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CHAPTER [6]

Alkalotolerant consortium as a potential degrader of dioxin-like compounds of pulp and paper mill wastewater

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ABSTRACT

Nowadays, the industrialized world is confronted with the contamination of soils, water sources, and air with hazardous and toxic xenobiotics. Polycyclic aromatic hydrocarbons (PAHs) are toxic pollutants that have accumulated in the environment due to a variety of anthropogenic activities . In this study, an alkalotolerant bacterial consortium was developed by continuous enrichment in the chemostat in presence of dibenzofuran (DBF) as sole carbon source. Six different types of bacterial isolates were isolated on agar plates. Among the six isolates tested for degradation of DBF, strain C of alkalotolerant bacterial community had better potency to degrade dibenzofuran. Alkalotolerant bacterial consortia introduced in soil microcosm for evaluation of survival of most suitable isolates and degradation of dioxin-like compound indicated more than 90% degradation of dibenzofuran after 45 days by the bacterial consortia enriched for 180 days in the chemostat at pH 10, however, microbial community, not enriched in the chemostat, was not competent to utilize even 50% DBF after day 30. This suggests that the microbial community adapted for a longer time in chemostat had better efficiency for the degradation of DBF. Degradation of dibenzofuran in soil microcosm indicates that the community is competent enough to survive and retain its degrading potency even *in*-situ conditions.

KEYWORDS

Alkalotolerant, Dibenzofuran, Consortia, Degradation, Microcosm

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Introduction

Pollution of the environment has been one of the largest concerns for science and the general public in the last years. Nowadays, the industrialized world is confronted with the contamination of soils, water sources, and air with hazardous and toxic xenobiotics. Polycyclic aromatic hydrocarbons (PAHs) are toxic pollutants that have accumulated in the environment due to a variety of anthropogenic activities (Barkovskii and Adriaens, 1996). Bioremediation using various microorganisms is one of the approaches tested for the removal of PAHs from the environment. Bioremediation is a process by which living organisms degrade or transform hazardous organic contaminants to less toxic compounds (Arun *et al*., 2008). Halogenated dibenzo-p-dioxin and dibenzofuran are the most toxic group of persistent organic pollutants (POPs) having carcinogenic, immunosuppressive, endocrine disruptive and teratogenic properties (Adriaens *et al*., 1996; Mandal, 2005). Dioxins are an unintentional by-product of various industrial activities. Pulp and paper industry is one of the major sources for dioxin contamination (Thacker *et al*., 2007). Very few microbial strains have the capability to metabolize dioxin-like compounds due to its planar structure, acute hydrophobicity, less bioavailability (Nojiri and Omori, 2002). Many bacterial strains, Pseudomonas sp. strain HH69 (Fortnagel *et al*., 1990); *Brevibacterium* sp. strain DPO 1361 (Strubel *et al*., 1989; Strubel *et al*., 1991), Sphingomonas sp. strain RW1 (Wittich *et al*., 1992), *Pseudomonas aeruginosa* and *Xanthomonas maltophilia* (Ishiguro *et al*., 2000), Terrabacter sp. strain YK3 (Iida *et al*., 2002), *Serratia marcescens* (Jaiswal and Thakur, 2007) and *Pseudomonas* sp. strain ISTDF1 (Jaiswal *et al*., 2011) have been isolated and catabolic potential for degradation of dibenzofuran has been evaluated. But very few reports are available for in-situ bioremediation of dioxin-like compounds in the environment (Haack *et al*., 1995; Kumar *et al*., 2019).

The highly alkaline nature of industrial effluent necessitates the search for bacteria that can survive and degrade dioxin in such extreme conditions. Because of the relatively low cost and minimal impact on the environment, dioxin degrading bacteria have been isolated by using dibenzofuran (DF) as a model substrate in enrichment culture (Fortnagel *et al*., 1996). The indigenous community is the actual player which persistently exists in such a hostile environment. Gradual adaptation of native bacteria under increasing alkaline conditions could be useful for efficient in-situ bioremediation of dioxin including other persistent organic pollutants (POPs) (Kao *et al*., 2001). The bioremediation of dioxin-like compounds under alkaline conditions is of interest because the alkalinity of effluent will change gradually after release in the environment which is a very critical factor for biodegradation. In addition, they have higher sorption capability in soil, and toxicity decreases as pH increases, owing to the increased conversion of undissociated (more toxic) to the dissociated form (Kishino and Bayashi, 1995). In general, populations of bacteria decline the following introduction into natural soil and the

growth of introduced populations is poor in microbiologically undisturbed soil**.** For successful bioaugmentation, there is the need to identify, and properly manage, the environmental conditions controlling the survival and activity of introduced micro-organisms. Microcosms are used to study biodegradation and the fate and effect of introduced micro-organisms (Wagner-Do¨bler *et al*., 1992). This can lead to a better understanding of the effect of factors controlling soil microbial inoculation, especially with regard to indigenous micro-organisms and to study the capability of inoculated micro-organisms to degrade certain chemicals.

Study area

Sludge effluent and sediment samples were collected from Century pulp and paper mill, Lalkua, Nainital, Uttaranchal, India (29°24' N, 79°28' E). The site was effluent discharging canals and premises of the industry. The sludge with liquid effluent was collected in clean plastic bags and stored at 4°C in a refrigerator until used for further analysis.

Characterization of effluent

The effluent was characterized for various physical and chemical parameters like pH, temperature, TDS, TSS, color, DO, BOD, COD and lignin content. pH was measured with the help of pH meter (Cyberscan 51), temperature by using portable digital multi-stem thermometer (Hanna Instrument Co. Italy) with external sensing probe on the sampling sites, TDS and TSS were estimated as described in APHA (2005), color by 2120 C Cobalt-platinate method (APHA, 2005), DO (Wrinkler's method), COD (5220 B open reflux method) and BOD were estimated as per APHA (2005). Lignin content was estimated according to Pearl and Benson (1940). Dibenzofuran was detected by GC-MS as described later.

Chemostat: enrichment of bacterial consortium

The reactor vessel consists of a 22×7 cm glass vessel, effective volume 1 litre, provided with an inlet for the entry of fresh sterile medium and an outlet for the removal of spent medium. Another inlet was provided specifically for the alkaline solution which maintained the pH of culture from 7 to 10. Sterile air was passed into the culture vessel by way of using aeration pump and sinister glass filter. The culture vessel was kept over a magnetic stirrer which was capable of maintaining the temperature at 28°C to 30°C. The composition of mineral salt medium (g/l) was: Na_2HPO_4, $2H_2O$. 7.8; KH_2PO_4, 6.8; $MgSO_4$, 0.2, ammonium ferric acetate, 0.01; $Ca(NO_3)_2$, $4H_2O$, 0.05; $NaNO_3$, 0.085, trace element solution with 4-chlorosalicyclic acid (CSA) (5Mm) / dibenzofuran (1Mm) as described (Thakur, 1995). The sediment and sludge containing bacterial cell populations served as inoculum in the chemostat. Initially, the bacteria were adapted in the presence of 4-CSA. After stabilization of the bacterial growth as determined by O.D. at 595 nm, the CSA in the medium was replaced by DBF (dibenzofuran). The pH of the chemostat was

gradually increased from pH 7 to pH 10. The culture medium was collected from chemostat at pH 7, 8, 9, and 10 after stabilization of the bacterial growth determined by O.D. at 595 nm. Four samples thus collected were centrifuged at 7000 rpm for 10 min. The bacterial pellet thus obtained was used for the determination of DBF utilization.

Monitoring of substrate depletion by gas chromatography

Utilization of dibenzofuran was tested in Erlenmeyer flasks containing mineral salt medium supplemented with 1mM dibenzofuran (DBF crystals dissolved in dimethylsulfoxide, 100 mg/L) as the sole source of carbon and energy, and incubated at 30^{O}C on an orbital shaker at 150 rpm. The samples were removed after 0, 6, 12, 24, 48, 120h growth of bacterial strains and the utilization of carbon source was determined. Bacterial cells were removed by centrifugation at 7000 rpm for 10 min. DBF concentration was determined by gas chromatography (GC). The culture medium (25 ml) was dissolved in DMSO and then extracted with double volume ethyl acetate (Jaiswal and Thakur, 2007). The organic phase (extract) was separated by a separating funnel, and the extract was finally concentrated on a rotary evaporator. Ethyl acetate was evaporated and the residue was re-dissolved in 100 µl ethyl acetate. The concentration of DBF was identified by using gas chromatography (GC) (GCPerkin Elmer) equipped with a capillary column (DB5 MS; 30 m ·0.25 mm film thickness ·0.25 mm I.D. ·30 m long). One µl 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 to 250°C and kept it at 250 °C for 4.5 min).

Isolation of bacteria from chemostat sample at pH 10

The enriched bacterial community from chemostat at pH 10 was diluted in tenfold serial dilution and spread on LB-agar plates. After 14 hrs incubation, colonies that appeared on Luria Bertani agar plates were characterized by morphological observation. Six dominant and morphologically distinct colonies were isolated.

Survival pattern of each strain

Morphologically distinct colonies were isolated and inoculated in MSM (Minimal Salt Medium) having dibenzofuran (1mM) dissolved in DMSO. Survival pattern of each colony was drawn on the basis of absorbance at 595nm on spectrophotometer Cary, 100 Bio (Varian Co., Australia) as described by Fortnagel *et al*. (1990). DBF utilization by each strain was determined by gas chromatography (Jaiswal and Thakur, 2007).

Bacterial growth and culture conditions

After adaptation in a chemostat, the bacterial consortium obtained at pH 10 was inoculated in

Luria Bertani medium at 5% (v/v). The culture was incubated till 1.0 optical density (OD) at 595 nm and then centrifuged at 6000 rotation per minute (rpm) for 6 minutes at 4°C. The bacterial pellet, thus obtained, was transferred to the minimal salt medium with dibenzofuran (1mM) at pH 10 for agitation in the orbital shaker. After 6, 12, 18, 24, 30 and 36 hours, samples were collected and OD was measured at 595 nm. MSM samples were centrifuged at 6000 rpm for 6 minutes. Its supernatant was taken for biodegradation studies.

Utilization of dibenzofuran

Culture supernatant was extracted with double volume ethyl acetate and split it into two equal volumes. One was acidified with 6N HCl to approximately pH 2.0 and other at pH 7 (Jaiswal and Thakur, 2007). The organic phase (extract) was separated by a separating funnel, and the extract was finally concentrated on a rotary evaporator. Ethyl acetate was evaporated and the residue was re-dissolved in 100 µl acetonitrile. The concentration of DBF was identified by using gas chromatography (GC) (GC-Perkin Elmer) equipped with a capillary column (DB5; 0.25 mM film thickness · 0.25 mm Internal diameter. 30 meters long). One µl 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 to 250°C and kept it at 250 °C for 4.5 min). Concentration was derived from the standard plot between peak area and concentration of DBF.

Microcosm soil analysis

The soil moisture content, water holding capacity (WHC), and pH were determined as described previously by Vinas *et al*. (2005). To determine the best soil water content for use in the microcosm experiments, five different water contents (5%, 20%, 40%, 60%, and 75% WHC) and autoclaved soil as an abiotic control were assayed for 15 days in triplicate in miniaturized microcosms. The best results were observed with 60% WHC (70% biodegradation of DBF). Thus, water content was established as a key factor for biodegradation activity, and 60% WHC was defined as the optimal water content for soil microcosm experiments.

Microcosm design

Microcosms were prepared according to Gautam *et al*. (2003) and modified as follows: soil grits (100 gm) were placed on the bottom of a sealed plastic jar, then sand (100 gm) formed the middle layer and soil (300 gm) formed the top later. Experiments were performed using sterile soil and non-sterile soil. Eight sets of microcosms were prepared as described in Table 1. The soil was treated as described by Megharaj *et al.* (1997). Four sets of soil microcosms were sterilized by autoclaving at 121 °C for 45 min on three consecutive days. Sterility was checked afterward by streaking dilutions of soil suspensions on LB agar plates. Sterile soil and non-sterile soil were then treated with either DBF at a final concentration of 1 mg/g soil from 50-mg/ml stock solutions

Table 1. Microcosms inoculated with the bacterial community which is non-adapted or adapted at different pH levels.

Label	Description
A	Control with Unautoclaved soil
B	Control with Autoclaved soil
C	Unadapted Bacterial Community + Unautoclaved soil
D	Unadapted Bacterial Community + Autoclaved soil
E	Bacterial Community Adapted at pH 7.0 + Unautoclaved soil
F	Bacterial Community Adapted at pH 7.0 + Autoclaved soil
G	Bacterial Community Adapted at pH 10.0 + Unautoclaved soil
H	Bacterial Community Adapted at pH 10.0 + Autoclaved soil

made in analytical grade acetone. The acetone solvent was evaporated and soils were rigorously mixed and allowed to equilibrate at 25°C for 6 h. Controls were similarly treated with acetone. Microcosms containing non-sterile soil were pre-incubated at 30°C, prior to the experiment, to allow the indigenous microorganisms to reach an equilibrium state, avoiding a thermal artifact at time zero of the experiment (Lafuente *et al*., 1996). Bacteria were grown with shaking at 30°C in Luria-Bertani (LB) broth to the late exponential phase. Cells were harvested by centrifugation, resuspended in mineral salt medium and then inoculated in the soil at a level of 10^7-10^8 cells/g dry soil, partly following recommendations of Comeau *et al*. (1993). Sterile distilled water was added to the soil to reach a final moisture content of 60% (v/wt), rigorously mixed and kept at 30 °C.

Sampling

Chemical, microbial, and molecular analyses were carried out on sampling days 0, 03, 07, 15, 30, 45, and 90. At each sampling time, 25 g of soil was extracted as a composite sample from five points in each microcosm and stored at -20°C prior to most analyses; the only exception was microbial counting, which was performed immediately after sampling.

Monitoring the depletion of dibenzofuran in soil microcosm

The extraction of metabolites was performed by a modified method described by Jaiswal and Thakur (2007). 25 ml of acetonitrile with 2% H_3PO_4 was added to 10gm of soil and agitated for 60 min. The particles were settled down, and the supernatant was passed through a polytetrafluoro-ethylene membrane filter (0.2 µm). One µl of each extract was analyzed by Gas chromatography-mass spectroscopy (GC-MS) (Varian) equipped with a capillary column (DB5 MS; 30m × 0.25um film thickness × 0.25mm I.D. × 30 meter long) at splitless mode; initial temperature 80°C for 1.5 min; temperature increased from 80 to 230°C at a rate of 20°C /min and 230 to 250°C and kept it

at 250°C for 4.5 min. The head pressure of the helium carrier gas was 80 kPa helium flow rate 1.1ml/min as described by Iida *et al*. (2002).

Physical and chemical properties of effluent

The effluent collected from pulp and paper industry, Lalkuan, Uttarakhand showed the following physical and chemical characteristics. It was dark in color with high COD, BOD and DO and was alkaline in nature. The effluent was found to be contaminated with dibenzofuran (2 ppm) (Table 2). Each value represents the mean of three replicates ± SEM.

Utilization of dibenzofuran by the bacterial community at different pH

The effluent from the industry was used as inoculum in chemostat containing MSM with CSA, followed by MSM with DBF where the bacterial community was adapted for a pH range from 7 to 10. Samples collected at each pH were inoculated in MSM with DBF and monitored for the depletion of DBF. Gas Chromatogram of samples collected from MSM at different time intervals revealed adaptation has enhanced the degradation ability of the indigenous bacteria. Adapted culture degraded more dibenzofuran than non-adapted culture. While non-adapted culture degraded only 35% DBF in 264 hrs, culture adapted at pH 7 degraded 45%, pH 8 degraded 50%, and pH 9 degraded 70%. The culture at pH 10 showed maximum degradation potential, which is more than 90% in 264 hrs (Figure 1).

Isolation of bacteria from pH 10 sample

The sample collected of pH 10 culture was used as inoculum to spread on LB plates. Six colonies were selected on the basis of morphological differences. The colonies were labeled as Strain A,

Table 2. Physico-chemical characterization of effluent used in this study.

Parameter	Value
pH	10.4 ± 0.2
Temperature	30.5 ± 01°C
TDS (mg l^{-1})	1243 ± 11.3
TSS (mg l^{-1})	285.9 ± 1.9
Color of effluent (coloring units)	62385 ± 70.23
DO (in ppm)	0.0 ± 0.0
BOD (in ppm)	53216 ± 13
COD (in ppm)	204358 ± 81.20
Lignin (in ppm)	153741 ± 91.20
Dibenzofuran (in ppm)	2.00 ± 0.32

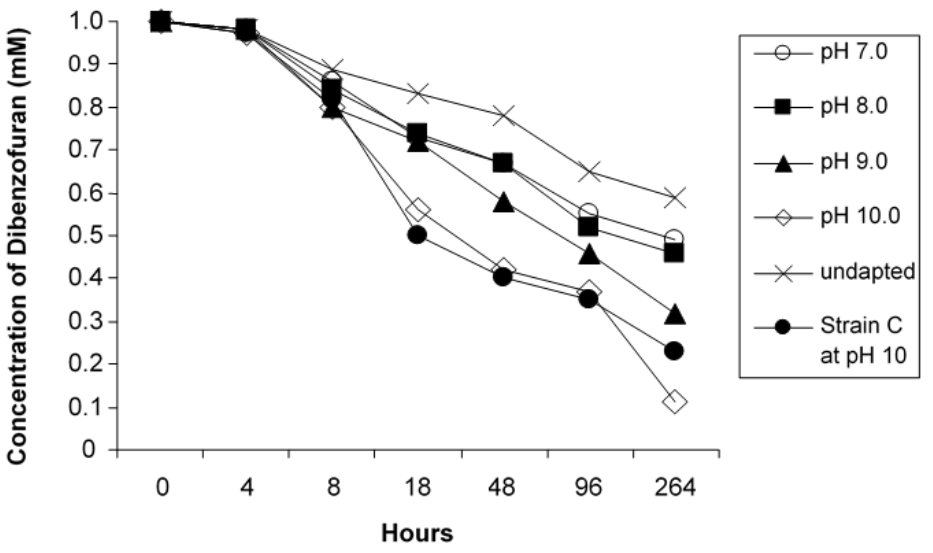

Figure 1. Comparison of utilization of dibenzofuran by total bacterial community enriched at gradually increasing pH

Strain B, Strain C, Strain D, Strain E, Strain F.

Utilization of dibenzofuran

The utilization of dibenzofuran by all six strains was then analyzed. Each strain was inoculated in MSM containing DBF and samples were collected at different time intervals and the concentration of DBF was calculated by peak formed during GC. The concentration of DBF was plotted against the time interval as shown in Figure 2. The survival pattern of each strain was also estimated and Strain C was found to be most effective. It degraded approximately 50% DBF in 260 hrs. As shown in Figure 2, strain C gives a maximum peak of absorbance indicating maximum growth. Strains A and F were found to be least effective on DBF while strains B, D, and E gave good results.

Thus, the bacterial community obtained through chemostat was found to be capable of growing and degrading DBF at pH 10. The community consists of strains A, B, C, D, E, and F. Among which strain C was found to be most potent DBF degrader followed by B, D and E. Strains A and F were not found to be efficient DBF degrader. Still, they are present in the stable bacterial community. This indicates that they might be involved in the degradation of lower metabolites.

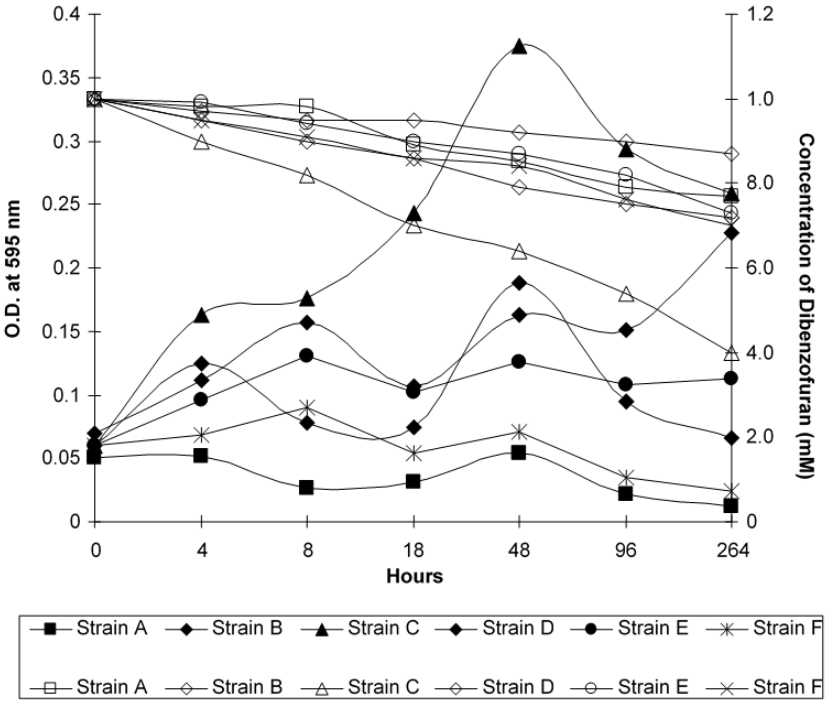

Figure 2. Growth pattern of six bacterial isolates from pulp and paper industry effluent determined at 595 nm. pH 7.0. A, B, C, D, E and F represents bacterial strains in minimum salt medium and dibenzofuran (1mM) as sole carbon source in the chemostat.

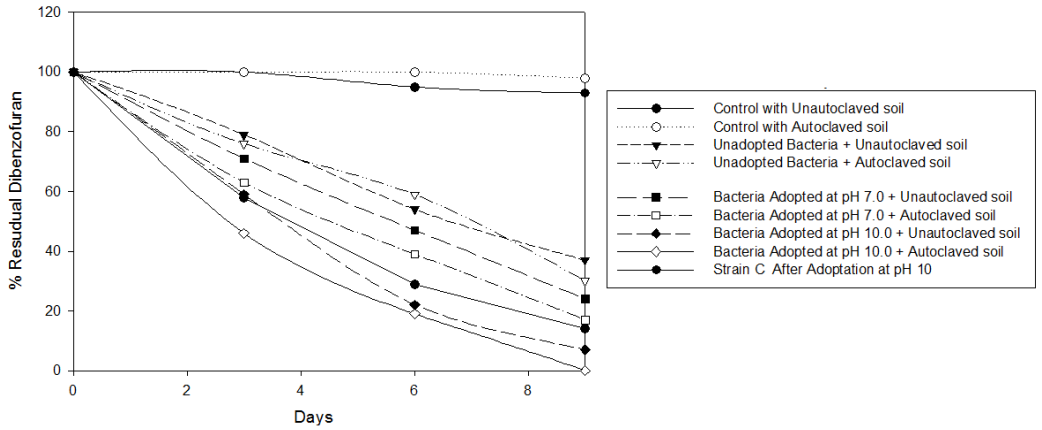

Figure 3. Degradation of dibenzofuran under different plans in microcosm along with controls.

Biodegradation of dibenzofuran in soil microcosm

The *in vivo* efficiency of the community was tested through soil microcosm. After 90 days, the amount of dibenzofuran left in the soil was tested in each microcosm. Almost, no degradation was found in the case of control with autoclaved soil (microcosm A). The concentration of DBF in the soil at 0 days was taken as 100%. Control with autoclaved soil (microcosm B) showed the presence of 70% DBF. Likewise, microcosm labelled as C, D, E, F, G, H showed 36.8%, 36.59%, 9.7%, 7.5%, 2.1% and 1.9% DBF respectively. Maximum degradation was seen in the case of microcosm inoculated with consortia adopted at pH 10 as shown in Figure 3.

Conclusion

Microbial community isolated from sludge and sediment of pulp and paper mill was enriched in a chemostat with gradually increasing pH from 7 to 10. Based on morphological dissimilarity, six strains were isolated from the chemostat at pH 7.0 with the highest diversity on LB plates. One of these strains, strain C, enriched at pH 10.0 was found to be most efficient evaluated by growth rate and dibenzofuran degrading potency. The microbial community adapted in chemostat for different time duration was inoculated in soil microcosm. 180 days adapted community showed maximum degradation which makes it sufficient time for adaptation. GC analysis reflects that within 90 days, almost all dibenzofuran were metabolized. The best degradation results were shown by 180 days of adapted strain in a chemostat. Whereas 45 days and 90 days adapted community also showed significant results but not better than 180 days adapted community in a chemostat. The unadapted community is also degrading but not significantly as compared to the aforementioned community. This suggests that the microbial community adapted for a longer time in chemostat had better efficiency for the degradation of DBF. Degradation of dibenzofuran in soil microcosm indicates that the community is competent enough to survive and retain its degrading potency even *in*- situ conditions.

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