Chapter 2

Degradation of petroleum hydrocarbon by Geobacillus stearothermophilus PS11

2.1 Introduction

Soil contamination remains to be one of the major environmental pollution that requires attentions and one of the major potential contaminants are petroleum hydrocarbons containing several toxic aromatic (toluene, benzene and its derivatives, xylene, etc) and aliphatic (hexane, kerosene oil, etc) compounds. Petroleum hydrocarbon contaminants are released in the environment by accidental spillage of oil or leakages and sometimes due to human waste disposals or huge amount of oil sludge generated from refineries and industries

As they are recalcitrant, they remain in the environment for long periods affecting the natural flora of the entire ecosystem. Some of them are also reported to be mutagenic and carcinogenic and can accelerate tumor formation. US Environmental Protection Agency (EPA) has listed some of these petroleum hydrocarbons as priority pollutants.

The alternative physical and chemical removal processes have yielded toxic byproducts that were recalcitrant. The only eco-friendly, cheap way for the removal of these petroleum hydrocarbon wastes includes the use of bacterial population in a process called biodegradation. Biodegradation of organic wastes is a useful side effect of microbial metabolism, thus the fundamental principles of biodegradation are integrally linked to microbial physiology. A wide variety of micro-organisms, mostly bacteria and fungi found in the environment, has the ability to use a wide range of organic compounds as sources of carbon and energy hence they are able to biodegrade a variety of organic compounds.

A microbial community having prior exposure to hydrocarbons, either from natural sources or from other sources such as accidental spills or waste oil disposals etc, is important in determining how rapidly hydrocarbons are degraded. The number of hydrocarbon utilizing microorganisms increase upon exposure to other hydrocarbon pollutants and that the levels of hydrocarbon utilizing microorganisms generally reflect the degree of contamination of the ecosystem. Microorganisms are easily capable of degrading the hydrocarbons having the similar structure.

Therefore, this study was taken up to isolate a bacterium that can effectively degrade petroleum hydrocarbons along with other solvent wastes. Screening the potential bacterial gene involved in the degradation was also studied in this chapter.

2.2 Materials and methods

2.2.1 *Isolation of petroleum hydrocarbon degrading microorganisms from soil sample*

Soil sample was collected from the proximity of a petroleum contaminated (car garage, car service station) sites in Siliguri, India. One gram soil sample from each source was suspended in 10 ml of sterile normal saline and vortexed. The suspension was allowed to settle down and 2.5 ml of supernatant was used as inoculum in 50 ml of MS broth containing 1% hydrocarbons (crude oil) and incubated for 72 h at 37°C on a rotary shaker at 150 rpm. After incubation, 0.1 ml of culture was spread plated on MS agar plates supplemented with 1% of crude oil. The plates were incubated at 37°C for one week.

The isolated strains were preserved in 25% (v/v) glycerol solution at -20°C and sub-cultured at an interval of 30 days. For day to day experimentation, strains were maintained on nutrient agar slants at 4°C in refrigerator.

2.2.2 *Screening of the best petroleum hydrocarbon degrading microbial strain*

The best petroleum hydrocarbon degradingbacterial strain was screened by agar plate assay. Each pure colony was spread plated on MS agar plate supplemented with sterile 1% (v/v) crude oil. The plates were incubated at 37°C for 72 h. Among all the colonies, only the colony exhibiting the highest zone of hydrocarbon utilization was selected for further works.

2.2.3 *Phenotypic and Biochemical characterization of the selected bacterial strain*

The morphological characterization of the selected bacterial strains was determined by gram staining and other microscopic examinations. Presence of endospore was detected by endospore staining with malachite green and saffranine. The motility of the bacteria was determined by hanging drop technique. To determine the optimum temperature of the bacterial growth PS11 was grown in the temperature range of 30°C to 60°C. The biochemical characterization was

determined by performing following biochemical tests: IMViC test, catalase test, nitrate reduction test, carbohydrate fermentation, oxidation of sugars, starch hydrolysis, lipid hydrolysis and casein hydrolysis (Cappuccino and Sherman, 2004). All these tests were performed in duplicates.

2.2.4 *Antibiogram characterization of the selected bacterial strain*

Overnight grown PS11 culture was spread plated on Mueller Hinton agar plate. Standard disc of 10 antibiotics were placed with the help of forceps and pressed gently to ensure full contact with the media. Plates were incubated at 37° C for 24 h. The antibiogram pattern of bacterial isolate was expressed in terms of sensitivity (S) and resistant (R).

2.2.5 *Phylogenetic characterization of the selected bacterial strain*

2.2.5.1. Isolation of genomic DNA

Genomic DNA was isolated by modified Marmur's procedure (Marmur, 1961). Ten ml. of nutrient broth was inoculated with PS11cells and grown for overnight. Bacterial cells were harvested at 8000 rpm for 10 min and washed with 1:1, 0.1M EDTA: 0.15M NaCl solution. Cells were again pelleted by centrifugation at 8,000 rpm for 5 min and suspended in 2-3 ml of 1:1, 0.1M EDTA: 0.15M NaCl solution and was stored at -20 °C at least for 4 h. Cells were thawed at 55°C still dissolution and 1.5 mg/ml solution of lysozyme was added and incubated at 37°C for 30min.To this SDS was added and incubated at 55°C for 15 min. Protein was removed by treatment with Proteinase-K (4 μg/ml) at 55°C for 30 min. The DNA preparation was then extracted with equal volume of Tris-saturated phenol followed by extraction of aqueous phase with equal volume of 1:1 mixture Tris-saturated phenol and chloroform. To the aqueous phase, equal volume of chloroform was added and centrifuged at 10,000 rpm for 10 min. Upper aqueous phase was aspirated and double volume of ethanol was added. DNA was spooled out and dissolved in autoclaved distilled water.

2.2.5.2. Agarose gel electrophoresis of genomic DNA

1% agarose in 1X TAE (Tris-acetate-EDTA) buffer was melted in microwave and then cooled to 50- 60° C. It was then supplemented with 5µg/ml ethidium bromide. The melted agarose was then poured in a casting tray fitted with a Teflon comb forming wells. Genomic DNA sample was mixed with DNA loading dye (5X) prior to loading in the wells. Electrophoresis was performed in a horizontal electrophoresis tank using 1X TAE buffer. DNA bands were visualized on a UV- transilluminator (Genei, India).

2.2.5.3. PCR amplification of 16S rRNA gene and sequence analysis

The 16S rRNA gene was amplified by using universal primers 27F (5'-AGAGTTGATCCTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACTT-3'). A 25 ml reaction mix contained 2.5 ml 10X Taq buffer (Promega), 2.5 µl 25mM $MgCl_2$, 50 pmol of each forward and reverse primers, 2µl of 10mM dNTPs, 100 ng of template DNA and 2.5 Units of Taq DNA polymerase (Promega). Thirty cycles of amplification were carried out at 94°C for 1 min, 55°C for 1min, 72°C for 1min with a 7 min final extension at 72°C. The PCR product was analysed by electrophoresis on 1% agarose-TAE gel and visualized in transilluminator. The PCR product was cloned in pGEMT-easy vector (Promega, USA) following manufacturer's instructions and sequenced by dideoxy method using T7 and SP6 universal primers. The identity of isolate was confirmed by phylogenetic analysis of 16S rRNA sequence using the software package Mega4(Tamura *et al.*, 2007).

2.2.6 ***Determination of growth and dry cell mass of PS11strain***

For bacterial growth, the inoculum was prepared by inoculating a loopful of PS11 cells from slant into nutrient broth followed by incubation at 50 °C and 140 rpm. One ml of overnight grown culture having 10^6 cfu/ml was used to inoculate 100 ml of MS broth overlaid with 10% (v/v) crude oil in Erlenmeyer flasks. To prevent the evaporation of crude oil, flasks were sealed with butyl rubber stoppers. The incubation was carried out at 50 °C with constant shaking at 140 rpm in an orbital shaker for 96 h. The bacterial culture growing under similar conditions in

absence of petrol served as control. Growth was determined by recording absorbance at 660 nm after a constant interval of 6 h till 96 h.

For dry cell mass measurement, 1 ml of PS11 culture broth was centrifuged at 10,000 rpm at 4 °C for 10 min. The cell pellet was washed twice with distilled water and dried at 90°C to achieve constant mass.

2.2.7 *Tolerance of PS11 cells to other solvents*

The tolerance of the microorganism to other organic solvents (log P_{ow} values-0.28 to 4.5), such as isooctane, dimethylsulphoxide (DMSO), xylene, acetonitrile, toluene, benzene, chloroform, 1-butanol, 2- propanol and ethanol, was determined. For this the isolated PS11strain was inoculated in Erlenmeyer flasks containing MS broth overlaid with either of the organic solvent (10 % v/v) and incubated at 50 °C with shaking at 140 rpm. Evaporation of solvent was prevented by plugging the flasks with butyl-rubber stoppers. The bacterial culture growing in absence of organic solvent under similar conditions served as control.

For dry cell mass measurement, 1 ml culture broth was centrifuged at 10,000 rpm at 4 °C for 10 min to pellet down the cell mass. The pellet was washed twice with distilled water and dried at 90°C to achieve constant mass.

2.2.8 *Transmission electron microscopy (TEM) of PS11 in presence of petroleum hydrocarbon:*

The effect of petroleum hydrocarbons on intracellular changes in PS11 was studied using transmission electron microscope. Specimen for transmission electron microscopy was prepared by growing the PS11cells for 48h in culture medium in absence or presence of crude oil (10% v/v). Cells were harvested by centrifugation at 5000 rpm for 10 min and then fixed overnight in a solution containing 2.5% (w/v) glutaraldehyde in 0.1M Na_2HPO_4/KH_2PO_4/phosphate buffer (pH 7.2) at 4°C and post fixed with 1% osmium tetroxide (OsO_4). The cells were then dehydrated with ethanol and embedded in Spurr. The cells were stained with 1% (w/v) uranyl acetate and 1% (w/v) sodium citrate and examined under electron microscope (Philips model CM10) at an accelerating voltage of 80KV.

2.2.9 *Determination of membrane fatty acid adaptive profile in presence of petroleum hydrocarbons*

2.2.9.1. Growth conditions

PS11 was inoculated in MS broth containing crude oil (10% v/v) and grown at 50°C. 100 µl of crude oil was added at every 3 h of bacterial growth to maintain continuous exposure to the solvent. All treatments were replicated three times. Bacterial cells growing in absence of petrol served as control.

2.2.9.2. Extraction of bacterial membrane lipid

Lipids were extracted using the method of Bligh-Dyer (1959) with modifications. Two hundred milliliters of bacterial culture was centrifuged. The cell pellet was extracted twice using 2 ml of methanol:chloroform (1:1). The chloroform layer was extracted twice, combined and centrifuged. The supernatant fraction was filtered (Whatman no. 40) and evaporated under nitrogen. Lipids were redissolved in 0.50 ml chloroform stabilized with amylenes for both phospholipid analysis and fatty acid methyl ester (FAME) derivatization.

2.2.9.3. Phospholipid analysis

The lipid classes were separated by TLC on silica gel H plates (0.5 mm thickness). Solvent mixture chloroform: methanol: water (65:25:4 v/v) was used as mobile phase. Phospholipids were detected by dipping the plate into 10% phosphomolybdic acid (Sigma) in ethanol. The glycolipids were detected by spraying the plate with α-naphthol–sulfuric acid reagent followed by charring for 15 min at 110°C (Jacin and Mishkin 1965). Lipid classes were identified by comparing it with standards run along with the samples on the TLC plates. The phospholipid bands were scraped from the plates and quantified by standard procedures (Nahaie *et al.,* 1984). Diphosphatidylglycerol, phosphatidylglycerol (PG), and lysylphosphatidylglycerol (LPG) (Sigma) were used as standards for determining phospholipids.

2.2.9.4. Analysis of fatty acid composition by gas chromatography

Analysis of FAME in hexane was performed using a quadruple GC System (HP5890, Hewlett & Packard, Palo Alto, USA) equipped with a split/splitless injector. A CP-Sil 88 capillary column (Chrompack, Middelburg, The Netherlands; length, 50 m; inner diameter, 0.25 mm; 0.25 μm film) was used for the separation of the FAME. GC conditions were: Injector temperature was held at 240°C, detector temperature was held at 270°C. The injection was splitless, carrier gas was He at a flow of 2 ml min^{-1}. The temperature programme was: 40°C, 2 min isothermal; 8°C min^{-1} to 220°C; 15 min isothermal at 220°C. The pressure programme was: 27.7 psi (=186.15 kPa), 2 min isobaric; 0.82 psi min^{-1} (5.65 kPa min^{-1}) to the final pressure 45.7 psi; 15.55 min isobaric at 45.7 psi (310.26 kPa). The peak areas of the carboxylic acids in total ion chromatograms (TIC) were used to determine their relative amounts. The fatty acids were identified by GC and co-injection of authentic reference compounds obtained from Supelco (Bellefonte, USA).

2.2.10 *Detection of total petroleum hydrocarbons (TPH) and some individual petroleum hydrocarbon degradation by Gas Chromatography (GC)*

The degradation of TPH and some other individual hydrocarbons, such as benzene, toluene, ethyl benzene and xylene (BTEX) by PS11 was analyzed by GC. Enrichment of PS11 cells was done by growing the cells in presence of crude oil. Enriched PS11 culture (1%)was inoculated in five different 1000 ml capacity serum bottles filled with 300 ml of nutrient broth overlaid separately with of crude oil, toluene and benzene (10% v/v each), xylene and ethyl benzene (5% v/v each). The bottles were then closed with Teflon-coated septa and aluminum caps and the degradation study was preformed for a period of 15 days at 50°C under 180 rpm. On 15^{th} day of degradation, residual oil content in the culture was extracted using 1:1 proportion of n-hexane (liquid-liquid extraction) and sample. The extracted oil was then analyzed to study the degradation percentage of hydrocarbon fractions using Perkin–Elmer 900 gas chromatograph equipped with a flame ionization detector. Separation was carried out on 181m X 0.76 mm stainless steel open tubular column. The temperature was programmed 20°C - 130°C at 2 °C/min after

an initial isothermal period of 6 min. The injection temperature was 120°C and detector temperature was 140°C. A Perkin-Elmer PEP1 data processor was used for quantification of volatile compound as parts per billion (ppb v/v). Response factor according to Dietz (1967) was used. The cultures in absence of the solvent and uninoculated media enriched with solvents were used as control. All the experiments were carried in duplicate.

2.2.11 *Characterization of the gene responsible for petroleum hydrocarbon degradation.*

2.2.11.1. Isolation of plasmid DNA and its restriction digestion profile

A single colony of PS11 was inoculated into a 10 ml LB broth containing 100 µg/ml tetracycline as selective marker and incubated for 12-16 h at 50°C with vigorous shaking followed by centrifugation at 6000 rpm for 10 min. Plasmid was isolated by plasmid isolation kit (QIAprep® Spin Miniprep Kit, QIAGEN, USA) following the manufacturer's instruction. The bacterial cell pellet was suspended in 250 µl P1 buffer containing containing lysozyme (1mg/ml) and RNase A solution and lyse blue. To it 250 µl P2 buffer was added and mixed thoroughly by inverting the tube 4-6 times. It was immediately followed by addition of 350 µl of N3 buffer. The mixture was centrifuged for 10 min at 14000 rpm. The supernatant was applied to the QIA Prep spin column and centrifuged for 1 min. The column was washed with 0.5 ml PB buffer and was centrifuged for 1 minute at 12000 rpm. The column was washed again by adding 0.75 ml PE buffer containing ethanol (96-100%) and was centrifuged for 1 min at 12000 rpm. The flow was discarded and the column was centrifuged for additional 1 minute to remove residual wash buffer. The QIA Prep spin column was placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 50 µl EB buffer (10 mM Tris-Cl, pH 8.5) was added to centre. It was allowed to stand for 1 minute and was centrifuged for 1min. DNA sample was mixed with DNA loading buffer and subjected to electrophoresis on 0.8% agarose-TAE gel and visualized in transilluminator. The plasmid was restriction digested with Bam H1 by restriction digestion kit (NEB, UK) following the manufacturer's instruction.

2.2.11.3. Curing of plasmid DNA

To evaluate the role of plasmid in petroleum hydrocarbon degradation plasmid curing was performed by growing the PS11cells in LB medium in the presence of acridine orange (100, 150 and 200 μg/ml) at 50 °C. During the exponential phase, the flasks were withdrawn and the colonies were isolated by dilution technique. These isolated colonies were sub cultured on LB agar slants and plasmid DNA was isolated as described in section 10.1. Presence of plasmid DNA in the isolated colonies was determined by agarose gel electrophoresis.

2.2.11.4. Growth of plasmid cured and wild PS11 strain in presence of petroleum hydrocarbon

The growth of plasmid cured and wild strain of PS11 in presence of petroleum hydrocarbons was checked in Erlenmeyer flasks containing nutrient broth overlaid with organic solvents (10% v/v). Organic solvents with log P_{ow} values ranging 0.28-4.5, such as isooctane, dimethylsulphoxide (DMSO), xylene, acetonitrile, toluene, benzene, chloroform, 2-butanol, 2-propanol, ethanol and petrol were used. The flask was incubated at 50°C with shaking at 140 rpm. Evaporation of solvent was prevented by plugging the flasks with butyl-rubber stoppers. The bacterial culture growing in absence of organic solvent under similar conditions served as control.

For dry cell mass measurement, 1 ml PS11 culture was centrifuged at 10,000 rpmat 4 °C for 10 min to pellet down the cell mass. The pellet was washed twice with distilled water and dried at 90°C to achieve constant mass.

2.2.11.5. Preparation of competent E. coli JM109

E. coli JM109 cells were grown overnight in 10 ml LB broth. 1 ml of overnight culture was inoculated in fresh LB broth (10 ml) and incubated for 2-3 h at 37°C to an approx OD of 0.4. The culture was centrifuged at 4,000 rpm for 5 min. The cell pellet was washed with 10 ml of ice cold $CaCl_2$ (100 mM) and centrifuged. The cell

pellet was suspended in 5 ml of ice cold $CaCl_2$ and incubated on ice for 30 min followed by centrifugation. The cell pellet was resuspended in 2 ml of ice cold $CaCl_2$ (100 mM) for future use.

2.2.11.6. Transformation of plasmid in JM109 and screening of transformed cells

50ng of plasmid DNA of PS11 was added to 50µl of $CaCl_2$competent JM109 cells and mixed thoroughly by gently swirling the vial. The vials were stored on ice for 30 min. The cells were subjected to heat shock by placing the vial in a water bath at 42°C for 90 sec and then immediatelytransferred on ice for 15 min. To this, 800 µl of LB medium was added and incubated at 37°C for 1.5 h at 150 rpm. Cells were centrifuged for 5 min at 1500 rpm in microfuge. The supernatant were discarded.

In order to screen the transformants, cell pellet was resuspended in 100µl of LB broth and were spreaded on 50 µg/ml tetracycline and incubated overnight at 37°C. Overnight grown single colony from tetracycline containing LB agar plates were picked and reinoculated in minimal salt broth having 0.1% of catechol.It was incubated overnightat 37°C. Transformant cells having the capability to degrade petroleum hydrocarbon was screened by observing the color change in the media. . Presence of plasmid DNA in the transformant cells was determined by agarose gel electrophoresis.

2.2.11.7. Degradation of Total petroleum hydrocarbons (TPH) and some individual petroleum hydrocarbon by transformed JM109 cells

The capability of transformed JM109 to degrade TPH and some other individual hydrocarbon such as benzene, toluene, ethyl benzene and xylene (BTEX) was analyzed using 1% of the transformed JM109 culture. The degradation experiment was carried out under similar conditions as described in section 6. Degradation of TPH and BTEX was determined by gas chromatography method. The wild strain of PS11 and non transformed JM109 cultures grown in media enriched with the solvent were used as control. All the experiments were carried in duplicate.

2.2.11.8. PCR amplification of plasmid borne gene responsible for degradationand sequence analysis

As the transformant were screened on the basis of catechol metabolism, a common intermediate of aromatic hydrocarbon meta degradation pathway, the gene of catechol metabolism pathway i.e. catechol 2, 3 dioxygenase, was amplified by PCR using plasmid isolated from transformed cells as template. PCR amplification was also done using plasmid as template isolated from wild PS11 cells. The following set of primers was used to amplify the specific gene:

Primer set: **Forward:** 5'-ATGGCTATTATGCGG-3'

Reverse: 5'-TTATGTCAGCGCC-3'

PCR conditions:

DNA templates were prepared by method described in section 10.1.PCR was conducted for 40 cycles. The PCR condition was as follows: denaturation at 94°C for 60s, annealing at 54 °C for 30s and extension for 30s. Aliquots of PCR products were analyzed by agarose gel electrophoresis stained with 0.5μg/mlethidium bromide.

PCR amplified DNA was further cloned into pGEMT-Easy vector. For cloning the PCR product was extracted from agarose gel by using Gel Extraction Kit (QIAGEN, Germany). The ligation reaction mixture in a total volume of 10μl contained: 2X rapid ligation buffer, 5 μl; pGEMT-Easy vector, 50ng (1 μl); PCR product, 2μl; T4 DNA ligase (3weiss unit/μl), 1 μl and H_2O (1 μl). The reaction mixture was incubated over night at 4°C and was transformed into competent *E. coli* JM109 cells by heat shock at 42°C for 90s. The transformed cells were revived for 1.5 h at 37°C in a shaking incubator. The transformants were selected in ampicillin (50μg/ml) agar plate supplemented with 20% IPTG and 2% (w/v) X-gal by incubating overnight at 37°C. Plasmid was isolated from the recombinant colonies by alkaline lysis method (Birnboirm and Dolly, 1979). The recombinant plasmid was analyzed for the presence of DNA inserts by restriction digestion. The cloned PCR product was subjected to sequence analysis.

Similarity searches of the translated catechol 2, 3 dioxygenase sequences were performed compared with sequences available at the NCBI data base ("Blastp") using the BLAST algorithm. Multiple protein sequence alignments based with hierarchical clustering were carried out using MULTALIN.

2.3 Results

2.3.1 Isolation of petroleum hydrocarbon degrading microbial strain

Generally, almost all of the microbial species in the environment have genetic ability to utilize the hydrocarbons as carbon source. This property of microorganisms comes into expression when they inhabitin hydrocarbon rich environment. Therefore, soil from petroleum oil contaminated sites could be a potential source of microbial strains with hydrocarbon degradation capability. Hence, soil sample was collected from the proximity of high petroleum contaminated (car garage, car service station) sites in Siliguri, India.

To isolate the efficient petroleum degrading bacteria, soil sample in normal saline was vortexed and the supernatant was inoculated in MS broth containing 1% crude oil. It was incubated at 37 °C under shaking. After 72 h of incubation, 0.1 ml of the culture was spread plated on MS plates overlaid with 1 % of crude oil. Forty different bacterial colonies that grew in presence of hydrocarbon were isolated after one week incubation at 37 °C (Table1).

2.3.2. Screening of best petroleum hydrocarbon degrading microbial strain

The isolated microbial strains were screened for best hydrocarbon degrading capacity. For this, each bacterial pure culture was spread plated on MS agar plate supplemented with 1% crude oil.The plates were incubated at 37°C for 72 h.

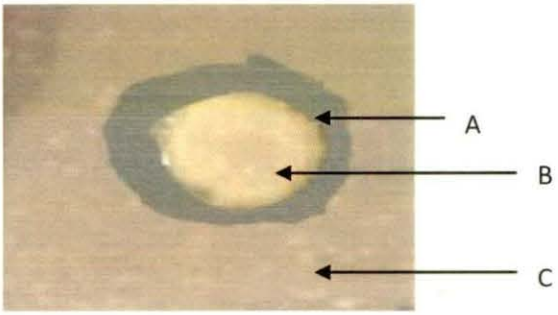


Fig 1. Strain PS11 showing zone of hydrocarbon (crude oil) utilization. (A) Clear zones of hydro-carbon (crude oil) utilization, (B) Single colony of PS11 strain, (C) MS agar plate surface-coated with crude oil (magnification, X 12.5).

Among the forty bacterial strains only thirty two strains showed the zone of hydrocarbon utilization. Among them, the strain PS11 exhibited the highest clearance zone of hydrocarbon utilization (Fig 1) and thus was selected for further studies.

Table 1: Isolation of bacterial colonies from petroleum contaminated sites

Sample	Colony character	Area ofhydrocarbon utilization (sq. cm)
PS1	White, round, dry, regular margin	1.12±0.34
PS2	Small submerged	1.03±0.40
PS3	White, dry, defined margin	0.76±0.45
PS4	Transparent, pin head, moist	0.33±0.22
PS5	White, small, round, glistering	
PS6	White, big, flat, irregular margin	0.95±0.45
PS7	White, moist, regular margin	0.40±0.29
PS8	White, dry, irregular	0.21±0.23
PS9	White, leafy irregular margin	0.34±0.14
PS10	White, moist, regular margin	
PS11	**White, round, moist, regular margin**	**1.57±0.76**
PS12	Greenish	0.95±0.23
PS13	White, round, dry , defined margin	0.67±0.11
PS14	White, flat, dry, defined margin	1.04±0.23
PS15	Moist lawn	1.08±0.32
PS16	Slight yellowish, moist, irregular margin	1.23±0.21
PS17	White, irregular, leafy margin	
PS18	White, small dots, dry, flat	1.32±0.12
PS19	Round, dry, regular margin	
PS20	White, moist lawn	1.37±0.19
PS21	Yellow, round, dense centered, regular margin	
PS22	Round, slimy, defined margin	0.65±0.39
PS23	Round, dry, leafy irregular margin	0.99±0.40
PS24	Yellow, dots, slimy	1.12±0.50
PS25	Round, dry, irregular margin	1.37±0.34
PS26	White, pin head	0.52±0.23
PS27	Round, big, moist	0.97±0.44
PS28	White, big, dry, irregular margin	1.21±0.32
PS29	White, round dots, submerged, dry	1.33±0.29
PS30	White, scaly, irregular margin	
PS31	White, round, moist centered	
PS32	White, round, dry, defined margin	0.32±0.13
PS33	White, round, glistering, opaque, defined margin	0.34±0.19
PS34	Round, dry, opaque, regular margin	1.50±0.79
PS35	White, small, moist, slimy, regular margin	1.21±0.34
PS36	White, regular, moist at margin	0.23±0.78
PS37	Round, pin head, glistering	
PS38	Yellowish, big, dry, regular margin	0.51±0.56
PS39	White, irregular margin	0.23±0.43
PS40	Round, pin head, opaque, regular margin	1.03±0.34

2.3.3. *Phenotypic, biochemical and phylogenetic characterization of the selected strain*

The isolated strain PS11 was found to be gram positive rods arranged in chains or clusters (Fig 2). Its phenotypic characteristics are summarized in Table 2.

- The isolated PS11 strain has terminal and sub terminal endospore and was found to be motile.
- PS11 could grow in a wide range of temperature from 37°C to 65°C. But the optimum temperature was 50°C for its growth.
- Starch hydrolysis was performed to test the ability of PS11 strain to produce α-amylase that hydrolyzed starch. A clear zone around the PS11 bacterial colony indicated that the organism hydrolyzedstarch by producing α-amylase.
- Casein hydrolysis test was done to examine the production of caseinase which hydrolysed casein, a complex protein present in milk agar. PS11 inoculated plate didn't produce any clear zones which indicate the negative result.
- Tributyrin hydrolysis test was done to examine the production of lipase which hydrolysed tributyrin, a lipid present in agar. PS11 inoculated plate produced clear zones which indicate the positive result.
- Gelatin test was performed to check the ability of PS11 to produce gelatinase that hydrolyzed gelatin present in the media. PS11 strain inoculated tubes liquefied the solid gelatin present in the media. It represented production of gelatinase to hydrolyse gelatin into smallerpolypeptides, peptides, and amino acids that can cross the cell membrane and be utilized by the organism.
- In oxidase test broth of PS11strain changed colour to blue which indicated that bacterial cells produced cytochrome oxidase that participate in the electron transport chain by transferring electrons from a donor molecule to

oxygen. The colour change was due to the oxidation of a reducing agent, chromogenic present in oxidase reagent. So the test result was positive.

- The indole test was performed to determine the ability of the organism to split indole from the tryptophan. Yellow color in the surface alcohol layer of the PS11 strain inoculated broth represented the result as negative. It may be due to lack of production of intracellular enzymes tryptophanase that split indole from the amino acid tryptophan.

- Methyl-red test was performed to examine the mixed acid fermentation by PS11cells grown on MR-VP broth containing glucose, peptone and phosphate buffer. Organisms that perform mixed-acid fermentation produce enough acid to overcome the buffering capacity of the broth, so a decreasein pH results. After incubation with PS11 cells, the pH indicator Methyl Red was added to the broth that showed pink color. So it was considered as positive result.

- Voges-Proskauer test was done to determine whether organisms can produce acetyl methylcarbinol (acetoin) from fermentation of glucose. After incubating the MR-VP media with PS11 cells when alpha-naphtol (5%) and potassium hydroxide (40%) was added, it didn't produce any pink reddish colour which indicated negative result.

- Citrate test was performed to detect the ability of a microorganism to use citrate as the sole source of carbon. PS11 are inoculated on a medium containing sodium citrate and a pH indicator bromothymol blue is used. The culture tubes produced blue colour which indicated the production of enzyme citritase that break down citrate to oxaloacetate and acetate. Oxaloacetate is further broken down to pyruvate and carbon dioxide (CO_2). Production of sodium bicarbonate ($NaHCO_3$) as well as ammonia (NH_3) from the use of sodium citrate and ammonium salts results in alkaline pH. This positive result was indicated bychange of the medium's color from green to blue.

- In nitrate reductase test broth of PS11 strain remain colourless which indicated the bacterial cells were able to reduce nitrate (NO_3^-) to nitrite (NO_2^-) using anaerobic respirationand the result was positive.

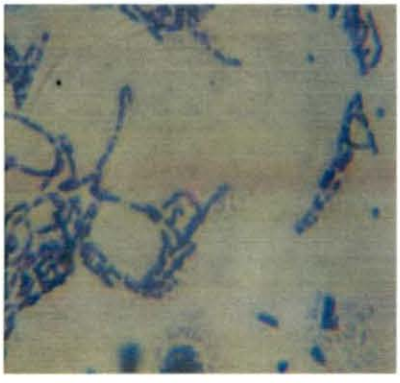


Fig 2: Microscopic (100X) view of gram stainedcells of PS11 strain

Table 2. Comparision of phenotypic and biochemical characteristics of PS11 with *Geobacillus* sp.

Phenotypic Characterization	**PS11**	***Geobacillus* sp.** (adapted from Bergey's manual)
Gram straining	+ rods	+ rods
Endospore	+	+
Motility	+	+
Optimum Temperature	50°C	55°C
Starch hydrolysis	+	+
Casein hydrolysis	-	-
Tributyrin hydrolysis	+	+
Urease	-	-
Indole	-	-
Methyl red	+	+
VP	-	-
Citrate utilization	+	+
Nitrate reduction	+	+
Catalase	+/-	-
H_2S production	-	-
Lysine- decarboxylase	-	-
Arginine –dexydrolysis	+	-
Phenylalanine dezaminase	-	-
Tryptophan Deaminase	-	-
B galactosidase	+	+
Oxidation & fermentation		
Glucose	+	+
Xylose	+	+
Mannitol	+	+
Sucrose	+	+
Rhamnose	-	-
Ionisitol	-	-
Sorbitol	-	-
Arabinose	+	+

- Catalase test was performed to check the ability of PS11 strain to produce catalase that breaks hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2).

- PS11 was found to utilize arginine but couldnot utilize phenylalanine, tryptophane and lysine. It also could not produce H_2S. The isolate could utilize galactose by producing β galactosidase.

- When the PS11 strain was inoculated bubbling was seen which is due to the evolution of O_2 gas. It indicated that the test result waspositive. The organism PS11 could further ferment glucose, xylitol, sucrose, arabinose and manose.

Based on the phenotypic characterization the similarity coefficient of the isolated strain PS11 with *Geobacillus* sp. was found to be 94%.

2.3.4 Antibiogram of the selected strain

PS11 was tested for its susceptibility to eleven different antibiotics (Table 3). The antibiogram of PS11 showed that the isolated strain was sensitive to all the 10 antibiotics expect tetracycline. This indicated the presence of tetracycline resistance gene in PS11 strain. Thus, this antibiotic was later used as a marker for the screening of plasmid cured bacterial colonies.

Table3. Antibiogram of *G. stearothermophilus* PS11

Antibiotics	Growth
Penicillin G	S
Amoxicillin	S
Tetracycline	R
Bacitracin	S
Vancomycin	S
Rifampicin	S
Neomycin	S
Azithromycin	S
Kanamycin	S
Novobiocin	S
Chloramphenicol	S

2.3.5 Phylogenetic characterization of the selected strain

Phylogenetic characterization of PS11 strain was performed by analysis of 16S rRNA sequence. The 16S rRNA gene of PS11 strain was PCR amplified from

genomic DNA using universal primers 27F (5'-AGAGTTGATCCTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACTT-3') and the 1.4 kb PCR product was cloned in pGEMT-easy vector and sequenced using T7 and SP6 universal primers. The identity of isolate was confirmed by phylogenetic analysis of 16S rRNA gene sequence.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007).

Phylogenetic characterization based on 16S rRNA (Fig3.) identified the bacterial isolate as *Geobacillus stearothermophilus.* Hence, it can be concluded from the phenotypic and phyogenetic analysis that isolated PS11 strain is *Geobacillus stearothermophilus.* The 16S rRNA sequence was submitted to Gene Bank with the accession no. **KC311354.**

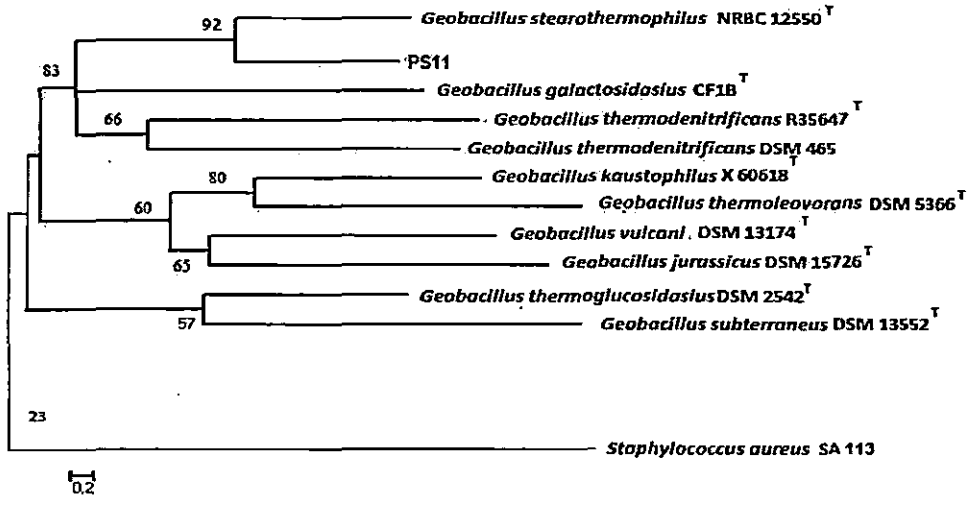


Fig 3. Phylogeneticanalysis of 16S rRNA sequence. (The evolutionary history was inferred using the Neighbor-Joining method. Phylogenetic analyses were conducted in MEGA 4, The numerials are the boot strap values)

2.3.6. *Determination of growth and dry cell mass of G. stearothermophilus PS11*

The growth curve of *G.stearothermophilus* PS11in the absence and presence of crude oil (10 %, v/v) is shown in Fig 4. Incorporation of crude oil into the growth media served as factor for screening of petroleum tolerant strain. PS11 showed delayed growth profile in presence of crude oil with a prolonged lag phase of 6h, a shorter log phase of just 12h and subsequently entered the stationary phase. In contrast, the bacterial strain growing in absence of crude oil exhibited as shorter lag phase of less than 6h and continued to remain in log phase till the end of incubation time.

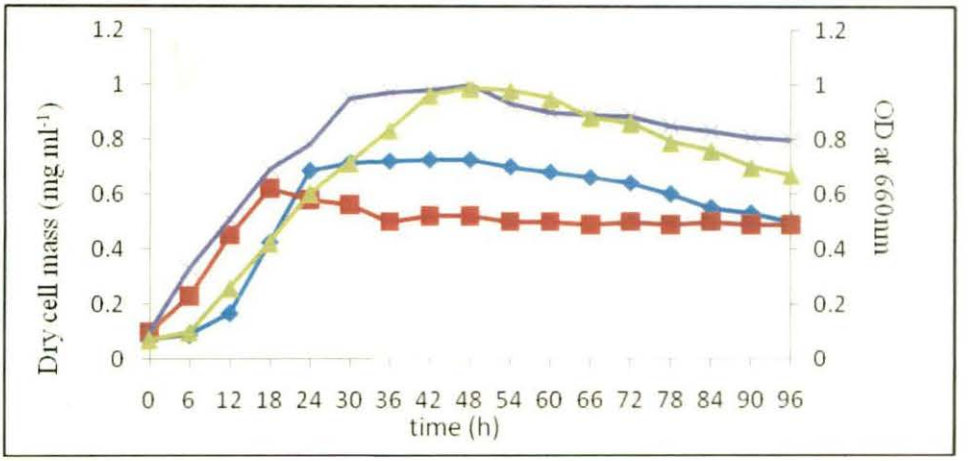


Fig 4. Growth of *Geobacillus stearothermophilus* PS11in the absence and presence of crude oil. Bacterial growth in absence of crude oil: $OD_{660\ nm}$, (▲); dry cell mass, (╲) and growth in presence of crude oil: $OD_{660\ nm}$, (♦); dry cell mass, (■). The experiment was carried out in triplicates and the difference in the individual results was less than 3%.

2.3.7. *Tolerance property of G. stearothermophilus PS11 to other solvents*

The response of *G.stearothermophilus* PS11towards other organic solvents was studied by monitoring its growth in medium over laid with solvents of varying logP_{ow} values. The log P value is the index for measuring the toxicity of solvents. It is defined as logarithm of solvents partition coefficient in n-octanol and water. The

results summarized in Table-3show that PS11 grew well in solvents with higher log *P* value, such as, DMSO, toluene and cyclohexane with least growth inhibition in presence of benzene (1.52%) and DMSO (7%) respectively. The growth was almost negligible in presence of solvents with low log *P* value like alcohols.

Inhibition of growth was observed in presence of acetonitrile (88.8%) and chloroform (50.76%). PS11 strain did not grow in the presence of isopropanol, 1-butanol and ethanol thus indicating its tolerance tohydrophobic solvents rather than hydrophilic.

Table 3. Growth of PS11strain in presence of organic solvents

Solvent	log *P*	OD $_{660\,nm}$	Dry cell mass (mg ml^{-1})	% of growth inhibition
Control[b]		1.97	1.09	
Isooctane	4.5	-*	-*	
DMSO	-1.35	1.83	0.46	7
Xylene	3.1	1.21	0.7	38.5
Acetonitrile	0.03	0.221	0.19	88.8
Cyclohexane	3.2	1.67	0.4	15.2
Toluene	2.5	1.34	0.56	31.9
Benzene	2	1.94	0.86	1.52
Chloroform	2	0.97	0.49	50.76
1-Butanol	0.8	-*	-*	
2-Propanol	0.28	-	-	
Ethanol	-0.24	-	-	

* O D $_{660}$ value < 0.1 and dry cell mass (mg ml^{-1})< 0.05 after 48h of growth
[a]After 48h of growth, [b]without solvent

2.3.8. Transmission electron microscopy(TEM) analysis of G. stearothermophilus PS11 in presence of petrol

To determine the effect of petroleum hydrocarbon at intracellular level, Specimen for transmission electron microscopy was prepared by growing the cells of PS11 for 48h in culture medium in absence or presence of crude oil (10%v/v). TEM image showed that thegrowth of *G. stearothermophilus* PS11cells in the presence of 10% (v/v) crude oil resulted in convolution and disorganization cell membrane and

accumulationthe solvent within the cytoplasm (Fig 5). It is clearly visible that the crude oil accumulation inside the cell was initially increased occupying the entire cytosolic region at 24h of incubation (Fig 5b). Gradual change in the cell shape from rod to semi circular was obseved after 24h of incubation in presence of crude oil (Fig. 5c). The decline in crude oil accumulation and reorganization of cell membrane were observed on further incubation till 48 hours (Fig 5d).

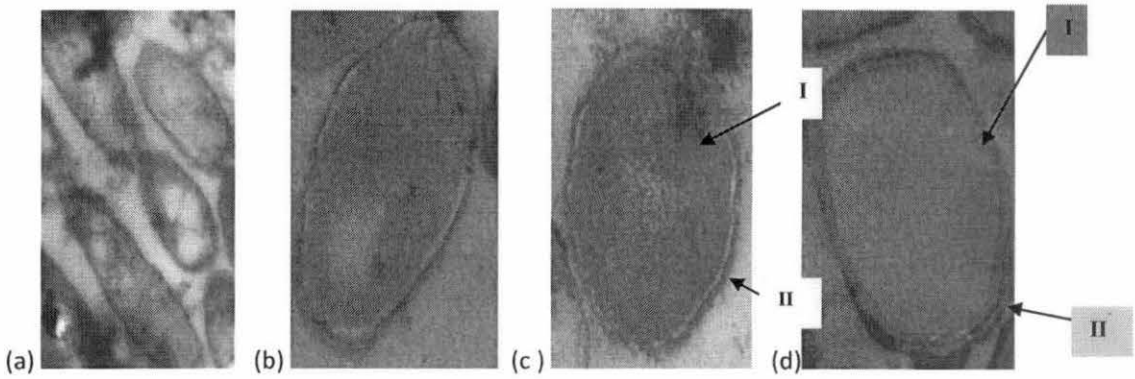


Fig 5. Transmission electron micrograph of *Geobacillus stearothermophilus* PS11cells. **(a)**in the absence of crude oil (exposure 21,000); **(b & c)** in the presence of 10% crude oil (exposure 21,000) after 24 hours incubation - (I) accumulation of solvents and (II) convoluted and disorganized cell membrane; change in cell shape **(d)** in the presence of 10% crude oil (exposure 21,000) after 48 hours incubation. (I) and (II) regeneration of cytoplasm and cell wall.

2.3.9. Determination of membrane fatty acid adaptive profile in presence of petroleum hydrocarbons

2.3.9.1. Phospholipid analysis

The membrane lipid composition of *G. stearothermophilus* PS11 grown in the absence (control) and in the presence of 10% (v/v) crude oil was examined by chromatographic techniques to detect alterations in the lipid composition induced by the addition of crude oil to the growth medium. In the control sample, the polar lipids constituted about 80-90% of the total lipid extract of bacterial membranes. As summarized in Table 4 lipid fraction contained phosphatidylethanolamine (PE), diphosphatidylglycerol (DPG), phosphatidylglycerol (PG) , phosphoglycolipid (PGL) and trace amounts of two unidentified phospholipids (X_1 and X_2). The major class PE

made about 57 % of the total phosphorus content while DPG, PG and PGL accounted for 20, 14 and 3.5 %, respectively. The remaining 5.5% of phospholipid was distributed among $X_1$and X_2 by 2.2 and 2.8% respectively.

Table 4.Phospholipid composition of *G. stearothermophilus*PS11 membranes, as affected by crude oil

	Membrane phospholipid*					
	PE	PG	DPG	PGL	X_1	X_2
Control	57±1.23	14±0.56	20±1.45	3.5±0.67	2.2±0.76	2.8±1.5
10% crude oil	51±0.34	12±0.45	23±1.09	7.99±0.77	2.8±1.2	3.2±.98

Values of phospholipid contents for control culture and culture grown in the presence of 10% (v/v) crude oil were compared by Student's t-test. They indicate whether the phospholipid differences observed between cells grown with and without petrol were statistically significant ($P< 0.05$).

*Values are given as % of total phosphorus content± S.D. of three independent determinations

As listed in table 4, crude oil present in the media altered the membrane phospholipid composition of *G. stearothermophilus* PS11. Relative to control, presence of crude oil decreased PE by almost 11% and PG by 14% whereas increased DPG and PGL by 15 and 128% respectively. Therefore, the significant change in polar lipid content was induced by 10% (v/v) crude oil with increase in PGL and parallel decrease in PE and PG.

2.3.9.2. Analysis of fatty acid composition

The acyl chain composition of *G. stearothermophilus* PS11 polar lipids is listed in Table 5.The dominant fatty acid was palmitic(nC16, 22%), followed by 13-methyltetradecanoic (iC15, 21.5%),14-methylhexadecanoic (aC17, 18.5%), 15-methylhexadecanoic (iC17, 13.4%), 14-methylpentadecanoic (iC16, 9.12%), 12-methyltetradecanoic (aC15, 7%) and myristic (nC14, 2%). The membrane fatty acid composition of PS11 strain was altered in presence of 10% (v/v) crude oil.The proportions of aliphatic chains changed significantly. Eachisomer of iC15 and iC17 was increased by 20.9% while aC17, iC16, and aC15 were decreased by 13.5, 12.2 and 28.5% respectively. A marked decrease of 50% and 15% in myristic acid (C14) and palmitic acid (C16) respectively was noted in presence of crude oil.

Branched chained iso- and anteiso-fatty acids were the predominant aliphatic components of the polar lipids and constitute about 70.9% of total membrane fatty acid (Table 6) in control PS11cells. Among the branched chained fatty acid, iso-class was the major group and its relative proportion was about 45.4% of the total fatty acids. The rest 25.5% was branched anteiso-fatty acids.

Table5. Fatty acid composition of polar phospholipid fraction of *G. stearothermophilus* PS11 as affected by crude oil

*Fatty acid	Control	In presence of 10% (v/v) crude oil	% change in polar phospholipid
iC14	0.75±1.23	0.53±0.98	29% decrease
nC14	2±1.22	1.0±0.65	50% decrease
iC15	21.5±0.45	28±1.23	21% increase
aC15	7±0.33	5±1.09	28% decrease
nC15	2±0.56	1.0±0.77	50% decrease
iC16	9.12±1.02	8±1.2	12% decrease
nC16	22±0.98	18.7±0.43	18% decrease
iC17	13.4±1.12	18.2±0.79	21% increase
aC17	18.5±0.17	16±1.3	14% decrease
nC17	0.61±0.87	0.9±0.60	48% increase
iC18	0.64±1.04	0.85±0.43	33% increase
nC18	1.27±0.34	1.0±0.28	21% decrease
C 18:1	0.58±0.2	0.55±1.2	5% decrease
C19:1 trans	0.63±0.34	0.72±1.03	14% increase

*Fatty acid analysis of polar lipid extracts from cultures grown in the absence (controls) and in the presence of 10% v/v crude oil. Values are average % ± S.D.of three independent determinations. (i and a denotes branched chained iso- and anteiso-fatty acids respectively)

Presence of crude oil increased the proportion of branched chained fatty acid (8%) while decrease in straight chained fatty acid (17.8%) was noted. The ratio of branched chain : straight chain fatty acids increased from 2.43 (control cultures) to 3.20 (cultures adapted to crude oil). A relative increase (22.4%) of iso

fatty acids was noted in cell membrane of PS11 adapted to 10% (v/v) crude oil whereas a simultaneous decrease of the anteiso-acids (17.6%) was observed.

Table 6. Fatty acid categories as affected by crude oil

	*Fatty acid composition			
	Total straight chain	Total branched chain	Branched iso-acids	Branched anteiso-acid
Control	29.09±1.58	70.91±0.54	45.41±0.78	25.5±1.02
10%(v/v) crude oil	23.92±0.93	76.58±1.02	55.58±1.34	21±0.56

*Values of fatty acid contents for control culture and culture grown in the presence of 10% (v/v) crude oil were compared by Student's t-test. They indicate whether the fatty acid differences observed between cells grown with and without crude oil were statistically significant ($P < 0.05$). The values are the sums of % of fatty acids represented in Table 6, arranged in chain categories.

2.3.10. Detection of Total petroleum hydrocarbons (TPH) and some individual petroleum hydrocarbon degradation by Gas Chromatography (GC)

In order to correlate the growth of PS11 strain in presence of different petroleum hydrocarbon (BTEX) with its degradation capabilities, 1% of the enriched bacterial culture was inoculated in five different 1000ml capacity serum bottles filled with 300 ml of nutrient broth overlaid with 10% v/v of crude oil, toluene & benzene, 5% xylene and ethyl benzene separately. The bottles were then closed with Teflon-coated septa and aluminum caps and the degradation study was preformed for a period of 15 days at 50°C under 180 rpm. The total petroleum hydrocarbon (TPH) and the individual residual component of BTEX in the medium were analyzed by GC after 15 days incubation. The degradation rate was determined as the ratio of the amount of hydrocarbon degraded to the initial amount. The analysis revealed that PS11 strain degraded TPH and individual components of BTEX compounds at different ratesin the same time span. TPH chromatogram showed that PS11could degrade aromatic compounds more readily compared to that of alkanes(Fig 6a). After 15 days of incubation, almost all the

aromatics were completely degraded while 30% of alkanes were present as residual components in crude oil .

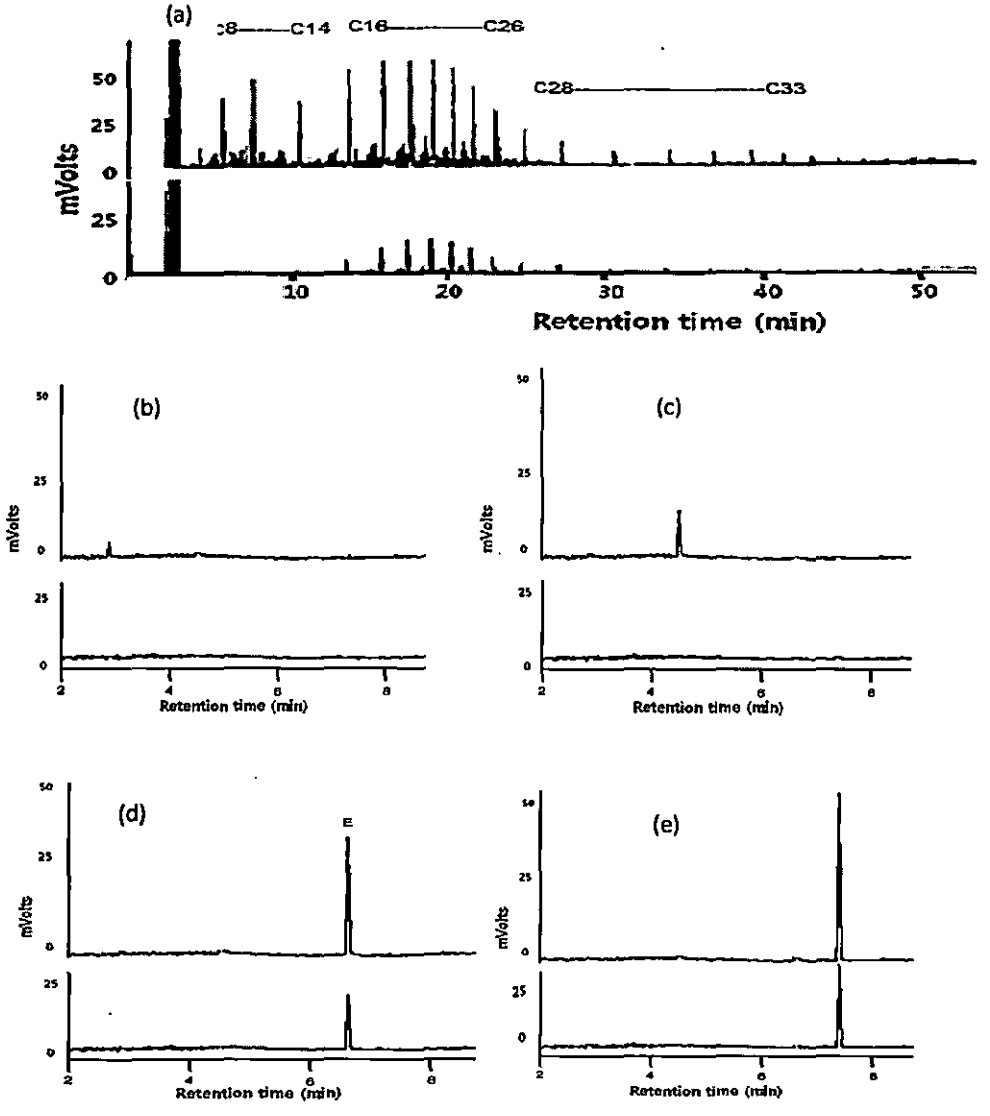


Fig 6: Gas chromatographic pattern of residual total petroleum hydrocarbons (TPH) and BTEX as exhibited by PS11cells after 15 days of incubation.Degradation of 10% v/v TPH (a), 10% v/v benzene(b), 10%v/v toluene(c),5% v/v ethyl benzene (d)and xylene 5% v/v (e) after 15 days incubation.

The selected strain could completely degrade benzene (10% v/v) and toluene (10% v/v) present in the growth medium. 100% degradation was confirmed by the

absence of the benzene (Fig 6b) and toluene (Fig 6c) peak in the GC chromatogram while ethyl benzene (5% v/v) and xylene (5% v/v) was degraded up to 70% and 50% respectively (Fig 6c, 6d).

2.3.11. Characterization of the gene responsible for petroleum hydrocarbon degradation.

2.3.11.1. Isolation of plasmid DNA

Plasmid DNA was extracted from *G. stearothermophilus* PS11by using QIA prep Spin miniprep kit (Qiagen, USA). The size of plasmid was determined by agarose gel electrophoresis. The size of the mega plasmid DNA was 20 kb which was determined by comparison with Lambda *Hind III* digest DNA marker (Fig. 7). Similar report of harboring a 21 kb catabolic plasmid capable of degrading isopropyl benzene was found in *P. putida* (Eaton *et al.,* 1998). Restriction digestion of plasmid DNA from PS11 strain showed presence of five restriction endonuclease sites when digested with Bam H1.

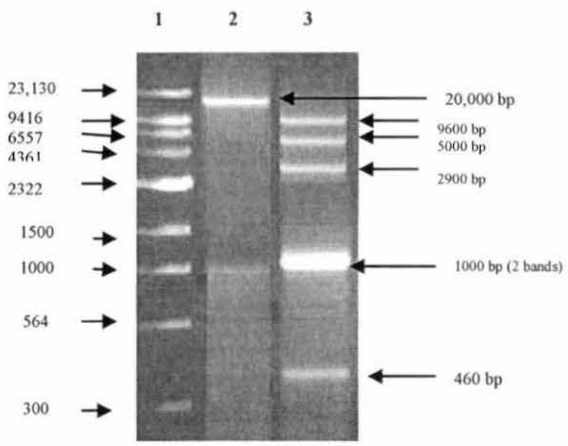


Fig 7. Isolation and restriction digestion pattern of PS11 plasmid DNA. Lane1: Lambda *Hind III* digest DNA marker, Lane 2: Plasmid DNA of PS11, lane 3: BamH1 digest plasmid DNA of PS11

2.3.11.2.Curing of plasmid DNA

Curing of plasmid DNA was performed by growing *G. stearothermophilus* PS11 in the presence of various concentrations of acridine orange (100, 150 and 200

μg/mL). It was concluded that 200 μg/mL of acridine orange effected complete curing of the plasmid DNA as observed from the subsequent electrophoresisof the cell lysate (Fig 8).

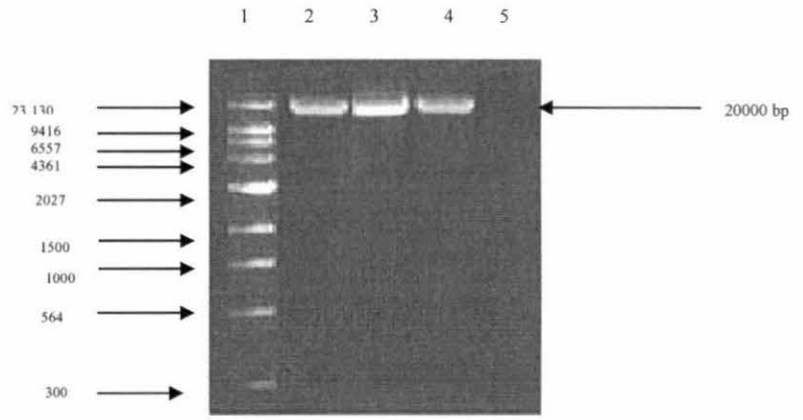


Fig 8. Curing of plasmid with acridine orangeLane1.Lambda*Hind III* digest DNA marker, 2. control plasmid, 3. plasmid with 100 μg/ml acridine orange, 4. plasmid with 150 μg/ml acridine orange, 5. plasmid with 200 μg/ml acridine orange.

2.3.11.3. Growth of plasmid cured strain and wild type strain in presence of petroleum hydrocarbon

In order to confirm whether the plasmid DNA is involved in degradation of petroleum hydrocarbon, the plasmid cured PS11 strain and the wild type PS11 strain was grown medium broth overlaid with solvents of varying log P_{ow} values.

Table7. Growth of wild type and plasmid cured PS11strain in presence of organic solvents

Solvent	log *P*	*Wild type PS11[a]	*Plasmid cured PS11[a]
Control[b]		1.97	1.93
Petrol		0.725	-
DMSO	-1.35	1.83	-
Xylene	3.1	1.34	-
Acetonitrile	0.03	0.221	-
Cyclohexane	3.2	1.67	-
Toluene	2.5	1.21	-
Benzene	2	1.94	-
Chloroform	2	0.97	-

* O D $_{660}$ value, [a]After 48h of growth, [b]without solvent

2.3.9.2. Transformation of plasmid in JM109 and screening of transformed cells

Plasmid transformation provided a conclusive proof for the involvement of plasmidDNA in the degradation of petroleum hydrocarbon especially BTEX.The plasmid DNA from *G. stearothermophilus* PS11 was successfullytransformed into *E. coli* JM109. The transformed cells were able to utilize 0.1% of catechol (Fig 9). Culture broth became yellow in presence of 0.1% of catechol. Catechol, a common intermediate of aromatic hydrocarbon meta degradation pathway, was converted to 2-hydroxymuconic semialdehyde by catechol 2, 3 dioxygenase produced by the transformed JM109 cells.

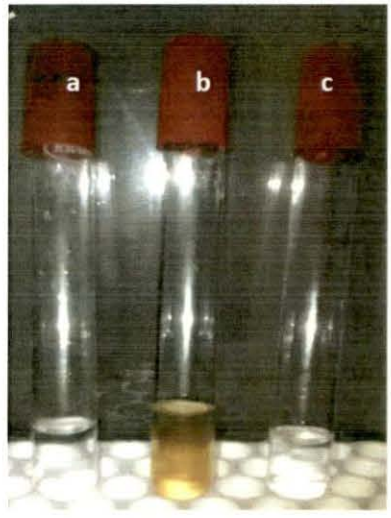


Fig 9. Catechol utilization by transformed JM109 cells. (a) Control w/o inoculam, (b) transformed JM109 cells, (c) non transformed JM109 cells

Agarose gel electrophoresis of the plasmid DNA isolated from the transformed JM109 cells show that it contained megaplasmid of approximately 22000bp (Fig 10) which was similar to that of the plasmid harbored by wild type *G. stearothermophilus* PS11 cells.

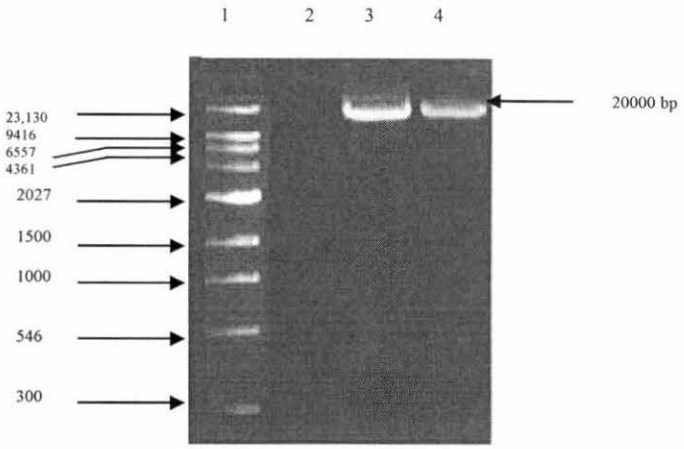
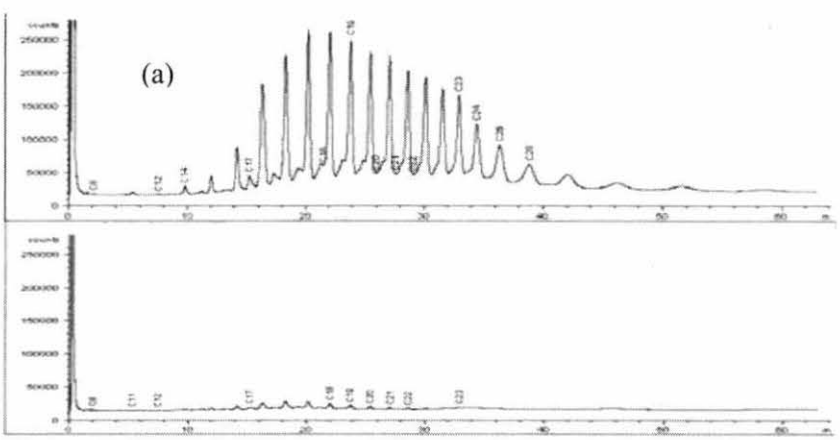


Fig 10. Transformation of *E.coli* JM109 with plasmid from PS11.Lane 1.Lambda *Hind III* digest DNA marker, Lane 2. Wild type JM109, Lane 3.Transformed *E.coli* JM109 cells, Lane 4. Wild type PS11

2.3.9.3. Degradation of Total petroleum hydrocarbons (TPH) and some individual petroleum hydrocarbon by transformed JM109 cells

GC analysis revealed that transformed JM109 cells degraded different BTEX compounds and TPH at different rate in the same time span. The degradation rate was determined as the ratio of the amount of hydrocarbon degraded to the initial amount.TPH chromatogram showed that transformed JM109 cells could degrade both aromatic and alkanes (Fig 11a) in 15 days.



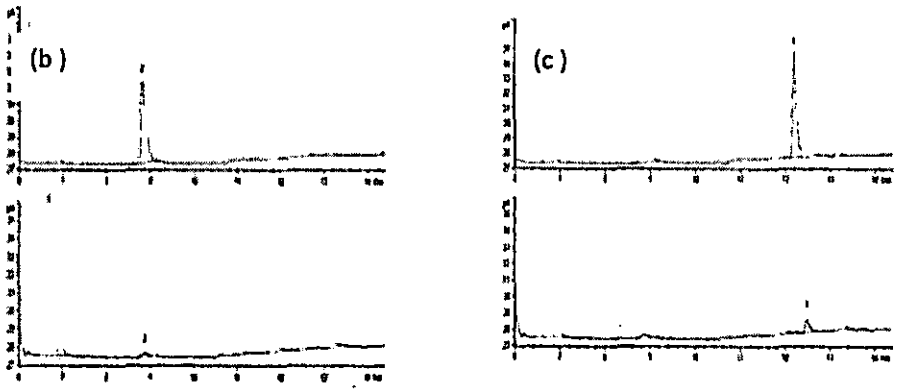


Fig 11. Gas chromatographic pattern of residual total petroleum hydrocarbons (TPH), benzene and ethyl benzene as exhibited by transformed JM109 cells after 15 days of incubation.Degradation of 10% v/v TPH (a), 10% v/v benzene(b) and 5% v/v ethyl benzene (c) after 15 days incubation.

After 15 days of incubation, almost all the aromatics were completely degraded while 5-10% of alkanes were present as residual components in crude oil. The transformed JM109 cells could completely degrade benzene (10% v/v) and toluene (10% v/v) present in the growth medium. 100% degradation was confirmed by the absence of the benzene (Fig 11b) and toluene (data not shown) peak in the GC chromatogram while ethyl benzene (5% v/v) and xylene (5% v/v) was degraded up to 82% and 65% respectively (Fig 11c).

2.3.9.4. PCR amplification and sequencing of plasmid borne gene responsible for degradation

The transformant were screened on the basis of catechol metabolism, a common intermediate of aromatic hydrocarbon meta degradation pathway. The gene of catechol metabolism pathway i.e. catechol 2, 3 dioxygenase, was amplified by PCR using plasmid isolated from transformed cells as well as wld PS11 cells as template.

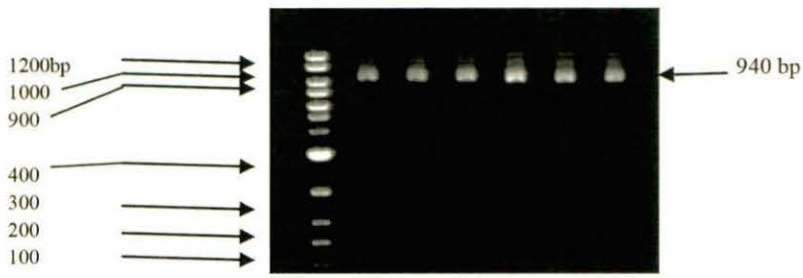


Fig 12.PCR amplification of C23O.Lane 1:100bp ladder, lane 2-4: amplified DNA of C2,3O from transformed cells, lane 5-7: amplified DNA of C2,3O from wild PS11 cells

A PCR product of about 900 bp was successfully obtained with plasmid DNA as template using the primers (Fig. 12).Sequencing of that PCR product resulted in a 915-nucleotidesequence (DDBJ accession no **AB983479**) .The open reading frame (ORF) corresponding to C2,3O gene was composed of 915 bp with an ATG initiation codon and a TAA termination codon. The determined nucleotide sequence and deduced aminoacid sequence of C2,3O are shown in Fig. 13. Nucleotide sequence of the PCR product showed 100% homology with catechol 2,3 dioxygenase of *G. stearothermophilus* DSMZ6285. The C2,3O gene exhibited 49.18% of G +C content. The open reading frame could encode a polypeptide chain containing 304 aminoacid.

Fig 13. Nucleotide sequence of the C2, 3O gene of *G. stearothermophilus* PS11.The open reading frame of the C2,3O gene is indicated in capital letters, The nucleotide sequence has been deposited in theDDBJ database under Accession No.AB983479. The termination codon of C2,3O is indicated by *.

2.3.9.5. Sequence comparison of C23O with other extradiol dioxygenases.

C2,3O from PS11 exhibited significant sequence homology with other reported C2,3Os at amino acid levels (Table 8). The highest sequence homology with C2,3O from *G. stearothermophilus* PS11was identified with corresponding enzyme from *G. stearothermophilus* DSMZ6285 exhibiting 100% indentity at amino acid sequence.

Table 8. Sequence identity of C2,3 O from *G. stearothermophilus* PS11 with other reported C2,3Os

Name of Organism	Amino acid Identity	Accession no
Geobacillus stearothermophilus DSMZ 6285	100%	AAZ76884
Geobacillus thermoglucosidasius NBRC 107763	99%	GAJ45377
Alicyclobacillus acidoterrestris ATCC 49025	77%	EPZ41034
Geobacillus thermoglucosidasius A7	74%	AAF66550
Geobacillus caldoxylosilyticus	74%	WP_017437487
Geobacillus thermoglucosidasiusTNO-09	73%	WP_003249975
Bacillus sp. 1NLA3E	72%	WP_015593673
Bacillus azotoformans LMG 9581	70%	WP_004432417
Pseudomans putida	48%	WP_037273918
Rhodococcus rhodnii	50%	WP_032490117

Amino acid sequence of C2,3O exhibited 99% identity with *G. thermoglucosidasius* NBRC 107763. It showed more than 70% identity with other C2,3Os of *Geobacillus* sp. 48% and 50% amino acid sequence similarity was also noted with *Pseudomonas putida* and *Rhodococcus rhodnii.*As indicated in Fig 14, the deduced amino acid sequence of the C23O of *G. stearothermophilus* PS11 was well aligned with other six catechol 2, 3 dioxygenases. Evolutionary conserved amino acid sequences present abundantly were identified as histidine, tyrosine, phenylalanine, aspartic acid, glutamic acid, alanine, arginine, lysine, proline and glycine.

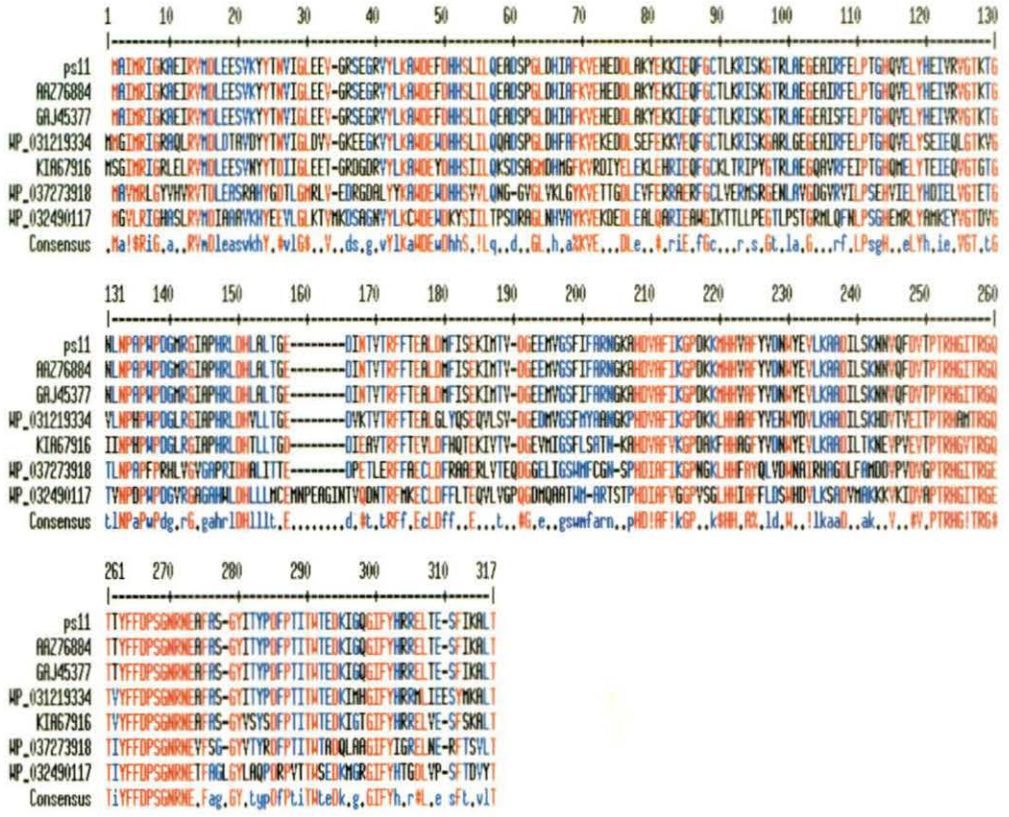


Fig 14. Comparison of deduced amino acid sequence of catechol 2,3-dioxygenase of *G. stearothermophilus* PS11 with other catechol 2,3-dioxygenase. The catechol 2,3-dioxygenase (C23O) of *G. stearothermophilus*PS11was aligned to corresponding enzyme of *G. stearothermophilus* DSMZ6285(AAZ76884), *G. thermoglucosidasius* NBRC 107763(GAJ45377), *Alicyclobacillus acidoterrestris* (WP_031219334), *Bacillus* sp. SgZ-8 (KIA67916), *Pseudomans putida* (WP_037273918), *Rhodococcus rhodnii* (WP_032490117). The closely identical amino acid residues are in blue letter, non identical residues are in black letters and conserved amino acid residues are in red letter.

2.4. Discussion

Uncontrolled discharge of petroleum hydrocarbons during the last century has affected the flora and fauna of land and water bodies. The health risks to humans and the possible carcinogenic effects of petroleum hydrocarbons in the environment arealso of concern. Because of their genotoxicity, discharge levels of 16 differentpetroleum hydrocarbons are marked as prioritypollutants and their presence are continuously monitored in industrial effluents (US EPA).Despite decades of research, successful bioremediation of petroleum hydrocarbon contaminated soil remains a challenge (Rahman *et al.,* 2003).Thisinterest has led to the isolation of several indigenous microorganisms that could be used in the development of various technologies to detoxify PAH-contaminated wastes.

In the present work, soil samples from thepetroleum contaminated sites were screened for petroleum hydrocarbon degrading microbes. Although forty heterotrophic bacteria grew on crude oil supplemented MS agar medium, thirty two petroleum degrading bacteria were distinguished as colonies surrounded by clearzones due to crude oil utilization. Similar process was reported for isolation and screening of pyrene degrading bacteria (Heitkamp *et al.,* 1988). Since all the bacteria in the present study were isolated from a petroleum contaminated soil sample, they survived in the oil-supplemented media very easily as also reported by other authors (Rahman *et al.,* 2003). Based on the highest zone of crude oil utilization, PS11 strain was selected for further work. Biochemical characterization and phylogenetic analysis confirmed strain PS11 to be *Geobacillus stearothermophilus.*

The bacterial strain PS11 utilized crude petroleum-oil but showed delayed growth pattern in its presence. Since butyl rubber covered flasks were used in both cases, availability of oxygen may not be responsible for lesser growth in the presence of solvent. The reason may be the direct effect of crude oil on cells. The dry cell mass of the culture in the presence of crude oil was about double of that in its absence. Similar growth behavior was reported in the case of *P. aeruginosa* PST-01 while 23% cyclohexane was incorporated in the media (Ogino *et al.,* 1995, 1999).*B. subtilis* DM-04, *P. aeruginosa* M and NM strains were reported to utilize crude petroleum-oil hydrocarbons as sole source of carbon and energy, which was evident

from the increase in bacterial dry biomass, protein content and cell density after 120 h of incubation (Das and Mukherjee, 2007). Toledo *et al.,* (2006) reported about fifteen bacterial strains isolated from solid waste oil samples that have the capacity of growing in the presence of petroleum hydrocarbons. Majority of the strains belonged to *Bacillus pumilus* (eight strains) and*Bacillus subtilis* (two strains). Besides, three strains were identified as *Micrococcus luteus*, one as *Alcaligenes faecalis* and one strain as *Enterobacter* sp.

The response of *G. stearothermophilus* PS11towards various other solvents of varying log P_{ow}values were studied. The toxicity of a solvent is determined by its log*P* value. It is defined as the partition coefficient of aparticular solvent in an equimolar solution of octanol and water (Sikkema *et al.,* 1995). Generally, log*P* value is inversely proportional to the polarity of solvent. Hence, solvents with lower log *P* value have greater ability to mediate a toxic response throughmembrane partitioning. Solvents with log P_{ow} values between two and four are considered highly toxic for microorganisms (Torres *et al.,* 2009). Every organism posses its own intrinsic tolerance level for organic solvents thathas been genetically acquired and sometimes influenced byenvironmental factors (Kobayashi *et al,* 1998). Tolerance level of eachmicroorganism has been represented by the index solvent and the indexvalue. The index value is the log *P* value of the most toxic organic solvent (index solvent). Results summarized in Table 3 show that the isolated strain PS11 could grow in solvents (such as toluene, benzene) having higher log *P*-value. Similar finding was observed in *Anoxybacillus* sp.PGDY12 that was able to tolerate 100% solvents such as toluene, benzene andp-xylene in plate overlay method (Gao *et al.,*2011). A strain *Bacillus* EEZMo-3 reported bySegura *et al.* (2008) was found to be more tolerant to different organic solventsand also was able to survive in 100% benzene. It might be that organic solvent emulsifying substances or solubilising enzymes play an important role in diminishing solvent toxicity in gram-positive bacteria (Sardessai and Bhosle 2004). In contrast, alcohols having very low log *P*-value inhibited the growth of PS11 cells.The inhibitory effect of acetone and butanol on the growth of organisms might be due to the chaotropic effects of the alcoholic solvents on the cell membranes (Ezeji *et al.*, 2010).

Transmission electron micrograph of *G. stearothermophilus* PS11cells in the presence of 10% crude oil showed accumulation of solvents and convoluted, disorganized cell membrane. Similar cellular changes have been reported for *Pseudomonas* sp. cells grown in *p*-xyleneand *Enterobacter sp.* grown in the presence of cyclohexane (Gupta *et al.*, 2006).Solvents are reported to damage the integrity of cell membrane structure resulting in loss of permeability regulationsand asan energy transducer concomitantly leading to damages ofthe cellular metabolism, growth inhibition, and sometimes celldeath (Isken and de Bont 1998). In certain cases leakage of cell RNA, phospholipid and protein also take place (Sikkema *et al.*, 1995). Moreover, it was clearly visible that the solvent accumulation changed the cell size and shape. A number of reports highlight that increasedcell size on exposure to toxicorganic compounds. Similarly, *P. putida* and *Enterobacter* sp. increasedin size when exposed to phenol, 4-chlorophenoland butanol (Neumann *et al.*, 2005). Thus, it can be inferred from the result that surface-to-volume ratio of the cellseems to be the major factor responsible for these structural changes.The decline in solvent accumulation and reorganization of cell membrane was observed on further incubation till 48 hours (Figure 5c). Structural changes of PS11 as observed in TEM cells suggest that organic solvent affected the membrane system. The solvent tolerant nature of the bacterial strain was evident from the reorganization of cell membrane and decline in cytosolic solvent accumulation during prolonged exposure. Hence, solvent adaptation property of *G. stearothermophilus* PS11 seems to be related to both restoration of membrane fluidity and metabolic transformation of hydrophobic solvent to less toxic products. As observed in the present study, *Bacillus* sp. also showed cell morphology alterations and filamentous growth in response to environmental stress, including organic solvents (Toress *et al.*, 2009).

Several mechanisms of solvent tolerance in bacteria were reported earlier. Efflux pumps are reported to be one of the main mechanisms of solvent tolerance and this mechanism may have the effect of diminishing the solvent concentration in the cytoplasm. In gram positive bacteria, efflux systems are either secondary transporters or ATP binding cassette (ABC) type transporters (Bolhuis *et al.*, 1997). Solvent tolerant cells also adapt by making changes in fatty acid composition and protein/lipid ratio in cell membrane to restore the fluidity. They also have capability

of metabolic transformation of toxic compound into non-toxic products (Sardessai and Bhosle, 2003). Some *Bacillus* species are reported to produce active hydrolases in non-aqueous media, indicating that they are genetically armored to challenge the effects of harsh organic solvents (Sareen and Mishra 2008; Gupta and Khare, 2009). Although, there are a variety of mechanisms that could confer bacterial adaptation to organic solvents, the bacterial solvent tolerance is notpossible by a single mechanism (Heipieper *et al.,* 2007). It is very likely that the combination of different metabolic strategies leads to cellular solvent tolerance.

The membrane phospholipid composition of *G. stearothermophilus* PS11 was altered when grown in the presence of 10% (v/v) crude oil. Presence of crude oil decreased phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) with parallel increase in cardiolipin (DPG) and diphosphatidylglycerol (PGL). Similar result was observed in case of *P. aeruginosa* IBB_{P010} when grown in presence of organic solvents (Stancu, 2011). This increase in cardiolipin (sugar containing lipid) and PGLconcentration indicated increased hydrogen bonding capacity ofthe membrane lipid bilayer. The hydrogen bonding between hydroxyl group of the sugar rings and the surrounding water or the phospholipid contributed to structural stabilization of the membrane (Murinova *et al.,* 2014). So when crude oil gets incorporated into the membranes of *G. stearothermophilus* PS11, the glycolipid enrichment provided the cell membrane with an increased stability against the perturbations induced by crude oil which gets incorporated into the bilayer (Schlame, 2008). The electrostatic and hydrophobic forces determine the membrane interactions in fluid phase. Therefore, the presence of crude oil has been expected to largely affect the equilibrium between the electrostatic interactionsof the polar heads and the hydrophobic interactions of the acyl chains of phospholipids. Also, the changes in the proportions of the phospholipid area represented cell adjustment in order to maintain the interactive and repulsive properties at the membrane surface. These alterations also contribute to the reorganization of biological water, i.e., water hydrogenbonded to organic molecules.The decrease in PE may contribute to higher bilayer stability to counteract the effect of crude oil (Weber and de Bont, 1999).

While PS11did alter its membrane fatty acidcomposition in presence of crude oil, it did so in a manner opposite to gram-negative bacteria. PS11 cells increased its

membrane fluidityby increasing (8%) its level of branched straight-chain fatty acids while decreasing (17.7%) its level of straight-chain fatty acids. A higher percentage of iso branched fatty acids (55.58%) resulted in a more fluid membrane. As the iso-branched chains are loosely packed than straight chains, the enrichment of adapted membranes in iso-branched chains compensated for the ordering effect promoted by crude oil in the fluid state of polar lipids, when added directly to the lipid dispersions.Simultaneously, the ratio of branched chain: straight chain fatty acids also increased resulting in the decrease of membrane rigidity.According to Kim *et al.,* (2002) and Shabala and Ross, (2008) the role of these membrane fatty acids might be very important in protection against aromatic compounds stress as well as many environmental factors.

The degradation of total petroleum hydrocarbon (TPH) and BTEX by PS11 strain was analyzed using gas chromatography. As evident from the result, all aromatic compounds in TPH were more readily degraded compared to that of alkanes after 15 days incubation. The present observation is in agreement with microbial flora from activated sludge that could extensively degrade aromatic compounds compared to alkanes present in gasoline in 25 days (Serena *et al.,* 1999). On the contrary, preferential degradation of *n*-alkanes (C_{14}–C_{30}) compared to PAHs present in crude petroleum-oil was demonstrated in case of *Bacillus subtilis* DM-04 and *Pseudomonas aeruginosa* M (Das and Mukherjee, 2007). After 15days of incubation, *G. stearothermophilus* PS11 could completely degrade benzene and toluene, however, in the same time period it partially degraded ethyl benzene and xylene. In a similar type of study, *Pseudoxanthomonas spadix* BD-a59completely degraded BTEX as reported by Kim *et al.,* (2008).

Reports suggested that plasmid DNA may play an important role in genetic adaptation as it representsa highly mobile form of DNA, which can be transferred via conjugation or transformation and can impart novel phenotypes including hydrocarbon-degrading abilityto the microorganisms (Sentchillo *et al.*, 2000). In order to ascertain the role of plasmid in petroleum hydrocarbon degradation, plasmid was cured by acridine orange in sub-lethal doses, in bacterial populations, that led to the elimination of plasmid DNA without harming the bacterial chromosome and

maintained the ability to reproduce and generate off spring (Dam *et al.*, 2012). Plasmid cured culture of PS11 strain could not grow in presence of crude oil or any of the solvents. The inability to grow in presence of crude oil or other solvents might be due to the removal or inactivationof gene(s) responsible for petroleum hydrocarbon degradation from *Geobacillus stearothermophilus* PS11, thus, confirming that the petroleum hydrocarbon degrading gene is harbored by the plasmid. Role of plasmid in the degradation of petroleum hydrocarbon by *Pseudomonas fluorescens* NS1 was also reported by Vasudev *et al.*, (2007). Several other reports documented that catabolic pathways that encode different aromatic hydrocarbon degradation routes are frequently located on plasmids. But localization of degradative genes can be on either chromosome or plasmid (Mesas *et al.*, 2004; Gokhan *et al.*, 2005). It was interesting to note the presence of approximately 22 kb mega plasmid in *Geobacillus stearothermophilus* PS11. Earlier, mega plasmid was detected in anthracene degrading *Geobacillus stearothermophilus* AAP7919 (Kumar *et al.*, 2012) and PAH degrading *Sphingomonas sp.* strain KS14 (Cho *et al.*, 2001).

Plasmid transformation experiments were conducted to provide a conclusive proof for the involvement of plasmid DNA in the degradation of petroleum hydrocarbon. The plasmid DNA from *Geobacillus stearothermophilus* PS11was successfully transformed into *E. coli* JM109.The resultant transformed cells were able to grow in presence of catechol, a common intermediate of PAH (poly aromatic hydrocarbon) meta degradation pathway, as the sole source of carbon and energy. The wild *E. coli* JM109 showed no significant growth in presence of 0.1% of catechol, which indicates the expression of the catabolic genes in the transformed *E. coli* JM109 strain. This finding was further concluded by agarose gel electrophoresis that showed the presence of mega plasmid of 20000bp in transformed *E. coli* JM109 which was similar to that of the plasmid harbored by wild *G. stearothermophilus* PS11 cells.

GC analyses of the transformed *E. coli* JM109 showed similar pattern of TPH and BTEX degradation like that of wild *G. stearothermophilus* PS11 cells. Similar findings was reported by Mervat (2009) who successfully transformed *E. coli* K12 DH5α with plasmid encoding for methomyl degradation from *Stenotrophomonas maltophilia* M1 strains and the transformed cells were able to grow on the

contaminant. The crude oil degrading potentials of transformed *E.coli* K12 DH1 with the extracted plasmids from *Klebsiella pneumonia* and *Serratia marscencens* were confirmed by Akpe *et al.*, (2013).

Catechol 2, 3 dioxygenase is involved in the metabolism of catechol, a common intermediate of aromatic hydrocarbon meta degradation pathway. Several researchers have reported that catechol 2, 3-dioxygenase encoding gene is locatedon plasmid (Jiang *et al.*, 2004). In our study, PCR amplification using plasmid as template isolated from transformed cells as well as wild PS11 cells successfully yielded a DNA of about 900 bp. The comparison of deduced amino acid sequences with reported sequences of the related enzymes is of particular interest, because conserved amino acid residues may be important for enzyme function.Therefore the derived amino acid sequence of C2,3 0 was compared to that of the various extradiol dioxygenases reported earlier. Sequence analysis of 917 bp PCR product showed 100% homology with catechol 2, 3 dioxygenase of *Geobacillus stearothermophilus* DSMZ 6285 at the nucleotide and amino acid levels. The conserved amino acids are important for enzyme activity. Comparision of C2, 3O with other reported sequences of the related enzymes showed high frequency of glycine and proline. Their presence suggested that the tertiary structures will prove to be highly conserved among the extradiol dioxygenases. The active site of C2,30 contains a Fe^{2+} centre that has been implicated in the binding and catalytic activation of molecular oxygen (Mabrouk *et al.*,1991). High conservation of six histidines and six tyrosines was found in all enzymes compared. It has been suggested that presence of histidine and/or tyrosine may be the iron-binding residues in C2,30 (Tatsuno *et al.*,1980). It has been noted that a single cystein residue is present in all the enzymes compared except the C 2, 3 O from *Rhodococcus rhodnii.* Taira *et al.* (1988) suggested that cysteine might be functionally important due to the formation of disulphide linkages between the subunits of extradiol dioxygenases.The extradiol dioxygenase from a gram-positive PS11 strain showing almost 50% homology with enzyme from the gram-negative *Pseudomonas putida* is quite an interesting fact to note. This homology of nucleotide and amino acid sequence of catechol 2, 3 dioxygenase from Gram-positive and Gram-negative strains was first reported by Candidus *et al.* (1994)for *Rhodococcus rhodochrous* CTM. The possible reason for such high homologyof nucleotide

sequences might be a very recent commongenetic origin of all these enzymes. The horizontal transfer of the gene coding for catechol 2,3-dioxygenasein many bacterial strains could be another possible reason. Such horizontal transfer of the C23O gene was alsoobserved by Wang *et al.* (2007) between endophytic andrhizosphere bacteria and by Jussila *et al.* (2007) between *Pseudomonas* and *Rhizobium*.

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