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Biodegradation of Benzene under Anaerobic Condition using Enriched Microbial Culture

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ABSTRACT

Benzene is a highly toxic organic contaminant having carcinogenic potential and a serious ground water contaminant. Refinery industry is a major source of benzene contamination in the environment. The present paper focuses on biodegradation of benzene under anaerobic condition by using enriched microbial consortium. Enriched bacterial isolates, identified by 16S rRNA sequencing technique were taxonomically 99% similar to *Bacillus cereus strain BC2, Citrobacter freundii strain : A11-1, Bacillus cereus strain SS263-3, Brevibacterium linens strain S402003.* A bioreactor was designed & developed for the study of biodegradation of benzene with different concentrations (5, 10, 25 ppm). 94.3 % degradation was observed in 5 ppm followed by 10 and 25 ppm i.e. 67.08 % & 51.3 % respectively. Results of this study indicated that these genera may play an active role in bioremediation of benzene.

Keywords: Biodegradation, Benzene, Anaerobic, Enriched, Microbial Culture

I. INTRODUCTION

Industrialization and technological advancement has resulted into generation of huge amount of liquid and solid waste which is a concern to environmentalist. Manufacturing exploration, extraction processes use raw materials and consumables from each component of environment, and release of hazardous waste which results into deleterious effects on the surrounding environment. The main sources of contaminants are the chemical industries, e.g. petrochemical industry which involves the production of several chemicals, basic raw materials and key intermediates, generate complex hazardous waste. The most common aromatic organic compound found in the waste discharge by the industry is benzene. Benzene is among the most prevalent organic contaminants in groundwater and is of major concern due to its toxicity and relatively high solubility [1, 2]. It possesses a major threat to human health and environment due to its toxicity and carcinogenic effects. Clean-up of the contaminant is needed in order to avoid public health hazard. Conventional methods of treatment of volatile organic wastes have been largely physical or chemical but these can also lead to secondary pollutant problems. The ecologically acceptable disposal of organic waste remains a major challenge to the petrochemical industry. Bioremediation is an increasingly popular alternative to conventional methods for treating waste compounds using natural microbial activity. It is expected to be an energy efficient, economical and environmentally sound approach. With this view the present study was undertaken with the refinery effluent to isolate promising bacteria for bioremediation of benzene.

II. METHODS AND MATERIAL

2.1 Sample Collection

Wastewater sample was collected from effluent treatment canal of Indian Oil Corporation limited (IOCL), located at Koyali near Vadodara, Gujarat, India, with the following location coordinates: Latitude; 22.37425, Longitude; 73.11948.

2.2 Physico-Chemical and Microbial Characterization

Physical and chemical parameters were analyzed by standard methods [3]. Total organic carbon and heavy metals were analyzed by total organic carbon (TOC)

analyzer and atomic absorption spectroscopy (AAS) by acid digestion method respectively. Microbial status was analyzed by colony forming unit (CFU) count, gram staining, biochemical test, carbohydrate test, antibiotic sensitivity test. Results were interpreted by Bergey's manual of systematic bacteriology (vol, 2C, 3, 4) [4,5,6].

2.3 Preparation of Enriched Microbial Culture

For enrichment of anaerobic benzene degrading bacteria, 100 ml of collected water sample and mixed with 40 ml of the mineral medium (NH_4Cl 1g/l,KH_2PO_4 1g/l, $MgCL_2.5H_2O$ 0.1g/l $CaCl_2.2H_2O$ 0.05g/l, $NaNO_3$ 1.5g/l). The medium was supplemented with 100 µl of benzene. The incubation was performed at 20°C in an anaerobic chamber that contained mixture of carbon di-oxide, hydrogen and nitrogen gas and all the media and solutions were prepared under anaerobic conditions. Then, 0.5 ml of the incubated samples were added to 100-ml glass bottle, which contained 80 ml mineral medium containing 1% (V/V) trace mineral solution and 1% (V/V) ($COCl_2.6H_2O$ 30mg/L, $CUCl_2$ 0.15mg/L, H_3BO_3 5.7mg/L, $MnCL_2.4H_2O$ 20mg/L,$Na_2MoO_4.2H_2O$ 2.5mg/L, $NiCl_2.2H_2O$ 1.5mg/L, $ZnCl_2$ 2.1mg/L). Vitamin 1% Biotin 20 mg/l, Folic acid 20 mg/l, Pyridoxine HCL 100mg/l, Riboflavin 50 mg/l, Thiamin 50 mg/l, Nicotinic acid 50 mg/l Pantothenic acid 50 mg/l, Cyanocobalamin 1mg/l, p-Aminobenzoic acid 50 mg/l, Thiotic acid 50 mg/l, Reasazurin redox indicator 1g/l). In addition, 2 µl each of benzene were added. After 7–9 d, transfers were done by adding 1 ml of incubated inoculum to 9 ml mineral medium in sterile 20-ml serum bottles. After it was transferred 8 times every 7–9 d [7].

2.4 Preparation of Bacterial Consortium

The microbial consortium was prepared from the isolated bacterial cultures through enrichment process. Microbial consortia were prepared on the basis of compatibility with each other, the mixed bacterial consortium for anaerobic degradation of benzene was obtained. An aliquot (1 ml) of each bacterial culture was transferred to a 250 ml Erlenmeyer flask containing a thyoglycollate medium base and mineral salt medium (MSM) incubated at 37°C in 120 rpm. The combination of bacterial isolates was based on permutation combination. Three possible combinations were made for the preparation of microbial consortia. The compatibility of the bacterial strains within the consortia was analyzed by increasing optical density at 600 nm by spectrophotometer from 0 hr to 168 hr.

2.5 Identification of Microbes by 16S Rrna Gene Sequencing

The 16S rDNA gene sequence was used for the identification of bacterial strains and carry out BLAST with the non-redundant (nr) database of NCBI genebank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 5 (Sequencing was done by Xceleris laboratory, Ahmedabad, Gujarat).

2.6 Experimental Setup for Bioremediation of Benzene

Biodegradation of benzene was studied in a closed glass reactor consisting of one injection and sampling port containing 500 ml of bacterial consortium (48 hr incubated). Different concentrations of benzene dissolved in methanol (5, 10, 25 ppm) were injected in the reactor through injection port after 24 hr of reactor setup for the establishment of anoxic condition. . The bioreactor was maintained at a temperature range of between 25-27˚C with help of thermostat. Samples were withdrawn hourly up to 5 hours. Samples were transferred to a 5 ml capacity vial and capped with Teflon-coated septa prior to high performance liquid chromatograph (HPLC) analysis. The bioremediation conditions i.e. temperature, pH, chemical oxygen demand (COD), biological oxygen demand (BOD), nitrate (NO_3^-), nitrite (NO_2^-) were also monitored [8].

2.7 Sample Preparation and Analytical Procedure

Samples were centrifuged (10 min, 10000 rpm) to separate cell mass and the supernatant. The samples were extracted in organic solvent (n-hexane) for analysis [8, 9]. The extracted samples were injected in a high-performance liquid chromatograph system HPLC Agilent 1260 series LC equipped with UV-VIS Detector. All data for quantification of the benzene were obtained by applying the gradient elution program (Column C18, Flow rate 1 mL/min, Injection volume 5µL, Mobile phase A=Acetonitrile (10%), B= deionized water (90%), Retention time 3 minute, at wavelength 201 nm). High performance liquid chromatography had clearly showed

a benzene peaks at a retention time of 1.3 and reduction in a peak area.

III. RESULTS AND DISCUSSION

3.1. Physico-Chemical Characterization

The wastewater used in this study was alkaline in nature with a pH value of 8.3. Refinery effluent is contaminated with many heavy metals and organic aromatic compounds. Results of AAS analysis shows that different heavy metals were present in range from 0.02 ppm – 0.07 ppm in the sample (Table 1) Benzene concentration in effluent was below 1 ppm. TDS was found high in comparison with other chemical parameters. Similarly high concentration of TDS, TSS, COD, DO, BOD, $PO4^{3-}$, $SO4^{2-}$, Cl^{-} and NO^{3-} were also observed in previous research study on petrochemical effluent [10]. Higher values of electrical conductivity in the water sample was observed; results into high turbidity which may be due to the presence of dissolved suspended solids which can cause problem in water purification processes such as flocculation and filtration, hence increases the treatment cost [11].

Table 1: Physico-Chemical Characterization of Wastewater

Parameters	Mean value ± SD
pH	8.33±0.12
OIL and grease (mg/l)	4.61±0.22
DO (mg/L)	4.8±0.11
Bod 5 days,20° c (mg/L)	124±0.81
COD (mg/L)	306.33±1.69
Total Suspended Solids (mg/l)	141.33±0.94
Phenols (mg/l)	3.73±0.21
Nitrate (mg/l)	27.31±0.89
Nitrite (mg/l)	23.36±0.70
Ammonia as N (mg/l)	67.48±3.0
Organic phosphorus (mg/l)	14.9±0.63
Inorganic phosphorus (mg/l)	26.44±1.02
Volatile fatty acid (mg/l)	3.23±0.04
Sulphate (mg/l)	23.29±0.63
Chloride (mg/l)	3433.967±73.38
Hardness total (mg/l)	338.3±8.4
Mg hardness (mg/l)	161.66±2.3
Ca hardness (mg/l)	176.66±6.2
Fixed solid (%)	80.27±1.6
Volatile solid (%)	19.41±0.6
Total organic carbon (mg/l)	23.88±0.23
Total dissolve solid (ppm)	212.6±10.37
Electrical conductivity (µS)	403±2.9
Total alkalinity (mg/l)	1661±28.43
Benzene (ppm)	<1
Nickel (ppm) at 341 nm	0.026 ± 0.015
Cadmium (ppm) at 326 nm	0.076 ± 0.016
Zinc (ppm) at 213.9 nm	0.062 ± 0.013
*values indicate the average of triplicate samples and standard deviation	

3.2 Microbial Characterization and Enriched Microbial Culture

The microorganisms present at contaminated site, capable of using contaminants or just resisting their toxicity can be found [12]. Nine different bacterial cultures were isolated from the selected sites and identified on the basis of biochemical test simultaneously antibiotic resistivity test were performed for all the isolates (Table 2, 3). Antibiotic resistivity test shows the sensitivity of an organism to different antibiotics. In the present study *Bacillus cereus strain BC2 (P1)* and *Tatumella* (C1) were found sensitive to all antibiotics except to amoxyclav (AMC), whereas *Pantoea* (F1) showed resistance to most of the antibiotics. Results were compared by National committee for clinical laboratory standards [13] (Table 4). During enrichment technique among the nine, four different bacterial cultures were found benzene tolerant, which are facultative anaerobic in nature (Fig 1). Four isolated bacterial cultures were able to survive in combination. The isolated strains were further characterized by 16S rRNA sequencing and showed 99% similarity with *Bacillus cereus strain BC2*, *Citrobacter freundii strain:A11-1*, *Bacillus cereus strain SS263-3*, *Brevibacterium linens strain S402003*. The phyla %, similarity accession number and evolutionary relationship of these isolates are presented in (Table 5, fig 1, 2 & 3). The identity of hydrocarbonclastic bacteria has been determined in earlier studies [14, 15]. As earlier *Bacillus cereus* was isolated from gasoline contaminated soil for degradation of benzene [16]. *Bacillus cereus* A, *Bacillus cereus* B and *Bacillus* sp. ZD are the most promising hydrocarbon degraders. *Bacillus cereus* A showed maximum diesel oil degradation (82.41% and 81.56% of aliphatic and aromatic hydrocarbons) after 2 days incubation under shaken condition, at pH 7 [17]. Therefore, this study screens the dominant bacterial strains for pollutant biodegradation and evaluates their biodegradation efficiency in order to assess their efficacy. Catechol is formed from benzene through the sequential action of toluene dioxygenase and toluene cis-glycol dehydrogenase, and L-DOPA is synthesized from the resulting catechol in the presence of pyruvate and ammonia by tyrosine phenol-lyase cloned from *Citrobacter freundii* [18]. Similarly the ability of three bacterial isolates (*Bacillus spp*, *Micrococcus spp* and *Proteus spp.*) was studied, isolated from two rivers and

refinery effluent to degrade two Nigerian Crude oils. Bacterial isolates showing increase in cell number and optical density as pH decreases and observed as potential hydrocarbon degraders [19]. As mentioned in previous research studies *Brevibacterium spp* was isolated from industrial rejection of refinery and selected due to the capacity of growing in the presence of hydrocarbon. Growth of the bacterial culture was realized in mineral liquid media supplemented with petroleum oil as sole carbon source [20].

Table 2: Bacterial strains present in wastewater samples

Bacterial strains	**Gram positive/ Gram negative**	**Shape**
Bacillus cereus strain BC2 (P1)	Positive	Short rods
Enterococcus(B1)	Positive	Cocci
Brevibacterium linens strain S402003(P)*	Positive	Short rods
Citrobacter freundii, strain: A11-1 (P4)	Negative	Rods
Bacillus cereus strain SS263-34 (P5)	Positive	Short rods
Pantoea (F1)	Negative	Cocci
Streptococcus (G1)	Positive	Cocci
Staphylococcus (H1)	Positive	Cocci
Vibrio (I1)	Negative	Short rods

Table 3: Biochemical Tests of Bacterial Isolates

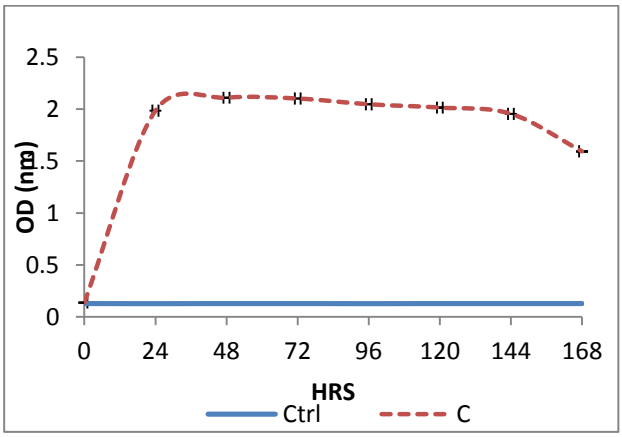
Name of test	***Bacillus cereus strain BC2 (P1)***	***Enterococcus (B1)***	***Brevibacterium linens strain S402003(P*)***	***Citrobacter freundii, strain: A11-1 (P4)***	***Bacillus cereus strain SS263-34* (P5)**	***Pantoea (F1)***	***Streptococcus (G1)***	***StaphylococcuS (H1)***	***Vibrio(I1)***
OXIDASE TEST	-	-	+	-	-	-	-	-	+
TSI	K/A	K/A	K/A	A/A	K/A	NF	K/A	A/A	NF
VP TEST	-	-	-	-	+	-	-	-	-
MR TEST	-	-	-	+	+	+	-	+	+
CITRATE TEST	-	-	+	-	+	-	-	-	-
DEHYDROGENASE	-	+	-	-	+	-	-	-	-
CATALASE TEST	-	-	+	-	-	-	-	+	+
UREASE TEST	-	-	-	+	+	-	-	-	-
INDOLE ACETIC ACID PRODUCTION	-	-	-	+	-	-	-	-	-
NITRATE	+	+	+	+	+	+	+	+	+
MOTILITY	+	+	+	-	+	+	+	+	+
SULPHATE REDUCTION	-	+	-	-	-	-	-	-	-
AMMONIUM PRODUCTION	+	-	+	+	+	+	+	+	+
H_2S PRODUCTION	-	+	-	-	+	+	-	-	-
O/F TEST	-/+	+/+	-/-	+/+	+/+	-/+	-/+	-/+	+/-

- + = present, - = absent, K/A –**Glucose fermentation, peptone catabolize, **A/A**- Glucose and lactose fermentation, **NF**- Non fermenting,-/+ oxidative metabolism positive, -/- non sachrolytic organisms, +/+ fermentative metabolism positive.

Table 4: Antibiotic sensitivity test of bacterial isolates

Bacterial strains	CX(30 mcg)	P (10 unit)	TE(30 mcg)	L(2 mcg)	GEN(10 mcg)	CTX(30 mcg)	AMC(30 mcg)	CD(2 mcg)	CEP(30 mcg)	OX(1 mcg)	E(15 mcg)
Bacillus cereus strain BC2 (P1)	3	2.8	3	3.5	3	2	R	S	S	R	S
Enterococcus (B1)	S	S	S	S	S	S	S	S	S	R	0.5
Brevibacterium linens strain S402003(P)*	S	S	S	2.6	2.5	S	S	S	S	S	S
Citrobacter freundii, strain: A11-1 (P4)	S	S	S	S	3	3.5	2.5	S	S	1.5	S
Bacillus cereus strain SS263-34 (P5)	1	R	2.5	R	S	S	R	R	<3	1.5	R
Pantoea (F1)	2.3	R	3.5	R	2.5	3	R	R	R	R	R
Streptococcus (G1)	S	1.5	4	R	S	S	R	S	S	S	R
Staphylococcus (H1)	1.5	R	2.3	R	1.5	S	S	S	>2.5	R	R
Vibrio (I1)	S	R	S	R	S	S	S	S	>1.7	S	1

- mcg- microgram, all numeric values are in centimeters (cm), R = resistant (complete growthon media), S = sensitive (>3.5cm), **CX- cefoxitin**, P-** penicillin G**, TE-**tetracycline**, L-** linomycin**, GEN-** gentamycin**, CTX-** cefotaxime**, AMC-** amoxyclav**, CD-** Clindamycin**, CEP-** cephalothin**, OX-** oxacillin**, E-** erythromycin



Bacterial combination	Bacterial strains
A	P4 + P* + P1 + P5
B	P4 + P* + P1
C	P4 + P1 + P5

Where *Bacillus cereus strain BC2 (P1), Citrobacter freundii, strain: A11-1 (P4),\ Brevibacterium linens strain S402003 (P*), Bacillus cereus strain SS263-34 (P5)*

Figure 1: Growth of the enriched bacterial consortium (A)
(**Data presented are the mean of triplicates with standard error (5%))

Table 5: Genotypic characterization and phylogeny of enriched bacterial isolates

Description	Accession number	Base pair	Similarity
Brevibacterium linens strain S402003 (P*)	KJ019204.1	1450	99%
Bacillus cereus strain BC2 (P1)	KM111173.1	1448	99%
Citrobacter freundii, strain: A11-1 (P4)	AB244300.1	1454	99%
Bacillus cereus strain SS263-34 (P5)	JX429824.1	1474	99%

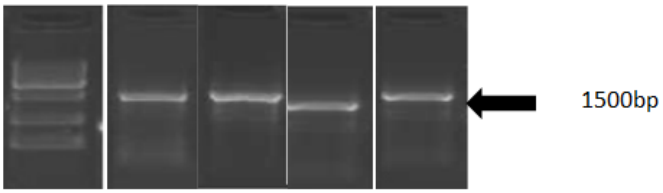
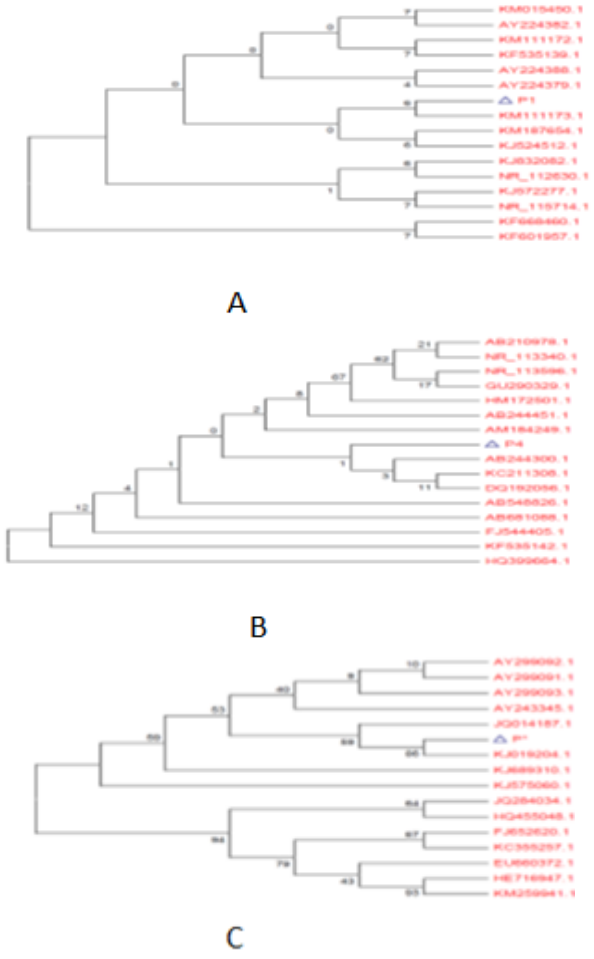


Figure 2: 1.2% Agarose gel showing single 1.5 kb of 16S rDNA amplicon

(**Where; 1-DNA marker 1kb ladder, 2 - 16S rDNA amplicon of *Brevibacterium linens strain S402003* (P*), 3- 16S rDNA amplicon of *Bacillus cereus strain BC2* (P1), 4- 16S rDNA amplicon of *Citrobacter freundii, strain: A11-1* (P4), 5 - 16S rDNA amplicon of *Bacillus cereus strain SS263-34* (P5))



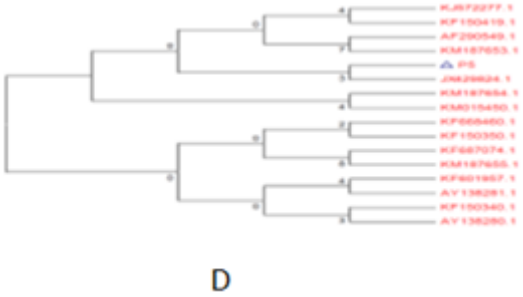


Figure 3: Evolutionary relationships of 11 taxa of A- Bacillus cereus strain BC2 , B- Citrobacter freundii, strain: A11-1, C- Brevibacterium linens strain S402003, D- Bacillus cereus strain SS263-34

3.3. Biodegradation of Benzene: Physico-chemical variation

For several biological processes, pH has a significant impact on bioremediation. Maximum variation was found in 5 ppm benzene followed by 10 ppm, 25 ppm (fig 4); which is desirable since biodegradation rate is highest at near neutral pH [8, 21]. Microbial activity is severely disturbed by any deviation from an optimum pH range. Due to neutrophilic (optimum pH is 7) behavior of the microorganism, maximum degradation observed for benzene was in the pH range of 7.5 and 8.0 [22]. During the study nitrate was found to decrease, major decrease was observed in 5 ppm followed by 10 ppm, 25 ppm. This showed that nitrate serve as e^- acceptor for removal of benzene [23, 24]. During denitrification active bacterial culture, oxidize resazurin which confirms denitrification. The results of denitrification agree with the study conducted previously [16] which also confirms benzene degradation under denitrifying condition. According to thermodynamic principles, the higher energy yielding reaction takes place when microbes utilize nitrate as electron acceptor. Nitrate is much more soluble than oxygen hence microbes may require less time for the degradation of contaminant. Therefore nitrate was a more favorable electron acceptor for mixed culture study [25, 16]. Simultaneously nitrite accumulation occurred with nitrate reduction, but doesn't have any inhibitory effect on benzene degradation. Similar results were observed during anaerobic degradation of BTEX (benzene, toluene, ethyl benzene, xylene) using adapted consortia [26]. This result may also attributed to the presence of fermentative bacteria which possess oxidation-reduction pathway in which nitrite formed as intermediate.

Biodegradability of benzene was analyzed by determining the BOD and COD. The observed decrease in BOD (difference in final dissolve oxygen and initial dissolve oxygen) is primarily due to the consumption of oxygen by the microbial biomass for their growth and proliferation. The results indicate that decrease in BOD was proportional to the growth of microorganisms. The decrease in COD was directly proportional to the rate of degradation of compound studied [8]. The decrease in COD with increasing time duration was observed at 5, 10, 25 ppm of benzene (fig 4).

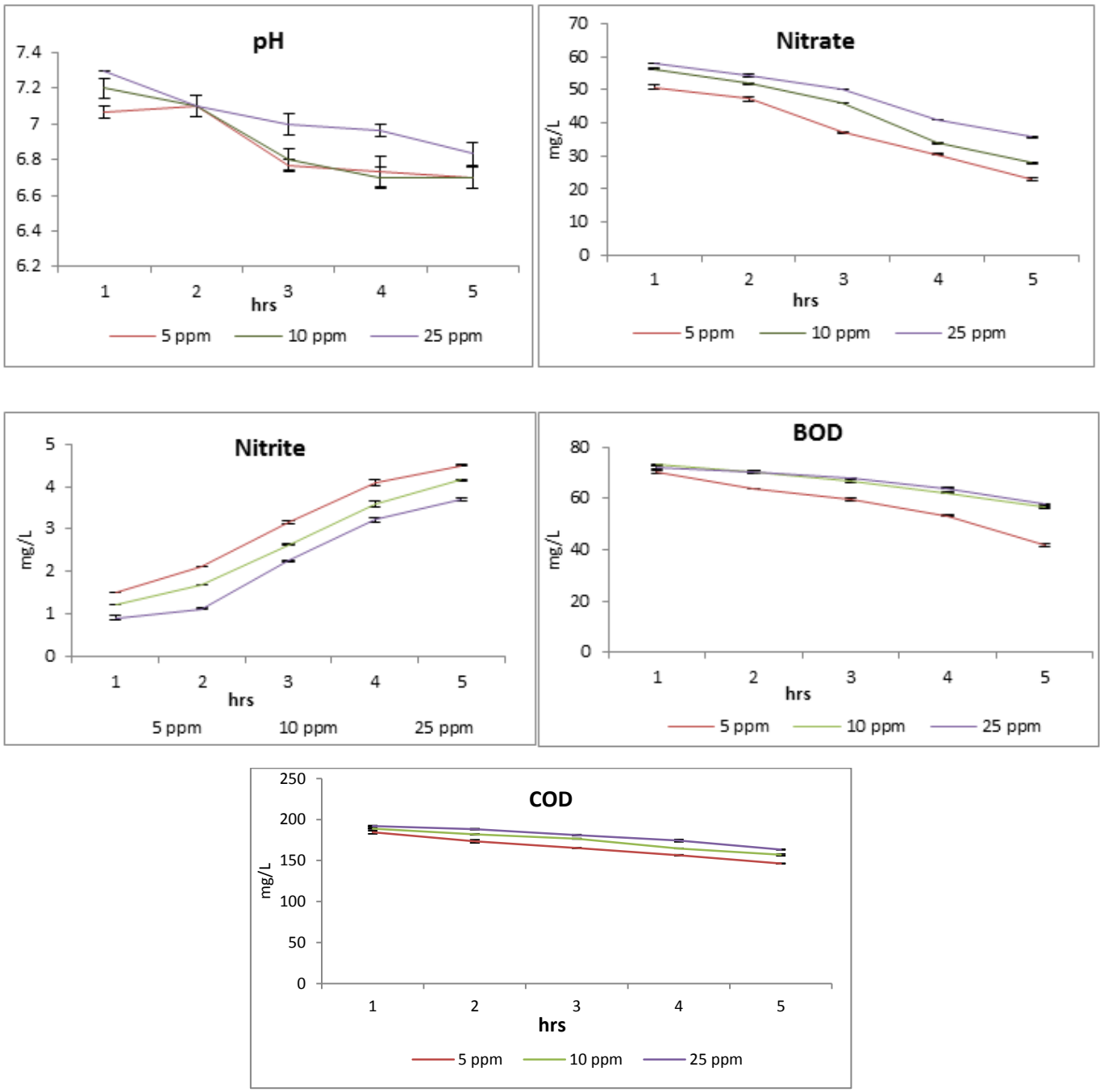


Figure 4: Variation of environmental conditions during bioremediation (**Data presented are the mean of triplicates with standard error (5%))

3.4. Biodegradation of Benzene

Analysis of the contaminant concentration throughout the experiment period showed significant increase in rate of degradation (fig 5). Similarly optimization of conditions to improve the biodegrading activity of the isolated strains was studied using different concentration of petroleum oil [20]. The effectiveness of benzene

biodegradation depends upon the application of selected bacterial communities adapted to utilize any single organic compound [27, 28]. Biodegradation of benzene indicate that in case of 5 ppm degradation started within 5 hr and was degraded up to 94.3 %. At 10 ppm of benzene, degradation within 5 hr and 67.08 % degradation had occurred during the experimental period. Higher concentration of benzene (25 ppm) was found less in rate of degradation and percentage degradation was observed up to 51.3 %. Bioremediation of benzene, toluene and o-xylene was also studied with the help of cow dung microbial consortium containing bacterial strains (*Pseudomonas* sp, *streptococcus* sp, *Sarcina* sp, *E.coli* sp), fungi (*Penicillium sp, Rhizopus sp, Mucor sp, Aspergillus sp*), benzene and toluene degraded upto 100% at 100 mg/L, whereas o-Xylene was degraded upto 97% at a concentration of 50 mg/L [8]. Biological agents are economical, energy efficient, and environmentally friendly approach that can be used even to treat the benzene contaminated environmental sites [29, 30]. The higher growth rate of the microbial consortium in the presence of benzene may ensure that bioremediation of monoaromatic hydrocarbons can be accelerated through application of microbial consortium rather than pure culture of individual bacterium from the same consortium. Therefore, benzene biodegradation potential of the formulated bacterial consortium in the present study may be well suitable for biodegradation of high concentration of benzene in soil as well as in water [31].

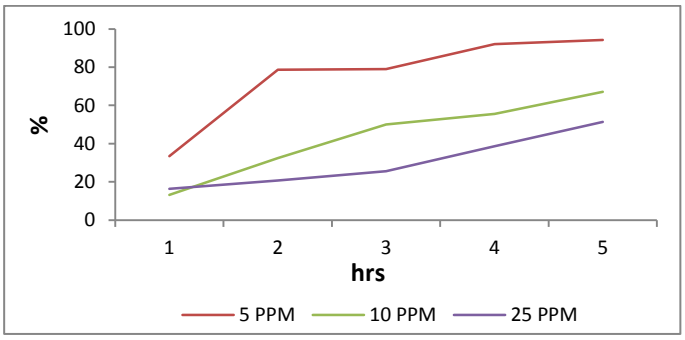


Figure 5: Percentage degradation of benzene

IV. CONCLUSION

Carcinogens and mutagens pose the greatest risk to human and environmental health and the earlier removal of these compounds would be beneficial to the bioremediation process. This study has demonstrated the benefit of nitrate reducing enriched bacterial consortium for benzene degradation under anaerobic condition, in order to carry out safe, efficient and sustainable bioremediation. Four isolated bacterial strains; *Bacillus cereus strain BC2, Citrobacter freundii strain:A11-1, Bacillus cereus strain SS263-3, Brevibacterium linens strain S402003* were found to be the most effective. Stimulating the anaerobic microbial community has the advantage of enhancing an active microbial population for the removal of any organic contaminant from the environment. Bioremediation under anoxic condition is still unexplored and require more efficient research work to be done in this field to assess the microbial community associated with degradation of any organic compound. Anaerobic biodegradation using enriched microbial culture could be a robust and sustainable approach to accelerate the natural attenuation of benzene in contaminated environment.

V. ACKNOWLEDGEMENT

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