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Protein Profile of the Bacterium Capable of Degrading Trichloroethylene

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ABSTRACT

A novel bacterial strain, *Bacillus cereus* 2479 was capable of degrading Trichloroethylene (TCE), grown on TCE containing medium. The total proteome was extracted and profiled through one-dimensional SDS PAGE. TCE has been recognized to be significant environmental pollutants in the soil, ground water and atmosphere. This strain was grown in different minimal medium either with peptone (0.2%) or TCE (0.2%) with peptone. This gel was further examined by densitometer tracing. Nine differentially expressed proteins were found in the bacterium grown in presence of TCE. Out of these nine proteins, three (97.1, 40 and 30 kDa) were found to react immunologically to the antibodies raised against TCE inducible proteins in Western blot analysis. It indicates that some *de novo* proteins were induced in presence of TCE.

Key words: Protein profiling, trichloroethylene, *Bacillus cereus* 2479, densitometer tracing, western blot

INTRODUCTION

Trichloroethylene (TCE) is a chlorinated solvent widely used in many industries including textile, metal processing, electronic, printing, pulp and paper. It is a common ground water pollutant throughout the United States (Amer *et al*., 2008). TCE also affects soil respiration and microbial biomass (Kiyota *et al*., 2006). Currently, there is concern over the presence of this compound of drinking water supplies because they are known to persist over time and many are toxic or carcinogenic or both for experimental animals (Richmond *et al*., 2001; Ma *et al*., 2002). In anaerobic condition, Vinyl Chloride (VC) is produced through TCE degradation which is more toxic than TCE. Bioremediation is one of the environment friendly means of degrading toxic chemicals. Therefore, extensive efforts have been made to study the biodegradation of TCE by bacteria. Although no microbial growth on TCE as the sole carbon source has been reported yet, we were the pioneer in reporting microorganism capable of growing on TCE as the sole carbon source (Dey and Roy, 2009). However, TCE was found to be cometabolize by some ammonia-oxidizing bacterial species and by some bacterial species able to grow on hydrocarbons such as methane (Hazen *et al*., 2009), propane and isoprene (Ewers *et al*., 1990). The uses of *Pseudomonas putida* are capable of degrading TCE in presence of phenol (Ferhan, 2003). *Pseudomonas putida* CEMB 10124 was best degrader of phenol as well as TCE (Ferhan *et al*., 2002). It was reported

that the Toluene Dioxygenase (TDO) enzyme catalyzes the degradation of TCE in presence of toluene of *Pseudomonas putida* F1. This is a multicomponent enzyme in which terminal oxygenase component is an iron-sulfur protein (ISP_{TOL}), has an $\alpha_2\beta_2$ subunit composition. The cellular processes are either regulated or directly carried out by proteins or protein complexes.

The aim of the study is to find out the proteins that are involved in TCE degradation in *Bacillus cereus* 2479. In the present work, we describe the changes in protein profiles of *B. cereus* 2479 which occur when cells grown in presence of TCE. A set of new genes can be express either some stress condition or altered condition.

MATERIALS AND METHODS

Bacterial strain: The strain 2479 was isolated from the soil of industrial belt, situated at Rajbandh (West Bengal, India) in 2006 where the use of polychlorinated hydrocarbons (including TCE) is abundant. The complete identification of the isolated organism was done by the polyphasic approach and ribotyping was classified as *Bacillus cereus* strain 2479 (Dey and Roy, 2009; Mitra and Roy, 2010).

Growth conditions: The *Bacillus cereus* 2479 was grown in M9 medium (Na_2HPO_4-6 g; KH_2PO_4-3 g; NaCl-0.5 g; NH_4Cl-1 g; $MgSO_4$-0.002 M; $CaCl_2$ -0.0001 M; Glucose-0.2%; H_2O-1L. pH-7.0). In this experiment, peptone (0.2%) replaced glucose. Further, TCE (0.2%) (Merck, India pvt. Ltd.) was added with peptone for inducible protein analysis. Incubation was done for 2-3 days with shaking at 31°C. The bacterium was grown on TCE with peptone for analyzing the TCE inducible proteins.

Measurement of TCE degradation: *B. cereus* 2479 could degrade TCE efficiently was analyzed by Fujiwara test. To performed this test, TCE (0.2%) with Peptone (0.2%) containing M9 media are inoculated with *B. cereus* 2479 and *E. coli*. The incubation was done for 48 h at 31°C with shaking. Optical density (OD_{470}) was measured at the beginning (just after inoculation) and repeated after 12 h, 24 h, 36 h and 48 h, respectively by UV-VIS Spectrophotometer (UV-1700 Pharma Spec, Shimadzu).

Preparation of cell extracts: The total proteome was extracted and profiled through one-dimensional SDS PAGE. The M9 media containing peptone (0.2%) or with both peptone and TCE (0.2%) grown cells were harvested at 10,000 g for 10 min at 4°C and then the cells denatured with 1% SDS and 1 mM phenylmethylsulfonyl fluoride, boiled it for 3 min.

Protein analysis: The extracted protein samples were boiled with Laemmli buffer (Laemmli, 1970) [1% SDS, 5% Mercaptoethanol, 0.05% Bromophenol blue in 25 mM Tris-HCl, 10% glycerol, pH 6.8] for 10 min and electrophoresed on 12% SDS-PAGE. Protein electrophoretic profile in gel was stained with Coomassie blue R-250 (0.2%) and the bands were scanned using a densitometer. It was calculated the relative mobility of proteins from TCE with peptone grown cells, pattern based on the migration rate of the standard molecular weight marker. Western-blot analysis was done on identical SDS-PAGE (without staining) using antibodies generated as follows:

Preparation of antibodies specific to TCE induced protein: The SDS extracted proteins from bacterial cells grown on TCE (as a sole carbon source) containing M9 medium were injected into

rabbits to obtain polyclonal antibodies. The titre of the antiserum was first determined by Dot-blot in different dilution of anti sera (1:500; 1:1500; 1:2000) by using the spotted antigens. The polyclonal anti-serum was pre adsorbed by nitrocellulose membrane (Sigma-Aldrich) on which total cellular proteins from peptone grown cells was immobilized. Thus the antibodies reacting to the common antigens found in both peptone grown and TCE grown cells were removed. The preadsorbed antiserum was found to react specifically with TCE induced proteins in Western blot.

Dot blot assay: The SDS extracted proteins (cells were grown in peptone and peptone with TCE) and BSA were spotted (in different volumes; 1, 2 and 3 μL) onto three nitrocellulose strips (Sigma-Aldrich Chemical Co, USA) allowed to dry at room temperature. Each the strips were incubated overnight in 3% skimmed milk in buffer- A (10 mM Tris-HCl pH-8.0; 0.9% normal saline; 0.2% Tween 20) to block the residual binding sites on the paper. The strips were rinsed for 10 min in same buffer and then incubated with anti sera diluted 1:500; 1:1500; 1:2000 in buffer for 90 min at 37°C. After incubation, the strips were washed by the use of four changes of washing buffer-A and were further incubated for 90 min at 37°C with a goat anti-rabbit IgG (Sigma-Aldrich Chemical Co, USA) diluted 1:30,000 in buffer-A. The strips were washed four times and stained with BCIP/NBT in alkaline phosphatase buffer-B (100 mM Tris with 5 mM Mg^{2+} at pH 9.5) then allowed to dry.

Western blotting (Immunoblotting): Following electrophoresis, the proteins (without staining) were electrotransferred onto nitrocellulose membrane for 3 h at 20 volts. The membrane was blocked with 3% skimmed milk for 1 h at room temperature and incubated with pre adsorbed antiserum (raised against TCE inducible proteins) for 1 h. After this reaction, methods were same as above (Dot blot assay).

RESULTS AND DISCUSSION

TCE degradation by Strain 2479: Fujiwara test was done to examine that the *B. cereus* 2479 (cells grown either in peptone or TCE with peptone containing medium) could degrade TCE efficiently. Fujiwara test was performed to estimate the concentration of the polychlorinated hydrocarbon in the medium (Moss and Rylance, 1966). In the test, TCE was treated with pyridine in an alkaline environment and the medium was heated. In presence of TCE the red aqueous upper phase was developed and determined at 470 nm by spectrophotometer. The absorbance of the red aqueous phase is proportional to the concentration of free TCE. Figure 1 shows OD value of red upper aqueous phase gradually decreases with time (h) in *B. cereus* 2479 grown in TCE with peptone medium during Fujiwara test. It indicates that the medium contained no free TCE. The plot shows no declination of OD value, in case of normal *E.coli* cells and uninoculated TCE containing medium. TCE concentration was not changed. The mean value and standard deviation regarding data of OD was calculated after triplicate observation. From the observations in Fujiwara test, it can be concluded that the compound, TCE was metabolized by the bacterium *B. cereus* strain 2479.

SDS-PAGE and densitometry: The protein profiles of *B. cereus* 2479 with and without exposure to TCE are shown in Fig. 2a.

Analysis of proteomes revealed that nine new proteins were produced responding to TCE that was not expressed by the same strain in the absence of TCE. The major polypeptides in the TCE

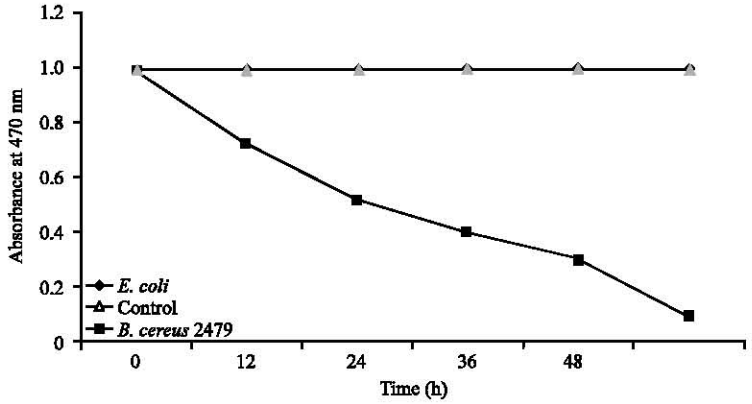

Fig. 1: Optical density of the products of Fujiwara test with *B. cereus* 2479 and *E.coli* cells grown in peptone with TCE and the uninoculated M9 medium (Control) recorded at different time (h) intervals, measured at OD_{470}. The data are means and standard deviations of triplicate reading

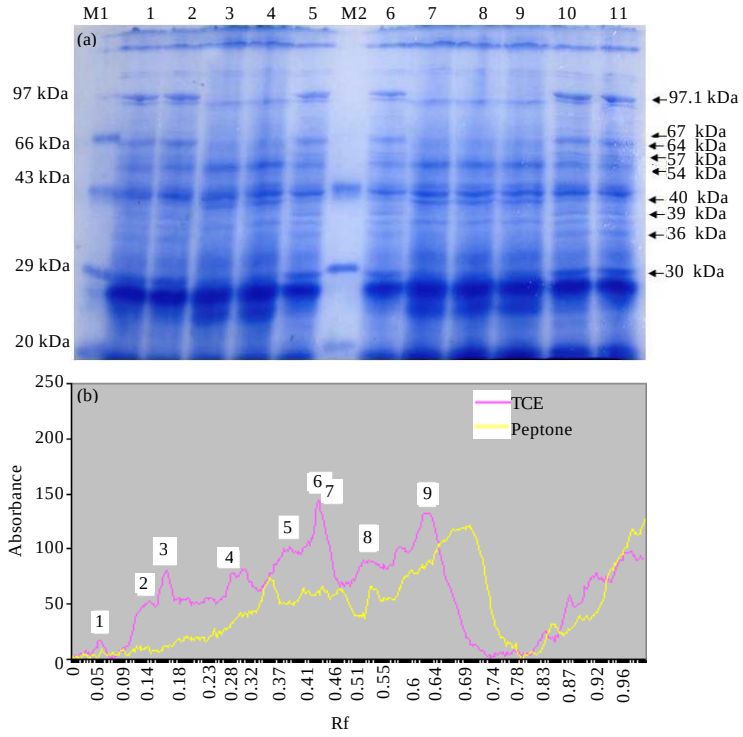

Fig. 2: (a) SDS-PAGE profile of *B. cereus* 2479 grown in peptone or peptone with TCE. Lane 1, 2, 5, 6, 10, 11 shows proteins from cells grown in peptone with TCE and Lane 3, 4, 7, 8, 9 are protein samples obtained from bacterium grown in peptone as a sole carbon source. Lane M1 and M2 are protein molecular weight marker, high and low range, respectively and (b) densitogram of stained proteins from *B. cereus* 2479 grown in peptone and TCE with peptone after separation on a 12% polyacrylamide gel

with peptone grown cells had molecular masses of 97.1, 67, 64, 57, 54, 40, 39, 36, 30 kDa. These nine newly expressed proteins may be involved in TCE degradation. It occurs when the bacteria's medium composition varies even in the same strain and same condition, causing different sets of protein expression (Neto and Yamamoto, 1993). Densitometer tracing of the same gel showed the presence of possible 9 high peaks in TCE containing medium that indicates these were induced in presence of TCE. Such peaks were not found in protein sample extracted from peptone grown cells (Fig. 2b). Hence, it may suggested that alterations in cultural conditions may affected the proportion of the different proteins in the cytoplasm.

Immunoblotting: In Dot blot, a clearly defined blue spot at the both lane (peptone and peptone with TCE grown cells proteins) where the antigens were spotted was considered a positive result and no such spots were found in BSA spotted site, considered a negative result (Fig. 3a). It is concluded that before preadsorbtion of antiserum was found to be reacted with both proteins from peptone and TCE grown cells. In Western Blot studies, three proteins (97.1, 40 and 30 kDa) from *B. cereus* 2479 specifically reacted with pre-adsorbed antiserum against TCE inducible protein (Fig. 3b). It suggested that the three proteins with molecular masses 97.1, 40 and 30 kDa are directly involved in TCE metabolism.

Proteomics or the systematic analysis of the proteins expressed by the genome is not only a powerful tool for describing complete proteomes at the organelle, cell, organ or tissue levels (Porubleva and Chitnis, 2000) but also for comparing proteomes as affected by different physiological conditions, such a those resulting from exposure to TCE or several other environmental factors. It has been reported that when *Escherichia coli* (Jenkins *et al*., 1988; Groat *et al*., 1986) and *Pseudomonas putida* (Givsov *et al*., 1994) *Methylocystis* sp. M. are starved,

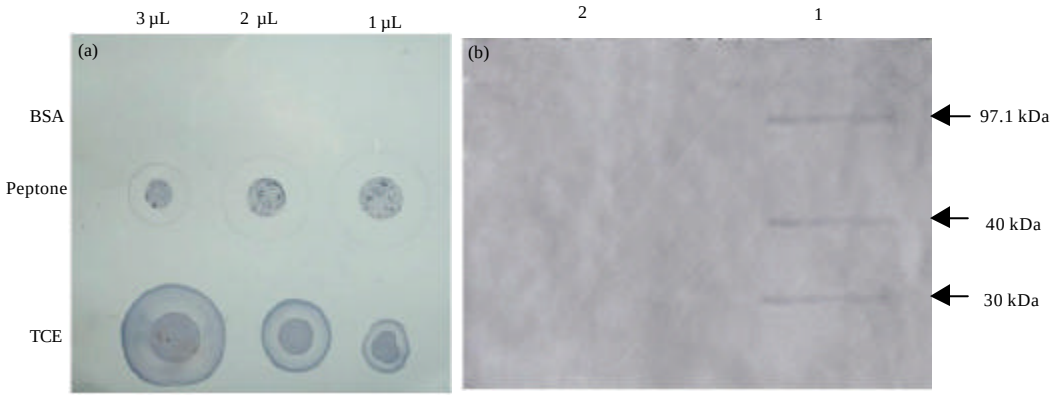

Fig. 3: (a) The antigens were spotted onto each lane, strips were developed by the technique described in Materials and Methods. Lane BSA (negative control) shows no spot, lane peptone and TCE shows spots in different volume (1, 2 and 3 µL). No reaction was found in lane BSA and (b) In Western blot: lane 1 shows specific reaction of anti-serum against TCE inducible proteins with proteins from TCE with peptone grown cells and lane 2 shows no such reaction with proteins from peptone grown cells

the cells acquire enhanced resistance to a variety of stresses, thereby enhancing survival. Even enhancing the amounts of stress proteins induced by the chemicals (especially TCE) may increase the TCE degradation activity and therefore, facilitate enhanced degradation ability.

CONCLUSION

The change of culture media would induce some proteins which may be involved in TCE degradation. It may be suggested that the pattern of protein profile showed differences with exposure to the TCE in polyacrylamide gel electrophoretogram. In this study, the investigations reveal that 9 differentially expressed polypeptides are found in presence of TCE. Three of them are reacted immunologically to the antiserum raised against TCE inducible proteins. It may be concluded that some *de novo* proteins may have been synthesized in *B. cereus* 2479 which involved in TCE degradation.

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