Biotechnology and Bioprocess Engineering **2005**, 10: 40-46 ©KSBB

Evaluation of Different Culture Conditions of *Clostridium bifermentans* DPH-1 for Cost Effective PCE Degradation

Afroze Syeda Humayra[1], Yuki Hasegawa[2], Izumi Nomura[2], Young C. Chang[1], Takeshi Sato[3], and Kazuhiro Takamizawa [1,2]*

[1] United Graduate School of Agricultural Science, Gifu University, Gifu 501-1193, Japan
[2] Department of Applied Life Science, Faculty of Applied Biological Sciences, Gifu University, Gifu 501-1193, Japan
[3] Department of Civil Engineering, Gifu University, Gifu 501-1193, Japan

Abstract *Clostridium bifermentans* strain DPH-1 has already been found to dechlorinate perchloroethylene (PCE) to *cis*-dichloroethylene (*cis*-DCE) *via* trichloroethylene (TCE). In this study, our investigation on different culture conditions of this DPH-1 strain was extended to find a more efficient and cost effective growth medium composition for this DPH-1 strain in bioremediation practices. Temperature dependency of strain DPH-1 showed that the growth starting time and PCE degradation at 15°C was very slow compared to that of 30°C, but complete PCE degradation occurred in both cases. For the proper utilization of strain DPH-1 in more cost effective bioremediation practices, a simpler composition of an effective media was studied. One component of the culture medium, yeast extract, had been substituted by molasses, which served as a good source of electron donor. The DPH-1 strain in the medium containing molasses, in the presence of K_2HPO_4 and KH_2PO_4, showed identical bacterial multiplication (0.135 mg protein $mL^{-1}h^{-1}$) and PCE degradation rates (0.38 μM/h) to those of the yeast extract containing medium.

Keywords: culture conditions, PCE dechlorination, *Clostridium bifermentans* DPH-1, molasses, bioremediation

INTRODUCTION

Halogenated compounds are generally well known as toxic environmental pollutants. Of these chlorinated aliphatic compounds, such as ethylenes and ethanes, are the most common organic contaminants in ground waters. Chloroethylenes, such as tetrachloroethylene (perchloroethylene, PCE) and trichloroethylene (TCE), isomers of dichloroethylene (DCE) (*cis*-1,2-DCE and trans-1,2-DCE, and 1,1-DCE), vinyl chloride (VC) and other chloroaliphatics, occur in significant concentrations in the environment due to modern industrial activities. PCE and TCE are excellent solvents, which have been widely used as industrial degreasing solvents, dry-cleaning fluids and fumigants, and in a variety of other applications. They are especially used in the dry cleaning and textile industries in scouring machines, and for extracting fats [1] and stripping paint [2], mainly as nonflammable solvents [3]. This widespread usage, along with careless handling and improper storage and disposal, has made these chemical compounds ubiquitous ground water pollutants [4] due to their relatively high water solubilities. These compounds are highly mobile in soils and aquifer materials, and often easily infiltrate ground waters. As they are suspected carcinogens [5] and frequent ground water contaminants, they pose a serious public health problem through contaminated ground waters. Therefore, all these factors have led to an intense interest in their transformation in contaminated sites and remediation processes. An important interesting and novel category of transformations are those that occur under the influence of anaerobic microbial communities used in bioremediation techniques. Biological strategies, known as bioremediation, provide an attractive, relatively cost effective and environmentally compatible option [6].

PCE is an important model for the study of biodegradation of chlorinated aliphatic compounds due to its high halogen content and toxicity. PCE is recalcitrant under aerobic conditions because of its oxidized nature [7], but can be reductively dechlorinate by certain microbes under anaerobic conditions. Thus, there is growing interest in anaerobic biological systems for PCE decontamination. Mixed anaerobic enrichment cultures have been frequently reported to effect reductive dechlorination of PCE [2,7-10]. However, only a few PCE degrading monocultures, *e.g. Dehalospirillum multivorans* [11,12], *Desulfomonile tiedjei* [13], *Dehalobacter restrictus* [14], *Dehalococcoides ethenogenes* 195 [15,16], *Desulfitobacterium* sp. PCE-S [17] and *Desulfitobacterium* sp. Y51 [18], have been studied in detail and their dehalogenases

***Corresponding author**
Tel : +81-58-293-2906 Fax : +81-58-293-2906
e-mail: tak2003@cc.gifu-u.ac.jp

characterized.

In 1987, for the first time, Fathepure *et al.* [13] isolated an organism which could dechlorinate PCE. They also isolated two anaerobic organisms that could produce methane and reduce sulfates, respectively. Most anaerobic dehalogenases degrade PCE principally to *cis*-1,2-DCE; however, a novel PCE dehalogenase from *Dehalococcoides ethenogenes* 195 [15] can extensively detoxifying by its reductive dechlorination of the PCE to ethylene. Aerobic degradation of *cis*-1,2-DCE by *Rhodococcus rhodochrous* [19] and *Nitrosomonas europaea* [20] has been reported. Thus, *cis*-1,2-DCE accumulation in the anaerobic system can be eliminated by further degradation using such aerobic dehalogenases.

Previous research in our laboratory has identified and documented the bacterium *Clostridium bifermentans* DPH-1 as a PCE dechlorinating, gram (+), endospore forming, obligate anaerobic bacterium, isolated from ditch sludge, which can effectively dechlorinate high concentration of PCE to *cis*-1,2-DCE [21]. To utilize this bacterium for PCE degradation at actual contaminated sites in practical bioremediation, its capability for biodegradation at actual sites and the favorable conditions for its growth and PCE dechlorination have to be known. Previous laboratory experiments have been based on the optimum nutrient, temperature and pH conditions for the strain DPH-1. The environmental conditions at actual contaminated sites are far from those used in laboratory studies, *i.e.* the yearly average ground water temperature in Japan is around 15°C and the media used in these studies with strain DPH-1 are nutrient rich and costly. Knowledge on the capability of strain DPH-1 for PCE dehalogenation at 15°C, utilizing more simple and cheap nutrient conditions is important. In this paper these objectives are discussed using the growth and PCE dehalogenation rates as criteria.

MATERIALS AND METHODS

Chemicals

All chemicals used in this study were of analytical grade and purchased from Wako Chemical (Kyoto, Japan). The PCE was obtained from Kanto (Tokyo, Japan) and the other chlorinated chemicals, such as trichloroethylene (TCE) and *cis*-dichloroethylene (*cis*-DCE) from GL Sciences (Tokyo, Japan). The molasses was a gift from Nippon Beet Sugar MFG., Co. Ltd. and contained 78.6 g/L of glucose, 79.7 g/L of fructose and galactose, 170.9 g/L of sucrose, 10.2 g/L of raffinose and 339.4 g/L of fermentable sugar.

Microorganisms and Anaerobic Culture Conditions

Clostridium bifermentans DPH-1 was isolated from ditch sludge contaminated with wastes from an electric company in Gifu Prefecture, Japan [21]. The culture was maintained by weekly subculture on MY medium, with the following composition (in g/L): K_2HPO_4, 7.0; KH_2PO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 0.1; $(NH_4)_2$ SO4, 1.0 and yeast extract, 2.0, at pH 7.2. 9.5 mL of the medium was autoclaved in 26 mL-serum bottles, followed by the addition of 0.1 mL of filter sterilized vitamin solution (1 g of *para*-aminobenzoic acid and 1 mg of biotin per liter), 0.1 mL $FeSO_4 \cdot 7H_2O$ (2 g/L) and 300 μL DPH-1 inoculum. Before adding the vitamin and iron solutions, and the DPH-1 inoculum, the headspaces of the vial bins were flushed with pure nitrogen and sealed with Teflon-lined rubber septa and aluminum crimp caps.

Gas-chromatography Analysis of Chlorinated Aliphatic Compounds

Chlorinated aliphatic compounds were quantified using head space analysis [22] on a model GC-9A gas chromatograph (Shimadzu, Japan). High concentrations of PCE were determined gas chromatographically using the above machine equipped with flame ionization detection (FID) and a packed glass column (i.d. 3.2Ø, 2.1 m; silicone DC-550 20% Chromosorb W [AWDMCS] 80/100).

Different Temperature Regime

To find the effect of temperature on the DPH-1 growth and PCE degradation rates, batch cultures were prepared using MY medium and incubated at 4, 10, 15, 30 and 37°C, respectively. The O.D. of the subcultures at 660 nm was measured at different temperatures and time intervals using a UV-VIS spectrophotometer (Shimadzu UV-1200) with the inoculation time regarded as 0 h. This was followed by estimation of the protein concentrations in the cultures using the Bradford method [23] at 595 nm at specified time intervals. The PCE degradation rates were simultaneously measured by FID.

Determination of Simpler and Cheaper Nutrients Composition of Medium for Effective Growth and PCE Degradation

Lactic acid, formic acid, glucose, ethanol, pyruvic acid, and fumaric acid were tested as nutrient candidates without yeast extract, but none were found to work as electron donors, suggesting that organic nitrogen, minerals, vitamins and source of other factors were important to strain DPH-1. Therefore, molasses, which may contain these nutrients, including electron donors, was selected. The growth rate and PCE degrading efficiency of strain DPH-1 were compared between in both yeast extract containing media and molasses containing media. The media contained different concentrations of yeast extract (0.125~4.0 g/L) which were used to culture the strain DPH-1. In this study, the PCE was added to the medium at final concentration of 6 μM.

The growth and PCE dechlorination rates were studied in different molasses containing media at 30°C. The media combinations were: (i) molasses with K_2HPO_4 and $(NH_4)_2SO_4$, (ii) molasses with K_2HPO_4, KH_2PO_4 and $(NH_4)_2SO_4$, and (iii) molasses with K_2HPO_4, KH_2PO_4, $(NH_4)_2SO_4$, and $MgSO_4 \cdot 7H_2O$.

The specific growth and PCE degradation rates of strain DPH-1 were compared at 15 and 30°C, with 1.0, 2.0, 4.0, and 8.0 g/L yeast extract. To find the effects of molasses on the specific growth and degradation rates of strain DPH-1, similar experiments were conducted with different concentration of molasses, 1.0, 2.0, 4.0, 8.0 g/L were substituted for the yeast extract at both 15 and 30°C.

Determination of the Degradation Capacity of PCE by Strain DPH-1 in Molasses at 15°C

The molasses containing media was consisted with, molasses: 2.0 g/L, K_2HPO_4: 7.0 g/L, KH_2PO_4: 2.0 g/L, $(NH_4)_2SO_4$: 1.0 g/L, $MgSO_4 \cdot 7H_2O$: 0.1 g/L. The PCE degradation rates were studied by GC/FID at different time intervals, up to 11 days, in the above mentioned molasses media with 30, 60, and 600 μM PCE.

Effect of pH on the PCE Degradation by DPH-1 in Media Containing Molasses and Nutrients at 15°C

The bacterial growth and PCE dechlorination were studied under different culture media pH conditions. The medium containing 0.2 g/L molasses was adjusted to different pHs; 3.0, 5.0, 7.3, 9.0 and 11.0 with the addition of 6.0 μM PCE to each culture and the bacterial proliferation rates measured.

RESULTS AND DISCUSSION

Different Temperature Regime

DPH-1 was incubated at different temperatures. To find a suitable temperature for the growth and PCE degradation of strain DPH-1, cultures were incubated and observed at the different specified temperatures, with the exception of 4°C (data not shown). The growth and PCE degradation rates at each temperature were found to gradually increase after 24 h incubation. To compare the growth rate under two different culture conditions (15 and 30°C), the O.D. at 660 nm values in Fig. 1(a), were converted into cell protein concentrations from standard protein concentrations. In this type of observation of the growth, the rate of log phase is a very important parameter. Therefore, only the log phase O.D. value was extracted, and converted to the standard protein concentration, which was considered as equivalent to the cell numbers. The data from the log phase was analyzed by linear equation. At 15 and 30°C the linear equations are (y= 0.034x-0.204) and (y= 0.121x-0.311), respectively. From these equations the growth rates at 15 and 30°C were 0.034 and 0.121 mg protein mL^{-1} h^{-1}, respectively. The PCE degradation capacities at the two different temperatures are shown in Fig. 1(b). From Fig. 1(b), the PCE degradation rates at 15 and 30°C were estimated to be 0.183 and 0.426 μM/h, respectively. At 10 and 15°C, the PCE dechlorination rates were only 14 and 24%, respectively, but at 30 and 37°C these were 86 and 100%, respectively. From this finding it can be assumed that the

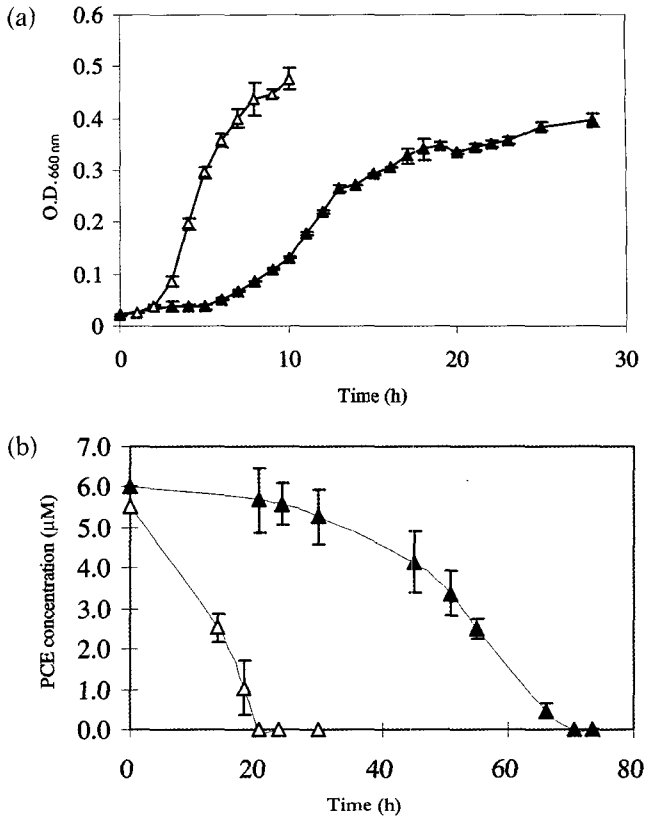

Fig. 1. Comparison of growth and PCE degradation by *Clostridium bifermentans* DPH-1 at 15 and 30°C condition [Media composition: Yeast extract (2.0 g/L), K_2HPO_4 (7.0 g/L), KH_2PO_4 (2.0 g/L), $(NH_4)_2SO_4$ (1.0 g/L) and $MgSO_4 \cdot 7H_2O$ (0.1 g/L)], -▲- 15°C, -Δ- 30°C, (a) Growth (b) PCE degradation. Data are expressed as mean ± S.D. n = 6.

PCE degradation was better at higher temperatures. Usually, the incubation or temperature condition for culturing DPH-1 is 30°C, but in nature a ground water temperature condition of 15°C prevails. At 15°C the growth and PCE degradation rates were 0.28 times Fig. 1(a) and 0.43 times Fig. 1(b) lower, respectively, than those at 30°C. It was also found that at 15°C, the initiation of growth and PCE degradation were very late (by 3 and 6 h, respectively) compared to 30°C condition, but in both cases the PCE was fully degraded. Okeke *et al.* [24] found that the dehalogenase of strain DPH-1 catalyzed the dechlorination of PCE, with an optimum temperature of 35°C. They also observed that the enzyme was relatively stable in the temperature range 20~35°C. Chang *et al.* [21] also found a similar temperature dependency for the dechlorination activity of the cell-free extract of strain DPH-1. Pietari [25] observed that two methanogenic enrichment subcultures were able to carry out complete reductive dechlorination of PCE or TCE to ethene in the temperature range 15~30°C. The present study showed good agreement with previous studies in relation to the temperature patterns of the dechlorination activity. From the above mentioned experiments, strain DPH-1 was

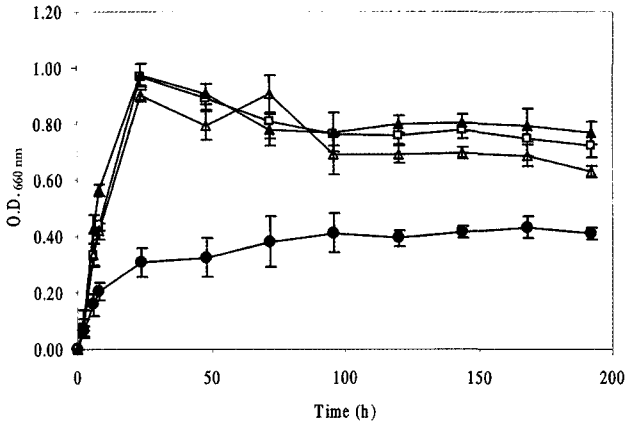

Fig. 2. Effect of media composition on the growth of *Clostridium bifermentans* DPH-1 at 30°C. -●- Only molasses (2.0 g/L), -□- Molasses (2.0 g/L), K_2HPO_4 (7.0 g/L) and $(NH_4)_2SO_4$ (1.0 g/L), -▲-Molasses (2.0 g/L), K_2HPO_4 (7.0 g/L), KH_2PO_4 (2.0 g/L) and $(NH_4)_2SO_4$ (1.0 g/L), -Δ- Control [Molasses (2.0 g/L), K_2HPO_4 (7.0 g/L), KH_2PO_4 (2.0 g/L), $(NH_4)_2SO_4$ (1.0 g/L) and $MgSO_4 \cdot 7H_2O$ (0.1 g/L)]. Data are expressed as mean ± S.D. n = 6.

found to be applicable for the dehalogenation of PCE at 15°C.

Determination of Simpler and Cheaper Nutrients Composition of Medium for Effective Growth and PCE Degradation

Yeast extract was one of the expensive components of the medium at approximately 30,000 kg^{-1}, whereas the price of molasses was approximately 1,500 kg^{-1}. Therefore, the bacterial culture conditions and PCE degradation rate were studied using molasses, a comparatively cheaper component than yeast extract. It was found that molasses was better for the bacterial growth compared with the yeast extract (data not shown) at 30°C.

To decide a simpler and efficient medium composition for strain DPH-1 for a cost effective bioremediation practice, the bacterial growth and PCE degradation patterns were studied using several different culture medium compositions with molasses. The patterns of growth are shown in Fig. 2. From this study it was found that molasses with inorganic nitrogen and phosphate enhanced the bacterial growth of *Clostridium bifermentans* DPH-1. In this experiment three different molasses media compositions were used to study the PCE degradation patterns as shown in Fig. 3. It was observed that with the medium containing molasses (2.0 g/L), K_2HPO_4 (7.0 g/L) and $(NH_4)_2SO_4$ (1.0 g/L), the rate of PCE (6 μM) degrada tion was 0.38 μM/h, as shown in Fig. 3(a). When the composition was modified by supplementation with 2.0 g/L KH_2PO_4, the bacterial growth rate rose to 0.135 mg protein $mL^{-1}h^{-1}$ as shown in Fig. 2, and the PCE dechlorination rate was also slightly increased to 0.39 μM/h, as shown in Fig. 3(b). This composition showed the best degradation pattern of the three compositions used. Ac-

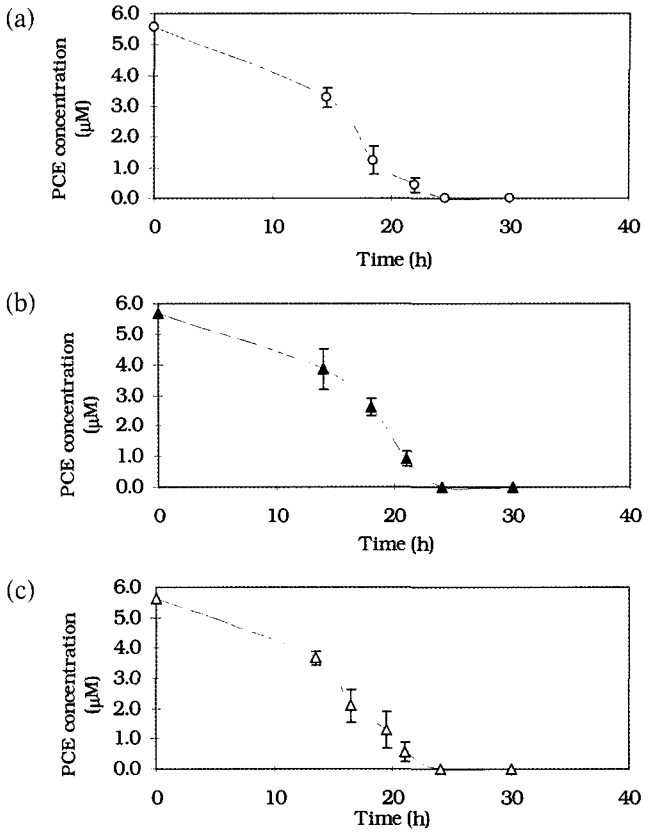

Fig. 3. Comparison of the PCE dechlorination in each molasses medium at 30°C. a) molasses (2.0 g/L), K_2HPO_4 (7.0 g/L) and $(NH_4)_2SO_4$ (1.0 g/L), b) molasses (2.0 g/L), K_2HPO_4 (7.0 g/L), KH_2PO_4 (2.0 g/L) and $(NH_4)_2SO_4$ (1.0 g/L), c) molasses (2.0 g/L), K_2HPO_4(7.0 g/L), KH_2PO_4, $(NH_4)_2SO_4$ (1.0 g/L) and $MgSO_4 \cdot 7H_2O$ (0.1 g/L). Data are expressed as mean ± S.D. n = 4.

tually, there was very little difference between two media compositions; (1) molasses with K_2HPO_4 and $(NH_4)_2SO_4$ and (2) molasses with K_2HPO_4, KH_2PO_4 and $(NH_4)_2SO_4$. From these experiments the medium with molasses (2.0 g/L), K_2HPO_4 (7.0 g/L) and $(NH_4)_2SO_4$ (1.0 g/L) can be regarded as the most simple medium composition for strain DPH-1. Harkness *et al.* [26] and Ellis *et al.* [27] stated that bacteria required basic nutrients, like nitrogen and phosphorus, in order to support their growth. The MY medium with yeast extract in the presence of inorganic nitrogen and phosphate, acts as an electron donor for the PCE dechlorination [28]. Similar PCE degradation performance was shown when only molasses was used instead of yeast extract, in the presence of inorganic nitrogen and phosphate, without the addition of an electron donor (*i.e.* methanol, ethanol, glucose, lactic acid, formic acid, fumaric acid and acetic acid) at 15 and 30°C condition (data are not shown). The degradation rates in the case of molasses at both the temperatures, but with different electron donors, showed similar results. Therefore, it can be concluded that only yeast or only molasses containing media in the presence of inorganic nitrogen

Table 1. Comparative study of the specific growth (μ) and dechlorination rates (k) of *Clostridium bifermentans* DPH-1 media containing in different concentrations of yeast extract and molasses at 30 and 15°C

	Specific growth rate (μ) (h^{-1})								Specific dechlorination rate (k) (h^{-1})	
	Different concentration of yeast extract (g/L)				Different concentration of molasses (g/L)				* Yeast extract containing media	** Molasses containing media
Temperature	1.0	2.0	4.0	8.0	1.0	2.0	4.0	8.0		
30°C	0.64	0.80	0.84	0.92	0.30	0.33	0.44	0.60	0.13	0.40
15°C	0.21	0.24	0.27	0.26	0.12	0.13	0.17	0.25	0.067	0.19

* Composition of yeast extract containing medium: Yeast extract (2.0 g/L), K_2HPO_4 (7.0 g/L), KH_2PO_4 (2.0 g/L), $(NH_4)_2SO_4$ (1.0 g/L) and $MgSO_4 \cdot 7H_2O$ (0.1 g/L)

** Composition of molasses containing medium: Molasses (2.0 g/L), K_2HPO_4 (7.0 g/L), KH_2PO_4 (2.0 g/L), $(NH_4)_2SO_4$ (1.0 g/L) and $MgSO_4 \cdot 7H_2O$ (0.1 g/L)

and phosphate, are enough to dechlorinate at both at 15 and 30°C. Harkness [29] reported that among others carbon sources, molasses can be used for promoting reductive dechlorination by anaerobic bacteria. It was also stated that molasses could be a suitable and inexpensive basic component of culture media for the growth of *Clostridium perfringens* type D [30]. Bradley *et al.* [31] also found that molasses itself could be used as an electron donor to stimulate the biodegradation of chlorinated solvents in ground water at contaminated sites.

In the case of the yeast extract containing medium, the specific growth and PCE degradation rates of strain DPH-1 at 15°C were between 0.3 and 0.5 times lower than those at 30°C (Table 1). When similar experiments were carried out with molasses instead of yeast extract in the media, the specific growth and PCE degradation rates at 15°C were found to be between 0.4 and 0.5 times lower, respectively, than those at 30°C (Table 1). From the specific growth and specific degradation rates, strain DPH-1 showed similar patterns with both the yeast extract and molasses containing media. From the findings of this and previous studies [26-31], it can be suggested that the nutrient composition with molasses, nitrogen and phosphorus might be used as an effective and inexpensive nutrient medium for strain DPH-1 for enhancing bioremediation in practice. Therefore, molasses can be recommended for use, in the place of yeast extract, as a cost effective and important organic component of media.

Study on the Effect of High Concentration of PCE Dechlorination on Molasses Containing Media at 15°C

The effect of high concentrations of PCE on the degradation ability of strain DPH-1 was examined in the molasses (2.0 g/L) containing media at 15°C and the result is shown in Fig. 4. The degradation of 30 μM of PCE to *cis*-DCE took 250 h (data not shown), as shown in Fig. 4, whereas that of 60 μM took 264 h. At a higher concentration of PCE (600 μM), no degradation was observed for up to 300 h. The effect of high concentrations of PCE on its dechlorination in molasses containing media at 15°C showed strain DPH-1 has no dehalogenase activity *i.e.* inhibited the dehalogenase activity

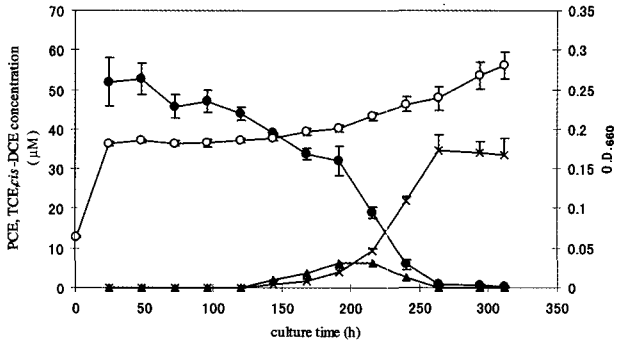

Fig. 4. PCE dechlorination and growth of *Clostridium. bifermentans* DPH-1, with an initial PCE concentration of 60 μM in the molasses medium at 15°C. -●- PCE concentration (μM), -▲- TCE concentration (μM), -X-*cis*-DCE concentration (μM), -○- O. D. at 660 nm. Data are expressed as mean ± S.D. n = 4

Table 2. Dechlorination of PCE by *Clostridium bifermentans* DPH-1 in the media containing molasses at different pHs

pH condition	Percentage of dechlorination
pH 3.0	0
pH 5.0	0
pH 7.3	97%
pH 9.0	97%
pH 11.0	0

Medium composition: Molasses (2.0 g/L), K_2HPO_4 (7.0 g/L), KH_2PO_4 (2.0 g/L), $(NH_4)_2SO_4$ (1.0 g/L) and $MgSO_4 \cdot 7H_2O$ (0.1 g/L).

[9,11,15]. Therefore, it was assumed that at low temperatures the dehalogenase activity of strain DPH-1 was inhibited by high concentrations of PCE.

Effect of pH on the Growth and Dechlorination Pattern with Molasses Containing Media at 15°C

As shown in Table 2, dechlorination only occurred under pH conditions of 7.3 and 9.0. The degradation rate

at pH 9.0 (0.0223 μM/h) was almost 1.3 times lower than that of pH 7.3 (0.028 μM/h). When the protein concentrations were compared between pH 7.3 and 9.0, it was found that although the protein concentration at pH 9.0 was not as high as at pH 7.3, but 97% of the PCE was still degraded. It can be assumed that the dehalogenase, which is responsible for the PCE dechlorination, was not active in the acidic pH range from 3.0~6.0. The optimum pH range of the enzymatic activity has already been found by Okeke *et al.* [6] and Chang *et al.* [30]. These two groups both reported maximum dehalogenase activity at pH 7.5 and that the enzyme was most stable at pH 7.5~8.0. Similar experiments were performed with *Desulfitobacterium tiedjei* [32], with the optimum pH for its dehalogenase determined as 7.2. With regard to this information, it can be assumed there will be no PCE degradation under acidic pH conditions. A similar result was obtained for the bacterium *Desulfuromonas michiganensis*, where the optimum pH for dechlorination ranged from 7.0~7.5 [33].

In this experiment, the inorganic ingredients, which have to be supplied with the molasses, have been specified, but their minimum concentrations remain to be determined. Also, no other source of nutrition, except molasses instead of yeast extract, has been found. It is hoped these experimental findings may contribute well in the conception of setting up cost effective bioremediation practices for PCE by strain DPH-1 of *Clostridium bifermentans*.

CONCLUSION

The findings of this present study can be concluded as: (i) the DPH-1 strain can take part in an aqueous based bioremediation process when an average temperature of 15°C prevails. (ii) Molasses (2.0 g/L), K_2HPO_4 (7.0 g/L), KH_2PO_4 (2.0 g/L) and $(NH_4)_2SO_4$ (1.0 g/L) can be recommended as more simple components in the formation of media for the effective growth and PCE degradation by the *Clostridium bifermentans* DPH-1. (iii) The substitution of molasses in the medium in the place of yeast extract will decrease the media cost, and give more effective growth and efficient dechlorination mechanisms.

Acknowledgements We would like to thank Nippon Beet Sugar MFG. Co. Ltd. for supporting our experiments by providing the molasses as gift. We also thank Miss. Miyuki Kojima for her technical assistance.

REFERENCES

[1] Neumann, A., G. Wohlfarth, and G. Diekert (1995) Properties of tetrachloroethene dehalogenase of *Dehalospirillum multivorans*. *Arch. Microbiol.* 163: 276-281.

[2] Distefano, T. D. (1999) The effect of tetrachloroethylene on biological dechlorination of vinyl chloride: Potential implication for natural bioattenuation. *Water Res.* 33: 1688-1694.

[3] Fetzner, S. (1998) Bacterial dehalogenation. *Appl. Microbiol. Biotechnol.* 50: 633-657.

[4] U.S. Environmental Protection Agency (1985) *Substances Found at Proposed and Final NPL Sites Through Update Number Three.* Document NPL-U3-6-3. US Environmental Protection Agency, Washington, D.C., USA.

[5] Infante, P. F. and T. A. Tsongas (1982) Mutagenic and oncogenic effects of chloromethanes, chloroethanes, and halogenated analogs of vinyl chloride. *Environ. Sci. Res.* 25: 301-327.

[6] Okeke, B. C., A. Paterson, J. E. Smith, and I. A. Watson-Craik (1997) Comparative biotransformation of pentachlorophenol in soils by solid substrate cultures of *Lentinula edodes*. *Appl. Microbiol. Biotechnol.* 48: 563-569.

[7] Ensley, B. D. (1991) Biochemical diversity of trichloroethylene metabolism. *Annu. Rev. Microbiol.* 45: 283-299.

[8] de Bruin, W. P., M. J. J. Kotterman, M. A., Posthumus, G., Schraa, and A. J. B. Zehnder (1992) Complete biological reductive transformation of tetrachloroethene to ethane. *Appl. Environ. Microbiol.* 58: 1996-2000.

[9] Holliger, C., G. Schraa, A. J. M., Stams, and A. J. B. Zehnder (1993) A highly purified enrichment culture couples the reductive dechlorination of tetrachloroethene to growth. *Appl. Environ. Microbiol.* 59: 2991-2997.

[10] Maymo-Gatell, X., V. Tandoi, J. M. Gossett, and S. H. Zinder (1995) Characterization of an H_2-utilizing enrichment culture that reductively dechlorinates tetrachloroethene to vinyl chloride and ethane in the absence of methanogenesis and acetogenesis. *Appl. Environ. Microbiol.* 61: 3928-3933.

[11] Neumann, A., H. Scholz-Muramatsu, and G. Diekert (1994) Tetrachloroethene metabolism of *Dehalospirillum multivorans*. *Arch. Microbiol.* 162: 295-301.

[12] Miller, E., G. Wohlfarth, and G. Diekert (1997) Comparative studies on tetrachloroethene reductive dechlorination mediated by *Desulfitobacterium* sp. Strain PCE-S. *Arch. Microbiol.* 168: 513-519.

[13] Fathepure, B. Z., J. P. Nengu, and S. A. Boyd (1987) Anaerobic bacteria that dechlorinate perchloroethylene. *Appl. Environ Microbiol.* 53: 2671-2674.

[14] Schumacher, W. and C. Holliger (1996) The proton electron ratio of the menaquinone-dependent electron transport from dihydrogen to tetrachloroethene in *Dehalobacter restrictus*. *J. Bacteriol.* 178: 2328-2333.

[15] Maymo-Gatell, X., Y. Chien, J. M. Gossett, and S. H. Zinder (1997) Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. *Science* 276: 1568-1571.

[16] Magnuson, J. K., R. V. Stern, J. M. Gossett, S. H. Zinder, and D. R. Burris (1998) Reductive dechlorination of tetrachloroethene to ethene by a two component enzyme pathway. *Appl. Environ. Microbiol.* 64: 1270-1275.

[17] Miller, E., G. Wohlfarth, and G. Diekert (1998) Purification and characterization of the tetrachloroethene reductive dehalogenase of strain PCE-S. *Arch. Microbiol.* 169: 497-502.

[18] Suayama, A. M., S. Yamashita, S. Yoshino, and K. Furukawa (2002) Molecular characterization of the PceA reductive dehalogenase of *Desulfitobacterium* sp. strain Y51.

J. Bacteriol. 184: 3419-3425.

[19] Malachowsky, K. J., T. J. Phelps, A. B. Teboli, D. E. Minnikin, and D. C. White (1994) Aerobic mineralization of trichloroethylene, vinyl chloride and aromatic compounds by *Rhodococcus* species. *Appl. Environ. Microbiol.* 60: 542-548.

[20] Vanneli, T., M. Logan, D. M. Arciero, and A. B. Hooper (1990) Degradation of halogenated aliphatic compounds by the ammonia-oxidizing bacterium *Nitrosomonas europaea*. *Appl. Environ. Microbiol.* 60: 542-548.

[21] Chang, Y. C., M. Hatsu, K. Jung, Y. S. Yoo, and K. Takamizawa (2000) Isolation and characterization of a tetrachloroethylene dechlorinating bacterium, *Clostridium bifermentans* DPH-1. *J. Biosci. Bioeng.* 89: 489-491.

[22] Gossett, J. M. (1987) Measurement of Henry's law constants for Cl and C2 chlorinated hydrocarbons. *Environ. Sci. Technol.* 21: 202-208.

[23] Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.

[24] Okeke, B. C., Y. C. Chang, M. Hatsu, T. Suzuki, and K. Takamizawa (2001) Purification and cloning, and sequencing of an enzyme mediating the reductive dechlorination of tetrachloroethylene (PCE) from *Clostridium bifermentans* DPH-1. *Can. J. Microbiol.* 47: 448-456.

[25] Pietari, J. M. H. (1999) *Development and Characterization of a Psychotropic Dechlorinating Culture and Temperature Response of a Mesophilic Dechlorinating Culture.* M.S. Thesis. University of Washington, WA, USA.

[26] Harkness, M. R., A. A. Bracco, M. J. Jr. Brennan, K. A. DeWeerd, and J. L. Spivack, (1999) Use of Bioaugmentation to stimulate complete reductive dechlorination of trichloroethene in Dover soil columns. *Environ. Sci. Technol.* 33: 1100-1109.

[27] Ellis, D. E., E. J. Lutz, R .J. Odom, Jr. Buchanan, M. D. Lee, C. L. Bartlett, M. R. Harkness, and K. A. Deweered (2000) Bioaugmentation for accelerated *in situ* anaerobic bioremediation. *Environ. Sci. Technol.* 34: 2254-2260.

[28] Chang, Y. C., M. Hatsu, K. Jung, Y. S. Yoo, and K. Takamizawa (2000) Degradation of a variety of halogenated aliphatic compounds by an anaerobic mixed culture. *J. Ferment. Bioeng.* 86: 410-412.

[29] Harkness, M. R. (2000) Economic considerations in enhanced aerobic biodegradation. pp. 9-14. In: G. B. Wickramananyake, A. R. Gavaskar, B. C. Alleman, and V. S. Magar (eds.). *Bioremediation and Phytoremediation of Chlorinated and Recalcitrant Compounds.* Battelle Press, Columbus, OH, USA.

[30] Silva, H. J., A. M. Giulietti, R. F. Segovia, and R. J. Ertola (1982) Use of molasses and whey in culture media for the development and production of a toxin from *Clostridium perfringens* type D. *Rev. Argent. Microbiol.* 14: 85-90.

[31] Bradley, P. M., F. H. Chapelle, and D. R. Lovley (1998) Humic acids as electron acceptors for anaerobic microbial oxidation of vinyl chloride and dichloroethene. *Appl. Environ. Microbiol.* 64: 3102-3105.

[32] Ni, S., J. K. Fredrickson, and L. Xun (1995) Purification and characterization of a novel 3-chlorobenzoate-reductive dehalogenase from the cytoplasmic membrane of *Desulfomonile tiedjei* DCB-1. *J. Bacteriol.* 177: 5135-5139.

[33] Sung, Y., K. M. Ritalahti, R. A. Sanford, J. W. Urbance, S. J. Flynn, J. M. Tiedje, and F. E. Löffler (2003) Characterization of two tetrachloroethene-reducing, acetate-oxidizing anaerobic bacteria and their description as *Desulfuromonas michiganensis* sp. *Appl. Environ. Microbiol.* 69: 2964-2974.

[Received October 12, 2004; accepted February 16, 2005]