Cow dung Bacteria offer an Effective Bioremediation for Hydrocarbon-Benzene

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Abstract
Characterization of cow dung microorganisms was carried out for determining its potential to degrade potent and widely accumulated petrochemical hydrocarbon Benzene. Physico-chemical and microbial parameters were studied and ten cultivable bacteria were isolated and identified. Out of these, five of the isolates survived at increasing concentrations of Benzene up to 100mg/L. Later, these five isolates were taken as a consortium and bioremediation of benzene at varying concentrations at 50, 100, 200 and 400mg/L was carried out. Benzene was found to be completely degraded at 50mg/L and 100g/L concentration within 96 hr and 144hr whereas, 99 percent degradation was observed for 200 mg/L and up to 69.2 percent for 400mg/L of benzene. These findings help to have an insight about indigenous source of biomass (like cow dung) and its effectiveness to develop an environmental friendly treatment technology for remediation of hydrocarbons like benzene.

Keywords — *benzene, cow dung, bacteria, bioremediation, biomass*

I. INTRODUCTION

In the last few decades the industrial revolution has led to an exceptional release of hazardous compounds into the environment, endangering the ecological balance of our planet. The release of hydrocarbons whether accidentally or due to human activity is a prime cause of water and soil pollution. Management of pollution is achieved by largely physico-chemical methods like incineration, adsorption or extraction [1]. However, these processes are usually difficult to employ or cannot be scaled up universally due to their prohibitive costs and also seldom result in the secondary pollution of the environment.

Benzene is a major mono-aromatic hydrocarbon produced by many manufacturing processes and present in petroleum based fuels. It is a constituent of motor fuels; a solvent for fats, waxes, resins, oils, inks, paints, plastics, and rubber; used in the extraction of oils from seeds and nuts; and in photogravure printing [2]. It is also used in the manufacture of detergents, explosives, pharmaceuticals, and dyestuffs [3]. Benzene is a common industrial pollutant and a component of gasoline. The US Environmental Protection Agency has classified benzene as a Group A human carcinogen. It was selected as a high priority candidate for consideration under Proposition 65 based on selection by a group of experts in reproductive toxicity combined with use, production and exposure data [4]. Benzene was also one of 14 high priority agents chosen by a Delphi committee of experts organized by OEHHA to prioritize candidate DARTs. Benzene is a radiomimetic and its exposure may lead progressively to aplastic anaemia, leukaemia and multiple myeloma [5]. Long-term exposure to benzene has the potential to cause chronic health effects including central nervous system (CNS) damages, cardiac effects, and lung cancers. The widespread use of benzene has led to presence in groundwater due to leaks in underground storage tanks and pipelines, improper waste disposal practices, inadvertent spills and leaching from landfills [6].

Conventional techniques like air sparging, air stripping, adsorption and vapour phase extraction are typically used for the removal of benzene. However, these methods are expensive and can lead to incomplete decomposition of contaminants. Bioremediation appears to be an economical, energy efficient and environmentally sound approach. It is defined as a technology that can transform contaminants into less harmful forms by microorganisms and their produced substances. The most effective merit of bioremediation is environmental friendliness [7].

Earlier research and data shows the effectiveness of many bacterial strains with regards to their benzene degradation ability. Microorganisms have shown to be able to degrade Benzene under aerobic, micro-aerobic or hypoxic, as well as anaerobic conditions [8] - [24]. It is commonly observed that, aerobic biodegradation is much faster and effective than anaerobic processes. Bioremediation of benzene in contaminated sites relies on the immense metabolic capacities of the microbial world for the transformation of pollutants into essentially harmless or at least less dangerous compounds [25] – [30]. It aims at the biological mineralization of organic compounds to CO_2 and water, or at least at transformation to less-toxic or innocuous forms [31], [32]. Reference [33] showed mineralization of benzene to equimolar amounts of methane and

carbon dioxide using methanogenic benzene degrading culture. The presence of a large number of diverse bacterial species in nature expands the variety of chemical pollutants that can be degraded and the extent to which polluted sites can be decontaminated. Microbial diversity offers an immense field of environment friendly options for mineralization of contaminants or their transformation into less harmful non-hazardous compounds.

Several pure cultures have also been studied for bioremediation of benzene in the past with strains like *Pseudomonas aeruginosa* [34]; *Geobacter metallireducens* [35]; *Mycobacterium cosmeticum* byf-4 [36]; *Planococcus sp.* strain ZD22 [37]; *Pseudomona putida* MHF3107 [8]; *Pseudomonas putida* F1 [38]; *Janibacter sp.* SB2 [39]. But, different microorganisms are able to degrade different contaminants depending upon the nature and concentration of contaminant and the metabolic needs of the microorganisms. The metabolic pathway of any contaminant offers presence of intermediates at each level of the catabolic process and use of mixed cultures or consortium would be an effective way to develop a bioremediation strategy. The presence of a large number of diverse bacterial species in nature expands the variety of chemical pollutants that can be degraded and the extent to which polluted sites can be decontaminated. Microbial diversity offers an immense field of environment friendly options for mineralization of contaminants or their transformation into less harmful non-hazardous compounds. Communities of microorganisms rather than single strains have often been considered more important in bioremediation as their metabolic diversity and sometimes metabolic redundancy may contribute to the robustness of process [40] – [42]. Powerful molecular biology technologies have revealed valuable insights in the identification of microbial species in diverse contaminated sites. A mixed microbial population offers participation of multiple degrading enzymes. The nature of this contaminant-microbial interaction can be studied in the laboratory, under stimulated conditions, corresponding to natural environments. Isolation of microbial cultures, mixed or pure, from natural sources, is therefore, instrumental in establishing the pathways for recalcitrant compound degradation, since microorganisms, and nutrients are essential for their maintenance at adequate levels over a long period [43] – [51].

Besides, microorganisms which show high efficiency biodegradation capabilities under laboratory conditions may not perform equally well than actual contaminated sites [52], [53]. The consortium for biodegradation may be based on exchange of specific nutrients, removal of growth inhibitory products and co-metabolism during bioremediation.

In the present study, we have used characterization of indigenous bacterial cultures to identify a potential microbial consortium for the bioremediation of petrochemical waste compound – Benzene. A prime effort to explore cow dung as a source of novel indigenous microorganisms for bioremediation of hazardous compound benzene has been carried out. Cow dung contains organic matter available for microbial energy recovery and it also contains three essential fertilizer elements (nitrogen, phosphorus and potassium) [54]; generally used as a source of fuel, fertilizer, heat source, purifier, floor coating, mud brick additive and as pond pH balancer [55]. The cow dung was collected from cattle shed from different sites and pooled together and later characterized for identifying culturable bacterial species using physico-chemical, biochemical and molecular biology tools i.e. 16SrRNA technique. Further, these isolates were studied for their bioremediation potential to degrade Benzene. The laboratory pilot scale study was carried out in stimulated conditions to develop methodology and treatment technology for remediation of industrial effluent waste contaminants.

II. MATERIALS & METHODS

A. Sample Collection and Physicochemical Analysis

The cow dung sample (N=5) was aseptically collected from cow sheds from distinct sites within the city of Mumbai, India. The breed of cows in the study was Sahiwal and all cows wherein the lactating phase. All samples were pooled and taken as a source of biomass. The samples were filtered through sieve (20µm) to remove suspended particles. The samples were processed on the day of collection and its physicochemical parameters such as pH, temperature, alkalinity, acidity, calcium, organic carbon, phosphorus, magnesium and sulphate levels were analysed. The physicochemical parameters were analysed as prescribed in standard methods in the 17th edition of APHA [56], [57].

B. Microbial Characterization

To identify and isolate the microbiome present in the cow dung, serial dilution method was used. For isolation of bacteria, 1 gm of the pooled sample was added to 9ml of sterile deionized water and 100ul of the diluted sample was plated onto Nutrient Agar medium (Himedia Laboratories, India) incubated at room temperature for 24 hrs. Plates showing well separated, isolated bacterial colonies were taken for further analysis. In case of fungus, colonies were counted after 48-72 hrs. Ten bacterial colonies were randomly chosen with no biases and their colony characteristics and biochemical properties were analysed in accordance to Bergey's Manual of Determinative Bacteriology [58]. Isolated

colonies were further plated on NA slants to obtain pure bacterial cultures.

C. Identification of bacterial isolates for its potential to degrade Benzene

Selected bacterial isolates were cultured in 100ml Minimal Salt Medium (MSM) (K_2HPO_4 (1gm/L), (NH_4) SO_4 (0.5gm/L), $MgSO_4$.7H2O (0.2gm/L), $FeCl_3.6H_2O$ (0.034gm/L), $CaCl_2$ (0.1gm/L), Glucose (0.1gm/L), Peptone/yeast extract (250mg/L), and 1.5% agar as gelling agent, pH 7) with Benzene (25 mg/L) (Sisco Research Laboratories Pvt. Ltd, Mumbai, India) as a sole carbon source. Flasks were incubated at 30^0C.at 150 rpm for 72 hrs. pH and optical density at 600nm were measured daily to analyse bacterial growth. Further, to evaluate the bacterial isolates with higher benzene degrading capability, bacterial cultures were exposed to higher benzene concentrations of 50mg/L, and subsequently to 75mg/L, up to 100mg/L. The bacterial isolates showing luxuriant growth were further identified by 16SrRNA technique.

DNA was isolated from pure cultures and used as template. The 16SrRNA gene fragment was amplified from genomic DNA by PCR technique using 16SrRNA gene universal primers (Sigma Aldrich). 50µl reaction mixture containing primer 16SrRNA template, buffer, $MgCl_2$, dXTPs and Taq-polymerase was prepared and PCR reaction was carried out in a thermal cycler (Eppendorf Thermal Cycler) with 30 cycles at 95^0C for 2min, 52^0C 30 sec., 72^0C 90 sec. conditions. PCR products were subjected to electrophoresis on a 2% agarose gel. The PCR product was sequenced bi-directionally using the forward and reverse internal primer. The sequence data were aligned and analysed on the NCBI, BLAST database to identify the bacteria and their closest neighbors. Based on maximum identity score and query coverage, the best 10 highly identical sequences were selected and aligned using multiple alignment software program Clustal W. The evolutionary history was inferred using the Maximum Parsimony and Neighbor-Joining method and phylogenetic tree was drawn to analyse evolutionary relationships among sequences of isolated microorganism and nearest neighbors using Phylogeny FR software. The sequences were then submitted to NCBI-Genbank.

D. Generating microbial consortium to evaluate its benzene degrading capacity

A consortium of 5 bacterial isolates was generated from strains that showed higher potency to degrade benzene. Bioremediation of benzene was carried out in a 500ml glass sealed bottle with sufficient headspace containing sterile MSM (constituents as described earlier) and benzene concentrations of 100mg/L, 200mg/L and 400mg/L. 1ml of 0.6 optical density of each bacterial strain was seeded in each flask. The flasks were incubated at room temperature in a rotary shaker at 130rpm for a period of 7 days. The bioremediation was assessed using parameters such as BOD, COD, pH, temperature, microbial count and concentration of benzene were analysed each day. Un-inoculated MSM along with benzene served as a control.

E. Colony Forming Unit (CFU) counting

Culture broth was collected at regular intervals under sterile conditions. Supernatant was centrifuged and bacterial cells were allowed to settle. The supernatant was discarded and the pellet was resuspended in 0.1 ml saline, diluted and plated onto nutrient agar plates for CFU counting.

F. Detection of Benzene

Culture supernatants were centrifuged (10min, 10000rpm) to separate cell mass. Supernatants was extracted in organic solvent (n-hexane) for analysis. The extracted samples were injected in a high-performance liquid chromatograph system (HPLC, Jasco LC 2000 plus, Japan) equipped with a UV-VIS Detector and C-18 column. The samples were analysed using the following programme: mobile phase Acetonitrile-water 75:25, wavelength 254nm, flow rate 0.7 ml/min, isocratic run for 10 min [59].

G. Statistical Analysis

Data represented as mean +/- SE. Unpaired t-tests was used for comparison among the control (un-inoculated medium). Values of $p<0.05$ were considered statistically significant.

III. RESULTS

A. Physico-chemical and microbial enumeration of cow dung samples

Firstly, we started with the analysis of physico chemical parameters of all the cow-dung samples that were used in the study. Since, the breed of the cows was same and within the same geographical area, we hypothesized very little or no significant difference in their properties. As evident from Table 1, the various physico-chemical characteristics were not apparently difference within the samples and hence a pool of the five samples was used in the study. We further characterized the physico-chemical parameters of the pooled sample. We did not observe any significant difference in the pooled sample as compared to the individual sample.

TABLE 1. Physio-chemical characterization of pooled cow dung sample

Parameter	Results
pH	7.3
Dissolved Oxygen	6.4 mg/L
Temperature	25.9°C
% Organic Carbon	0.67%
Biological Oxygen Demand	19.83 mg/L
Chemical Oxygen Demand	195.2 mg/L
Phosphorus	0.23 mg/L
Sulphate	38.5 mg/L

*Values are taken as average of three replicates

Pooled sample was plated onto the agar plate to enumerate and analyze the microbiome present in the cow dung sample. The total number of bacterial, fungal and yeast colonies were counted using colony counter. As documented in Table 2; cow dung possesses a huge microbial load.

TABLE 2. Microbial enumeration of the cow dung sample

Total viable count	2.29 X 10^8 cells/ml
Total Fungal count	1.16 X 10^7 cells/ml
Yeast count	7.5 X 10^6 cfu/ml

TABLE 3. Biochemical assay for the ten isolates

Colony Characters	1	2	3	4	5	6	7	8	9	10
Shape	Slightly Irregular	Circular	Circular	Circular	Circular	Circular	Almost circular	Irregular	Irregular	Irregular
Margin	Undulate	Raised	Slightly raised	Convex	Raised	Raised	Umbonate	Umbonate	Umbonate	Umbonate
Color	Creamy	Butyrous	Mucoid	Opaque/ Yellowish	Mucoid/ Butyrous	Creamy	Beige/ Opaque	Creamy/ Off-white	Creamy	Off-white
Elevation	Flat	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Undulate	Entire
Texture	Wrinkled	Smooth	Gummy/Wet	Smooth	Smooth	Shiny	Shiny	Rough	Rough	Rough
Motility	Motile	Motile	Less Motile	Non-motile	Motile	Motile	Motile	Motile	Motile	Motile
Gram's Staining	Gram negative rods	Gram negative rods	Gram negative coccobacillus	Gram positive cocci	Gram negative rods	Gram negative rods	Gram negative rods	Gram negative rods	Gram negative rods	Gram negative rods

TABLE 4. Colony characterization of the ten bacterial isolates

Biochemical Test	1	2	3	4	5	6	7	8	9	10
ONPG	-	-	-	-	-	+	-	-	-	-
Lysine Utilization	-	-	-	-	+	-	-	-	-	-
Ornithine Utilization	+	-	-	-	+	+	-	-	-	-
Urease	-	-	-	-	-	+	-	-	-	-
Phenylalanine Utilization	-	-	-	-	+/-	-	-	-	-	-
Nitrate Reduction	+	+	+	-	+	+	+	-	-	-
H_2S Production	-	-		-	-	-	-	-	-	-
Citrate Production	+	+	+/-	-	+	+	-	+	-	+
Voges Proskauer's Test	+	-	-	-	-	-	-	+	-	-
Methyl Red	-	-	+	-	-	-	-	-	-	-
Indole	-	-	+	-	-	-	-	-	-	-
Malonaten Utilization	-	-		-	+	-	-	-	-	-
Esculin Hydrolysis	-	-		-	-	+	+	-	-	-
Arabinose	-	-	+	-	-	-	-	-	+	+
Xylose	-	+	+	-	+	+/-	-	-	-	-
Adonitol	-	-		-	-	-	-	-	-	-
Rhamniose	-	+	+	-	-	-	-	-	-	+
Cellobiose	-	-	+	+	-	-	+/-	-	-	-
Melibiose	-	-	+	-	+/-	-	-	-	-	-
Saccharose	-	-	-	-	-	+/-	+/-	-	-	-
Raffinose	-	-	+	-	-	+/-	-	+	-	-
Trehalose	-	-	-	-	-	+/-	-	-	+	-
Glucose	+	+	+	+	+	+	-	+	-	+
Lactose	+	-	+	+	+/-	+	-	-	+	-
Oxidase	+	+	-	-	+	-	Delayed +	-	-	-

Indications: + Growth Observed; +/- Variable Growth; - No Growth

B. *Isolation and characterization of bacterial isolates from cow dung*

With no bias, ten well isolated bacterial colonies were selected and their colonies characteristics were studied. As reported in Table 3, most were motile and 6 were Gram negative whereas 4 stained positive for Gram's staining. Biochemical tests were carried to identify *Enterobacteriaceae sp*. to determine the genus of various isolates. Analysis revealed isolated bacteria belonged to *Pseudomonas sp., Escherichia sp., Staphylococcus sp., Pseudomonas sp., Pseudomonas sp., Aeromonas sp., Bacillus sp., Enterobacter sp.* As mentioned in Table 4. Analysis of fungal colonies revealed Yeast, *Rhizopus sp.*, Nocardia origin on Sabouraud's agar slants.

C. *Five bacterial isolates successfully degraded Benzene*

To evaluate the potential of these selected ten bacterial isolates, we subjected them for growth on MSM with benzene as a sole carbon source. As observed from Table 5., five of the ten bacteria showed luxuriant growth.

Further, enhancing benzene concentrations unto 100mg/L revealed that these tolerate high benzene concentrations and may have effective benzene degrading capability.

16SrRNA sequencing demonstrated their identity up to species level viz.

Isolate 1 - *Enterbacter cloaceae,*
Isolate 2 - *Pseudomonas pseudoalcaligenes,*
Isolate 5- *Archromobacter sp.,*
Isolate 7- *Aeromonas hydrophila,*
Isolate 8- *Bacillus pumilis*

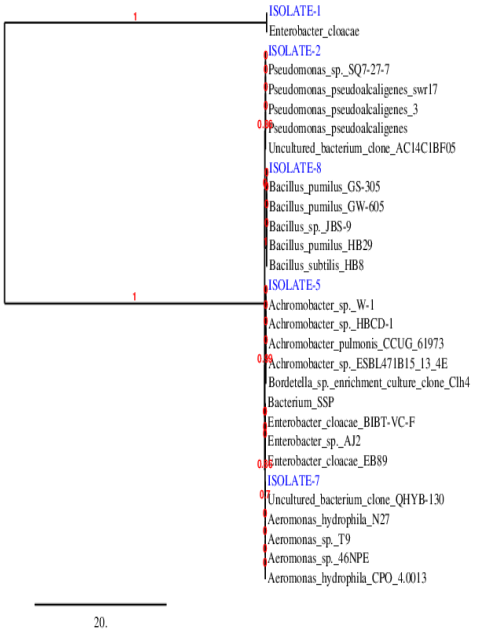
TABLE 5. Growth of ten isolates at varying concentrations of benzene

Isolate	25mg/L	50mg/L	75mg/L	100mg/L
1	+++	+++	+++	+++
2	+++	+++	+++	+++
3	-	-	-	-
4	-	-	-	-
5	+++	+++	+++	+++
6	-	-	-	-
7	+++	+++	+++	+++
8	+++	+++	++	++
9	-	-	-	-
10	-	-	-	-

Indication: +++ Luxuriant growth; ++ Moderate Growth; - No Growth

The genetic relatedness was linked by generating a phylogenetic tree as shown in Figure 1.

FIGURE 1. Phylogenetic tree representing relatedness linking the five bacterial isolates

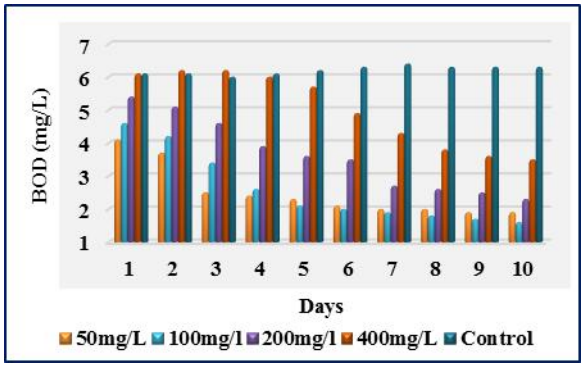


D. *Microbial consortium effectively degraded benzene with higher potency*

During biodegradation of benzene, the isolates surviving at 100mg/L concentration of compound were taken as a potential consortium and then studied to observe their interaction and growth as a biomass. The bioremediation was carried out at different concentrations i.e. 50mg/L, 100mg/L, 200mg/L and 400mg/L and control for the study included sample with no microbial consortium at 400mg/L compound in MSM. The environmental parameters like temperature, BOD, COD, pH and microbial growth using cfu/ml were studied during the bioremediation process for 14 days.

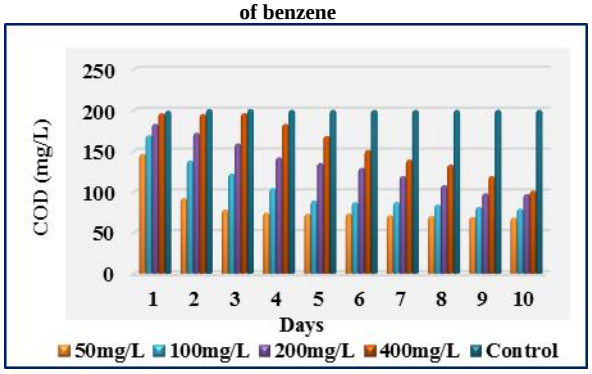
It was observed that during bioremediation, process temperature varied between 25^{o}C to 27^{o}C and there was an increase in temperature over time which correlates with the findings [60,8], that with the increase in temperature, the rate of bioremediation increases. It was observed as there were changes in the growth pattern of the cells there were changes in BOD as shown in Figure 2. In case of 50mg/L, decrease in BOD up to day 4 was observed and then remained unchanged; whereas for 100mg/L decrease up to day 5 and then unchanged. For 200mg/L there was decrease seen from day 3 till day 7 and for 400mg/L decrease in BOD was observed from day 4 up to day 10 and then remained unchanged. This indicated that as there was demand for oxygen with cell growth decrease in BOD observed, cells reach the log phase the BOD remained unchanged.

FIGURE 2. Biological Oxygen Demand during bioremediation of benzene



Another parameter studied was COD (Chemical Oxygen Demand) which is the indicator of rate of degradation of compound i.e. bioremediation of Benzene. It was observed that as there was decrease in the compound concentration there was a change in COD as shown in Figure 3. For 50 and 100mg/L gradual decrease in COD was observed since day 1, whereas for 200mg/L it was from day 4 and for 400mg/L from day 5. Negligible decrease for control was seen throughout the study.

FIGURE 3. Chemical Oxygen Demand during bioremediation of benzene



The microbial growth during the bioremediation was studied by Colony Forming Unit (CFU)/ml method as shown in Figure 4. The colony count on the plates was observed. The maximum cell growth was observed for 200mg/L of compound concentration indicating high growth and high degradation rate. The variation in pH was also determined all through the process of bioremediation which was in accordance with the changes during bioremediation as given in Figure. 5 [61]. pH values from 7.0 decrease up to 6.2 and maximum decrease was observed for 400mg/L concentration.

All through the bioremediation process, samples were taken for analysis on compound concentration and the degradation of compound was studied using HPLC peaks. Benzene was found to be completely degraded at 50mg/L and 100mg/L concentrations after 96hr and 144hr respectively, whereas for 200mg/L about 99 percent degradation after 240hr and for 400mg/L up to 69.2 percent degradation was observed. The details of the benzene degradation at varying concentration is shown in Figure and Table 6.

FIGURE 4. Microbial growth during bioremediation of benzene

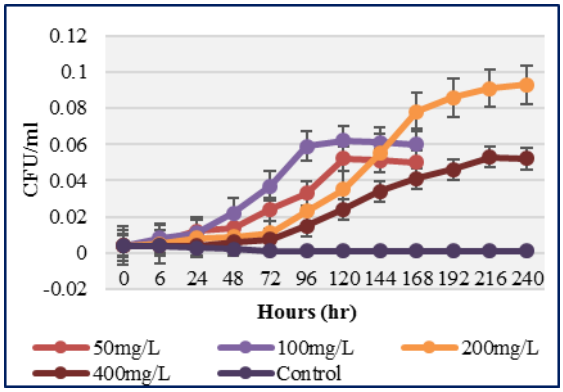
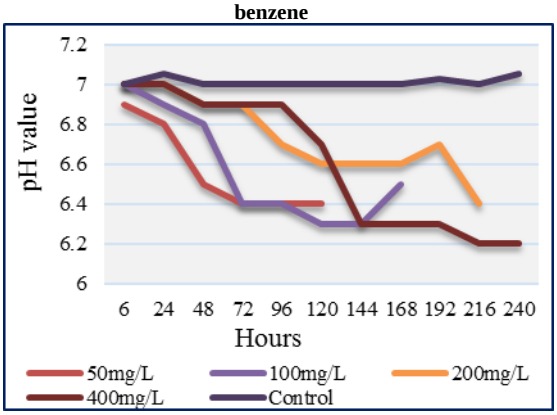


FIGURE 5. pH variation during bioremediation of benzene



This indicates that compound concentration of benzene at 400mg/L was found to be inhibitory for the microbial consortium. As complete degradation of benzene takes place at 50 and 100mg/L concentration indicates that degradation started within 6hr and degraded below the limit of detection within 96hr.

FIGURE 6. Percentage degradation for benzene bioremediation

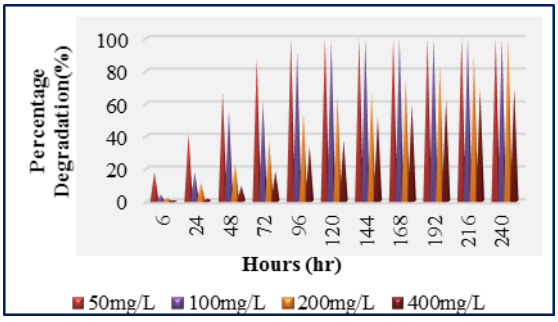


TABLE 6. Degradation of Benzene

Benzene Concentration	Degradation Percentage (%)	Hours
50 mg/L	100	96
100mg/L	100	144
200mg/L	99.1	240
400mg/L	69.2	240

IV. DISCUSSION

Environmental pollution caused by petroleum and its associated products through accidental or deliberate release results in the calamitous consequences within the ecosystem. Fuel products such as benzene, toluene ethyl benzene, and xylenes (BTEX) and fuel additives such as methyl tert-butyl ether (MTBE) are among the organic pollutants commonly detected in water resources. Out of this group of aromatic compounds, benzene, being listed as one of the USEPA priority pollutant [62], is highly volatile, flammable, toxic, and carcinogenic compound [63] commonly found as contamination linked to human activities [64] – [69]. When benzene is released into the atmosphere, it can be transformed or degraded by physicochemical reactions [70]. Benzene is of great environmental and human health concern since it has been identified as gene-toxicant as well as animal carcinogen. Benzene emissions represent an environmental hazard negatively affecting public health and productivity. Presence of benzene extensively in industrial wastewaters, gasoline and petroleum contaminated groundwater, has become a universal problem [71]. Because of their high water solubility and toxicity, it forms a serious risk for human health and the environment. As a result, much attention is paid to removal of benzene from the environment, e.g. by bioremediation [72]. It is an attractive and useful method of remediation of soils contaminated with petroleum hydrocarbons because it is simple to maintain, applicable in large areas, is economic and enables an effective destruction of the contaminant. Bioremediation processes are among the best approaches to restoring contaminated soils; its success depends on the ability of microbial degraders to remain active in the contaminated environment and on the bioavailability of the contaminants to microorganisms [28].

Microbiological activity is affected by many environmental factors including energy source, donors and acceptors of electrons, nutrients, pH, and temperature. These parameters influence how quickly microorganisms adapt to the environment [73]. Hydrocarbon degradation by microbial population in natural environment is influenced by physical, chemical and biological factors that contribute to the degradation of petroleum and individual hydrocarbons. Rate of biodegradation depends greatly on the composition, state, and concentration of the oil or hydrocarbons, with dispersion and emulsification enhancing rates in aquatic systems and absorption by soil particulates being the key feature of terrestrial ecosystems. Naturally occurring microbial communities that respond to the presence of contaminating hydrocarbons normally have more than one type of hydrocarbon utilizing microorganisms. Presence of nutrients, diverse classes of microorganisms in cow dung and its availability, cost effectiveness could be exploited as a very unique and environmental friendly source of biomass for biodegradation. Nutrient is one factor that can hinder biodegradation if not handled properly and could limit the rate of hydrocarbon degradation in the terrestrial environment [74].

Cow dung comprises of digested residue of herbivorous matter which is acted upon by symbiotic bacteria residing within the animal's rumen. The resultant faecal matter is rich in minerals and contains diverse classes of microorganisms. Presently it is used as a source of fuel, fertilizer, heat source, purifier, floor coating, mud brick additive, pond pH balancer, pot cleaner, etc. In our study we have tried to explore cow dung as a source of indigenous biomass. Five different cow dung samples were taken for the study from distinct places in Mumbai. These samples were taken such that the breed of the cow viz. Sahiwal (local variety of cow) and age of the cows were maintained same. The cows were selected particularly in the lactating phase of their life cycle. The physicochemical characterization data of these five samples showed the presence of high content of nutrients especially macro as well as micronutrients. This data was in accordance to the study conducted by [40]. These five samples were pooled to get a consistent data set and physicochemical characterization of pooled too showed rich nutrient content. Due to its high nutritional value, it helps growth of different microorganisms including Bacteria, Fungi as well as yeast. The microbiome study was conducted by serial dilution method and enumeration of viable count was conducted for bacterial, fungal and yeast. The focus of the study was confined to evaluate the bacterial population of the cow dung so as to further explore its biodegrading ability. The bacterial isolates were obtained by studying different colony morphology for basic identification. The total viable count for the presence of aerobic bacterial population was carried out using serial dilution method and it showed very high CFU/ml values. Fungal and yeast population was also determined. The method used for the study included determining only culturable microbial biomass. Among the bacterial population further isolates were identified by serial dilution and 10 different species were studied. The isolates were initially distinguished from each other based on their colony characteristics and gram nature as presented in Table 3. We got 7 of the isolates showed gram negative cocci and bacilli species whereas remaining 3 were gram positive rods. The biochemical tests to identify the *Enterobacteriaceae sp.* were carried using HiMedia Kit which helped to determine the genus of various isolates. The microorganisms found to be present were *Yeast, Rhizopus sp., Nocardia, Enterobacter sp., Pseudomonas sp., Escherichia sp., Staphylococcus sp., Pseudomonas sp., Pseudomonas sp., Aeromonas sp., Bacillus sp.* from the cow dung

sample assessed. Among the identified bacterial isolates, the potential microbial consortium has been developed and isolated by scaling up the benzene concentration wherein; microbial isolates were each adapted to higher concentrations of benzene gradually up to 100mg/L. The bacterial isolates were grown in Minimal salt medium (MSM) with selected petrochemical compounds viz. benzene, toluene and xylene separately as sole carbon source at varying concentration of 25 mg/L, 50mg/L and 100mg/L. Flasks were incubated at 30^0C and 150 rpm for 72 hr. pH was adjusted to 7.0 before inoculation. pH and optical density at 600nm were measured daily to analyse bacterial growth. Likewise, five different isolates were identified using 16SrRNA technique were *Enterobacter cloeceae, Pseudomonas pseudoalcaligenes, Achromobacter sp., Aeromonas hydrophila, Bacillus pumilis* were selectively found surviving at even 100mg/L concentration of benzene. These culture dependent studies have explored the diversity of culturable organisms in the environments. Such information on indigenous microorganisms has been considered to be useful in defining biostimulation and/or bioaugmentation based bioremediation strategies. It was observed that during bioremediation, process temperature varied between 25^0C to 27.5^0C and there was an increase in temperature over time which correlates with the findings [75], [76], [8], that with the increase in temperature, the rate of bioremediation increases. Reference [75] observed that highest rate of bioremediation in aqueous environment at 20-30^0C. As the microbial biomass initiated the growth from lag to log phase growth there was decrease in dissolved oxygen (DO) level. DO is an indicator of growth and proliferation microorganism in a medium. As the cell growth takes place there is a demand for available oxygen in the surrounding eventually decreasing the oxygen level. Thus, the BOD (Biological Oxygen Demand) was calculated. It was observed as there were changes in the growth pattern of the cells there were changes in BOD (Fig.1). In case of 50mg/L, decrease in BOD up to day 4 was observed and then remained unchanged; whereas for 100mg/L decrease up to day 5 and then unchanged. For 200mg/L there was decrease seen from day 3 till day 7 and for 400mg/L decrease in BOD was observed from day 4 up to day 10 and then remained unchanged. This indicated that as there was demand for oxygen with cell growth decrease in BOD observed, cells reach the log phase the BOD remained unchanged. Another parameter studied was COD (Chemical Oxygen Demand) which is the indicator of rate of degradation of compound i.e. bioremediation of Benzene. It was observed that as there was decrease in the compound concentration there was a change in COD (Fig.2). For 50 and 100mg/L gradual decrease in COD was observed since day 1, whereas for 200mg/L it was from day 4 and for 400mg/L from day 5. Negligible decrease for control was seen throughout the study. The microbial growth during the bioremediation was studied by Colony Forming Unit (CFU)/ml method as given in Figure 3. The colony count on the plates was observed. The maximum cell growth was observed for 200mg/L of compound concentration indicating high growth and high degradation rate. The variation in pH was also determined all through the process of bioremediation which was in accordance with the changes during bioremediation [61]. pH values from 7.0 decrease up to 6.2 and maximum decrease was observed for 400mg/L concentration. All through the bioremediation process, samples were taken for analysis on compound concentration and the degradation of compound was studied using HPLC peaks. Benzene was found to be completely degraded at 50mg/L and 100mg/L concentrations after 96hr and 144hr respectively, whereas for 200mg/L about 99 percent degradation after 240 hr and for 400mg/L up to 69.2 percent degradation was observed. This indicates that compound concentration of benzene at 400mg/L was found to be inhibitory for the microbial consortium. As complete degradation of benzene takes place at 50 and 100mg/L concentration indicates that degradation started within 6 hr and degraded below the limit of detection within 96hr. These findings can be effective in developing a treatment methodology for biodegradation of Benzene and other similar petrochemical hydrocarbons using identified source of bacterial microbial biomass. Therefore, the present research study on bioremediation of hazardous compounds using the cow dung bacterial consortium has provided an innovative research in the area the of bioremediation.

V. CONCLUSION

The study has established a unique strategy for biodegradation of one of the most potent and widely accumulated pollutant, benzene. The use of simple and easily available waste, cow-dung harbor a range of microbes that show a great potential to degrade benzene. These bacteria in isolation or as a consortium utilize and multiply in presence of high benzene concentrations. Our laboratory level bioremediation strategy has successfully provided a proof of concept that the consortium generated can be further employed at industrial scale to reduce the burden of toxic benzene from the environment and curb its health hazard.

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