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# Benzene bioremediation using cow dung microflora in two phase partitioning bioreactor

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ABSTRACT

Bioremediation of benzene has been carried out using cow dung microflora in a bioreactor. The bioremediation of benzene under the influence of cow dung microflora was found to be 100% and 67.5%, at initial concentrations of 100 mg/l and 250 mg/l within 72 h and 168 h respectively; where as at higher concentration (500 mg/l), benzene was found to be inhibitory. Hence the two phase partitioning bioreactor (TPPB) has been designed and developed to carryout biodegradation at higher concentration. In TPPB 5000 mg/l benzene was biodegraded up to 50.17% over a period of 168 h. Further the *Pseudomonas putida* MHF 7109 was isolated from cow dung microflora as potential benzene degrader and its ability to degrade benzene at various concentrations was evaluated. The data indicates 100%, 81% and 65% degradation at the concentrations of 50 mg/l, 100 mg/l, 250 mg/l within the time period of 24 h, 96 h and 168 h respectively. The GC-MS data also shows the presence of catechol and 2-hydroxymuconic semialdehyde, which confirms the established pathway of benzene biodegradation. The present research proves the potential of cow dung microflora as a source of biomass for benzene biodegradation in TPPB.



## 1. Introduction

Petrochemical industry is the fastest growing industry, manufacturing variety of chemicals beneficial for the other chemical processes and operations. However the waste generated during the manufacturing of basic raw material and intermediates are of the prime concern to the environmentalist. The petrochemical industry has been classified as hazardous group of industries under the Factories Act, 1948 Section 2(cb) and requires the compliance within the prescribed standards [1]. The wastes generated is commonly found to contain the complex hazardous compounds like benzene, toluene, xylene and phenol, etc. Benzene is one of the pollutants of petrochemical waste which is highly toxic and carcinogenic. Human exposure to benzene is a global environmental problem. After inhalation or absorption, benzene targets organs viz. liver, kidney, lung, heart and brain, etc. Benzene causes haematotoxicity through its phenolic metabolites that act in concert to produce DNA strand breaks and chromosomal damage [2]; hence benzene requires advance treatment to bring treated waste according to the prescribed level [3,4]. In spite of the present physico-chemical and biological treatment of waste the hazardous contaminants are still found persisting in the environment resulting into an increased level of pollution and causes environmental impact [5,6]. The ecologically acceptable treatment method for benzene and other waste hydrocarbons is a major challenge confronting to the petrochemical industries as well as other chemical industries.

Thus the recent advances in bioremediation techniques for the treatment of toxic waste will be of high significance [7]. Bioremediation techniques are typically more economical than traditional methods of waste treatment such as incineration, absorbent/adsorbent techniques, catalytic destruction, etc. Bioremediation technologies are improving as greater knowledge and experience are being gained in the field. Bioremediation application can be more effective where environmental conditions permit microbial growth and activity; its application often involves the manipulation of environmental parameters to allow microbial growth and degradation to proceed at a faster rate [8].

Many researchers have undertaken fundamental investigations for the degradation of benzene by microorganisms [9,10]. Bioremediation of benzene has been studied using activated sludge, pure and mixed microbial cultures [11–14]. The search for cost effective and environmentally friendly methods of benzene removal from contaminated sites needs to be further investigated [15]. In order to investigate some unique microbial source for bioremediation study, animal waste-cow dung has been used as a novel source. The presence of abundant macro and micronutrients provide suitable conditions for microbial growth in the cow dung, which could be an effective source for bioremediation of hazardous wastes [16,17].

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Toxicity level of hazardous wastes is the biggest hurdle in the way of bioremediation technology. Addition of the substrate (hazardous wastes) at too high concentration inhibits or even kills the microorganism where as substrate addition at too low rate can cause the microbial cell to starve and result in a sub optimal process performance. To overcome this difficulty, a two phase partitioning bioreactor system is designed and developed. The TPPB concept is based on the use of a water immiscible and biocompatible organic solvent that is allowed to float on the surface of a cell containing aqueous phase. The solvent is used to dissolve large concentrations of hazardous wastes (benzene), which then partition into the aqueous phase at low levels. Thus, although very high amounts of hazardous organic wastes can be added to the bioreactor, the microorganism experiences very low (sub inhibitory) concentrations [18].

The potential of various organisms to catabolize and metabolize organic compounds has been recognized as potentially effective means of disposing of hazardous wastes [19]. Although most microorganisms have detoxification abilities (i.e. mineralization, transformation and/or immobilization of pollutants) particularly bacteria, play a crucial role in it [20]. Bacteria have developed strategies for obtaining energy from virtually every compound under oxic or anoxic conditions (using alternative final electron acceptors such as nitrate, sulfate, and ferric ions). Several bacteria have been isolated and characterized which possess the ability to act on aromatic hydrocarbons. Some *Pseudomonas* species have also been identified as being efficient for degradation of aromatic compounds including benzene and its derivatives [21].

The aim of the present research study was to investigate and establish the biodegradation potential of cow dung microflora for benzene in single phase (direct contact with benzene) and TPPB (indirect contact with benzene). The potential benzene degrader present in cow dung microflora was also isolated and identified by different morphological, physiological, biochemical assays and 16S rDNA technology. Further the benzene biodegrading capability of isolated *Pseudomonas putida* MHF 7109 was assessed and the metabolites present during bioremediation were identified.

## 2. Material and methods

### 2.1. Preparation and activation of cow dung slurry

The cow dung taken as a source of biomass was diluted with water in the ratio 1:25 and filtered through a sieve (20 μm) to remove suspended particles. The prepared cow dung slurry was aerated and activated in a glass vessel for a week at room temperature (30–35 °C). After activation the physico-chemical characteristics of the cow dung were determined according to Soil Chemical Analysis (Jackson, 1973) and Standard Methods for the Examination of Water and Wastewater (APHA, 1998) before the activation process [22,23]. The aseptically collected cow dung was characterized for the assessment of its microbial diversity. The cow dung slurry was grown on nutrient agar plate and different microbial colonies were isolated based on morpho-colonial characteristics. The isolated microorganism after preliminary isolation and identification was further identified by conventional microbiological and biochemical techniques as described in Bergey's Manual of Systematic Bacteriology [24]. The different standard physiological and biochemical tests were performed using KB002 and KB003 kit (KB002, KB003 Hi25, Himedia, India). The basal salt solutions were supplemented to cow dung slurry as a source of inorganic nutrients (C:N:P) for growth and proliferation of microbial biomass. The constituent of basal salt solutions were $(NH_4)_2SO_4$—0.1 mg/ml, dextrose—0.2 mg/ml, $K_2HPO_4$—0.1 mg/ml, $KH_2PO_4$—0.1 mg/ml.

### 2.2. Design and development of TPPB

The TPPB has been designed and developed (glass and SS material having dimension 20 cm × 20 cm × 25 cm) with a provision to supply the air (12 mg DO/l), to maintain the aerobic condition [16]. The stirrer is provided for agitation of biomass and making the close contact of microorganism with contaminant. The bioreactor has also been provided with sampling port in both the aqueous and organic phase. In the bioreactor system vaporization take place simultaneously with bioremediation. In order to avoid the vaporization a condenser and cooling system have been provided to condense the vapor and trap the contaminant in organic phase.

### 2.3. Experimental setup for benzene degradation in single phase

The biodegradation of benzene was performed in the 160-ml glass bottles containing 100 ml activated cow dung slurry at 25 °C and continuous shaking at 100 rpm (shaker incubator, Neolab instruments) [10]. The glass bottles were tightly sealed with Teflon-coated rubber septa and screw cap to prevent the release of benzene by evaporation [25]. In a lab setup 100 mg/l, 250 mg/l, 500 mg/l concentrations of benzene were taken in activated cow dung microbial consortium. Biodegradation was assessed by comparing the disappearance of benzene in sample and control over time. Sterile control containing benzene was prepared for each concentration to discern volatilization and adsorption losses. Benzene concentration was monitored over time in order to compare lag periods and biodegradation rates for different concentrations. The lag period was determined as the time during which benzene concentration remained relatively constant. The environmental parameters were monitored throughout the experiment. Samples were withdrawn hourly starting from 0 h to 6 h, followed by every 24 h over a period of 168 h. The samples were transferred to 10-ml vials and capped with Teflon-coated septa prior to HPLC analysis.

### 2.4. Solvent selection for TPPB

n-Hexadecane was selected as organic solvent [18] for biodegradation of benzene. The solvent was tested to ensure that organic solvent is not taken up by the cow dung microflora as a carbon source. 5 ml of solvent was added to 125-ml Erlenmeyer flask containing 50 ml of activated cow dung slurry. Glucose was added to one flask, instead of solvent, as a positive control. The number of colony forming unit (CFU)/ml was counted at the interval of every 24 h. The CFU was counted by aseptically plating the 100 μl of serially diluted samples on sterile minimal agar plates containing benzene as single carbon source. The agar plates were incubated for 24 h at 37 °C. The number of CFU/ml was counted by applying the following formula [26]:

$$\text{No. of microbial colonies/ml} = \text{no. of colonies} \times \text{df/volume of the sample, ml}$$

df = dilution factor.

### 2.5. TPPB system and its operation for bioremediation study

In a designed and developed bioreactor 1 l activated cow dung slurry was taken as aqueous phase and 500 ml of n-Hexadecane was taken as organic phase. The concentration of benzene in organic phase was 5000 mg/l, which partitioned in aqueous phase at the concentration of 40 mg/l. The bioreactor was maintained at 25 °C, agitated at 250 rpm (magnetic stirrer, Remi equipments, ZMLH), and dissolve oxygen (DO) 12 mg/l. The bioremediation conditions like pH, temperature, DO were monitored through out the reaction. A control experiment with biomass-free aqueous phase was

also prepared in this manner to assess the possible loss of benzene through volatilization. Number of CFU/ml of microbial consortium was counted by plating the serial diluted cow dung consortium. Samples from both aqueous and organic phase were withdrawn through separate sampling ports similarly as described in Section 2.3.

### 2.6. Isolation and identification of potential microorganism from cow dung microflora

One ml aliquot of activated cow dung was inoculated to a 250-ml of Erlenmeyer flask containing 100 ml mineral salt medium (MSM). The composition of MSM per liter was 5 g $K_2HPO_4$; 4.5 g $KH_2PO_4$; 2 g $(NH_4)_2SO_4$; 0.3 g $MgSO_4$; 200 μl of trace element consisting of 16.2 g/l $FeCl_3 \cdot 6H_2O$; 9.44 g/l $CaHPO_4$; 0.15 g/l $CuSO_4 \cdot 5H_2O$ was added to 1 l of MSM as micronutrients. The sole carbon source was benzene at a concentration of 250 mg/l. One flask containing cow dung and MSM without benzene was taken as negative control. Flasks were incubated at 37 °C and 150 rpm for a period of 48 h [27,28]. Grown cultures were serially diluted and spread on sterile mineral agar plate containing benzene as a carbon source. Plates were incubated at 37 °C for 48 h after which isolated colonies were selected for further isolation. Individual isolated colony was re-streaked on mineral agar plates for identification. The isolated colony was gram stained and different standard morphological, physiological and biochemical tests were performed using KB003 kit (KB003 Hi25, Himedia, India). Further the genomic DNA from isolated microorganism was extracted using phenol:chloroform extraction procedure [20,29]. The 16S rDNA was PCR amplified using universal primers [30]. The PCR mixture contained 5 μl of 10× PCR buffer, 4 μl of 25 mM $MgCl_2$, 1 μl of 10 mM dNTP, 100 pmol of each primer, 1 μg of template DNA, 2 U of Taq DNA polymerase and volume made up to 50 μl with nuclease free distilled water. Amplification was performed with thermal cycler and following program—step 1, initial denaturation of 5 min at 94 °C, step 2, 35 cycles consisting of 60 s at 94 °C, 1.5 min at 52 °C, 1.5 min at 72 °C, step 3, 72 °C at 10 min. The PCR product was sequenced bidirectionally using the forward and reverse internal primers. The 16S rDNA sequence was aligned using CLUSTAL W software. The 16S rDNA sequence used for phylogenetic analysis was compared with the other 16S rDNA bacterial sequences available in EMBL/Genbank database. Phylogenetic tree was constructed using neighbor joining method.

### 2.7. Bioremediation of benzene by P. putida MHF 7109

Bioremediation of benzene by *P. putida* MHF 7109 was carried out in a 160-ml glass bottle containing 100 ml MSM at 25 °C and continuous shaking at 100 rpm. The composition of MSM per liter was 5 g $K_2HPO_4$; 4.5 g $KH_2PO_4$; 2 g $(NH_4)_2SO_4$; 0.3 g $MgSO_4$; 200 μl of trace element consisting of 16.2 g/l $FeCl_3 \cdot 6H_2O$; 9.44 g/l $CaHPO_4$; 0.15 g/l $CuSO_4 \cdot 5H_2O$ was added to 1 l of MSM as micronutrients [25]. In a batch biodegradation experiment 50 mg/l, 100 mg/l, and 250 mg/l benzene were taken as a single carbon source in 100 ml of mineral media. Biodegradation was assessed by comparing the disappearance of benzene in sample and controls over time. Sterile controls containing benzene were prepared for each concentration to discern volatilization and adsorption losses. Number of CFU/ml of *P. putida* MHF 7109 was counted by plating the serially diluted samples under sterile conditions (ref. Section 2.4). Samples were withdrawn similarly as described in Section 2.3, and transferred to 10-ml vials and capped with Teflon-coated septa for HPLC analysis. Optical density (600 nm) of the samples was also determined for monitoring the growth and proliferation of the microorganism.

**Table 1**
Microbial status of cow dung microflora.

| Microorganisms | Species |
|---|---|
| Bacteria | *Bacillus* sp.<br>*Pseudomonas* sp.<br>*Streptococcus* sp.<br>*Sarcina* sp.<br>*E.coli* sp.<br>*Acinetobacter* sp.<br>*Micrococcus* sp.<br>*Staphylococci* sp. |
| Fungi | *Penicillium* sp.<br>*Rhizopus* sp.<br>*Mucor* sp.<br>*Aspergillus* sp. |
| Actinomycetes | *Nocardia* sp. |

### 2.8. Sample preparation and analytical procedure

Samples were centrifuged (10 min, 10,000 rpm, Plasto crafts, Rota 6R-V/Fm) to separate cell mass and the supernatant and extracted in organic solvent (n-Hexane) for analysis. The samples were injected in HPLC system (Jasco, Model UV-2075 Plus, Japan) equipped with a UV–vis detector and C-18 column. The samples were analyzed using following programme: mobile phase acetonitrile–water 75:25, wavelength 254 nm, flow rate 1 ml/min, isocratic run for 10 min [25]. Benzene was further identified by borwin software, comparing UV spectra and retention times with the standards. For the TPPB, samples were withdrawn from both aqueous and organic phases and analyzed on HPLC as mentioned above. The samples were also analyzed by mass spectrometry to identify benzene and its metabolites. The samples were analyzed by mass spectrometer (Q-TOF micromass, YA-105, water's Ltd.) using ionization mode: ESI (electron spray ionization) and masrlynx 4.0V software.

## 3. Results

The primary objective of this work was to evaluate the potential of cow dung microflora for the degradation of benzene at various concentrations and enhancement of the biodegradation process at higher concentration of benzene using designed and developed two phase portioning bioreactor. Various concentrations of benzene were added to assess bioremediation in the single phase and TPPB; activated cow dung slurry served as a source of microbial biomass. The microbial characterization of cow dung microflora shows the presence of bacteria, fungi, and actinomycetes, etc. (Table 1). The physico-chemical characteristics of cow dung biomass are presented in Table 2, which shows the presence

**Table 2**
Physico-chemical characterization of activated cow dung slurry.

| Physico-chemical parameters | Quantity[a] |
|---|---|
| pH | 7.2 |
| Dissolved oxygen | 6.5 mg/l |
| Temperature | 26.1 °C |
| % Organic carbon | 0.31% |
| Phosphorus | 0.13 mg/l |
| Kjeldahl nitrogen | 14 mg/l |
| Sulphate | 34 mg/l |
| Calcium | 9.8 mg/l |
| Magnesium | 127 mg/l |
| Potassium | 159 mg/l |
| Sodium | 90 mg/l |
| Biological oxygen demand (BOD) | 9.50 mg/l |
| Chemical oxygen demand (COD) | 184 mg/l |

[a] The values represent the average of three replicates.

of macro- and micronutrient such as carbon, nitrogen, phosphorus, sulfate, calcium, magnesium, and sodium, in the cow dung slurry. Initially, the potential of the cow dung microflora was assessed for benzene degradation in single phase. In the single phase, the cow dung microflora remained in direct contact with the benzene and degraded benzene up to a concentration of 250 mg/l. In the bioremediation experiment, initial concentrations 100 mg/l, 250 mg/l, 500 mg/l of benzene were taken. The experimental findings indicated that in case of 100 mg/l benzene, degradation started within 4 h i.e. lag period observed at this concentration was very short. This benzene concentration was degraded below the limit of detection within 72 h. Similarly for 250 mg/l of benzene, 67.5% degradation was observed over a period of 168 h. Here degradation started after 24 h i.e. lag phase observed for this concentration was 24 h. Higher concentration of benzene like 500 mg/l was found relatively inhibitory for cow dung microflora as very little decrease in concentration was observed up to 168 h. The degradation pattern of benzene with time in single phase by the cow dung microflora is presented in Fig. 1. The biodegradation rates were about 1.4 mg/l/h and 1.1 mg/l/h for 100 mg/l and 250 mg/l benzene respectively. Biodegradation rates were estimated as the ratio of BTX removed (corrected for sterile controls) to the corresponding time after the lag period [31]. The essential environmental parameters responsible for bioremediation were monitored throughout the experiment. The data show an increase in temperature from 26 °C to 28 °C, supporting the bioremediation process. Decreases in pH (from 7.2 to 6.5) and DO (from 6.5 mg DO/l to 3 mg DO/l) were observed during the bioremediation process. During bioremediation in single phase the chemical oxygen demand (COD) and biological oxygen demand (BOD) were also monitored as indicator for bioremediation and microbial growth (Fig. 2). Fig. 2 demonstrates the decrease in COD levels over a period of bioremediation, which indicates the degradation of benzene by microorganisms present in the cow dung consortium. The decrease in BOD values indicates the growth of microorganisms in the various concentrations of benzene. The present bioremediation study of benzene in single phase shows that the cow dung microflora is able to degrade the toxic contaminant up to 250 mg/l. Thus, this system is effective for degradation of lower concentrations of contaminant. To overcome this limitation, a TPPB was designed and developed in which 5000 mg/l benzene was dissolved into the organic phase, providing an initial concentration of 40 mg/l in the aqueous phase. n-Hexadecane was selected as organic solvent as it has biocompatible properties. The biodegradability of n-Hexadecane by cow dung consortium was checked. It was found that the CFU/ml of cow dung consortium did not increase as compared to positive control in presence of n-Hexadecane. The concentration of benzene partitioned into

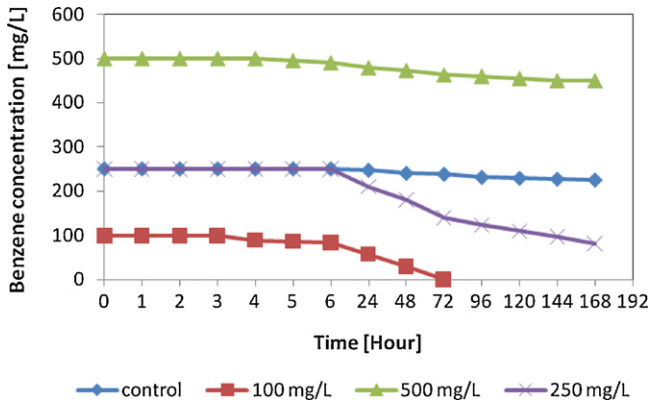


**Fig. 1.** Concentration of benzene during bioremediation by cow dung microflora in single phase.

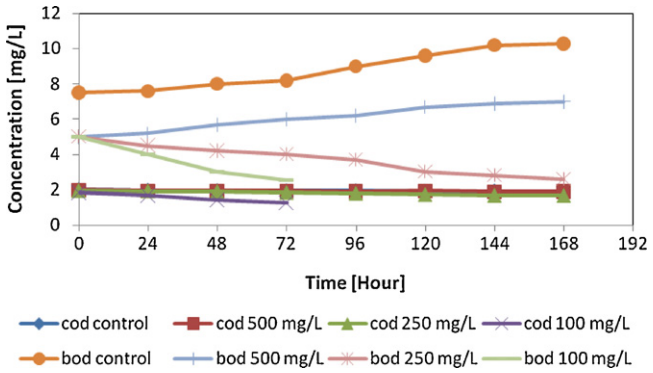


**Fig. 2.** Biological oxygen demand (BOD) and chemical oxygen demand (COD*) during bioremediation of benzene by cow dung microflora. *Values represented: concentration × $10^2$.

aqueous phase was much below the inhibitory concentration of benzene observed in single phase experiment. As presented in Fig. 3, the system experienced a lag phase of 24 h after which biodegradation started. Over the 168-h period 50.17% of the benzene was degraded. The biodegradation rate of benzene in TPPB was about 17.34 mg/l/h. Number of CFU/ml (Fig. 3) indicates that growth and multiplication of microorganism started after 24 h. The number of microorganism increased until the end of the experiment. Thus, this concentration of benzene (5000 mg/l) was not inhibitory for microorganisms in the TPPB. During bioremediation of benzene in the TPPB, the temperature increased from 26.4 °C to 28.5 °C, supporting the bioremediation process even at higher concentration of benzene. The pH was decreased from 7.4 to 6.1 and the DO from 6.8 mg DO/l to 3.1 mg DO/l.

The active and potent benzene degrader from cow dung microflora was isolated, and the bacterial colony that survived at higher concentration of contaminant was selected. In order to identify the isolated microorganism, different morphological, physiological and biochemical tests were conducted. The MHF 7109 isolate was gram negative rod with positive catalase and oxidase activity (Table 3). It assimilated glucose and xylose as well as showed the negative urease activity test. The microbial isolate also showed the positive test for citrate utilization and negative test for indole production. The characteristics indicated that the MHF 7109 isolate is a member of the genus *Pseudomonas*. For further identification the genomic DNA of bacteria was isolated, and 16S rDNA was amplified using universal primers. A 1.4-kb PCR product was obtained from the 16S rDNA. This PCR product was sequenced and sequence was aligned using CLUSTAL W software. The 16S rDNA sequence used for phylogenetic analysis was compared with the

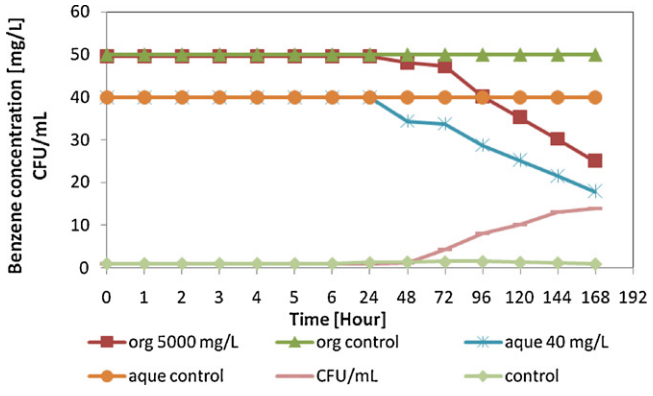


**Fig. 3.** Time course plot of benzene concentration in the organic phase, aqueous phase and number of colony forming units (CFU/ml × $10^7$) during bioremediation of benzene by cow dung microflora in the two phase partitioning bioreactor.

**Table 3**
The morphological, physiological and biochemical characteristics of *Pseudomonas putida* MHF 7109.

| Characteristics | Principal | Results[a] |
|---|---|---|
| Gram staining | Selective staining of cell wall | – |
| Cell form | | Rod |
| ONPG | Detects β-galactosidase activity | – |
| Lysine decarboxylase | Detects lysine decarboxylation | + |
| Ornithine decarboxylase | Detects ornithine decarboxylation | + |
| Urease | Detects urease activity | – |
| Phenylalanine deamination | Detects phenylalanine deamination activity | V |
| Nitrate production | Detects nitrate reduction | + |
| $H_2S$ production | Detects $H_2S$ production | – |
| Citrate utilization | Detects capability of organism to utilize citrate as a sole carbon source | + |
| Voges Proskauer's | Detects acetoin production | – |
| Methyl red | Detects acid production | – |
| Indole | Detects deamination of tryptophan | – |
| Malonate | Detects capability of organism to utilize sodium malonate as a sole carbon source | + |
| Esculin | Esculin hydrolysis | – |
| Arsbinose | Arsbinose utilization | – |
| Xylose | Xylose utilization | + |
| Adonitol | Adonitol utilization | – |
| Rhamnose | Rhamnose utilization | – |
| Cellobiose | Cellobiose utilization | – |
| Melibiose | Melibiose utilization | V |
| Saccharose | Saccharose utilization | – |
| Raffinose | Raffinose utilization | – |
| Trehalose | Trehalose utilization | – |
| Glucose | Glucose utilization | + |
| Lactose | Lactose utilization | V |
| Oxidase | Detects cytochromoxidase production | + |
| Catalase | Detects catalase production | + |

[a] V = 11–89% positive, + = positive reaction (more than 90%), – = negative reaction (more than 90%)

other 16S rDNA bacterial sequences available in EMBL/Genbank database. The BLAST search of available data in the EMBL/Genbank database showed a high similarity (99%) with *P. putida* IFO 14671 (Fig. 4). The sequence data reported in this paper have been submitted to the NCBI GenBank under accession number FJ975149. We designated this bacterium as *P. putida* MHF 7109 and its potential for bioremediation of benzene (50 mg/l, 100 mg/l, 250 mg/l) was evaluated. The isolated *P. putida* MHF 7109 strain started biodegradation of 50 mg/l benzene within 4 h and degraded it completely within 48 h. For 100 mg/l benzene, a lag phase of 24 h was observed, and 81% was degraded within 96 h. Similarly, 250 mg/l benzene was consumed 65% after 168 h by *P. putida* MHF 7109. A lag phase of 48 h was observed in this case (Fig. 5). The biodegradation rates of benzene degradation by *P. putida* MHF 7109 were about 1.13 mg/l/h, 1.30 mg/l/h, 1.35 mg/l/h respectively for 50 mg/l, 100 mg/l and 250 mg/l of benzene. The OD of the bioremediation sample (at 600 nm) was measured from 1 h up to 168 h (Fig. 6). The


**Fig. 4.** Phylogenetic tree showing the genetic relatedness of bacteria isolated from cow dung microbial consortium.

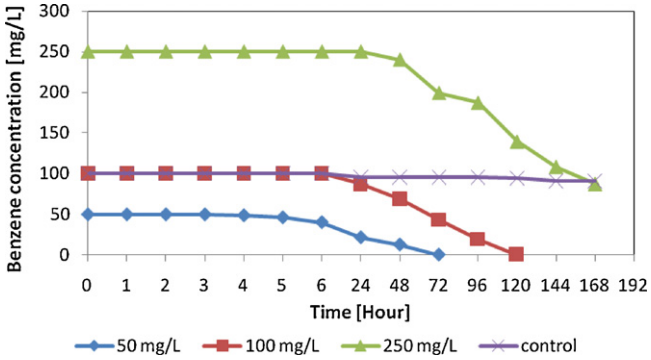


**Fig. 5.** Concentration of benzene during bioremediation by *Pseudomonas putida* MHF 7109.

data indicate the growth and development of the microorganism during the bioremediation of benzene. The viable count (CFU/ml) of *P. putida* MHF 7109 during benzene bioremediation confirms the growth and proliferation of the microorganisms. The viable counts of *P. putida* MHF 7109 in 50 mg/l, 100 mg/l, and 250 mg/l of benzene were increased from $1.8 \times 10^5$ to $4.1 \times 10^6$, $1 \times 10^5$ to $5.9 \times 10^6$, $0.6 \times 10^5$ to $7.1 \times 10^8$ respectively. The bioremediation samples were analyzed on mass spectrometer for detection of its metabolites or intermediates. The mass spectrometry analysis of bioremediation samples showed the presence of catechol and 2-hydroxymuconic semialdehyde during the bioremediation (Fig. 7a–c) [14]. In the present research study the potential of cow dung microbial consortium was evaluated for degradation of benzene in various ways. The results prove the effectiveness of present bioremediation strategy using cow dung microflora and TPPB for the degradation of benzene at various concentrations.

## 4. Discussion

Environmental pollutants have become a major global concern due to their undesirable recalcitrance and acute toxicity. One group of environmental pollutants, which is considered to be particularly troublesome due to their recalcitrant properties, is the petroleum hydrocarbons. Petrochemical industry generates hazardous wastes in significant quantities such as acetaldehyde, benzyl chloride, phenol, nitrobenzene, benzene, toluene, xylene, trichloroethylene, etc. Out of these benzene is of the most concern because of its high toxicity and solubility in water [32,33]. Hence the recent advances in bioremediation techniques for the treatment of petrochemical waste will be of high significance.

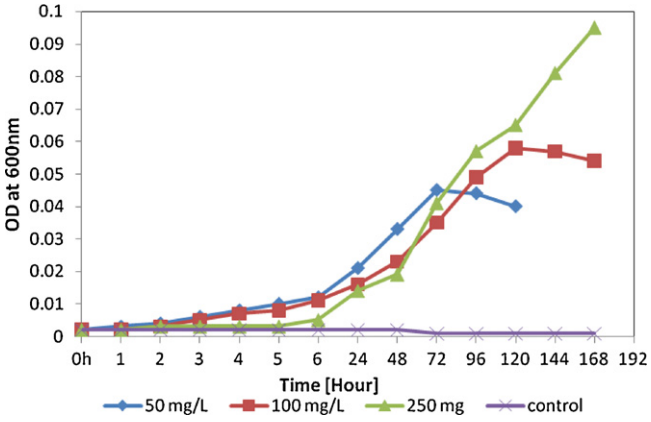


**Fig. 6.** Variation in optical density of bacterial culture during bioremediation of benzene by *Pseudomonas putida* MHF 7109.

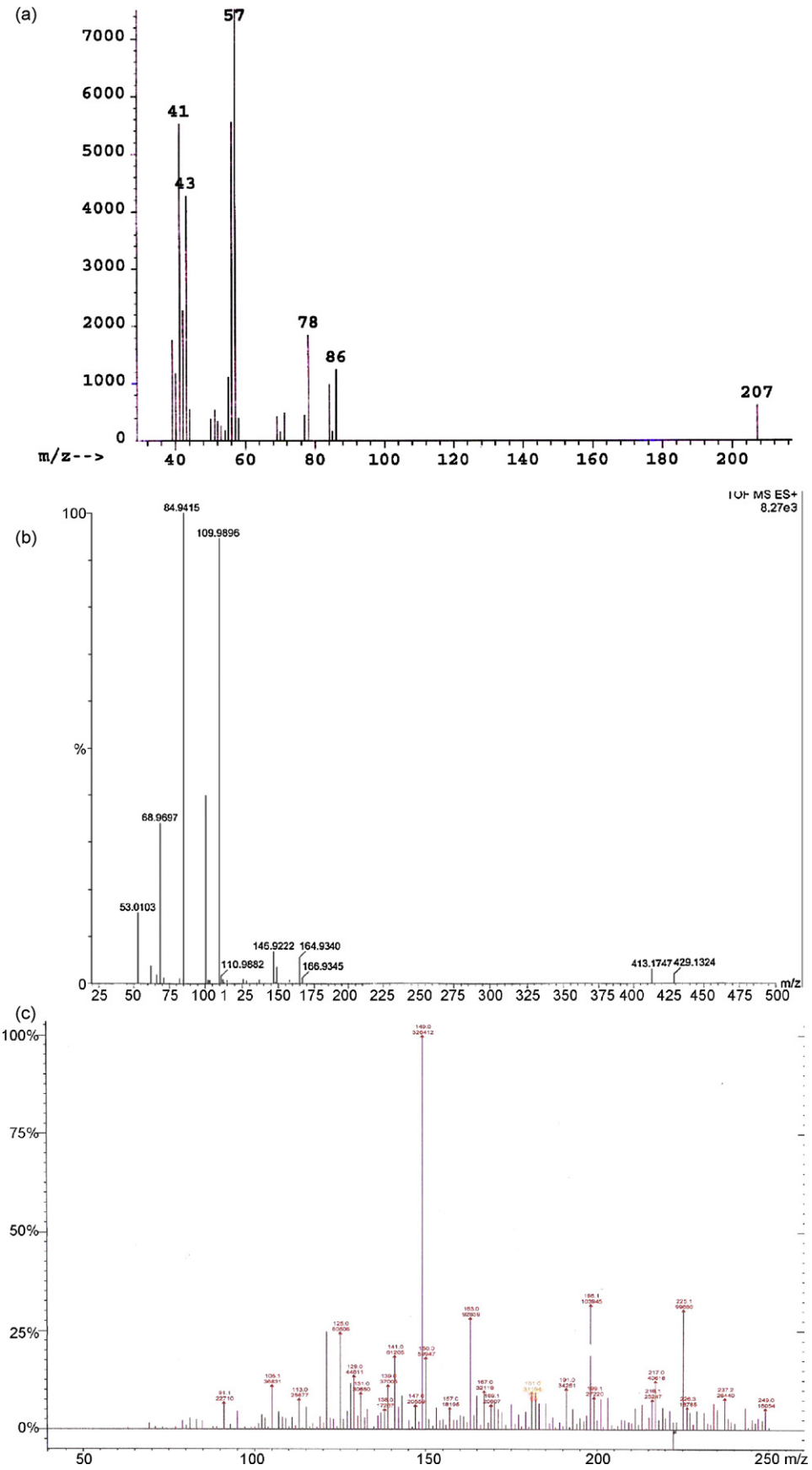


**Fig. 7.** (a) Mass spectrum of benzene ($m/z$ identification 78). (b) Mass spectrum of catechol ($m/z$ identification 109.98). (c) Mass spectrum of 2-hydroxymuconic semialdehyde ($m/z$ identification 141).

The purpose of this study was to investigate and establish the effective bioremediation methods for benzene biodegradation using cow dung microflora in TPPB and also to identify potential microorganism from cow dung consortium for benzene biodegradation.

In the present research study, cow dung microflora was used as the source of biomass and its potential for biodegradation of benzene at various concentrations (50 mg/l, 100 mg/l, 250 mg/l, 500 mg/l, 5000 mg/l) was assessed. The bioremediation of benzene was carried out using aqueous cow dung slurry directly as single phase. The advantages and limitations of the single phase bioremediation were assessed. Further for the advancement of bioremediation process, the TPPB has been designed and developed to carry out the bioremediation of benzene at higher concentrations. Single specific microorganism was also isolated as potential benzene degrader from same cow dung microflora and used for bioremediation study. The cow dung is a rich source of nutrients and contains variety of microorganisms [34,35]. The microbial assessment of cow dung microflora indicated the presence of *Bacillus* sp., *Pseudomonas* sp., *Streptococcus* sp., *Sarcina* sp., *E.coli* sp., *Acinetobacter* sp., *Micrococcus*, *Staphylococci* sp., *Penicillium* sp., *Rhizopus* sp., *Mucor* sp., *Aspergillus* sp., and *Nocardia* sp. Our findings are in agreement with findings of Akinde and Obire [35] who reported the presence of *Acinetobacter* sp., *Bacillus* sp., and *Pseudomonas* sp., in cow dung. The physico-chemical characterization of cow dung shows presence of carbon, nitrogen, potassium, phosphorus, sulfate, calcium, magnesium, and sodium which tallies with the findings of Bwembya and Yerokun [34]. They have also reported the presence of carbon, nitrogen, potassium, and phosphorus in cow dung. These macro- and microelements serve as a nutrient source for the growth and maintenance of microbial community. Bioremediation potential of cow dung microflora for benzene remediation has not been reported till date, only Satsangee and Ghosh [36] have reported the anaerobic phenol degradation using adapted mixed cultures derived from cow dung and sewage sludge and found a high degradation rate (2500 mg/l/d). Therefore, the present research study was carried out to evaluate the effectiveness of cow dung microflora for aerobic degradation of various concentrations of benzene. The interaction of cow dung microflora with the benzene in single phase led to the degradation up to 250 mg/l concentration of benzene within a period of 168 h. However, microbial growth was inhibited when the microorganisms come into direct contact with higher concentration (500 mg/l) of this contaminant in the single phase. Alvarez and Vogel [20,31] reported that mixed aquifer microbial culture degrade 50 mg/l benzene after 8 days having 25 mg/l/d degradation rate and 2 days lag period [20]. In comparison to this results cow dung microflora proved more effective in benzene degradation as it degraded 100 mg/l benzene in 72 h (3 days). The lag period observed in this case was only 4 h and degradation rate1.4 mg/l/h. In another set of experiment the designed and developed TPPB [12] has been used to biodegrade benzene using the concept of partitioning the toxic contaminant between the aqueous and organic phases [37]. In this technique microorganism assess the compound from organic phase and remain in aqueous phase where by higher concentration of contaminant can be degraded. In TPPB benzene was added in n-Hexadecane and the cow dung microbial consortium was kept in aqueous phase. Aerobic conditions were maintained by supplying air through an aeration port. The microorganisms present in aqueous phase progressively degrade the benzene partitioning into this phase from organic phase. This system has been found to degrade the benzene at a concentration of 5000 mg/l, with 50.17% degradation being observed within a period of 168 h. Study conducted by Yeom and Daugulis also reported the biodegradation of benzene at higher concentration (14,000 mg/l) using TPPB [38]. The cooling system attached to the bioreactor condensed vapors during the agitation and prevented the benzene loss due to vaporization. The viable count (CFU/ml) assessed during the bioremediation of benzene in the TPPB also suggests that the microorganism undergoes constant growth. The concentration of benzene used in the TPPB was much higher than in the single phase. Considering the fact that several factors such as pollutant concentration, temperature, active biomass concentration, pH, availability of inorganic nutrients, dissolve oxygen influence the rate and extent of bioremediation, these environmental parameters involved in the bioremediation were monitored throughout the bioremediation process. The data show an increase in temperature which is in agreement with the finding of Abdulsalam and Omale [39], who reported the variation of temperature from 25 °C to 29 °C during bioremediation. This temperature range fell within the optimum required for effective bioremediation process [39]. The pH was found to be near neutrality, keeping the fact in view that the biodegradation rate is highest at a pH near neutrality [40]. DO was also found decreasing, which indicates growth and proliferation of the microorganisms. Our findings confirm that the catabolic diversity of microbes in cow dung have the capability to degrade benzene. Apart from evaluation of biodegradative potential of cow dung microflora as a mixed microbial culture for benzene remediation, the single specific microorganism present in cow dung having benzene catabolizing property was also evaluated. Hence *P. putida* MHF 7109 was isolated and identified from cow dung microflora as a potential microorganism for bioremediation of benzene. *P. putida* is a very versatile microorganism, it can adapt to divers substrates and possess several catabolic pathways capable of acting on recalcitrant substances. This isolated microorganism was found to degrade benzene up to the concentration of 250 mg/l with the rate 1.35 mg/l/h. The result obtained for the benzene bioremediation by *P. putida* MHF 7109 is in agreement with findings of Shim et al., who also reported that inhibition of cell growth at 500 mg/l benzene concentration when *P. putida* and *Pseudomonas fluorescens* were exposed to this concentration [41]. Similarly Alvarez and Vogel found that 50 mg/l benzene was degraded with a rate of 10 mg/l/d by *Pseudomonas* sp. CSF-215 and lag phase of 5 days was observed [31]. The potential of *P. putida* MHF 7109 shows that it can be used to degrade hazardous compounds such as benzene. In another study Otenio et al. reported that *P. putida* CCMI 852 was not able to metabolize the benzene [14]. Hence *P. putida* MHF 7109 proves its effectiveness in comparison to other strains of *P. putida*. The GC-MS data shows the presence of catechol and 2-hydroxymuconic semialdehyde during the bioremediation of benzene. This result is in agreement with the findings of Haibo et al. and Otenio et al. (2005) who reported the catechol and 2-hydroxymuconic semialdehyde as a major inter mediate of benzene biodegradation [9,14,42,43]. The long term acclimatization of microbial biomass with the contaminants and their intermediates would convert into environmental friendly compounds.

## 5. Conclusion

TPPB designed and developed for bioremediation has been found effective for degradation of hazardous compounds at higher concentration using cow dung as a novel source of biomass. Further the identified microbial strain *P. putida* MHF 7109 has been found to be a potential organism for enhancing bioremediation of hazardous compound. The study of this microorganism in particular genomics and proteomics would further provide molecular approaches for bioremediation. Therefore the present research study on bioremediation of hazardous compounds using the cow dung consortium and *P. putida* MHF 7109 as potential organism in TPPB has provided an innovative research in the area the of bioremediation.

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