethylene. The observation that cis- and trans-DCE which have not been industrially used were found in soil/groundwater is an evidence for that TCE (PCE) is actually dechlorinated in the contaminated sites. A significant decisive defect of the anaerobic dechlorination is that it generates and accumulates, as catabolic intermediates, DCE isomers and/or VC, which are more toxic or carcinogenic than the parental compound. Furthermore, as the reaction generally proceeds very slowly, the anaerobic degradation seems undesirable for the bioremediation of chlorinated ethylenes.

On the other hand, aerobic degradation of chlorinated ethylenes leads to complete mineralization into cell materials and/or carbon dioxide. Aerobic TCE degradation is initiated by formation of TCE epoxide, which spontaneously or enzymatically breaks down to form dichloroacetic and glyoxylic acids and/or simple one-carbon compounds such as carbon monoxide and formate. These metabolites are not or much less toxic or carcinogenic compared with the parental compound, and will be readily utilized by various types of microorganisms. The reaction proceeds much faster than the anaerobic dechlorination. Therefore, the aerobic degradation is preferable for efficient and safe bioremediation, if possible.

For aerobic TCE degradation, three major groups of microorganisms, methanotrophs, aromatic compound degraders represented by toluene- or phenol-degrading bacteria, and ammonia oxidizers, have been known to be applicable. Amongst them, a high potential of phenol-degrading bacteria in bioremediation of TCE has been recognized. For instance, Hopkins et al. (1993) demonstrated that stimulation of phenol-utilizing microorganisms led to TCE degradation at a considerably higher rate than the stimulation of methanotrophs or ammonia oxidizers in *in situ* field studies. In this presentation, degradation of TCE by phenol-degrading bacteria will be described for discussing their availability in the bioremediation of TCE.

MATERIALS AND METHODS

Enumeration and characterization of phenol-degrading bacteria in soil

A variety of soil samples, which had never received contamination of TCE or other chlorinated solvents, were collected from the depth between 20 and 25 cm, and numbers of phenol-degrading bacteria in them were enumerated by using a minimal salt medium supplemented with phenol (phenol medium) as a selective medium (K_2HPO_4 1 g, $(NH_4)SO4$ 1 g, $MgSO4 \cdot 7H_2O$ 0.2 g, $FeCl_3$ 0.01 g, $CaCl_2$ 0.01 g, NaCl 0.05 g, Agar 17 g, phenol 125 mg or 25 in 1 liter of deionized water; pH=7.2). For comparison, viable counts of heterotrophic bacteria in soil samples were also measured with CGY medium (bacto-casitone 5 g, glycerol 5 g, bacto-yeast extract 1 g, agar 17 g in 1 liter of deionized water; pH=7.2). Fifty milligrams

of the soil sample were added into 450 ml of sterile sodium tripolyphosphate solution (50 mg/l), vigorously mixed by a homogenizer. The resultant soil suspension was plated onto the phenol medium, and all the colonies appeared on the plate were examined for their phenol-degrading capability by transferring them onto the new medium (replica plate technique) as to eliminate the counts of false-positive colonies. Two different concentrations of phenol was used for preparing the phenol medium, since preliminary studies indicated that the counts of phenol-degrading bacteria depended on the phenol concentration of the medium.

Further, in another series of investigations on the phenol-degrading bacterial population, we have isolated about 100 morphologically-different colonies from 11 soil samples, and have investigated their fundamental physiological properties. Physiological tests performed included gram stain, motility, oxidase, catalase, Hugh and Leifson (O-F) test, cell form and motility.

TCE degradation tests of phenol-degrading bacteria

Fourteen different phenol-degrading bacteria shown in Table 1 were examined for their TCE-degrading capability. Amongst them, *Rhodococcus* sp. PN1 and strain WP1 are gram-positive, and the others are gram-negative. They were isolated from a variety of environmental samples by using different substrates as sole carbon and energy sources. The bacterial strains were cultivated in the phenol medium containing phenol as a sole carbon and energy source to late-log or stationary growth phase, washed with phosphate-potassium buffer (pH 7.5), and suspended at an approximately turbidity of 2.0 at 600 nm (resting cell). TCE degradation (ca. 1 mg/l) by the resting-cells were monitored during incubation at 25 °C with reciprocal shaking at 100 rpm in sealed vials. TCE was measured by head-space analysis with an electron capture detector-equipped gas chromatography (Fujita et al., 1995).

Table 1 Phenol-degrading bacteria used for TCE degradation tests

Strain	Source	Substrate for isolation
Acinetobacter calcoaceticus AH	Activated sludge	Phenol
Pseudomonas putida BH	Activated sludge	Phenol
Pseudomonas putida PpG1064	Unknown	Naphthalene
Acinetobacter sp. YAD	Activated sludge	Aniline
Acinetobacter sp. YAF	Activated sludge	Aniline
Alcaligenes sp. YAJ	Activated sludge	Aniline
Rhodococcus sp. PN1	Activated sludge	*p*-Nitrophenol
Strain WP1	Activated sludge	Phenol
Strain A-4	Forest soil	Phenol
Pseudomonas fluorescens A-5	Forest soil	Phenol
Strain A-7	Forest soil	Phenol
Strain B-6	Solvent-contaminated soil	Phenol
Pseudomonas putida M-1	Field soil	Phenol
Pseudomonas putida Y-3	Field soil	Phenol

RESULTS AND DISCUSSION

Phenol-degrading bacteria in soil environment

Although a high potential of phenol-degrading bacteria in TCE bioremediation has been recognized, their microbial or ecological aspects have not been fully elucidated. For successful *in situ* bioremediation, the quantitative and qualitative characterization of phenol-degrading bacteria which are present in the polluted sites is required so as to establish rational strategies for effectively improving their population size. In order to evaluate the population size of phenol-degrading bacteria in soil environment, plate count technique was used, because the viable counts or colony forming units (cfu) enumerated by this methodology can express active bacterial population to a certain degree. On the purpose of bioremediation, non-active or non-growing bacterial population seems meaningless.

The counts of phenol-degrading bacteria in 6 forest and 2 field soil samples are summarized in Table 2 in comparison with the counts of heterotrophic bacteria. The population of phenol-degrading bacteria

enumerated on the phenol medium containing 125 mg/l of phenol accounted for 6 to 20 % of the heterotrophic bacterial populations in all the tested soil samples: they existed at the population sizes between 1.1 x 10^6 and 3.6 x 10^6 cfu/g of dry soil against the backgrounds of the heterotrophic bacteria between 9.2 x 10^6 and 3.1 x 10^7 cfu/g of dry soil. Phenol-degrading bacteria counted on the phenol medium containing a higher concentration of phenol (250 mg/l) were at a little lower levels, however, accounted for a considerable portion (4 to 13 %) of the heterotrophic bacteria. Phenol at a high concentration (e.g., 125 mg/l) may have toxic effects on a portion of phenol-degrading bacteria, and some of the phenol-degrading bacteria seem to unable to grow on phenol without specific nutrients such as vitamins and amino acids, therefore, the counts of phenol-degrading bacteria enumerated here are considered to be more or less underestimated. Even so, the values (population sizes of phenol-degrading bacteria) obtained were very high, suggesting common and abundant presence of phenol-degrading bacteria in natural soil environment.

Table 2 Natural abundance of phenol-degrading bacteria in soil environment

Sample	Heterotrophic bacteria [a]	Phenol-degrading bacteria [b]	
		125 mg/l phenol	500 mg/l phenol
Forest soil-1	9.4 x 10^6	1.5 x 10^6 (16) [c]	1.2 x 10^6 (13)
Forest soil-2	3.0 x 10^7	2.4 x 10^6 (8)	1.3 x 10^6 (4)
Forest soil-3	1.9 x 10^7	3.1 x 10^6 (16)	1.9 x 10^6 (10)
Forest soil-4	1.9 x 10^7	3.6 x 10^6 (19)	1.4 x 10^6 (7)
Forest soil-5	2.4 x 10^7	2.9 x 10^6 (12)	1.8 x 10^6 (8)
Forest soil-6	9.2 x 10^6	1.9 x 10^6 (20)	1.1 x 10^6 (12)
Field soil-1	3.1 x 10^7	3.2 x 10^6 (10)	1.9 x 10^6 (6)
Field soil-2	1.7 x 10^7	1.1 x 10^6 (6)	9.4 x 10^5 (5)

Viable counts in 8 soil samples are shown as cfu/g of dry soil.

[a] Viable counts enumerated on CGY medium after 7-day incubation at 28 °C.

[b] Viable counts enumerated on phenol medium after 7-day incubation at 28 °C. Colonies were transferred onto new medium to confirm their phenol-utilizing ability.

[c] Values in parenthesis indicate the ratios of cfu of phenol-degrading bacteria to that of heterotrophic bacteria.

As well as quantitative assay of the phenol-degrading bacteria in soil environment, it is important to know what kinds of phenol-degrading bacteria exist there. Judging from the observation of colony morphology in the above-mentioned enumeration experiments, each soil sample seemed to contain a few to several different types of phenol-degrading bacteria as dominant populations. Based on the physiological tests, phenol-degrading bacterial strains isolated from 11 soil samples were classified into 15 different genera according to the diagnostic tables of bacteria proposed by Cowan and Steel (1973); *Micrococcus*, *Streptococcus*, *Bacillus*, *Lactobacillus*, *Corynebacterium*, *Rothia* and *Kurthia* as gram-positive genera, and *Pseudomonas*, *Flavobacterium*, *Acinetobecter*, *Aeromonas*, *Neisseria*, *Cardiobacterium*, *Haemophilus*, *Branhanella* and *Chromobacterium* as gram-negative genera. Up to date, numerous numbers of phenol-degrading microorganisms have been isolated and characterized , although most-studied phenol-degrading microorganisms are pseudomonads (gram-negative rods) such as *Pseudomonas*, *Alcaligenes*, and *Flavobacterium* species. Other various bacterial genera, fungi, and yeasts have been also reported. They were isolated from various environmental samples including fresh water, marine water, wastewater, activated sludge, and soil, and it is believed that microbial phenol degradation is unlikely to be recently-acquired trait. The experimental results obtained here confirmed that there exists a wide variety of phenol-degrading bacteria in soil environment.

TCE degradation by phenol-degrading bacteria

The previous section described that phenol-degrading bacteria are widely and abundantly present in soil environment, and that many different types exist. If all the phenol-degrading bacteria present in soil environment have high potentials for TCE degradation, what we have to do is only that we propagate and induce TCE degrading activity of the indigenous phenol-degrading populations. However, if there exists

no phenol-degrading bacteria which are effective for co-oxidation of TCE, biostimulation with phenol will result in failure, and to bioaugmentate effective TCE-degrading bacteria may be requisite. Therefore, it is necessary to know TCE-degrading capabilities of various types of phenol-degrading bacteria.

Among 14 distinct types of phenol-degrading bacteria examined, only one strain, *Rhodococcus* sp. PN1, could not degrade TCE at all, and the other strains showed TCE-degrading capability to a certain extent (Fig. 2). However, when these strains were cultivated in a nutrient medium without phenol, none of them could exhibit a significant TCE degradation. Other aromatic compounds could not induce TCE-degrading capability, also. Therefore, it was suggested that most of commonly-observed phenol-degrading bacteria can degrade TCE, and that the TCE degradation is mediated by their phenol-degrading enzymes. Although we have not characterized phenol-degrading enzymes in the tested bacterial strains excepting phenol hydroxylase of *Pseudomonas putida* BH (Takeo et al., 1995), tested strains seemed to possess different types of phenol-degrading enzymes judging from assimilability of various aromatic compounds (data not shown). One of the reason for this inference, for example, is as follows: although the phenol hydroxylase of *Pseudomonas putida* BH can catalyze oxidation not only of phenol but also of *o*-, *m*-, and *p*-cresols, and is active also for TCE, most of other bacterial strains which could degrade TCE were not able to degrade cresols. Therefore, it seems that various types or at least a few types of phenol-degrading enzymes (phenol hydroxylases) have ability to co-oxidize TCE.

On the other hand, strain PN1 which could not degrade TCE, even when grown on phenol, is different from the other strains in that it can grow on *p*-nitrophenol as a sole carbon and energy source. Strain PN1 was further examined for its TCE-degrading capability against lower concentrations of TCE (up to 0.1 mg/l), however, any detectable amounts of TCE degradation occurred. Further, although several modifications were added to the cultivation condition of strain PN1-1, TCE could not be degraded at all. It seems that phenol-degrading enzyme in strain PN1 cannot catalyze TCE co-oxidation. Since another gram-positive strain WP1 could degrade TCE, the lack in TCE-degrading ability may not be a common property of gram-positive, phenol-degrading bacteria.

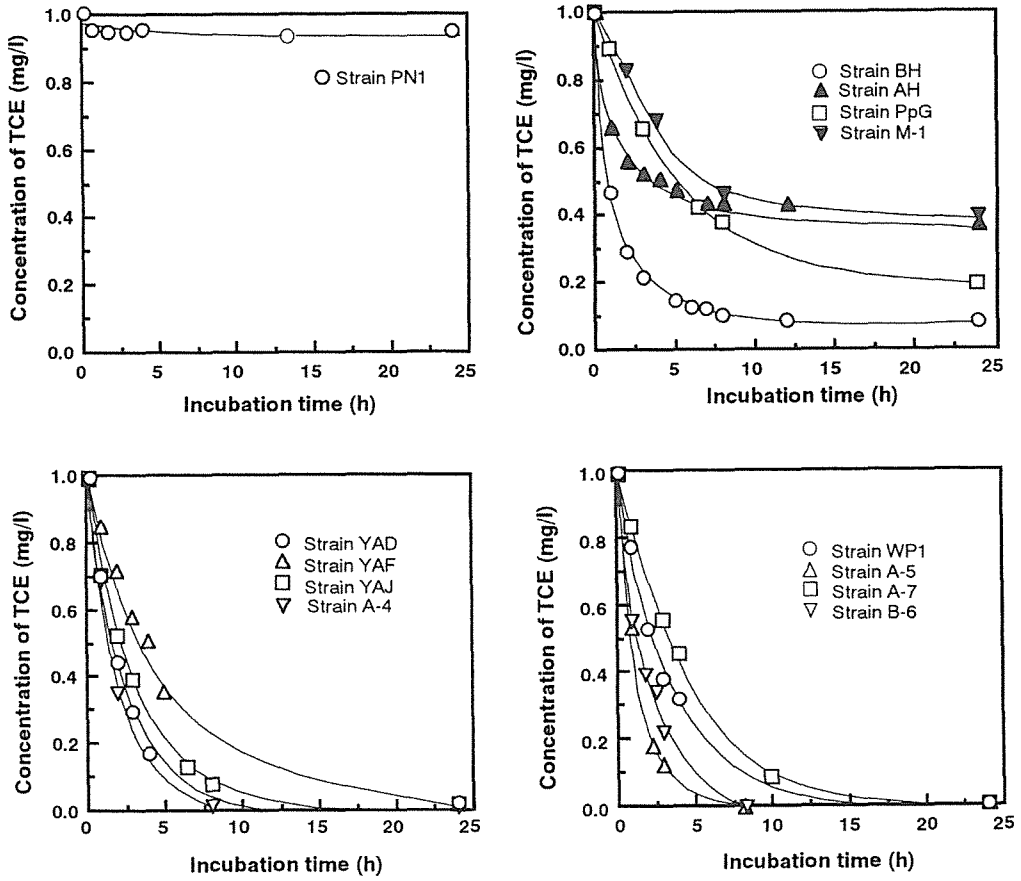

Fig. 2 TCE degradation by a variety of phenol-degrading bacteria

Relationship between phenol-degrading activity and TCE-degrading activity

Relationship between specific phenol-degrading and TCE-degrading activities in each bacterial strains were investigated by phenol-grown resting cells. Both activities were measured as the initial decrease rates by the resting cells, and expressed as micro-moles degraded per milligram of whole cell protein. Fig. 3 shows the results. A general trend was observed that bacterial strains with higher phenol-degrading activity showed higher initial TCE-degrading activity. However, although strains Y-3 and A-5 showed similar phenol-degrading activities, their TCE-degrading activities were very different. TCE-degrading activity of strain A-5 was about 3.5 times higher than that of strain Y-3, whereas the phenol-degrading activity was only about 1.2 times higher. This means that bacterial strain with higher phenol-degrading activity do not always show higher TCE-degrading activity, suggesting that TCE-degrading potentials of distinct types of phenol-degrading enzymes considerably differ from each other.

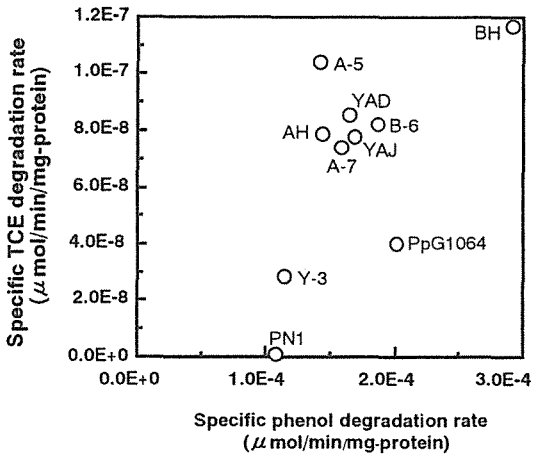

Fig. 3 Relationship between phenol-degrading activity and TCE-degrading activity

Until now, at least 3 considerably different types of phenol-degrading enzymes have been reported in bacteria. One major group is multicomponent oxygenases, and it was clarified that phenol hydroxylase of *Pseudomonas putida* BH belongs to this type (Takeo et al., 1995). Other types are single component oxygenases, and include at least 3 significantly different phenol-oxidizing enzymes, those from *Pseudomonas* sp. EST1001 (Kivisaar et al., 1990), from *Pseudomonas pickettii* PKO1 (Kukor and Olsen, 1990). What kinds of phenol-degrading enzymes are especially active for TCE degradation should be elucidated in future for easily selecting effective TCE-degrading bacteria. Amongst the bacterial strains tested here, strain BH exhibited the highest specific TCE-degrading activity. *Pseudomonas* sp. CF600 possessed multicomponent phenol hydroxylase identical (in amino acid sequence)to that of strain BH (Shingler et al., 1989), and it was demonstrated that the similar multicomponent type phenol hydroxylases were commonly found in *meta*-cleavage pathway utilizing phenol-degrading bacteria which were isolated from activated sludge, sea water, and freshwater mud. Therefore, biostimulation of soil with phenol seems to easily to lead to propagation of BH-type phenol-degrading enzymes which can effectively co-metabolize TCE.

Instability of TCE degradation

Though most of the phenol-degrading bacteria possessed TCE-degrading potentials, the extents of TCE degradation were considerably different: the ultimate TCE removal (TCE removal after 24 hours) varied between approximately 50 % and 100 %. TCE degradation by some strains apparently ceased in a few hours, and that by others proceeded extensively for more than 10 hours. In other words, some strains could not completely remove 1 mg/l of TCE (strains AH, BH, PpG1064, and M-1), but others could reduce TCE to under a detectable limit (strains YAD, YAF, YAJ, WP1, A-4, A-5, A-7, B-6). It should be especially emphasized that higher specific TCE-degrading activity did not necessarily cause higher ultimate removal of TCE. For instance, *Pseudomonas putida* BH exhibited the highest specific TCE-degrading rate in the initial 2 or 3 hours, its removal after 24 hours remained about 90 %. This indicated that TCE-

degrading potential in phenol-degrading bacteria depend not only on the magnitude of the degrading activity but also on the stability of the degrading-activity (enzymes)).

In order to investigate on the stability of TCE-degrading enzymes, bacterial strains were harvested at different growth phases on phenol (mid-log, late-log, and early stationary phases) and TCE-degrading activity of the resultant series of resting cells were compared. We assumed that the production of TCE (phenol)-degrading enzyme in the bacterial cells would reach maximum in the logarithmic growth phase and that the content of the enzyme would decrease during the stationary growth phase if it is not stable. *Acinetobacter* sp. YAD, *Acinetobacter calcoaceticus* AH, and *Pseudomonas putida* BH were used as the model strains for this experiment. Strain YAD was selected as a representative of stable TCE-degrader, and strains AH and BH as unstable ones. Results are shown in Fig. 9. The TCE-degrading activity of the resting cells was evaluated as the initial degradation rates of the TCE.

The TCE-degrading activity of strain YAD was mid-log phase < late-log phase < stationary phase, whereas that of strain BH was mid-log phase < late-log phase = stationary phase and that of strain AH was mid-log phase ≥ late-log phase > stationary phase. It seemed that strain YAD could actively produce its TCE-degrading enzyme throughout the growth (from mid-log to stationary phase) on phenol and that the enzyme was maintained stably and, consequently, accumulate to a high level in the cells. On the other hand, although the degrading enzyme in strain BH was accumulated during its logarithmic growth, its production seemed to cease or be balanced with its deterioration in the stationary phase. As for strain AH, it was considered that the enzyme production rate after the mid-log phase was lower than the rate of enzyme deterioration, resulting in the time-dependent decline of TCE-degrading activity. Thus, it was experimentally demonstrated that TCE (or phenol)-degrading enzymes can be stably maintained long in some strains but that may be naturally spoiled or degraded rapidly in some strains. It may be concluded that the degree of TCE degradation by phenol-degrading bacteria considerably depend on the stability of the enzyme, and the stability refers not only to the stability of the enzyme itself (as protein) but also to that of the induction system (regulatory system for the enzyme production).

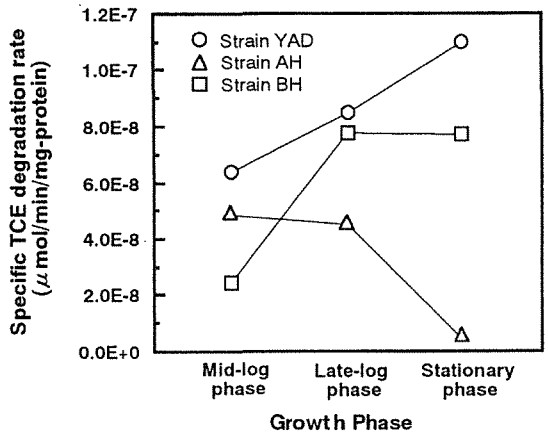

Fig. 4 Effects of growth phase on TCE degradation in strains YAD, AH and BH

CONCLUSIONS

The xeperimantal results suggested that the addition of phenol (stimulation of indigenous soil bacteria) can easily lead to propagation of phenol-degrading bacteria with high TCE-degrading potentials such as BH-like strains, consequently, will result in successful TCE bioremediation. However, in this strategy, TCE removal efficiency should considerably depend on the TCE-degrading properties of the phenol-degrading bacteria present in the targeted site. If there is no effective indigenous TCE degraders or if the injection of phenol causes predominant growth of ineffective TCE degraders, the biostimulation will result in failure. For such cases, we have to introduce effective ones which are screened in the laboratory (bioaugmentation). For screening effective TCE degraders, not only the activity but also the stability of TCE-degrading enzymes should be taken into consideration.

In order to develop system design or operation strategies for more effective *in situ* TCE bioremediation, further studies on microbial ecology or population dynamics of phenol-degrading bacteria in soil and their TCE-degrading enzymes are needed.

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