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RESEARCH ARTICLE

Bioremediation and Detoxification of Dioxin-like Compounds by Alkalotolerant Bacterial Consortium

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Abstract

Dioxins are a class of extremely toxic persistent organic pollutants which are known to have carcinogenic, immunosuppressive, endocrine disruptive and teratogenic properties. An alkalotolerant bacterial consortium from pulp and paper mill effluent was developed by continuous enrichment in the chemostat in the presence of 100 mg/l dibenzofuran (DF) as sole carbon source for 180 days. Soil microcosms were set up with DF (10 gram DF/kg of dry soil) to assess the biodegradation potential of the alkalotolerant bacterial community isolated from pulp and paper mill effluent. More than 90% degradation of DF by the enriched bacterial community in the microcosm occurred in 9 days. GC-MS analysis of the soil extract showed formation of various metabolites like Gentisic acid, catechol, 2-OH-HOPDA. The survival of competent bacteria monitored by DGGE showed the presence of *Pseudomonas* sp. strain ISTDF1 which was solely found to be responsible for degradation of 86% DF in the microcosm. Further toxicity assays using Hep G2 cell line showed significant detoxification. Biodegradation ability of the alkalotolerant consortium without the formation of toxic by-products reveals its potential in remediation of soil contaminated with dioxins.

Keywords

Bioremediation, Dibenzofuran, Dioxin-like compounds, Detoxification, Soil microcosms

Introduction

Dioxins are a class of persistent organic pollutants (POPs) known to be a major cause of concern due to their carcinogenic, endocrine disruptive, immunosuppressive and teratogenic properties (Kaiser, 2000; Landvik *et al,* 2007; Ozeki *et al,* 2011). It comprises of polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs). They are widespread in the environment formed as unintentional by-products during municipal incineration, synthesis of herbicides and pesticides and various other industrial activities (Alcock and Jones, 1996; Kuehl *et al,* 1987). Pulp and paper mill effluent is known to be one of the major sources of dioxin contamination (Peck and Daley, 1994). Planar structure, extremely hydrophobic nature and strong sorption to soil makes them highly recalcitrant to degradation and thus very few microbial strains have the capability to metabolize them (Field and Sierra-Alvarez, 2008).

Being a relatively soft carbon source than chlorinated dioxins, nonhalogenated dibenzofuran (DF) has been used as a model compound to study the biodegradation of dioxins. Like dioxins, DF is also a less bioavailable, recalcitrant, hydrophobic molecule with a planar structure. Some of bacterial strains that have been reported for DF degradation include *Pseudomonas* sp. *strain* HH69 (Fortnagel *et al,* 1990), *Brevibacterium* sp. strain DPO 1361 (Strubel *et al,* 1991), *Terrabacter* sp. strain YK3 (Iida *et al,* 2002), *Sphimngomonas* sp. strain HH69 (Harms *et al,* 1995), *Sphingomonas* sp. RW1 (Wittich *et al,* 1992),*Xanthomonas maltophilia* (Ishiguro *et al,* 2000), *Pseudomonas* sp. strain CA10 (Sato *et al,* 1997), *Janibacter terrae* strain XJ-1 (Shiwei *et al,* 2006), *Serratia marcescens* ISTDF2 (Jaiswal and Thakur., 2007). Different modes of initial dioxygenation are involved in the metabolism of DFs (Fortnagel *et al,* 1990). One is lateral dioxygenation, in which one of the aromatic rings is attacked at the lateral 1,2 or 2,3 positions, resulting in the formation of cis-dihydrodiols (Becher *et al,* 2000). The other type of dioxygenation is angular dioxygenation, which takes place at the angular positions 4 and 4a adjacent to the ether bridge (Nojiri *et al,* 2001).

However, application of these microorganisms as tools for in-situ bioremediation of dioxin-like compounds has shown little success (Haack *et al,* 1995).The highly alkaline nature of industrial effluents contaminated with dioxin compounds necessitates the search for alkalotolerant strains that can survive and degrade dioxins in such extreme conditions. Thus gradual adaptation of indigenous bacterial community under increasing alkaline condition could be a useful strategy for efficient in-situ bioremediation of dioxin-like compounds (Kao *et al,* 2001; Guffanti *et al,* 1986). Denaturing gradient gel electrophoresis (DGGE) is being popularly used for

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enumeration, identification and estimation of microbial community structure by using V3 region of 16 S rDNA (Rintala *et al,* 2001; Santegoeds *et al,* 1998; Kohli *et al,* 2011).

Though bioremediation is a promising tool for removal of pollutants from contaminated sites, but removal of contaminants may not always correspond to a reduction in health risk (Lemieux *et al,* 2009). In some cases substantial increase in toxicity following bioremediation have also been documented (Hughes *et al,* 1998; Gillespie *et al,* 2007). This may be either because of formation of toxic intermediary metabolites or increased bioavailabilty of native toxins over the course of bioremediation (Andersson *et al,* 2009). *In-vitro* models using human cancer cell lines have become well established for rapid and accurate evaluation of toxicity at acute, chronic and sub chronic levels with fair reproducibility (Chang *et al,* 2007; Tai *et al,* 1994). It has already been reported that DF binds to Aryl hydrocarbon receptors (AhRs) and induces cytochrome P450 monooxygenase (CYP) activity especially CYP1A2. Hepatic carcinoma cell line (Hep G2) has been shown to be very promising for detoxification studies of dioxins because of their ability to synthesize enzymes (CYP) especially CYP1A2 (Chaloupka *et al,* 1994).

The present investigation was designed to evaluate the potential of alkalotolerant bacterial consortium for degradation of DF in soil microcosm and to study the detoxification efficiency of the community using HepG2 cell line.

Materials and Method

Chemicals

Dibenzofuran and N, O-bis(trimethyl-silyl) trifluroacetamide (BSTFA) and all cell culture related reagents, i.e. Dulbecco's minimal essential medium (DMEM), fetal bovine serum, sodium pyruvate, non-essential amino acids, penicillin, streptomycin and trypsin/EDTA solutions were procured from Sigma–Aldrich (India). All solvents and other chemicals (GC–MS grade) were purchased from Merck, India.

Sampling and Sampling Site

Sediment core together with liquid effluent was collected (1:10 w/v) from effluent discharging canals and premises of Century pulp and paper mill, Lalkua, Nainital, Uttaranchal, India. A composite sample was prepared and stored at 4^C.

Microorganism and Culture Condition

Microorganisms were extracted from composite sample by centrifugation at 900 rpm for 10 min. The supernatant was used for enrichment in mineral salt medium in the chemostat.

Enrichment of microorganisms

A chemostat culture was set in a 2-l glass vessel, effective volume 1 l, with culture condition as stirring at 150 rpm; temperature at 30°C and pH 7 in the mineral salt medium (MSM). The composition of MSM (g/l) was: Na_2CO_3, 4; $Na_2HPO_4{\cdot}2H_2O$, 7.8; KH_2PO_4, 6.8; $MgSO_4$, 0.2, ammonium ferric acetate, 0.01; $Ca(NO_3)_2.4H2O$, 0.05; $NaNO_3$, 0.085, trace element solution with dibenzofuran(100 mg/l) as described (Jaiswal and Thakur, 2007). Dibenzofuran was dissolved with DMSO (100mg/l) in MSM (Gai *et al,* 2007). The bacterial cell population of supernatant served served as inoculums in the chemostat. The utilization of dibenzofuran in the chemostat was monitored by Gas Chromatography. Samples from the chemostat were removed under aseptic conditions and used for determination of the growth of bacterial cells and utilization of carbon source. After stabilization of community, MSM with pH 8 having 100 mg/l dibenzofuran was introduced in chemostat. Community stabilization was determined again by growth of the bacteria and utilization of dibenzofuran. This process was repeated for pH 8 to 9 and pH 9 to 10 too.

Isolation of alkalotolerant bacterial strains from chemostat

Since alkalotolerant microbial species was desired to be isolated, microbial cells from chemostat of pH 10 was diluted and streaked on MSM agar plates containing 100 mg/l DF. Morphologically distinct species were restreaked on different MSM agar plates till pure colonies were isolated.

Survival pattern and growth of the different bacterial strains

Bacterial cells were cultured from culture medium on LB-agar plates. Morphologically distinct isolates were grown in LB followed by MSM having dibenzofuran (1mM). Survival pattern for every colony were drawn on the basis of absorbance at 595nm on spectrophotometer Cary, 100 Bio (Varian Co., Australia) as described by Fortnagel *et al,* 1990.

Identification of bacteria by 16S rDNA method

Genomic DNA of pure bacterial strain showing degradation potential was isolated with the Genome DNA Kit (Qiagen Inc., USA) as described by the manufacturer. The 16S rDNA was selectively amplified from genomic

DNA by using PCR with oligonucleotide universal primers Eubac 27F, and Eubac 1495R. PCR amplification were done in thermal cycler under the following conditions: 10–20 ng template DNA, 5 µl 10x reaction buffer, 2·5 U Taq DNA polymerase, 1 µM forward primer, 1 µM reverse primer, 200 µM of each dNTP and H_2O combined in a total volume of 50 µl. The tubes were incubated at 94°C for 5 min and then subjected to the following thermal cycling programme: denaturation at 94°C for 2 min, primer annealing at 55°C for 30 sec, and chain extension at 72°C for 1 min with an additional extension time of 10 min on the final cycle, for a total of 30 cycles (Moore *et al,* 1993). The amplified DNA was purified using Qiaquick PCR Purification Kit (Qiagen) and sent to the M/S Banglore Genei, India for sequencing. Sequenced data was analysed and compared with existing database of Gene Bank, National Centre for Biotechnology Information. A phylogenetic tree was drawn by multiple sequence alignment with different species of bacteria through neighbor joining method using software Mega, version 3.1 for identification of the bacterial strain (Srivastava and Thakur, 2012).

Soil Microcosm and its composition

Microcosms were set up in sealed plastic jars according to Gautam *et al,* 2003. In microcosm, 100 gram of small grits were placed on bottom followed by 100 gram sand and on surface 20 gram of soil was placed. Soil was dried at 50°C for 3 days, sieved through a 0.4 cm sieve and sterilized in small packets by autoclaving three times on consecutive days at 121°C for 60 minutes. Sterile distilled water was added to the soil to reach final moisture content of 40% (volume/weight). Crystalline dibenzofuran (10 g DF/kg of dry soil) was added to the soil microcosm.

Table 1: Different Combination used in Microcosm Experiments

No.	Description
1	Control with Unautoclaved soil
2	Control with Autoclaved soil
3	Unadapted Bacterial Community + Unautoclaved soil
4	Unadapted Bacterial Community + Autoclaved soil
5	Bacterial Community Adapted at pH 7.0 + Unautoclaved soil
6	Bacterial Community Adapted at pH 7.0 + Autoclaved soil
7	Bacterial Community Adapted at pH 10.0 + Unautoclaved soil
8	Bacterial Community Adapted at pH 10.0 + Autoclaved soil
9	Strain C adapted at pH 10.0 + Autoclaved soil

Nine microcosms were prepared as summarized in Table 1. Microcosms were incubated at 30°C. After 0, 3, 6 and 9 days, samples were collected from microcosms for extraction and analysis of metabolites of dibenzofuran and for toxicological evaluation of intermediary metabolites.

Sample extraction and GC-MS analysis

Samples were dried for 16 h at room temperature and sieved (2 mm). Before extraction, an ortho-ter-phenyl acetone solution was added to 2 g of sieved, dried soil as a surrogate internal standard. The sample was extracted five times in an ultrasonic sonication (15 min for each extraction) with 10 ml of dichloromethane-acetone (1:1, vol/vol), and the extracts were pooled to obtain the total organic extract. The extracts were divided into two parts and both parts were dried over Na_2SO_4 and concentrated in a rotary evaporator (Vinas *et al,* 2005). First part was reconstituted with 1 ml of DMSO for toxicological analysis while the other was dissolved in ethyl acetate for GC-MS analysis.

The analysis was done using a using a Trace 2000 gas chromatograph (GC-Perkin- Elmer Instruments Model Auto System XL) equipped with a capillary column DB5 (dimension 0.25-mm film thickness × 0.25-mm internal diameter × 30 m in length). One microliter of each extract was analyzed by GC at condition (splitless mode; initial temperature 80°C for 1.5 min; temperature increased 80–230°C at a rate of 20°C min^{-1} and 230–250°C and kept it at 250°C for 4.5 min) (Iida *et al,* 2002). The concentration was derived from a standard plot between peak area and concentration of dibenzofuran as shown with GC with Flame ionizing detector (FID).

Toxicological analysis

Cell culture

The HepG2 cells were cultured at 37ˆC in a 5% CO_2 incubator with DMEM (Sigma), supplemented with 10% fetal calf serum, l-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 mg/ml). The cells formed a monolayer at confluence. Trypsin (0.25% (v/v) of media) with EDTA solution was used to detach the cells from the culture flask for plating and passing the cells as described previously (Jahroudi *et al,* 1990). The HepG2 cell cultures were maintained for 12 h in a 75-cm^2 surface area, 250 ml tissue culture flasks in 5% CO2 incubator at 37ˆC before various treatments.

Determination of cell number and viability

Cells and media were collected from tissue culture plates. Cell number was measured using a haemocytometer after trypsinization. Cell viability was assessed by the

traditional typan blue assay in 6 well plates as described by Tai *et al,* 1994.

Results and Discussion

Identification and Characterization of bacterial strains enriched in chemostat for the degradation of DF

For isolation of alkalotolerant bacterial species, the enriched bacterial community from chemostat maintained at pH 10 was streaked on MSM agar plates containing 1mM DF. Only six strains had the ability to survive on MSM plates containing DF as the sole carbon source. Each strain was designated as strains A, B, C, D, E and F. For growth curve and utilization studies of DF, each strain was grown in the presence of DF (1mM) with 5% inoculum in MSM broth. In this study, Strain C showed the maximum utilization (79%) followed by Strain A (28%); Strain B (23%); Strain D (16%); Strain E (16%) and lowest utilization was shown by Strain F (only 11%) in 36 hours (Fig. 1). Strain C was identified as *Pseudomonas* sp. strain ISTDF1 (gene bank accession number- EU834943) by 16S rDNA analysis (Jaiswal *et al,* 2011).

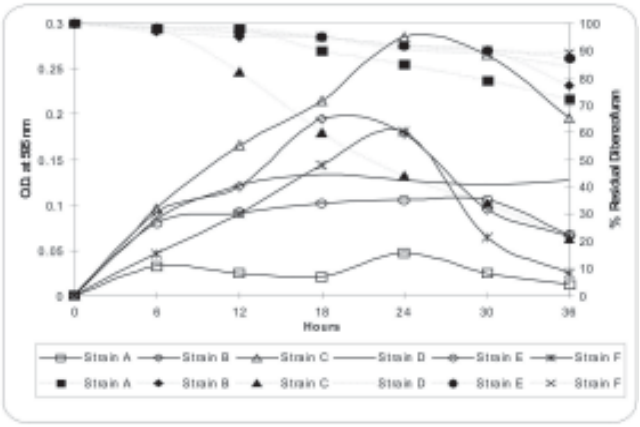

Fig.1. Growth of different bacterial strains in MSM containing 1mM DF as sole carbon source. At left y-axis, bold line represents bacterial growth measured as O.D. at 595 nm, at right y-axis, dotted line represents % degradation of dibenzofuran and at x – axis time (in hours) before adaptation on chemostat.

Biodegradation of DF in soil microcosm and GC-MS analysis

The soil samples were collected from soil microcosms containing 10gm DF/kg dry soil at intervals of 0 (T0), 3 (T3), 6 (T6) and 9 days (T9). The zero day sample is taken as control and compared with 3, 6 and 9 days of sample by GC-MS. The percentage degradation in different sets of microcosm has been summarized in Fig.2. From the figure it can be inferred that in control with unautoclaved soil there was almost no degradation and only 8%

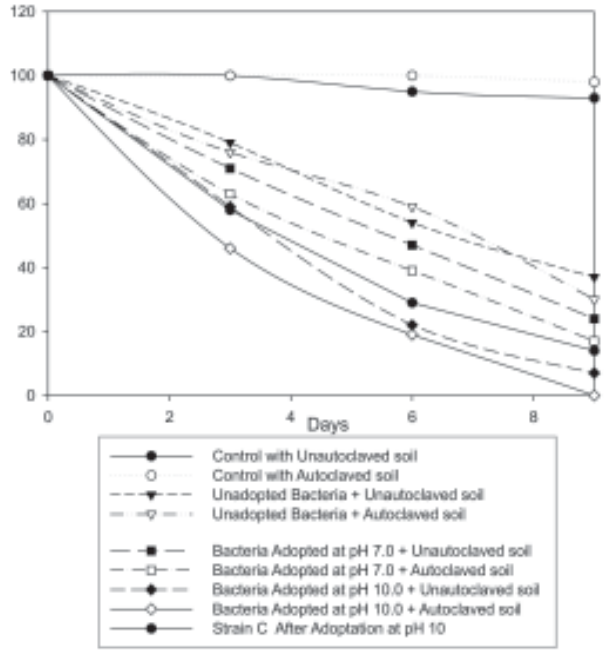

Fig. 2 .Degradation of Dibenzofuran under different plans in microcosm along with controls.

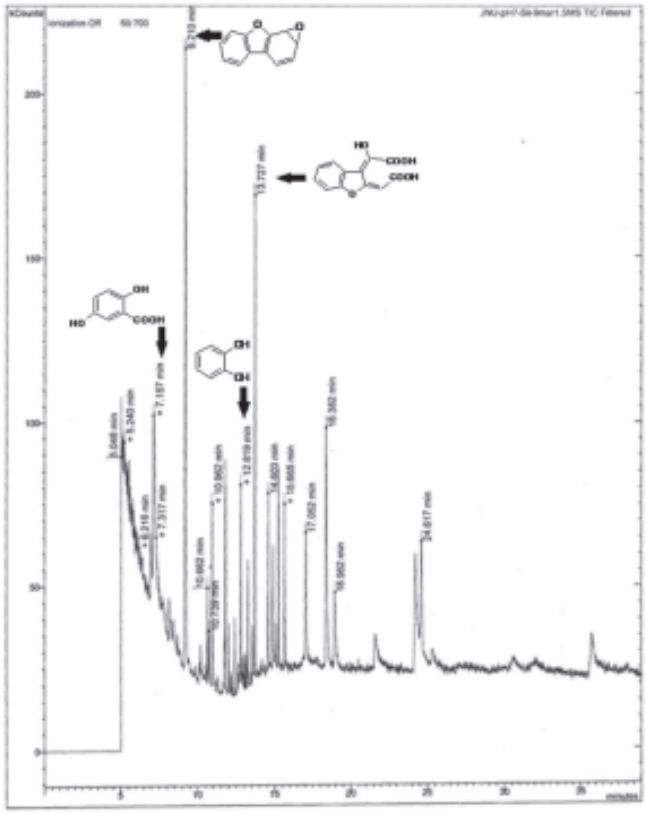

Fig.3. Gas chromatogram showing Dibenzofuran metabolites after 9 days of treatment with alkalotolerant microbial consortium

degradation in control with autoclaved soil. In 3rd,4th,5th,6th,7th and 8th plans, DF is degraded by 63%,70%,76%,83%,93%,96% and 86% respectively. Thus maximum degradation of DF was obtained after treatment with alkalotolerant microbial consortia. However, *Pseudomonas* sp. strain ISTDF1 alone was responsible for degradation of very high percentage (86%) of DF in the microcosm. The degradation products analysed through Gas chromatogram (Fig.3) with various metabolites formed after treatment with microbial consortia after 9 days has been shown. It can be inferred from this data that DF was degraded into many and diverse metabolites by the alkalotolerant consortia. On the basis of formation of intermediary metabolites like Gentisic acid, 2-OH-HOPDA and Catechol which are signature metabolites of angular dioxygenation and formation of 3t-HOBB which is a representative metabolite of lateral dioxygenation pathway, involvement of both the pathways for degradation of DF is proposed.

Cell viability

Cell count of HepG2 was found to attain the normal course as positive control (DMSO) after

toxicity attenuation by the alkalotolerant community after 9 days treatment as shown in Fig.4. Thus the treatment of 100mg/l of DF with alkalotolerant microbial consortium for 9 days in soil microcosm was efficient in reducing the toxicity of DF.

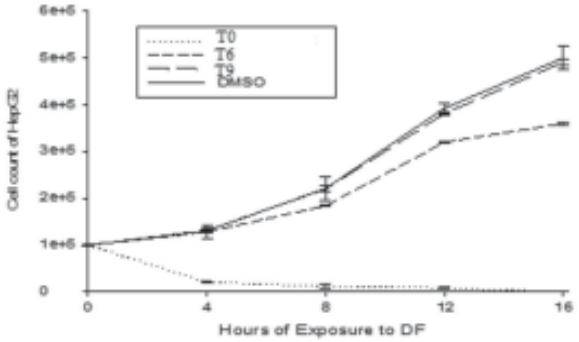

Fig.4. Cell count of HepG2 cells after 16h exposure with alkalotolerant bacterial community adapted at pH 10. T0, T6and T9 represent 0 day, 6 day and 9 day treated DF in microcosm respectively; whereas DMSO (without DF) was taken as positive control. Viability was estimated using trypan blue by hemocytometer. Results are expressed as the mean ± S.D. of the sample.

Conclusion

Microbial consortia was isolated from pulp and paper mill effluent and enriched in chemostat with gradually increasing pH from 7 to 10 for 180 days. The microbial community adapted at pH 10 showed maximum degradation (96%) of DF in soil microcosm in 9 days. Out of this 96%, alkalotolerant *Pseudomonas* sp. strain ISTDF1was solely responsible for degradation of 86% DF in the soil microcosm. This study indicates that the enriched alkalotolerant community is competent enough to survive and degrade dioxin-like compounds in *in-situ* conditions. Biodegradation along with detoxification reveals the potential of the community to be used for the remediation of soil contaminated with dioxin-like compounds.

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