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Physiological Adaptations Involved in Petroleum Hydrocarbon Degradation by *Geobacillus stearothermophilus* strain PS11

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Abstract—*Geobacillus stearothermophilus PS11 is capable of growing in wide range of toxic hydrophobic solvents (logP 1-4) such as petrol, BTEX as a sole source of carbon. TEM analysis of PS11 cells in the presence of 10% crude petrol showed convoluted cell membrane and accumulation of petrol in the cytoplasm. GC analysis showed that PS11 strain could degrade almost all aromatic compounds compared to that of alkanes in 15 days. The membrane phospholipids composition of PS11 was altered in the presence of 10% (v/v) petrol. Decrease in phosphatidylethanolamine (PE-11%) and phosphatidylglycerol (PG-14%) with parallel in increase cardiolipin (DPG-15%) and diphosphatidylglycerol (PGL-128%) was observed in presence of petrol. An increase (22.4%) of the iso-acids was noted in cell membranes adapted to 10% (v/v) petrol with concomitant decrease of the anteiso-acids (17.6%) and straight-chain saturated fatty acids (17.8%). Thus, the isolated strain Geobacillus stearothermophilus PS11 could be used as a tool towards green ecosystem.*

Keywords*: BTEX degradation, Bacterial membrane, solvent tolerance, Geobacillus stearothermophilus.*

1. INTRODUCTION

Petroleum industry involves its production, storage and transportation, refining and processing. During all these processes spillage of petroleum hydrocarbons often occur due to blowout accidents, leakage from oil pipelines and storage tanks and sometimes during refurbishing of refineries and petrochemical production equipment[1-2]. Biodegradation of organic wastes is a useful side effect of microbial metabolism, thus the fundamental principles of biodegradation are integrally linked to microbial physiology [3]. Since past few years, the application of bacteria to combat environmental pollutants has shown a promising approach because of its cost effective and eco-friendly nature [4]. A wide variety of micro-organisms, mostly bacteria and fungi found in the environment, has the ability to use a wide range of petroleum hydrocarbon compounds as sources of carbon and energy [5] hence they are able to biodegrade a variety of organic compounds [6].

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. Adaptation of its membrane phospholipids in presence of the toxic wastes was also studied.

2. MATERIALS AND METHODS

2.1 Isolation and screening of petroleum hydrocarbon degrading microorganisms from soil sample

Soil sample was collected from petroleum contaminated 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 minimal salt (MS) broth containing 1% hydrocarbons (crude oil) and incubated for 72 h at 37^{o}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^{o}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.

The best petroleum hydrocarbon degrading bacterial strain was screened by agar plate assay. Among all the colonies, only the colony exhibiting the highest zone of hydrocarbon utilization was selected for further works**.**

2.2. Identification of the selected bacterial strain

The morphological and biochemical characterization of the selected bacterial strains was determined by gram staining and other biochemical tests [7]. All these tests were performed in duplicates.

The identity of isolate was confirmed by phylogenetic analysis of 16S rRNA sequence using the software package Mega4 [8].

2.3. Determination of growth and dry cell mass of PS11strain

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. The incubation was carried out at 50 °C at 140 rpm for 96 h. 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 [10].

2.4. Tolerance of PS11 cells to other solvents

The tolerance of PS11 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 by growing the culture in MS broth overlaid with either of the organic solvent (10 % v/v) and incubated at 50 °C with shaking at 140 rpm. The bacterial culture growing in absence of organic solvent under similar conditions served as control.

2.5. 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) according to Hartman et al, (2010) [9].

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

2.6.1. 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.

2.6.2. Extraction of bacterial membrane lipid

Lipids were extracted using the method of Bligh-Dyer (1959) [11].

2.6.3. Phospholipid analysis

The lipid classes were separated by TLC on silica gel H plates (0.5 mm thickness) using the method of Jacin and Mishkin (1965) [12]. 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 [13]. Diphosphatidylglycerol, phosphatidylglycerol (PG), and lysylphosphatidylglycerol (LPG) (Sigma) were used as standards for determining phospholipids.

2.6.4. 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. 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).

2.7. 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 15th 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. The cultures in absence of the solvent and uninoculated media enriched with solvents were used as control. All the experiments were carried in duplicate.

3. RESULTS AND DISCUSSION

3.1. Isolation and screening of petroleum hydrocarbon degrading microorganisms from soil sample

Among thirty two isolated petroleum hydrocarbon degrading bacteria, PS11 strain was selected for further work based on highest zone of crude oil utilization. It was identified as *Geobacillus stearothermophilus* on the basis of phenotypic characteristics and phylogenetic analysis (Figure. 1). The 16S rRNA sequence was submitted to Gene Bank with the accession no. **KC311354**.

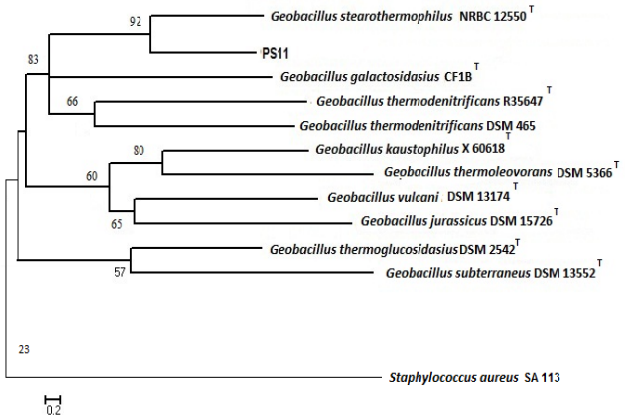


Figure 1: Phylogeneticanalysis of 16S rRNA sequence. (The evolutionary history was inferred using the Neighbor-Joining method. Phylogenetic analyses were conducted in MEGA 4, The numerials are the boot strap values)

3.2. Determination of growth and dry cell mass of G. stearothermophilus PS11

The growth curve of *G.stearothermophilus* PS11 in the absence and presence of crude oil (10 %, v/v) is shown in Figure 2. 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, control exhibited as shorter lag phase of less than 6h and continued to remain in log phase till the end of incubation time. Similar growth behavior was reported in the case of *Pseudomonas* spp. and *Bacillus* spp. while benzene and crude oil was incorporated in the media [14]. Hassan et al. (2012) [15] have reported the isolation of Alcanivorax sp. HA03 from soda lakes in Wadi E1Natrun capable of degrading benzene, toluene, and chlorobenzene as the sole sources of carbon.

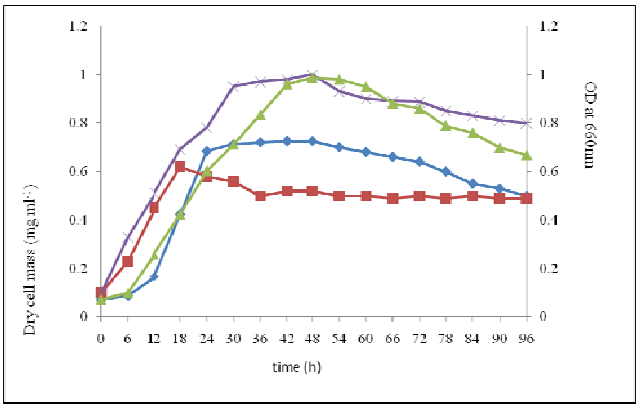


Figure 2: Growth of *Geobacillus stearothermophilus* PS11in the absence and presence of crude oil. Bacterial growth in absence of crude oil: $OD_{660\ nm}$, (▲); dry cell mass, (X) and growth in presence of crude oil: $OD_{660\ nm}$, (◆); dry cell mass, (■). The experiment was carried out in triplicates and the difference in the individual results was less than 3%.

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

The results summarized in Table-1 show 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. Similar finding was observed in *Anoxybacillus* sp.PGDY12 that was able to tolerate 100% solvents such as toluene, benzene and p-xylene in plate overlay method [16].

Table 1: Growth of PS 11strain in presence of organic solvents

Solvent	log *P*	$OD_{660\ nm}$	Dry cell mass (mg ml^{-1})	% of growth inhibition
Control[b]		1.9	1.09	
Isooctane	4.5	-*	-*	
DMSO	1.35	1.8	0.46	7
Xylene	3.1	1.2	0.7	38.5
Acetonitrile	0.03	0.22	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	-	-	

6* O D 60 value < 0.1 and dry cell mass (mg ml -1)< 0.05 after 48h of growth. a After 48h of growth, b without solvent

The inhibitory effect of alcohol might be due to the chaotropic effects of the solvents on the cell membranes [17].

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

TEM image showed that the growth 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 2b) at 24h of incubation resulting in permeability loss, growth inhibition, and sometimes cell death. Similar cellular changes have been reported for *Pseudomonas* sp. cells grown in *p*-xylene and *Enterobacter* sp. grown in the presence of cyclohexane [18].

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 2b).

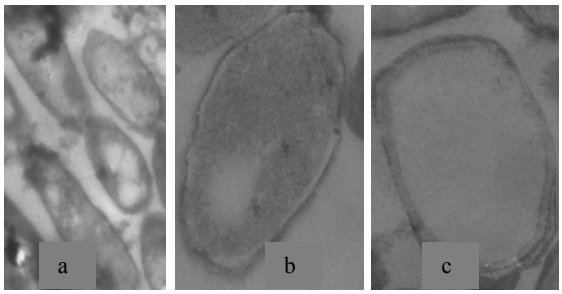


Figure 2: Transmission electron micrograph of *Geobacillus stearothermophilus* PS11cells. (a)in the absence of crude oil (exposure 21,000); (b) in the presence of 10% crude oil (exposure 21,000) after 24 hours incubation - accumulation of solvents and convoluted and disorganized cell membrane; change in cell shape (c) in the presence of 10% crude oil (exposure 21,000) after 48 hours incubation regeneration of cytoplasm and cell wall.

Gradual change in the cell shape from rod to semi circular was obseved after 24h of incubation in presence of crude oil. The decline in crude oil accumulation and reorganization of cell membrane were observed on further incubation till 48 hours (Fig 2c). Similarly, *Bacillus* sp. also showed cell morphology alterations and filamentous growth in response to environmental stress, including organic solvents [19].

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

3.5.1. Phospholipid analysis

In the control sample, the polar lipids constituted about 80-90% of the total lipid extract of bacterial membranes. 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. The major class PE is made about 57 % of the total As listed in table 2, 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.

Table 2: Phospholipid composition of *G.stearothermophilus* PS11 membranes, 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

*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

Similar result was observed in case of *P. aeruginosa* IBB_{P010} when grown in presence of organic solvents [20]. This increase in cardiolipin and PGL concentration indicated increased hydrogen bonding capacity of the membrane lipid bilayer that contributed to structural stabilization of the membrane against the perturbations induced by crude oil [21].

3.5.2. Analysis of fatty acid composition

The acyl chain composition of *G. stearothermophilus* PS11 polar lipids is listed in Table 3. The proportions of aliphatic chains changed significantly. Each isomer 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.

Table 3 Fatty acid composition of polar phospholipid fraction of *G. stearothermophilus* ps11 as affected by crude oil

	Membrane phospholipid*					
	PE	**PG**	**DPG**	**PGL**	**X_1**	**X_2**
Control	57±1.2	14±0.5	20±1.4	3.5±0.6	2.2±0.7	2.8±1.5
10% crude oil	51±0.3	12±0.4	23±1.0	7.9±0.7	2.8±1.2	3.2±.9

*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

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 4) 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.

Table 4 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±1.5	70.91±0.5	45.41±0.7	25.5±1.0
10%(v/v) crude oil	23.92±0.9	76.58±1.0	55.58±1.3	21±0.56

*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

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. A higher percentage of iso branched fatty acids (55.58%) resulted in a more fluid membrane. Reports suggest that the role of the membrane fatty acids might be very important in protection against aromatic compounds stress as well as many environmental factors [22].

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

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 4). After 15 days of incubation, almost all the aromatics were completely degraded while 30% of alkanes were present as residual components in crude oil.

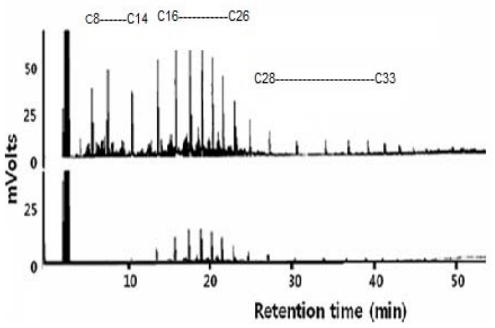
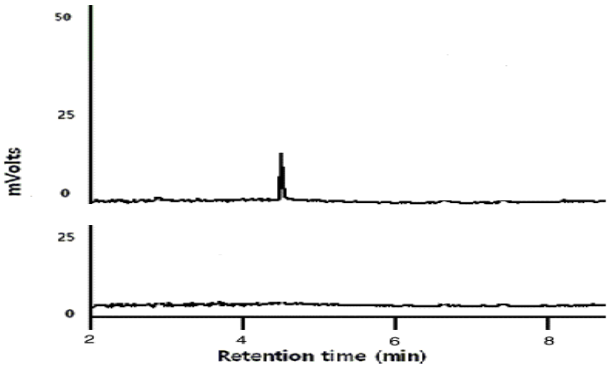
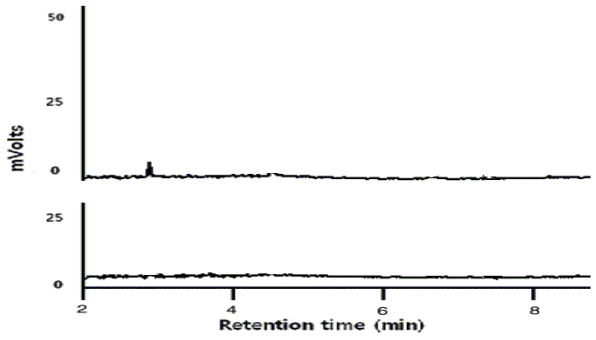


Figure 4. Gas chromatographic pattern of residual total petroleum hydrocarbons (TPH) as exhibited by PS11cells after 15 days of incubation.Degradation of 10% v/v TPH

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 5a) and toluene (Fig 5b) peak in the GC chromatogram.

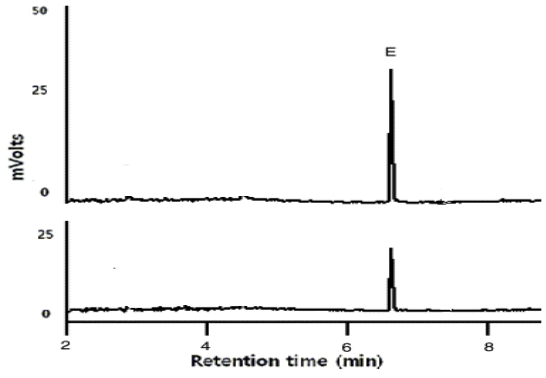


(5a)

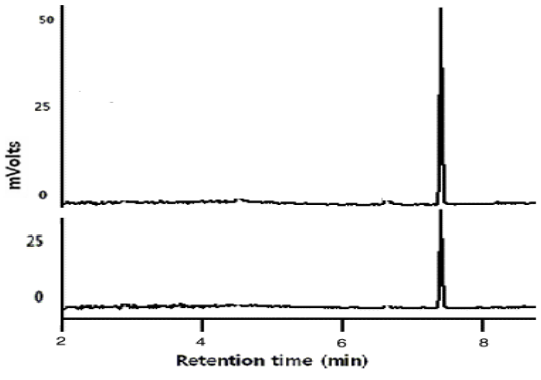


(5b)

while ethyl benzene (5% v/v) and xylene (5% v/v) was degraded up to 70% and 50% respectively (Fig 5c, 5d). . In a similar type of study, *Pseudoxanthomonas spadix* BD-a59 completely degraded BTEX [23].



(5c)



(5d)

Figure 5. Gas chromatographic pattern of BTEX as exhibited by PS11cells after 15 days of incubation.Degradation of (a), 10% v/v benzene(b), 10%v/v toluene(c),5% v/v ethyl benzene (d) and xylene 5% v/v after 15 days incubation.

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