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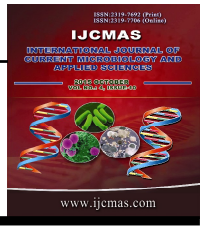

Original Research Article

Optimization of BH medium for efficient Biodegradation of Benzene, Toluene and Xylene by a *Bacillus cereus*

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Keywords

Bacillus cereus, Bioremediation, Benzene, tolune and Xylane (BTX), Catechol 1,2-dioxygenases

A B S T R A C T

The aim of this study was to isolate, characterize, and evaluate the potential of petroleum hydrocarbon (PHC) -degrading bacterial strains from oil-contaminated soil in the Coimbatore region. Among 40 oil-degrading bacterial cultures isolated from the oil-contaminated soil samples, 4 were identified as potential isolates such as *Bacillus cereus, Micrococcus spp, Pseudomonas spp* and *E. coli,* this potential isolates were selected on the basis of their capabilities to grow on Benzene, tolune and Xylane, (BTX) as their sole carbon sources and exhibited good growth as well as forms clear zone on BTX containing media. Among them**,** *Bacillus cereus* was highly active on BTX containing media. This strain was selected to check the ability of Catechol 1, 2 dioxygenase enzyme production. Moreover, formulate the medium condition for improving the degradation process. the better degradation observed when using the pH 7 media, while the optimum temperature was 35ºC. Ammonium chloride and Potassium dihydrogen phosphate were found to be the best nitrogen and phosphorous sources for the *Bacillus cereus* respectively. Optimization of the hydrocarbon enriched cultured medium stimulated the growth of above strain leading to the enhanced biodegradation of BTX.

Introduction

Nowadays Petroleum based products are the major source of energy for industry and daily life. The following potential sources for spills or releases of crude oil and its products to the soil; Exploration, production, storage, refining and transportation etc.,. These spills or release may be occurred by accident or due to human activities is a main cause of water and soil pollution. The oil polluted soil is a serious problem in both developed and developing countries (Anupama *et al*., 2009). Soil contamination with hydrocarbons causes extensive damage to the animals and plant (Guru *et al.*, 2013). Benzene, Toluene and Xylene are major aromatic hydrocarbon compounds in many petroleum products and threat to health and the environment (Anitha Singh *et al*., 2009).

Some women having breathed high levels of benzene for many months had irregular menstrual periods and a decrease in the size

of their ovaries. Men exposed to high levels of benzene are more likely to have an abnormal amount of chromosomes in their sperm (Gangula *et al.*, 2012). The number of technologies was commonly used for soil remediation includes mechanical, burying, evaporation, dispersion and washing, but these methods are not successful because these can also lead to secondary pollutant problems and expensive also.

Bioremediation is an increasingly popular alternative to conventional methods for treating waste compounds using natural microbial activity (Salini *et al*., 2015). Microbial remediation of a hydrocarbon–contaminated site is achieved by the help of a diverse group of microorganisms applying enzymes in their metabolism. Catechol 1,2-dioxygenases (1,2-CTDs) play important roles in the degradation pathways of various aromatic compounds and are ubiquitous in microorganisms (San *et al*., 2007).

Many reports concerning 1,2-CTDs, they are almost all about enzymes from bacterial spp and degrade the phenols. Previously, we isolated a strain of *Bacillus cereus*, which uses Benzene, tolune and Xylane as its energy source, from soil in an oil contaminated area in Coimbatore. Moreover the optimization process was carried out for improving the degradation process with different pH, T, Nitrogen and phosphorus sources.

Materials and Methods

Sample collection

Soil samples were collected randomly from oil contaminated area at Coimbatore area. Sample at a depth within 1-5cm from the surface of the soil using sterile spatula and were placed in pre sterilized polythene bags and tightly packed. Samples were immediately transferred to the laboratory for analysis and stored at 4°C for further processing.

Isolation of biodegrades

One gram of dried soil sample was dissolved in 9ml of distilled water and agitated vigorously. Soil sample was serially diluted upto 10^{-7} dilution and 0.1 ml from each dilution were selected and spread over Bushnell Hass-Agar medium (g/liter: 0.2 g MgSO4, 0.02 g CaCl2, 1.0 g KH2PO4, 1.0 g K2HPO4, 1.0 g NH4NO3, 0.05 g FeCl3, and the pH was adjusted to 7 - 7.2 with 0.01N HCl) supplemented with 2% of hydrocarbon such as Benzene, Toluene and Xylene. Incubation was done at 37˚C for upto7 days and growth was examined. Any isolate which grow on BHA plate were confirmed as degraders. Isolates were maintained on Nutrient agar for further analysis.

Screening of potential isolates

The confirmed isolates were inoculated into BH liquid media and incubated at 25°C for 7days. Prepare BH Agar plates and spread 100 µl of oil (Benzene, Toluene and Xylene), then prepare wells (8mm) and load 50 µl of broth culture and incubate plate at 37°C for 48hrs. The zone of clearance around the wells and measured the diameter of the zone.

Production of Catechol 1, 2 dioxygenase

LB medium (5g/l yeast extract, 10g/l casein peptone, 10g/l Nacl,) pH 7.0 was used to grow the isolate. The bacteria inoculam (10%) from LB culture were then transferred to 1000ml of freshly prepared mineral salts medium {2.75g/l of K_2HPO4, 2.225g/l of KH_2PO4, 1.0g/l of $(NH_4)2SO4$, 0.2g/l of $Mgcl_2.6H_20$, 0.1g/l of Nacl, 0.02g/l of $Fecl_3.6H_2O$ and 0.01g/l of $Cacl_2$} pH 7.0, supplemented with 1% v/v Benzene, Toluene and Xylene. The medium were

incubated at 30^{o} C on a rotary shaker at 200rpm (Olukunle *et al*., 2015).

Preparation of cell-free extract

The cells were harvested by centrifugation at 10,000 × g for 10 min and washed twice with 2 volumes of 0.9% NaCl. Wet cells (10 g) were resuspended in 50 mL of 50 mM sodium phosphate buffer (pH 7.4), and cells were disrupted with a Soniprep 150 Sonicator (MSE Sci. In., England) at 50 W for 2 min with ice-water cooling. The debris was removed by centrifugation at 12,000 × g for 20 min, and the supernatant solution was used as the cell-free extract.

Enzyme assay

Catechol 1, 2 dioxygenase activity was assayed spectrophotometrically using a Shimadzu UV-1800 UV-Vis Spectrophotometer. The standard assay of enzyme activity was performed by making an assay mixture containing 5µL of Catechol 1, 2 dioxygenase, 20 µL of 10 mM Catechol as a substrate and final volume adjusted to1 mL with 50 mM sodium phosphate buffer (pH 7.0). The enzyme activity was monitored by measuring the formation of cis, cis-muconic acid at 260 nm. One unit (U) of the enzyme activity was defined as the amount of the enzyme required to catalyze the formation of 1 µ mol of product per min at 25°C (Naiem *et al.*, 2011).

Identification of Microbe by 16SrRNA Gene Sequencing

The 16S rDNA gene sequence was used for the identification of bacterial strains and carry out BLAST with 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.

Effect of Temperature and pH

The effect of temperature and pH on the growth and degradation will be studied by using Bushnell-Haas broth supplemented with BTX (1%) will inoculate with the isolates and incubate at different temperatures (10°C, 20°C, 30°C, 40°C, 50°C) and different pH (5.5, 6.5, 7.5, 8.5 and 9.5) for this uninoculated tubes will be serve as controls. Growth and degradation of the organism will be assayed by gravimetric analysis.

Effect of different phosphorous sources

The following phosphates were used for the optimization process such as Potassium dihydrogen phosphate, dipotassium monohydrogen phosphate, disodium monohydrogen phosphate and diammonium hydrogen phosphate. The different phosphorus sources were added on Bushnell-Haas broth supplemented with BTX (1%) will inoculate with the isolates. All flasks were incubated at shaking conditions at 100 rpm at 37ºC for 7 days. Growth and degradation of the organism will be assayed by gravimetric analysis.

Effect of different nitrogen sources

Five different nitrogen sources were used for the optimization process. The following nitrogen sources were used such as Sodium nitrate, ammonium chloride, ammonium monohydrogen phosphate, ammonium sulphate and potassium nitrate on the growth of the *B.subtilis* isolates on BHB media. All flasks were incubated at 30ºC for 7 days at shaking conditions at 100 rpm.

Result and Discussion

The study was carried out for the optimization of media condition in the degradation process. The strain was isolated from oil contaminated soil areas, which was identified on the basis of biochemical character. In this study, 4 were identified as potential isolates such as *Bacillus cereus, Micrococcus spp, Pseudomonas spp* and *E.coli*, this potential isolates were selected on the basis of their capabilities to grow on BTX as their sole carbon sources and exhibited good growth as well as forms clear zone on BTX containing media. Among them, *Bacillus subtilis* were highly active on BTX containing media. Furthermore, this isolate were subjected to 16srRNA sequencing for confirmation.

The BLAST similarity search against database sequences revealed that the isolate had 94% similarity with a maximum score of 1081 bits to *Bacillus cereus* strain 16S ribosomal RNA gene partial sequence (Accession no. HQ 844643.1). A separate phylogenetic tree was constructed for isolate using the homologous sequences from the BLAST search, showing a relationship to *Bacillus cereus* strains (Fig.1). The strain was to be proven as an efficient hydrocarbon degraded in our previous study (Jayanthi and Hemashenpagam, 2015). Recently Murugan *et al*., (2013) observed the BTX degrading Bacillus species from waste water; Mukherjee and Bordoloi (2012) was also observed Bacillus spp from oil contaminated soils, Here, the reason for the dominant occurrence of gram-positive bacteria could be that these bacteria, due to their stronger cell envelope, can proliferate more easily than gram negative bacteria in the highly variable intertidal sediment environment (Ajeet *et al*., 2014).

Microbial remediation of a hydrocarbon–contaminated site is achieved by the help of a diverse group of microorganisms applying enzymes in their metabolism, especially the indigenous soil bacteria. Aerobic biodegradation of aromatic compounds involves their conversion into dihydroxylated intermediates (e.g., catechol or its alkyl- or chloro-substituted derivatives) which are then further metabolized by intradiol (catechol-1, 2-dioxygenase) or extradiol (catechol-2,3-dioxygenase) dioxygenases (Olukunle *et al*., 2015). Therefore, need to confirm the isolate that is able to secrete relevant enzyme with higher activity of degrading hydrocarbons. In this study three types of oils were used as sole carbon source and supplements such as Benzene, Toluene and xylene. The highest enzyme concentration was obtained (1.5833U/ml) when using benzene and followed by toluene (1.2777U/ml) (Table 1).

The culture conditions and composition of the growth medium are very important for the hydrocarbon degradation. Therefore, medium was improved with different pH, Temperature, carbon source, nitrogen source and phosphorus, which was considered to be optimized primarily as reported by many researchers (Trinetta *et al*., 2008). Most of the studies BH medium was utilized for the hydrocarbon degradation studies (Mandri and Lin, 2007). Therefore, optimization was carryout with BH broth supplemented with BTX. The $KH_2PO_4.2H_2O$ and $K_2HPO_4.2H_2O$ favored the growth due to their buffering capacity (Debajit *et al*., 2014). Moreover, phosphorus helps in the synthesis of ATP and DNA. $CaCl_2.2H_2O$, $MgSO_4.7H_2O$ and $FeCl_3.2H_2O$ favored the growth as Ca^{2+}, Mg^{2+} and Fe^{2+}ions has a well established role in enhancing the enzymatic action of hydrocarbon degrading enzymes. The degradation was measured by Gravimetric analysis according to the procedure of Anupama *et. al*., 2009.

Table.1 Production of catechol-1, 2-dioxygenase with BTX as substrate

S. No	Nature of the oil	Total activity (U/ml)	Total protein (mg/ml)	Specific activity (U/ml)
1	Benzene	29	28	1.5833
2	Toluene	23	18	1.2777
3	Xylene	19	12	1.0357

Fig.1 Phylogenetic tree of *B.cereus*

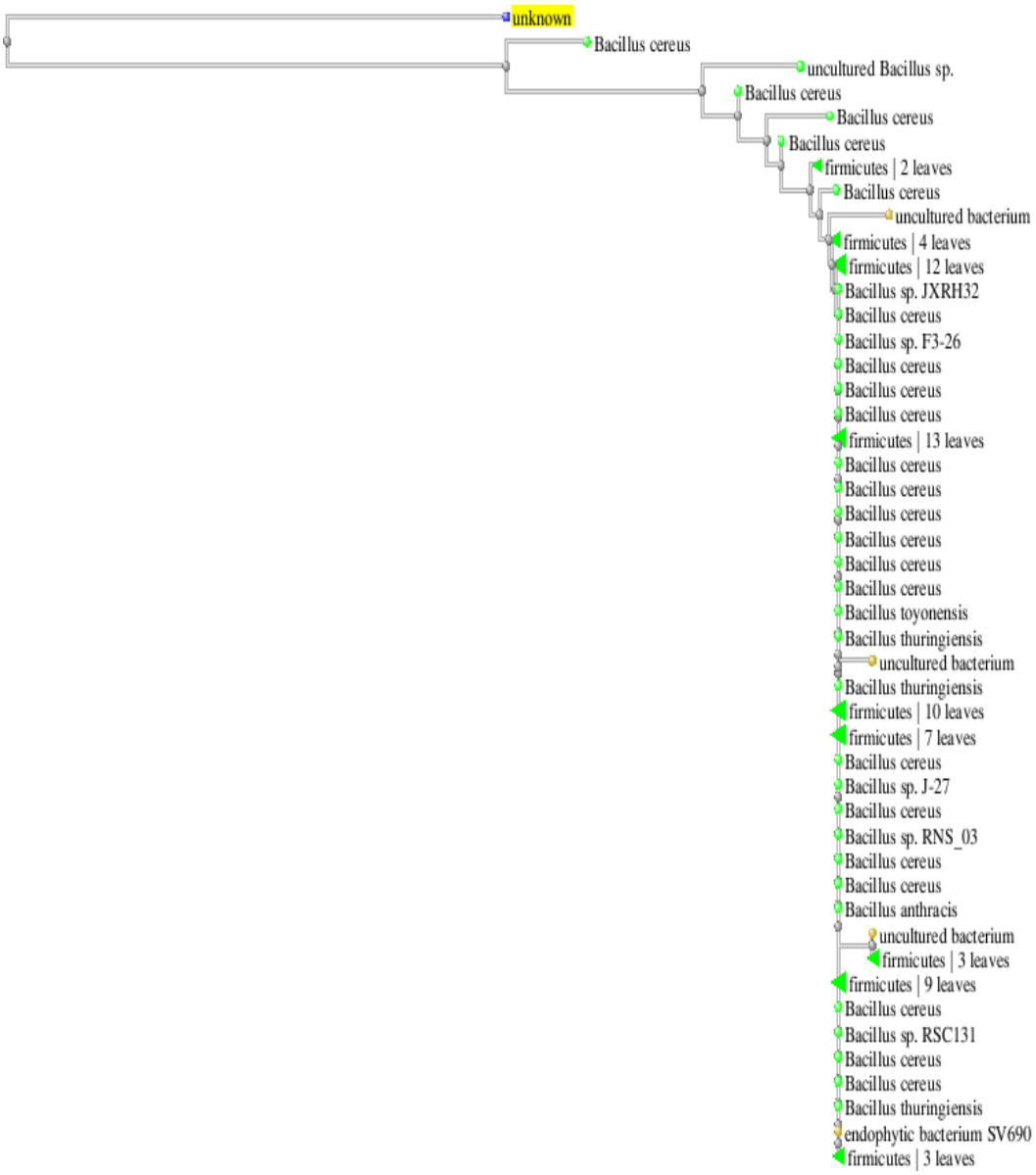


Fig.2 Effect of Temperature on degradation of BTX

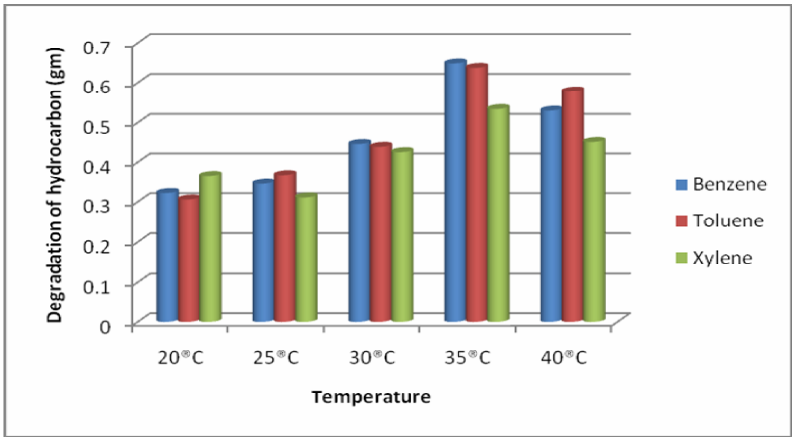


Fig.3 Effect of pH on degradation of BTX

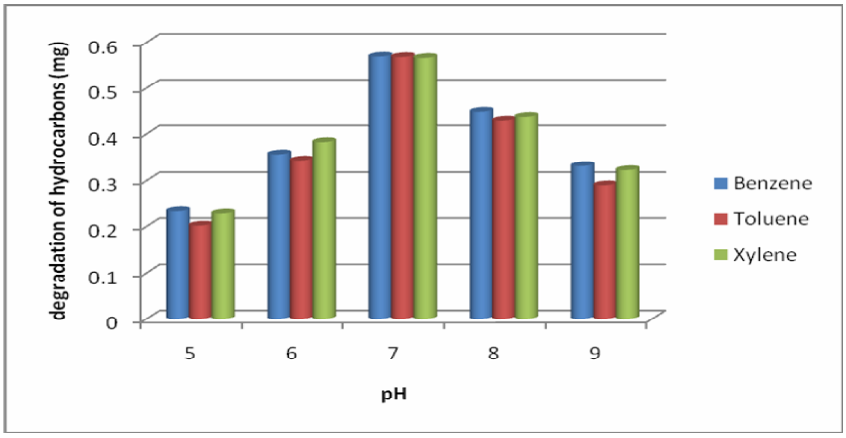


Fig.4 Effect of different types of Nitrogen sources on degradation of BTX

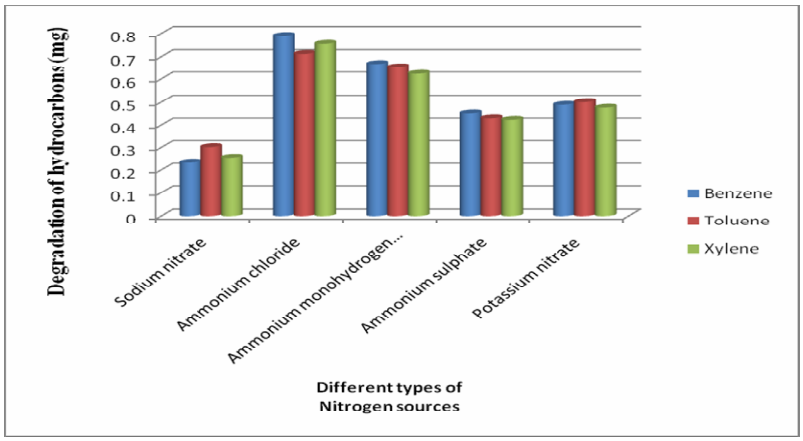
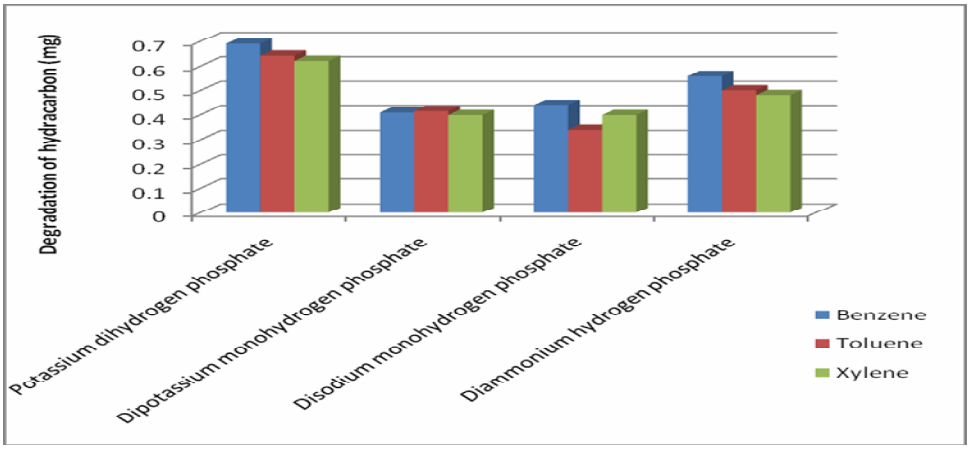


Fig.5 Effect of different types of phosphorous sources on degradation of BTX



In this study five different types of Temperature were utilized for the optimization process. The highest degradation process was occurring when using 35^0C and followed by 40^0C. The 0.648mg of benzene was degraded when incubate the medium with 35^0C. Among the 3 types of hydrocarbons, benzene was highly degraded by *Bacillus cereus* and followed by Toluene. The result was depicted in Fig.2.

Most microbes prefer to grow at temperatures in a range of about 10 to 38 °C. In this study, temperature tenure for the most efficient pollutant degradation was selected. It was found that the increase in temperature from 20 to 35 °C promotes degradation of substrate and it is highest at 35 °C, however, degradation level drop in the temperature at 40 °C.

Extreme values of pH can inhibit microbial growth by interfering the microbial metabolism. Most natural environments have pH values between 5.0 and 9.0. Consequently, this range is optimal for microbe-enhanced biodegradation of hydrocarbon (Elnara *et al.*, 2014). During the selection of an optimal medium pH, the maximum substrate degradation with *Bacillus cereus* strains was achieved at pH range 7, while more alkaline or acidic medium has decreased effective oil degradation (Fig.3).

This study was performed to examine the optimum nitrogen sources for the degradation. Among the 5 types of nitrogen sources, ammonium chloride, was highly degraded the hydrocarbon, especially Xylene and benzene (Fig.4). The second better result observed when using ammonium monohydrogen phosphate. Our result was similar to previous studies of Reda,*et al*., (2010). They were also observed the highest degradation when using ammonium chloride.

Five different phosphorous sources were utilized for degradation of hydrocarbon. When using Potassium dihydrogen phosphate in BM, which was highly degraded to Benzene (0.690mg) and followed by Toluene (0.642mg/ml) (Fig.5). This result was contrary to previous studies of Reda and Co workers. They were observed highest degradation when using Diammonium hydrogen phosphate. In our overall result, among the 3 types of oils, benzene was highly degraded compared to other oils. The current study showed successful hydrocarbon degradation by strain of *Bacillus cereus*. Furthermore,

optimization process proved to be improved the degradation process. Hence, it is suggested that the use of above strain under optimized conditions be considered as an alternative technology for effective and eco friendly technology for the degradation of PHCs.

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