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REDUCTIVE DECHLORINATION OF PERCHLOROETHYLENE BY *BACILLUS* SP. JSK1 ISOLATED FROM DRY CLEANING INDUSTRIAL SLUDGE

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Abstract: Perchloroethylene (PCE) is a major environmental pollutant that often persists in the subsurface soil and it highly recalcitrant under aerobic degradation. In this work we have isolated PCE degrading bacterial strain JSK1 from dry cleaning industrial sludge using anaerobic culture technique. Biochemical, physiological and morphological studies showed that the organism is rod shaped, highly motile, endospore forming, catalase positive and gram positive. 16S rRNA sequence of the strain showed a homology of 99% with *Bacillus* sp. IMR-B317 and confirmed as *Bacillus* sp. The strain JSK1 is able to tolerate PCE at concentration as high as 10 mM and degradation rates up to 2 mM. The rate of degradation of PCE via Trichloroethylene (TCE) to *cis*-1,2-Dichloroethylene (DCE) was 2.7 µmol of PCE·h^{-1} mg (dry weight) of $cells^{-1}$. The effect of electron donors for PCE degradation was studied with various electron donors instead of yeast extract. The organism showed the ability to degrade PCE in the presence of acetate, pyruvate, lactate, formate and glucose. The effectiveness of strain JSK1 in the biodegradation of PCE was observed under variable pH and temperature conditions. The maximum degradation was observed at pH 7.5 and temperature 30ºC respectively. These results indicated that, the strain JSK1 have great potential for remediation of soils contaminated with PCE.

Key words: *Bacillus* sp., Enrichment culture, Electron donors, Industrial sludge, Perchloroethylene

1. INTRODUCTION

Perchloroethylene (PCE) is a volatile organic compound that is extensively used as dry cleaning fluid, fumigant, industrial degreasing solvent and in variety of other applications. According to United States Environmental Protection Agency (US-EPA), PCE is a major pollutant under the list of 14 volatile organic compounds (Carter and Jewell, 1993) and the International Agency for Research on Cancer (IARC) has classified PCE as a group 2A carcinogenic compound. Like many chlorinated hydrocarbons such as polychlorinated biphenyl (Ganesh-Kumar et al., 2010), PCE can cause dizziness, nausea, headache, central nervous system, depression, spontaneous abortions and death (Kyyronen et al., 1989; Rowe et al., 1952; Stewart et al., 1970).

Microbial dechlorination is an effective and cheapest process that can be used for degrading chloroethenes present in contaminated environments. PCE is persistent under aerobic condition, because it is in a highly oxidized state (Fogel et al., 1986; Oldenhuis et al., 1989). Recently it has been reported that *Pseudomonas* sp. can degrade PCE aerobically (Jebakumar Solomon & Raymond, 2008). PCE dechlorination was predominately occurs under anaerobic condition and dechlorinating anaerobic bacteria can be used for more intensive clean-up of polluted sites (Yager et al., 1997). The rate and extent of in situ dechlorination are controlled by various interacting hydrological and geochemical parameters such as temperature, pH, organic matter content, redox potential, nature of the organic matter and the availability of electron donors (Distefano et al., 1992).

Dehalococcoides spp. is considered as the most important genus for the complete reductive dechlorination of chloroethenes (Maymo-Gatell et al., 1997). Several anaerobic bacteria were involved in the partial dechlorination and converted PCE to TCE (Egli et al., 1988; Fathepure & Boyd, 1987). The partial dechlorination of PCE to cis-DCE has also reported in various bacteria such as *Dehalobacter restrictus* (wild et al., 1996), *Desulfitobacterium* spp. (Gerritse et al., 1996). The reason behind partial dechlorination of PCE by some anaerobic microbes is not yet clearly understood, while others effect

complete dechlorination.

Limited numbers of organisms were investigated till now for PCE degradation this due to recalcitrant nature and unfavorable environmental condition, efficient organism is needed to remediate recalcitrant pollutants and able to tolerate the stressed environments. The objective of the present study is to investigate the capability of bacterium screened from dry cleaning industrial sludge. The ability of isolated organism to degrade PCE was estimated at various conditions and phylogeny of the isolate was studied. The effect of various electron donors, pH and temperature in the PCE degradation was also studied.

2. MATERIALS AND METHODS

2.1 Sampling site

The bacterial strain used in this study was isolated from a dry cleaning industrial sludge in southern India. Soil sample was collected at a depth of 30-60 cm using sterile screw capped bottle which was then sealed with anaerobic bag for transportation and stored at 4ºC before analysis.

2.2. Enrichment cultures

1 g (on a wet weight basis) of soil sample was added to 160 ml sterile serum bottle and filled with 100 ml minimal medium containing 2 g of NH_4Cl; 0.05 g of $CaCl_2.2H_2O$; 0.1 g of $MgCl_2.6H_2O$; 0.4 g of KH_2PO_4; 1.2 g of K_2HPO_4 and 1 g of yeast extract in 1000 ml of deionized water (pH 7.5). The head-space of the bottle was flushed with nitrogen gas for 15 min. The bottle was then covered with Teflon-Coated rubber stopper and aluminum crimp seal to maintain an anaerobic environment. PCE was injected into the medium at final concentration of 2mM using a Hamilton liquid-tight syringe. The bottle was incubated in inverted position in the dark at 30ºC for 30 days.

2.3. Isolation and characterization of the bacterial strain

After 30 days of incubation, the enrichment culture was diluted 10 fold with sterile minimal medium and smeared on minimal agar plates. Sterile cotton was packed into a micro tube (0.2 × 20 mm) and saturated with appropriate concentration of PCE, and then saturated tube was fixed inside the agar plate lid (Tokunaga et al., 1998). Thus the growing bacteria were continuously exposed to PCE vapor and the plates were incubated in an anaerobic incubator (VWR A-141-2, USA) at 30ºC for a week. After incubation, the isolates were repeatedly streaked and the pure cultures were analysed for the ability to degrade PCE as described above. The isolate that efficiently degraded PCE was named as JSK1 and morphological and biochemical characterization of the strain JSK1 was performed according to Bergey's manual (Holt et al., 1994).

2.4. 16S rRNA phylogenetic characterization of the isolate

Genomic DNA was isolated from strain JSK1 as described by Ausubel et al., (1995). The 16S rRNA gene was amplified using universal primers 27F 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R 5'-GGTTACCTTGTTACGACTT-3'. PCR was performed using a Bio-Rad MyCycler (Bio-Rad, USA). The purified 16S rRNA gene was sequenced using Applied Biosystems 3730XL DNA Analyzer with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The phylogenetic analysis was performed with RDP-II database (Cole et al., 2003), CLUSTAL W by Neighbor Joining method and PHYLIP 3.68 (Felsenstein, 2008) with bootstrapping over 1000 replicates. The phylogenetic tree was viewed with Tree View.

2.5. PCE dechlorination by strain JSK1

To study PCE dechlorination by the strain JSK1, the cells were grown in nutrient broth and bacterial cells were harvested during mid-logarithmic growth phase by centrifuging at 8000xg for 10 min at 4ºC (Tallur et al., 2009). The pellets were washed three times with 50mM phosphate buffer, pH 7.0 and resuspended in same buffer. 100 ml of a sterile minimal medium was dispensed into sterile 160 ml serum bottles and followed by 5% of cell suspension and in control heat killed cells were used as inoculum. The head-space of the bottles was flushed with nitrogen gas for 15 min, which were then covered with Teflon-Coated rubber stopper and aluminum crimp seal. The final concentration 2 mM PCE was added aseptically to all the bottles and incubated at 30ºC in the dark for one month without shaking. The concentration of PCE was monitored at various time periods. Experiment was performed in triplicates. Similarly, PCE toleration limit was studied at various PCE concentrations ranging from 10 μM to 10 mM (Bryant, 1972).

2.6. Effect of electron donors on PCE dechlorination

Electron donor plays a vital role in reductive

dechlorination of chloroethenes by anaerobes. Reductive dehalogenation involves the removal of a halogen substituent from a molecule with concurrent addition of electrons to the molecule, which requires electron donor (Mohn & Tiedje, 1992). In this study the effect of various electron donors on PCE dechlorination by strain JSK1 was performed. The following electron donors were added instead of yeast extract in the minimal medium: 20 mM each of acetate, formate, glucose, lactate, pyruvate, butyrate, propionate, citrate, succinate, methanol and ethanol.

2.7. Effect of pH and temperature on PCE dechlorination

In order to determine the effect of pH and temperature on dechlorinating potential of strain JSK1 on PCE, the strain was cultured in nutrient broth at 30°C for 24 h. The cells were collected by centrifugation at 8000xg for 10 min (4°C) and washed twice with 50 mM phosphate buffer (pH 7.0) and resuspended in different serum bottles with 100 ml minimal medium at different pH levels; 5, 5.5, 6, 6.5, 7, 7.5, 8, 9, and pH 10 using predetermined amounts of 1 mol/L HCI or 1 mol/L NaOH. PCE was added at final concentration of 2mM in each bottles and incubated at 25, 27, 30, 33, 35 and 35 °C for a week in darkness. After incubation, CFU (colony forming unit) and degradation results of PCE were monitored.

2.8. Gas chromatographic analysis

PCE degradation was analysed using gas chromatography (GC) equipped with flame ionization detector (FID) and DB-5 column. The operating conditions were: nitrogen as a carrier gas, flow rate 30 ml / minute, column temperature for 2 minutes at 35°C, then increased to 140ºC at a rate of 6 °C / minute. 100 µl of gas sample from the headspace of the culture bottles were drawn and injected manually using a Hamilton gas tight syringe (USA). Standard calibration curves were prepared for the quantification of PCE and its metabolites (Gossett, 1987).

3. RESULTS AND DISCUSSION

3.1. Isolation and identification of PCE dechlorinating strain

Five different bacterial colonies were identified from the enrichment culture of the dry cleaning industrial sludge. Pure cultures were grown on minimal agar plates exposed to PCE vapor. Among the five bacteria, only one grew well and was named as JSK1. Morphological and biochemical Characteristics of the isolate JSK1 was examined and results were summarized in the table 1. Based on the morphological and biochemical properties, strain JSK1 was assumed to be a *Bacillus* sp.

Table 1. Result of additional morphological, physiological and biochemical characters of strain JSK1

Experimental details	Observations
Gram stain	Positive
Shape	Rods
Arrangement	Single and in chains
Endospore	Positive
Motility test	Positive
Nitrate reduction	Negative
Indole production	Negative
Methyl red test	Positive
Voges-Proskauer test	Positive
Citrate utilization test	Positive
Gelatin hydrolysis	Positive
Triple sugar iron agar	Negative
Casein hydrolysis	Positive
Catalase activity	Positive

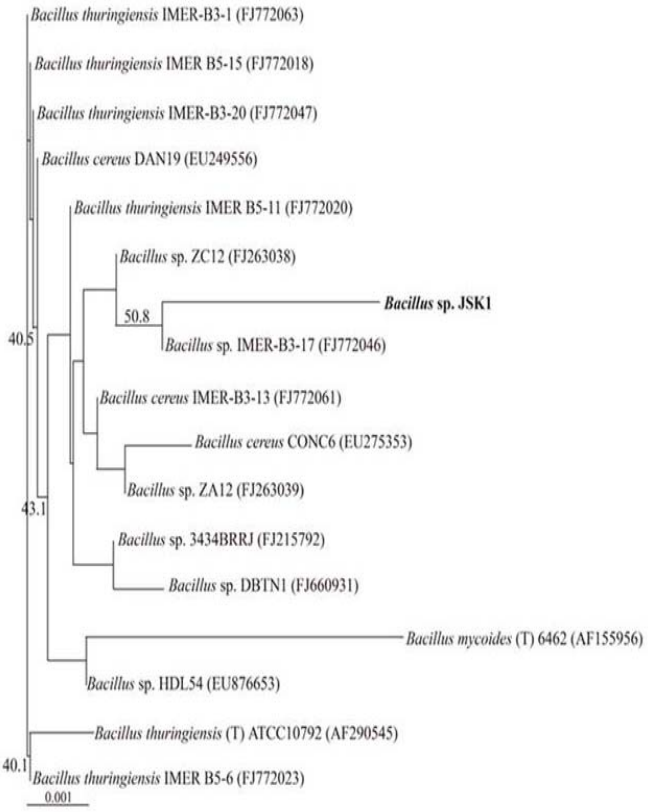

Figure 1 Neighbor-joining phylogenetic tree views of *Bacillus* sp.JSK1. The topology of tree was analyzed by bootstrapping with 1000 replicates 0.001 scale bar indicates the nucleotide substitution level.

3.2. 16S rRNA typing of the isolate JSK1

16S rRNA typing of JSK1 was performed and the phylogenetic analysis of the 16S rRNA gene sequence revealed that the isolated strain JSK1 belongs to the genus *Bacillus* sp. It has 99% similarity to the already identified 16S rRNA gene sequence of the *Bacillus* sp.IMR-B317 (FJ772046) in the nucleotide database (Fig. 1) and the strain was designated as *Bacillus* sp. JSK1. The 16S rRNA gene sequence (1441 bp) of strain JSK1 was deposited in GenBank, NCBI database (Accession No.: GQ527159).

3.3. PCE dechlorination by strain JSK1

The isolate JSK1 grown in the nitrogen purged minimal medium containing 2 mM of PCE was analysed for its PCE dechlorinating ability. At regular time intervals, the headspace gas from the anaerobic culture was analyzed in Gas Chromatography equipped with Flame Ionization Detector and results are presented in figure 2. The PCE dechlorination data reveals that the isolate JSK1 dechlorinates PCE via TCE to cis-1,2-DCE in 5 days at 30ºC in anaerobic condition. The major end product of PCE dechlorination was cis-1,2-DCE and vinyl chloride (VC) or ethylene were not produced.

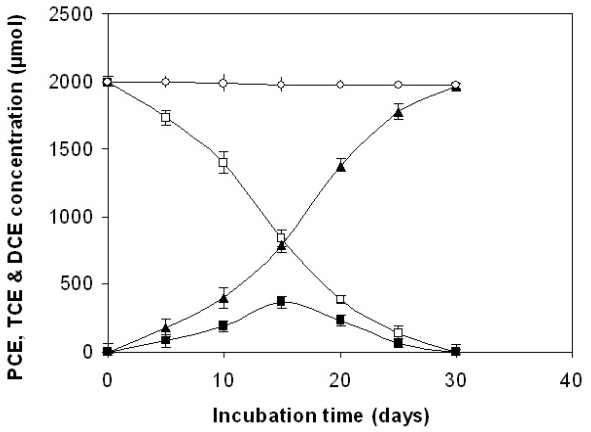

Figure 2. Degradation of PCE during time course experiment, anaerobic enrichment of *Bacillus* sp.JSK1 was incubated at 30°C with 2mM of PCE in the minimal medium. At every 5 days, test samples and controls (heat killed cells) were analyzed. Values plotted are means ± standard deviations for triplicate cultures. Symbols: control (○), PCE (□), TCE (■) and *cis*-1,2 DEC (▲)

Krumholz (1997) isolated a strictly anaerobic bacterium that can grow only in medium containing PCE and acetate or pyruvate, has tolerance level of 100 µM. The PCE toleration study shows that the isolated strain JSK1 is able to withstand up to 10 mM of PCE concentration. But at concentration above 2 mM, PCE dechlorination did not occur even after 30 days of incubation and production of TCE or DCE was not observed in autoclaved controls. According to the previous reports (Egli et al., 1988; Fathepure et al., 1987), the sulfate reducing and methanogenic bacteria dechlorinate PCE at very low rates (<0.001 µmol of PCE h^{-1}). PCE dechlorination data denotes that JSK1 can transform up to 2 mM of PCE to cis-1,2-DCE at the rate of 2.7 µmol of PCE·h^{-1}·mg (dry weight) of $cells^{-1}$. This PCE dechlorination study indicates that the isolated strain JSK1 can withstand high concentrations of PCE and is an efficient PCE dechlorinator.

3.4. Effect of electron donors for PCE degradation

Strain JSK1 was grown anaerobically with various electron donors instead of yeast extract. Previous reports suggest that electron donors play important role in PCE dechlorination. For PCE dechlorination, *Dehalococcoides* spp., utilizes hydrogen is an electron donor (Maymo-Gatell et al., 1997), where as, *Desulfidobacterium* spp. uses formate and pyruvate as electron donors (Miller et al., 1997). The strain JSK1 utilizes electron donors such as acetate, pyruvate, lactate, formate, glucose (Table 2). The results suggest that the isolated strain JSK1 is a versatile PCE dechlorinator which can utilize various electron donors for dechlorination.

Table 2. Effect of electron donors on dechlorination activities of strain JSK1

Electron donors	Strain JSK1
Pyruvate	+
Lactate	+
Butyrate	-
Propionate	-
Citrate	-
Formate	+
Acetate	+
Succinate	-
Methanol	-
Ethanol	-
Yeast extract	+
Glucose	+

3.5. Effect of pH and temperature on PCE dechlorination

In order to study the effect of pH on PCE biodegradation, a wide range of pH levels (5 - 10) were used. At pH 5 and 10, no bacterial growth was observed,

which indicated that high acidic and high alkali environment was inhibited bacterial growth. The pH levels from 6.5 to 7.5 supported the bacterial count as shown by CFU results taken at 107 dilution factor. The dechlorination in PCE at this pH level was due to the biodegradation of strain JSK1. There was a gradual increase in rate of reduction of the compound till pH 7.5 (67.1%), after which it declined till it reaches to the point when there was no more reduction at pH 10. these results indicated that, the growth and PCE degradation of strain JSK1 were maximum at pH.7.5 (Fig. 3). Similar study on the PCE dechlorinating strains of *Enterobacter, Desulfidofacterium* were observed maximum degradation in the pH range of 7.0 to 7.5 (Yoshida et al., 2007; Sharma and McCarty 1996).

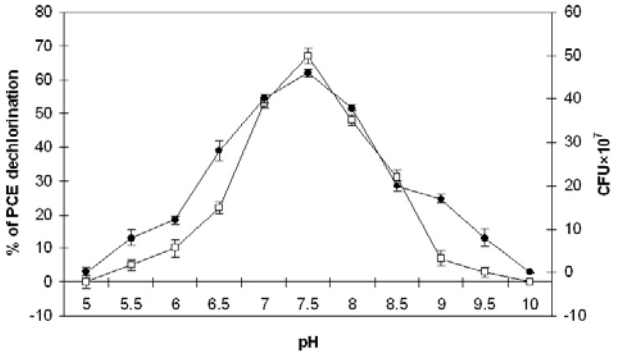

Figure 3. Effects of different pH levels on degradation of PCE by *Bacillus* sp.JSK1 after five days of incubation

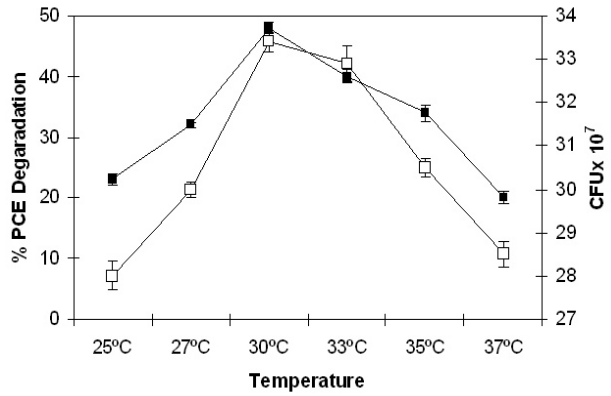

Figure 4. Effects of different temperatures on degradation of PCE by *Bacillus* sp.JSK1 after five days of incubation.

Effect of PCE degradation and growth of strain JSK1 were observed at various temperature ranges from 25 to 37°C. Degradation and growth of isolate were gradually increased when the temperature was increased. This was indicated that, the temperature play major role in growth and degradation process. The maximum PCE degradation and growth rate were observed at 30°C (Fig. 4). In addition, when the temperature was increased from 30 to 37°C, the PCE degradation and growth were reduced. These results suggested that, the optimum growth and degradation were at 30°C. A similar study on the dechlorination of PCE by *Desulfitobacterium* sp. observed that 30°C was the optimum temperature for dechlorination (Yoshida et al., 2007)

4. CONCLUSIONS

Bioremediation is an important process for removal of PCE from contaminated environmental sites. PCE is persistent under aerobic degradation; anaerobic degradation is an alternative technique to dechlorinate PCE. Unlike PCE and TCE, cis-1,2-DCE is believed to be non carcinogenic and has a higher U.S. Environmental Protection Agency drinking water maximum contaminant level. In addition, cis-1,2-DCE can be readily mineralized through aerobic co-metabolism. Our present study shows that the isolated bacteria *Bacillus* sp. strain JSK1 is able to transform PCE to cis-1,2-DCE. Some of the strictly anaerobic bacteria require hydrogen as an electron donor for PCE dechlorination. But the strain JSK1 can dechlorinate PCE to cis-1,2 DCE using simple electron donors like glucose, lactate, pyruvate etc. the optimum condition for PCE degradation was observed at pH 7.5 and temperature 30°C. From this study, it is inferred that the isolated strain JSK1 is an effective and versatile organism which can be exploited for the remediation of PCE contaminated environmental sites.

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