STUDIES ON BIODEGRADATION OF PETROLEUM HYDROCARBONS BY A SOIL BACTERIUM

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In
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By
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DECLARATION

I declare that the thesis entitled "**STUDIES ON BIODEGRADATION OF PETROLEUM HYDROCARBONS BY A SOIL BACTERIUM**" has been prepared by me under the guidance of Dr. Shilpi Ghosh, Assistant Professor of Department of Biotechnology, University of North Bengal. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE

I certify that Ms. Payel Sarkar has prepared the thesis entitled "**STUDIES ON BIODEGRADATION OF PETROLEUM HYDROCARBONS BY A SOIL BACTERIUM",** for the award of PhD degree of the University of North Bengal, under my guidance. She has carried out the work at the Department of Biotechnology, University of North Bengal.

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ABSTRACT

The increased generation of petroleum hydrocarbon waste has been stated as one of the most critical environmental problems. Though microbial bioremediation has been widely used for waste treatment but their application in petroleum hydrocarbon waste treatment is limited since they have toxic effects on the microbial cells.

In the present research work, soil samples from the petroleum contaminated sites were screened for petroleum hydrocarbon degrading microbes. Among the thirty two petroleum degrading bacteria, PS11 strain was selected for further work on the basis of highest zone of crude oil utilization. Phenotypic characterization and phylogenetic analysis confirmed strain PS11 to be *Geobacillus stearothermophilus*. The bacterial strain was able to grow in presence of petroleum hydrocarbons as evidenced by increase in cellular dry biomass after 6 h of incubation. However, PS11 cells exhibited a delayed growth pattern inthe presence of crude oil in the growth medium in comparison to those growing in absence of crude oil. The strain was also capable to grow in presence of wide range of other hydrophobic solvents with log *P*-values between1-4,whereasalcohols having very low log *P*-value had inhibitory effect on growth. Transmission electron micrograph of PS11 cells grown in the presence of 10% (v/v) crude oil for 24 h showed convoluted cell membrane and accumulation of crude oil in the cytoplasm, indicating the adaptation of the bacterial strain to the petrol. The mechanism of solvent tolerance of PS11 was ascertained by gas chromatography analysis of metabolic transformation products of crude oil and other solvents as well as membrane phospholipids. Results showed that PS11 cells growing in presence of crude oil (10 % v/v) for 15 days could degrade both alkanes and aromatic compounds. It degraded aromatic compounds more readily than alkanes. The membrane phospholipids composition of *G. stearothermophilus* PS11 was also altered in the presence of crude oil. Decrease in phosphatidylethanolamine (PE,11%) and phosphatidylglycerol (PG,14%) paralleled increase in cardiolipin (DPG, 15%) and diphosphatidylglycerol (PGL,128%) in presence of crude oil was noted. Significant changes were also observed in the membrane fatty acid. Similarly, an increase (22.4%) of the iso-fatty acids was noted in cell membranes adapted to 10% (v/v) crude oil with

concomitant decrease of the anteiso-fatty acids (17.6%) and straight-chain saturated fatty acids (17.8%). Hence, the 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.

Furthermore, the role of plasmid DNA in petroleum hydrocarbon degradation property of PS11 was determined. For this plasmid DNA was cured from the bacterial cell by treating with acridine orange. Plasmid cured culture of PS11 were unable to grow in presence of crude oil or any of the solvents indicating the involvment of plasmid encoded gene(s) in petroleum hydrocarbon degradation. The cured plasmid was subsequently transformed into competent*Escherichia coli*JM109 cells. The resultant transformants gained the ability to grow in presence of catechol, a common intermediate of aromatic hydrocarbon meta-degradation pathway. The role of plasmid in petroleum hydrocarbon degradation was further confirmed by PCR amplification of one of the gene of aromatic hydrocarbon metabolic pathway i.e. catechol 2, 3 dioxygenase, using primers designed from the sequence conserved region and plasmid DNA as template, which yielded aPCR product ofabout 900 bp.Homology analysis of the deduced amino acid sequence with the known catechol 2, 3 dioxygenase genes from other bacteria by BLAST programrevealed highest similarity score with *Geobacillus stearothermophilus* DSMZ6285 strain.

*Geobacillusstearothermophilus*PS11 was further employed for enzyme catalyzed biodiesel production. For this, an extracellular, organic solvent tolerant, alkaline lipase from isolated PS11 cells was produced. Optimization of production conditions by OVAT approach enhanced the lipase production by 2.46 folds. The 27KDa lipase was purified by 8.04 fold with 22.6% yield. Optimum pH for lipase was 10. It showed 100% stability in the pH range 8 to 11 for 2h. The enzyme showed maximum activity at 50°C and retained 50% activity at 70°C for 2h. Lipase was stable in presence of wide range of solvents including BTEX, methanol etc. Gas chromatography result confirmed the catalytic activity of lipase in biodiesel production by enzymatic trans-esterification of vegetable oils in presence of methanol.

Thus, *Geobacillusstearothermophilus*PS11 could serve as a potential tool for biodegradation of petroleum hydrocarbons and biodiesel production.

PREFACE

This thesis is the end of my journey in obtaining my Ph.D. I have not traveled in a vacuum in this journey. This thesis has been kept on track and been seen through to completion with the support and encouragement of numerous people and blessings of Almighty. At the end of my thesis, it is indeed a pleasant task to express my thanks to all those who contributed in many ways to the success of this study and made it an unforgettable experience for me.

At this moment of accomplishment, first of all I pay homage to my guide Dr. Shilpi Ghosh. I am very much thankful to her for picking me up as a student at the critical stage of my Ph.D. This work would not have been possible without her guidance, support and encouragement. Under her guidance, I successfully overcame many difficulties and learned a lot. Her unflinching courage and conviction will always inspire me and I hope to continue to work with her noble thoughts. I can only say a proper thanks to her through my future work.

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The road to my Ph.D started with initial research work at Amity School of Biotechnology, Amity University, New Delhi. I take this opportunity to say heartfelt thanks to Prof. R. Singh for providing very good training on latest microbiological techniques and building confidence in me to start my work. Thank you doesn't seem sufficient but it is said with appreciation and respect to her for her support, encouragement and care.

Some of the results described in this thesis would not have been obtained without a close collaboration with Microbiology Lab,

Amity University. I owe a great deal of appreciation to all the Ph.D. students of that lab. I extend my thanks to Mr. Anuj Kumar, Electron microscopy Department, AIIMS, New Delhi for helping me to carry out part of my research work using the TEM facility. He has spent his valuable time in critically observing the TEM images of my work.

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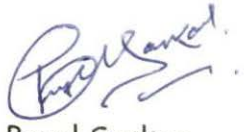
I am indebted to my many students for providing a stimulating and fun filled environment. My thanks go in particular to the students of 2010-12 session for encouragement during my tough times.

It's my fortune to meet Ashish and Jimmy during my stay in Delhi. They showed me that life is not a path laid with rose petals. I acknowledge the moments I shared with you that helped me to be stronger person to fight all odds of life. I express my appreciation to my friend Pramod for his support. He was always beside me during the happy and hard times, to push me and motivate me.

A journey is easier when you travel together. Interdependence is certainly more valuable than independence. I especially thank my mom, dad, and sister. My hard-working parents have sacrificed their small wishes for my sister and myself and provided unconditional love and care. It was my dad's wish to see me getting my doctoral degree.I would not have made it this far without them. My mom has been my best friend all my life and I love her dearly and thank her for all her advice and support. I know I always have my family to count on when times are rough. Special thanks to the newest additions to my family, Sayan, my husband as well as his family who all have been supportive and caring.

The best outcome from these past five years is finding my best friend, soulmate and husband. I married the best person out there for me. Sayan is the only person who can appreciate my quirkiness and sense of humor. He has been a true and great supporter and has

unconditionally loved me during my good and bad times. He has been non-judgmental of me and instrumental in instilling confidence. He has faith in me and my intellect even when I felt like digging hole and crawling into one because I didn't have faith in myself. These past several years have not been an easy ride, both academically and personally. I truly thank you, Sayan, for sticking by my side, even when I was irritable and depressed. I feel that we both learned a lot about life and that strengthened our commitments and determinations to each other and to live life to the fullest.



Payel Sarkar

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Chapter 1

General introduction and review of literature

1.1. Introduction

Every year spillage of tones of fuel and lubricating oil has become a major environmental hazard. In October 2014, a single oil spillage of 546 tones was reported at Mid-Valley Pipeline of Louisiana, United States. A recent incident highlighted how time consuming a cleanup operation of oil spillage can be. According to CNN International, in Nov 2013, oil-spillage occurred in Phillipines due to a typhoon and till date the cleaning up process is going on. There are also long-term effects on ecosystems related to the release of toxic components over a prolonged period as the oil breaks up and the concentration of toxicants in organisms towards the top of the food chain increases (Lin *et al.*, 2008).

These contaminations of the environment with petroleum hydrocarbons provides serious problems for many countries including India. Release of petroleum hydrocarbons into the environment whether accidentally or due to human activities, is a main cause of water and soil pollution. Soil contamination with hydrocarbons cause extensive damage to local system as accumulation of pollutants in animals and plant tissues results in death or mutations (Mehrasbi *et al.*,2003 and Head *et al.*, 2006). Several physical, chemical and thermal methods have commonly been employed to clean up the oil-contaminated sites (Frick *et al.*, 1999). However, these techniques are relatively expensive and also require site restoration (Lundstedt *et al.*, 2003).

Biodegradation of hydrocarbon-contaminated soils exploits the ability of microorganisms to degrade or detoxify organic contamination. It has been established as an efficient, economic, versatile and environmentally sound treatment (Bundy *et al.*, 2004).Microorganisms that biodegrade the components of petroleum hydrocarbons are isolated from various environments, particularly from petroleum-contaminated sites (Ghanavati *et al.*, 2010). Bacterial community composition can be correlated with ability to degrade target pollutants for the evaluations of indigenous microorganisms (Bestetti *et al.*, 2005).

Microorganisms have the capacity to degrade the majority of natural hydrocarbon components, especially the dominant saturated and unsaturated alkanes. The poly-aromatic hydrocarbons (PAHs), resins and asphaltenes are more recalcitrant to biodegradation and require a keen attention due to their

inherent mutagenic properties (Johnsen *et al.*, 2007). Hydrocarbon-degrading microbes must come into contact with their substrate for hydrocarbon uptake to occur and the insoluble nature of the majority of petroleum hydrocarbon molecules limits this contact (Hamme, 2003). The most widely recognized modes of hydrocarbon accession are direct microbial adherence to large oil droplets and interaction with pseudo solubilized (emulsified) oil. Hence, attempts to optimize or accelerate processes of hydrocarbons degradation include strategies for improving hydrocarbon accession by microorganisms.

Floodgate (1984) listed 25 genera of hydrocarbon-degrading bacteria and 27 genera of hydrocarbon-degrading fungi, which have been isolated from the marine environment; a similar compilation by Bossert and Bartha (1984) for soil isolates includes 22 genera of bacteria and 31 genera of fungi. Based on the reports the most common hydrocarbon degrading bacteria and fungi that are widely distributed in marine, freshwater, and soil habitats include *Pseudomonas, Marinobacter, Alcanivorax, Microbulbifer, Sphingomonas,Micrococcus, Cellulomonas, Dietzia,* and *Gordonia* groups *Aspergillus, Penicillium, Fusarium, Amorphoteca, Neosartorya, Paecilomyces, Talaromyces, Graphium* and the *yeasts Candida, Yarrowia* and *Pichia* have also been implicated in hydrocarbon degradation (Chaillan *et al.*, 2004; Brito *et al.*, 2006).). Reports of gram positive bacteria in hydrocarbon degradation are less common compared to gram negative bacteria. The underrepresentation of gram positive bacteria in hydrocarbon degradation may be due to the composition of cell envelope (Vermue *et al.*, 1999).

Microorganisms have adapted several mechanisms to survive and grow in the presence of toxic solvents. This includes modification of the membrane and cell surface properties, changes in the overall energy status or the activation of active transport systems for excluding solvents from membranes into the environment.

Since, the crude oil production in North east, India is a nage old routine process, therefore, the environmental contamination due to crude oil exploration is highly alarming. As a consequence, it is important to assess the native bacterial strains for bioremediation of crude oil contaminated site.

1.2 Objectives

Thus, this study was undertaken to achieve the following objectives:

- Isolation of petroleum hydrocarbon degrading bacteria from soil sample.
- Screening of the best petroleum hydrocarbon degrading bacterial strain
- Biochemical characterization and molecular identification of the particular bacterial strain.
- Determination of petroleum hydrocarbon degradation capability of the isolated strain
- Characterization of the gene responsible for petroleum hydrocarbon degradation.
- Optimization of lipase production from the isolated petroleum hydrocarbon degrading bacterial strain for biodiesel production
- Purification and characterization of lipase
- Application of the isolated petroleum degrading bacteria for biodiesel production.

1.3. Review of the literature

1.3.1 Structure and classification of petroleum

Petroleum is defined as any mixture of natural gas, condensate and crude oil. The crude oil in petroleum is a heterogeneous liquid consisting of hydrocarbons comprised almost entirely of hydrogen and carbon in the ratio of about 2 hydrogen atoms to 1 carbon atom (Ghanavati *et al.*, 2010). It also contains elements such as nitrogen, sulphur and oxygen, all of which constitute less than 3% (v/v). There are also trace constituents, comprising less than 1% (v/v), including phosphorus and heavy metals such as vanadium and nickel. The hydrocarbons in crude oil can be divided into four classes on the basis of the relative proportions of the heavy molecular weight constituents as light, medium or heavy (Okerentugba and Ezeronye, 2003):

- Saturated hydrocarbon
- Aromatics [benzene, toluene, ethylbenzene and xylenes; (BTEX) and polyaromatic hydrocarbons (PAHs)]
- Asphaltenes (phenols, fatty acids, ketones, esters, and porphyrins) and
- Resins (pyridines, quinolines, carbazoles, sulfoxides, and amides)

On a structural basis, the hydrocarbons in crude oil are classified as alkanes (*normal* or *iso*), cycloalkanes, and aromatics (Fig 1). Alkenes, which are the unsaturated analogs of alkanes, occur inmany refined petroleum products. Increasing carbon numbers of alkanes (homology), variations in carbon chain branching (*iso*-alkanes), ring condensations, and interclass combinations e.g., phenylalkanes, account for the high numbers of hydrocarbons that occur in petroleum.(Johnson *et al.*, 2011)

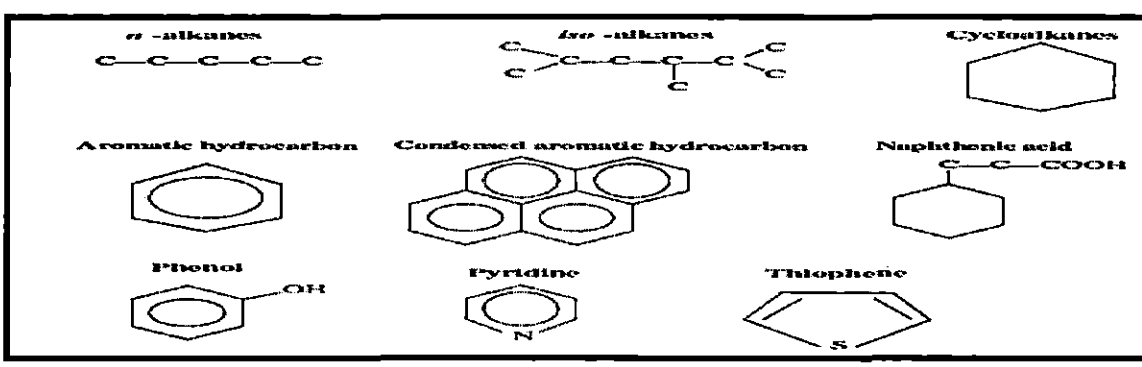


Fig 1. Structural classification of petroleum crude oil components. (Basha *et al*, 2010)

1.3.2 Recent scenario of petroleum spills and its hazardous effect

International scenario

In this current 2014, in different areas of USA alone almost 800 metric tones of oil was spilled due to bursting of pipelines. Some of the oil spill incidents that happened in last 5 years across the globe are listed in the table 1.

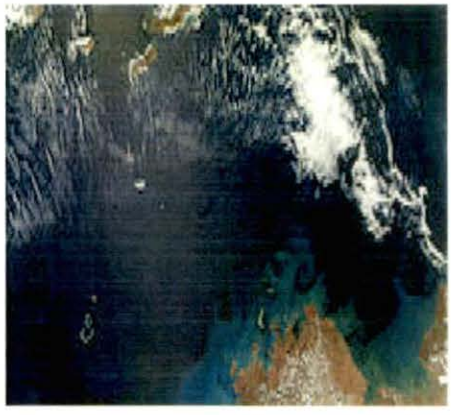
(a)  (b) 

Fig 2. Spread of oil slicks over the water surface (a), Satellite view of the wide spead oil slick on ocean due to oil spillage (b).

Table 1. World wide major oil spillage events in last 5 years

Spill / Vessel	Location	Dates	Tonnes
LA pipeline	California, USA	15 May 2014	160
MV *Miss Susan*/MV *Summer Wind*	Texas, USA	22 Mar 2014	546
North Dakota pipeline	North Dakota, USA	21 Mar 2014	110
North Dakota train collision	North Dakota,USA	30 Dec 2013	1,300
Rayong oil spill	Gulf of Thailand	27 Jul 2013	163
Lac-Mégantic derailment	Québec, Canada	6 Jul 2013	4,830
Sundre, Alberta	Canada, Sundre	8 Jun 2012	410
Guarapiche	Venezuela, Maturín,	4 Feb 2012	41,000
Nigeria	Nigeria, Bonga Field	21Dec2011	5,500
TK Bremen	France, Brittany,	16 Dec2011	220
Campos Basin	Brazil, Campos Basin,	7 Nov 2011	400
Rena oil spill	New Zealand	5 Oct 2011	350
North Sea oil	North Sea, UK	10 Aug2011	216
Yellowstone River	United States,	1 Jul 2011	140
Bohai Bay oil spill	China, Bohai Bay	4 June 2011	204
Little Buffalo oil spill	Canada, Alberta	29 Apr 2011	3,800
Fiume Santo power station	Italy, Sardinia,	11 Jan 2011	15

Barataria Bay oil spill	United States, Mexico	27 Jul 2010	45
Kalamazoo River oil spill	United States, Michigan	26 Jul 2010	3,250
Xingang Port oil spill	China, Yellow Sea	16 Jul 2010	90,000
Trans-Alaska Pipeline	United States, Alaska	25 May 2010	1,200
MT *Bunga Kelana 3*	Singapore,	25 May 2010	2,500
ExxonMobil	Nigeria,	1 May 2010	95,500
Deepwater Horizon	United States, Gulf of Mexico	Apr 2010	627,000
Great Barrier Reef / MV *Shen Neng 1*	Australia, Keppel Island	Apr 2010	4
Port Arthur oil spill	United States, Texas	Jan 2010	1,500
Yellow River oil spill	China, Chishui River	Jan 2010	130

Source: Wikipedia

National scenario

A number of incidents of oil spillage have been reported happening every year in the Indian shore line (Fig 2). Recently, on 21st January 2011, ONGC trunk oil pipeline burst at Uran, Mumbai. About40 to 45 metric tons of oil was spilled and spread around 4 sq km area. On 7thAugust, 2010 a foreign cargo ship, collided with another vessel about 10 km off Mumbai harbor, that spilled the oil in spite of the hectic effect made by Navy and Coast Guard to contain the leak, around 700 tons of fuel oil and 28 tons of diesel oil was spilled (*Times of India, 22nd Jan, 2011*).

Hazardous effect

These oil spill damage the waterways of birds and animals, interrupt breeding and fouling of breeding grounds. Besides, they thin bird and turtle egg shells and also damage the fish larvae, causing deformities. Soil ecosystem is disturbed and it effects the plant growth.

The primary effects of exposure to petroleum hydrocarbons are central nervous system, depression and polyneuropathy. The International Agency for Research on Cancer (IARC) has listed gasoline as possibly carcinogenic (2B) to humans (IARC 2000). Among the components of gasoline, N-heptane and cyclohexane are known to cause narcosis and irritation of the eyes and mucous membranes. Cyclohexane has been reported to cause liver and kidney changes in rabbits, and n-heptane has been reported to cause polyneuritis following prolonged exposure. The IARC, the National Toxicology program (NTP) and the Occupational Safety and Health Adminstration (OSHA) all classify benzene as a

human carcinogen. Other compounds of interest in petroleum are benzene toluene, ethyl benzene, and xylenes (BTEX) causes impairment of coordination and momentary loss of memory at exposures of 200–500ppm, and causes palpations at 500–1,500 ppm.

1.3.3 Remediation processes

1.3.3.1. Physico - chemical process

The various physical and chemical processes commonly used in the remediation of contaminated areas include recovery, dispersion, dilution, sorption, volatilization and abiotic transformations of hydrocarbon for its elimination. However, these types of treatment systems require heavy machinery and the environmental consequences of removing pollutants with these techniques may result in massive air pollution (Matsumiya and Kubo 2007). The rate of removal of petroleum hydrocarbon depend on the media in which the molecules are exposed including soil, water, presence or absence of light (Koch, 2011). Different petroleum sludge treatment technologies are categorized in table 2.

Photo-Oxidation:

Photo-oxidation is when sunlight in the presence of oxygen transforms hydrocarbons by photo oxygenation (increasing the oxygen content of a hydrocarbon) into new by-products. Oil can strongly absorb solar radiation and undergo transformation through photo degradation. In water and air, photo degradation of oil can occur rapidly when light is present; however in soil, photo degradation occurs to a very limited extent due to the low intensity of light. In water, this process can occur either directly by exposure to light at a wavelength less than 290 nm, or indirectly by exposure to oxidizing agents including OH radicals, O_3, and NO_3 (Douben 2003). Photo degradation plays an important role in enhancing the degradation of petroleum hydrocarbon by attacking the tertiary carbon atoms (a carbon atom bonded to three other carbon atoms with single bonds) in this. Atmospheric photochemical reactions transform hydrocarbons to less volatile and more polar derivatives, including nitrated, oxygenated and hydroxylated PAHs, which can lead to an increase in bioavailability and toxicity (Douben.2003).

Table 2. Evaluation of different petroleum sludge treatment technologies

Remediation	Technology	Comments
Bioremediation	Bioreactor	Application of natural and specialized microorganisms in controlled environmental and nutritional conditions, high biodegradation rates, accommodates variety of sludges, nonhazardous residues, on-site operation, cost-effective
	Landfarming	Uses natural microbial population and supplements of mineral nutrients, slow degradation rates, year-round operation difficult, potential to contaminate ground and surface water, cost-effective
	Biopiling	Uses natural microbial population and supplemented nutrients and air, slow degradation rates, year-round operation difficult, potential to contaminate ground and surface water
	Bioventing	A combination of advective soil venting and biodegradation method for in situ treatment of soils, most of the lighter hydrocarbons are volatilized
	Biostimulation/bioaugmentation	Application of mineral nutrients/surfactants and/or microorganisms to stimulate or supplement natural microbial population at contaminated site
	Phytoremediation	Uses plants and rhizospheric microorganisms for the treatment of contaminated soil, potential for removal of petroleum contaminants being evaluated, presumably cost-effective
Physicochemical	Incineration	High-temperature treatment, air pollution risks, expensive control equipment, high capital cost
	Thermal desorbtion	High-temperature oil removal and recovery method from oily solids, high capital and material preparation costs, nonhazardous residues
	Coker	Complicated sludge preparation for coker feed, some oil recovery, high capital and transportation costs
	Cement kiln	Complicated sludge preparation for use of fuel, high material preparation, transportation, and disposal costs
	Solvent extraction	Uses solvents and centrifugation or filtration for the separation of oil from sludges, safety hazard with solvent use, high capital cost

Chemical oxidation

Petroleum hydrocarbon can be chemically altered in soils by chemical oxidants including Fe and Mn metal ions, oxides and oxyhydroxides of Al, Mn, Si and Fe and by unsaturated fatty acids of plant residues (Koch, 2011).Common chemical oxidants that are used for biodegradation include ozone, hydrogen peroxide and permanganate. The addition of an oxidative agent such as hydrogen peroxide leads to the production of very strong non-selective oxidizing agents (hydroxyl radicals) that can react with hydrocarbons and induce transformation (Ferrarese *et al.*, 2008).

1.3.3.2. Biological process

Biological cleaning method involves natural processes in order to meet today's demands for cleaning and waste elimination without the use of potentially harmful chemicals. The biodegradation of these compounds is often a complex series of biochemical reactions and is often different when different microorganisms are involved. Biodegradation of organic wastes is a useful side effect of microbial metabolism, thus the fundamental principles of biodegradation are integrally linked to microbial physiology. Hydrocarbons differ in their

susceptibility to microbial attack. The susceptibility of hydrocarbons to microbial degradation can be generally ranked as follows: linear alkanes >small aromatics >branched alkanes >cyclic alkanes (Turki, 2009). Some compounds, such as the high molecular weight polycyclic aromatic hydrocarbons (PAHs) may not be degraded at all. The most rapid and complete degradation of the majority of organic pollutants is brought about under aerobic conditions. On the basis of mode of application bioremediation is classified in to two types namely In-situ bioremediation and ex-situ bioremediation.

In situ and ex situ methods

Bioremediation is broadly classified as ex situ and in situ methods. *Ex situ* methods are those treatments that involve the physical removal of the contaminated material for treatment process. In contrast, *in situ* techniques involve treatment of the contaminated material in place (Boopathy, 2000). Some of the examples of *in situ* and *ex situ* bioremediation are given below:

- Land farming: Solid-phase treatment system for contaminated soils: may be done in situ or ex situ.
- Composting: Aerobic, thermophilic treatment process in which contaminated material is mixed with a bulking agent; can be done using static piles or aerated piles.
- Bioreactors: Biodegradation in a container or reactor; may be used to treat liquids or slurries.
- Bioventing: Method of treating contaminated soils by drawing oxygen through the soil to stimulate microbial activity.
- Biofilters: Use of microbial stripping columns to treat air emissions.
- Bioaugmentation: Addition of bacterial cultures to a contaminated medium; frequently used in both in situ and ex situ systems.
- Biostimulation: Stimulation of indigenous microbial populations in soils or ground water by providing necessary nutrients.
- Intrinsic bioremediation: Unassisted bioremediation of contaminant; only regular monitoring is done.
- Pump and treat: Pumping ground water to the surface, treating, and reinjecting.

1.3.4.Factors affecting hydrocarbon degradation

A number of limiting factors have been recognized to affect the biodegradation of petroleum hydrocarbons. Typical examples of these factors are as listed in Table 3.

Table 3. Factors affecting petroleum hydrocarbon biodegradation.

Limiting factors	Explanation or examples
Petroleum hydrocarbon composition	Structure, amount, toxicity
Physical state	Aggregation, spreading, dispertion, adsorption
Weathering	Evaporation, photooxidation
Water potential	Osmotic forces
Temperature	Influence on evaporation and degradation rates
Oxidants	O_2 required to initiate oxidation,
Micronutrients	N, P, Fe may be limiting
Reactions	Low pH may be limiting
Microorganisms	PCB degraders may be low in number or absent

(Okoh, 2006)

Concentration of the Oil or Hydrocarbons:

The composition of the petroleum hydrocarbon pollutant is the first and most important consideration when the suitability of a remediation approach is to be evaluated. The heavier crude oils are generally much more difficult to biodegrade than lighter ones, just as heavier crude oils could be suitable for inducing increased selection pressure for the isolation of petroleum hydrocarbon degraders with enhanced efficiency. Also, Okoh *et al.* (2002) noted that the amount of heavy crude oil metabolized by some bacterial species increased with increasing concentration of starter oil up to0.6% (w/v), while degradation rates appeared to be more pronounced between the concentrations of 0.4 and 0.6%(w/v) oil. In another report (Rahman *et al.*, 2002), the percentage of degradation by the mixed bacterial consortium decreased from 78% to 52% as the concentration of crude oil was increased from 1 to 10%.

Chemical composition of the Oil

Biodegradability is inherently influenced by the composition of the oil pollutant. For example, kerosene, which consists almost exclusively of medium chain alkanes is, under suitable conditions, totally biodegradable. Similarly, crude oil is biodegradable quantitatively, but for heavy asphaltic-naphthenic crude oils, only about 11% may be biodegradable within are reasonable time period, even if the conditions are favorable (Okoh,2006). Maki *et al.*,(2005) reported that between 8.8 and 29% of the heavy crude oil *Maya* was biodegraded in soil microcosm by mixed bacterial consortium in 15 days, although major peak components of the oil was reduced by 65 - 70% . Also, about 89% of the same crude oil was biodegraded by axenic culture of *Burkholderia cepacia* RQ1 in shake flask within similar time frame, although petroleum biodegradation has been reported to be mostly enhanced in presence of a consortium of bacteria species compared to monospecies activities (Ghazali *et al.*, 2004).

Temperature

Temperature plays very important roles in biodegradation of petroleum hydrocarbons, firstly by its direct effect on the chemistry of the pollutants, and secondly its effect on the physiology and diversity of the microbial habitat (Venosa and Zhu, 2003). At low temperatures, the viscosity of the oil increases, while the volatility of toxic low-molecular weight hydrocarbons is reduced, delaying the onset of biodegradation (Yumoto *et al.*, 2002). Temperature also variously affects the solubility of hydrocarbons (Okoh, 2006). Hydrocarbon biodegradation can occur over a wide range of temperatures, the rate of biodegradation generally decreases with decreasing temperature. Hydrocarbon biodegradation can occur over a wide range of temperatures, the rate of biodegradation generally decreases with the decreasing temperature (Delille *et al.*, 2004). Figure 3 shows that highest degradation rates that generally occur in the range 30–40 °C in soil environments, 20–30°C in some fresh water environments and 15–20°C in marine environments (Pelletier *et al.*, 2004). Researchers have also reported that ambient temperature of the environment affect both the properties of spilled oil and the activity of the microorganisms. Significant

biodegradation of hydrocarbons have been reported in psychrophilic environments in temperate regions (Mrozik *et al.*,2010).

Oxygen

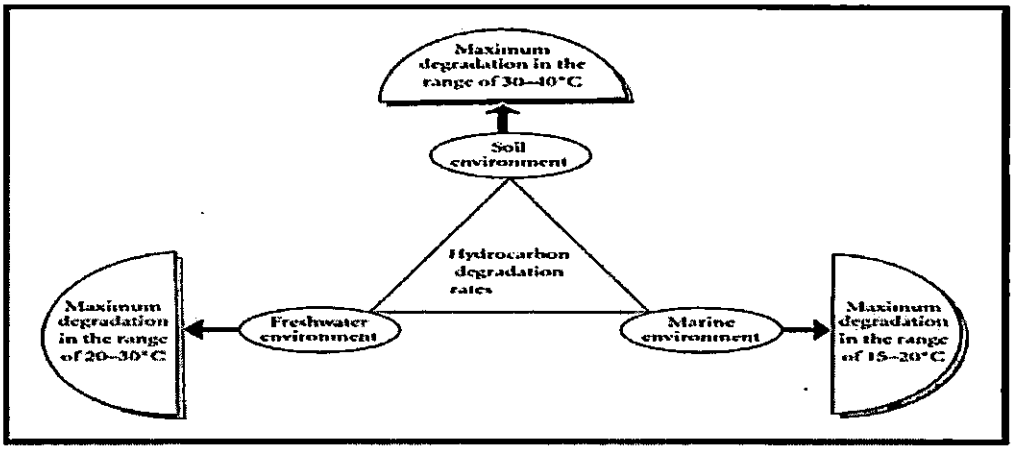


Fig 3. Hydrocarbon degradation rates in soil, fresh water, and marine environments. (Das and Chandran, 2011)

Microbial biodegradation of hydrocarbon requires an exogenous electron acceptor, with oxygen being the most common one. Aerobic conditions are therefore necessary for the microbial oxidation of hydrocarbons in the environment. The concentration of oxygen has been identified as the rate-limiting variable in the biodegradation of petroleum in soil. The availability of oxygen in soils is dependent upon rates of microbial oxygen consumption, the type of soil, whether the soil is waterlogged, and the presence of utilizable substrates which can lead to oxygen depletion (Bossert and Bartha, 1984).

Anaerobic degradation of petroleum hydrocarbons by microorganisms has been shown in some studies. In the absence of molecular oxygen, nitrate, iron, bicarbonate, nitrous oxide and sulfate have been shown to act as alternate electron acceptors during hydrocarbon degradation. The microbial degradation of oxidized aromatic compounds such as benzoate and of halogenated aromatic compounds such as the halo benzoates, chloro phenols and polychlorinated biphenyls has been shown to occur under anaerobic conditions (Chen *et al.,* 1988). Report indicates that microbial consortia from soil and sludge are capable of metabolizing unsubstituted and alkyl-substituted aromatics, including benzene,

toluene, xylene, 1, 3-dimethylbenzene, acenaphthene and naphthalene in the absence of molecular oxygen(Wilkes *et al.*, 2002).

Nutrients

Nutrients are very important ingredients for successful biodegradation of hydrocarbon pollutants especially nitrogen, phosphorus, and in some cases iron (Okoh, 2006). Some of these nutrients could become limiting factor thus affecting the biodegradation processes. Leo *et al.*(2011) reported that when a major oil spill occurred in marine and freshwater environments, the supply of carbon was significantly increased and the availability of nitrogen and phosphorus generally became the limiting factor for oil degradation. In marine environments, it was found to be more pronounced due to low levels of nitrogen and phosphorous in seawater. Freshwater wetlands are typically considered to be nutrient deficient due to heavy demands of nutrients by the plants. Therefore, additions of nutrients were necessary to enhance the biodegradation of oil pollutant. In nutrient limited waste waters, a BOD:N:P ratio of 100:5:1 is often used as a bench mark for nutrient addition.(Choi *et al.*, 2002; Kim *et al.*, 2005).On the other hand, excessive nutrient concentrations can also inhibit the biodegradation activity. Several authors have reported the negative effects of high NPK levels on the biodegradation of hydrocarbons especially on aromatics (Chaillan *et al.*, 2004).

Salinity

There is an inverse relationship between salinity and hydrocarbon solubility (Mailem *et al.*,2010). The substrate availability to microorganisms should be lower in saline than non saline environments. Some earlier reports indicated that even in the presence of high NaCl concentrations, actinomycetes (Al-Mueini *et al.*, 2007) and archaea (Tapilatu *et al.*,2010) could oxidize petroleum hydrocarbons. Other reports indicate that salinity did not affect microbial hydrocarbon biodegradation (Al-Mailem *et al.*,2010, 2013).

Pressure

Pressure is another important factor in the biodegradation of hydrocarbons. The degradation of tetradecane, hexadecaneand a mixed hydrocarbon substrate by a mixed culture of deep-sea sediment bacteria was monitored at 1 atm (ca.101 kPa) and 495 or 500 atm (ca. 50,140 or 50,650 kPa)

by Schwarz *et al.* (1974; 1975). Colwell and Walker (1976) have suggested that oil which reaches the deep ocean environment will be degraded very slowly by microbial populations and consequently, that certain recalcitrant fractions of the oil could persist for years or decades.

pH

Environmental parameters such as pH influence biodegradation of petroleum hydrocarbon. Extreme pH conditions are expected to have a negative influence on the ability of microbial populations to degrade the hydrocarbons. Microbial growth is higher in pH 6 and 7 but the biodegradation rate was higher at pH 7.0. Most heterotrophic bacteria and fungi favor a neutral pH (Venosa and Zhu, 2003). The pH may affect the solubility, bioavailability, and the chemical form of the pollutants and of macro-and micronutrients. Degradation of petroleum hydrocarbon increases with increasing pH, and that optimum degradation occur under slightly alkaline conditions (Guo *et al.*, 2005).

Production of biosurfactants by microorganisms

Biosurfactants are heterogeneous group of surface active chemical compounds produced by a wide variety of microorganisms (Ilori *et al.*, 2005, Kiran, 2009). It enhances solubilization and removal of contaminants due to increased bioavailability of pollutants. Biosurfactants actually can act as emulsifying agents by decreasing the surface tension and forming micelles. The microdroplets encapsulated in the hydrophobic microbial cell surface are taken inside and degraded. Table 4 summarizes the recent reports on biosurfactant production by different microorganisms. Bioremediation of oil sludge using biosurfactants has been reported.

Table 4. Biosurfactants produced by microorganisms

Biosurfactants	**Microorganisms**
Sophorolipids	*Candida bombicola*
Rhamnolipids	*Pseudomonas aeruginosa*
	Pseudomonas fluorescens
Lipomannan	*Candida tropicalis*
Surfactin	*Bacillus subtilis*
glycolipid	*Aeromonas sp.*
	Bacillus sp.

(Thavasi *et al.*, 2011)

by Cameotra and Singh (2008) in which a microbial consortium consisting of two isolates of *Pseudomonas aeruginosa* and one isolate *Rhodococcus erythropolis* was able to degrade 90% of hydrocarbons in 6 weeks in liquid culture. The ability of the consortium to degrade sludge hydrocarbons was tested in two separate field trials. The utilization of biosurfactants by *Pseudomonas* sp. for uptake of petroleum hydrocarbon is shown in Fig 4.

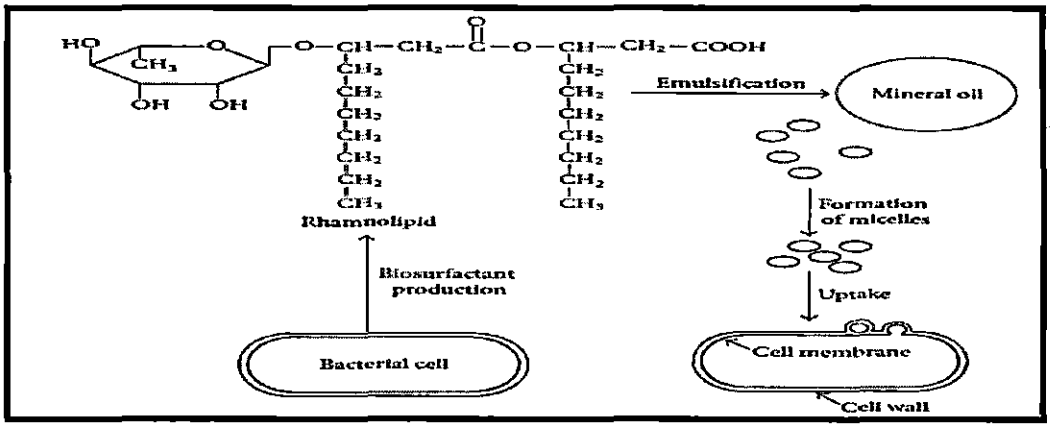


Fig 4. Involvement of biosurfactant (rhamnolipids) produced by *Pseudomonas* sp. in the uptake of hydrocarbons (Fritsche and Hofrichter, 2000).

The consortium degraded 91% of the hydrocarbon content of soil contaminated with 1% (v/v) crude oil sludge in 5 weeks. Separate use of any one additive along with the consortium brought about a 91–95% depletion of the hydrocarbon content in 4 weeks, with the crude biosurfactant preparation being a more effective enhancer of degradation. However, more than 98% hydrocarbon depletion was obtained when both additives were added together with the consortium.

1.3.5. Microorganisms involved in petroleum hydrocarbon degradation

Degradation of hydrocarbons by environmental micro flora involves microorganisms having specialized metabolic capacities. Bacterial strains isolated from petroleum contaminated waste water and soil can be good petrol and diesel degraders (Kashyap *et al.*, 2012).

These petroleum hydrocarbons degrading microorganisms share the following characteristics (Obire *et al.*, 2008):

- Efficient hydrocarbon uptake via special receptor sites for binding hydrocarbons and/or unique compounds that assist in the emulsification and transport of hydrocarbons into the cell.
- Enzymes that introduce molecular oxygen into the hydrocarbon and generate intermediates that subsequently enter common energy-yielding catabolic pathways.
- Inducer specificity: Exposure to petroleum and its constituents activate the two systems above.

Floodgate (1984) listed 25 genera of hydrocarbon-degrading bacteria and27 genera of hydrocarbon-degrading fungi which have been isolated from the marine environment; a similar compilation by Bossert and Bartha (1984) for soil isolates includes 22 genera of bacteria and 31 genera of fungi. Based on the reports the most common hydrocarbon degrading bacteria and fungi that are widely distributed in marine, freshwater, and soil habitats include *Pseudomonas, Marinobacter, Alcanivorax, Microbulbifer, Sphingomonas,Micrococcus, Cellulomonas, Dietzia,* and *Gordonia* groups (Brito *et al.*, 2006), *Aspergillus, Penicillium, Fusarium, Amorphoteca, Neosartorya, Paecilomyces, Talaromyces, Graphium* and the *yeasts Candida, Yarrowia* and *Pichia* have been implicated in hydrocarbon degradation (Chaillan *et al.*,2004). However, the actual numbers of hydrocarbon degraders are at variance with one another because of the methodological differences used to enumerate petroleum-degrading microorganisms. A list of microorganisms involved in degradation of petroleum hydrocarbon is summarized in Table 5.

Most of the microorganisms in the environment are alkene-degraders (Ting *et al.*, 2009), but it is the isolates that can degrade both aliphatic and aromatic hydrocarbons that are highly desirable, monocyclic hydrocarbons (Scragg, 2001), aromatic hydrocarbons (Ting *et al.*, 2009), resins and asphaltenes. One of the most extensively studied species of hydrocarbon degraders is the *Pseudomonas* species, known to degrade petroleum and a variety of polycyclic aromatic hydrocarbons crude oil (Bento *et al.*, 2003). Among the many *Pseudomonas* species, *P. aeruginosa* (Ijah and Antai, 2003), *P. alcaligenes* (Mahony *et al.*, 2006) and *P. putida* are more thoroughly investigated and their effectiveness as

biodegradative agents for hydrocarbon bioremediation is well-established. A number of studies have reported the ability of *Arthrobacter* sp. to degrade hydrocarbon. GC-MS analysis of metabolites produced during petroleum degradation by RKJ4 suggested that degradation occurs via the production of the conventional lower pathway intermediates *o*-phthalic acid and protocatechuic acid (Seo *et al.*, 2006). The ability of *Arthrobacter* sp. F101 isolated from sludge at an oil refinery wastewater treatment plant to utilize fluorene as a sole source of carbon and energy has also been reported (Koch, 2011).Several studies have reported on the roles of *Bacillus* sp. in hydrocarbon biodegradation. It was postulated that *Bacillus* sp. are more tolerant to high levels of hydrocarbons in soil due to the irresistant endospores (Ghazali *et al.*, 2004). The ability of *Bacillus* to accelerate the petroleum degradation significantly in various conditions indicates the potentials of this microorganism in clearing oil spills (Lin *et al.*, 2009).*Bacillus* has demonstrated the capacity of degrading petroleum effectively in contaminated sea water and sand with a significant higher degradation rate (Mandri and Lin, 2007). *Alkaligenes sp.* which are found within the vicinity of produce water discharge zone are capable of degrading produce water hydrocarbons and most especially the sparingly soluble components such as petroleum hydrocarbons that are very difficult to remove with the conventional mechanical treatment presently in use. Reports suggest that *Alkaligenes sp.* could degrade petroleum hydrocarbons from its original concentration of 1407 mg/l to 19.58mg/l (Amund *et al.*, 2010).This is a sure indication that the *Alkaligenes* sp. that dominates microbial activities at the discharge zone of produce water effluents can be beneficial to the environment because of its excellent hydrocarbon degradation potential. Different strains of *Acinetobacter* have adopted varying strategies to adhere to petroleum drops. In *Acinetobacter* the interaction between the petroleum droplets and the cell envelope are a complex process during which n-alkanes induces glycosylation of membrane proteins involved in oil uptake (Baldi *et al.*, 2003) and in biofilm formation due to cell-to-cell contact and synthesis of a composite material constituted by exopolysaccharides (EPSs) and n-alkanes. The cell-to-cell aggregation is parallel to an increase in cell envelope hydrophobicity and is then followed by internalization of petroleum droplets (Mara *et al.*, 2012). A completely different strategy is adopted by in-depth-studied *Acinobacter,* whose cells produce a strong

biosurfactant, the lipopolysaccharide emulsion that interfaces between cell membranes and oil (Mercaldi *et al.*, 2008). They solubilize hydrocarbons in vesicles composed of proteins, phospholipids and lipopolysaccharides (Leahy *et al.*,1990). Enzymatic degradation of hydrocarbons, is usually catalyzed by the alkene mono oxygenase complex formed by three different subunits: i) alkene hydroxylase (encoded by alkM); ii) rubredoxin; and iii) rubredoxin reductase. This complex has been characterized in detail in *P. putida*GPo1, where the alk genes are operonically organized into the octane utilization (OCT) plasmid (Van Beilen *et al.*, 2007). A different type of organization was found in some *Acinetobacter* strains, such as *Acinetobacter* baylyi ADP-1 (Hamme *et al.*, 2003) and *A. venetianus* VE-C3, where genes responsible for degradation of n-alkanes are located in the bacterial chromosome. However, analysis of Alk mutants suggested that genes involved in hydrocarbon uptake were also present on the two plasmids pAV1 (10,820 bp) and pAV2 (15,135 bp) (Decorosi *et al.*, 2006).The petroleum hydrocarbon degrading *Mycobacterium* grew in nutrient-supplemented artificial seawater with a heavy fuel oil as the sole carbon source, causing the complete removal of all linear (C_{12} to C_{40}) and branched alkanes from the aliphatic fraction, as well as an extensive degradation of the three- and four-ring polycyclic aromatic hydrocarbons (PAHs) (Vila *et al.*, 2010).*Rhodococcus* strain removed 50% of the aliphatic fraction of petroleum, completely depleting all linear alkanes (up to C_{20}) and pristane but showing limited action on higher-molecular-weight alkanes and phytane (Kim *et al.*, 2010).

Among the fungi *Aspergillus,* one of the commonest and the most abundant fungal specie found river and it helps to degrade petroleum hydrocarbons (Amund *et al.*, 2010). This ubiquitous fungal could degrade various fractions of petroleum hydrocarbons especially the recalcitrant PAHs and it help to reduce the problem of bioaccumulation of these organic compounds in the marine animals, and also the resultant risks of potential health hazards associated with the consumption of the contaminated sea foods. *Penicillium* species were the most efficient metabolizers of hydrocarbons (Obire *et al.*, 2008).This strain has the ability to produce extracellular enzymes and degrade petroleum hydrocarbons (Leitao, 2009).

Penicillium is able to grow at high concentrations of salt as well as in its absence, while also processing high resistance to hydrocarbon degradation efficiency, could be used as agents for abatement of these pollutants in hypersaline conditions, as well as in non-saline environment. *Fusarium* sp. F092 produced extra and intra-cellular enzymes, especially in liquid medium which helps in the rapid degradation of hydrocarbons (Tachibana, 2011). *Trichoderma* preferably degrades longer chain hydrocarbons (20-40°C) after 9 days of incubation with optimal physical and nutrient parameters.

Table 5.Selected microorganisms used in biodegradation of petroleum hydrocarbon. (Mrozik and Seget, 2010).

Microorganisms	Contaminants treated	References
Single strain		
Comamonas testosteroni BR60	Crude oil, PAHs	Gentry et al. (2001)
Arthrobacter chlorophenolicus A6L	4-Chlorophenol	Jernberg and Jansson (2002)
Absidia cylindrospora	Fluorene	Garon et al. (2004)
Pseudomonas sp. ST41	Marine gas oil	Stallwood et al. (2005)
Pseudomonas aeruginosa WatG	Diesel oil	Ueno et al. (2006)
Sphingobium chlorophenolicum ATCC 39723	Pentachlorophenol	Dams et al. (2007)
Burkholderia sp. FDS-1	Fenitrothion	Hong et al. (2007)
Aspergillus sp. LEBM2	Phenol	dos Santos et al. (2008)
Aspergillus sp. LEBM1 and LEBM3	Chlorobenzene	dos Santos et al. (2008)
Gordonia sp. BS29	Aliphatic and aromatic hydrocarbons	Franzetti et al. (2009)
Pseudomonas putida ZWL73	4-Chloronitrobenzene	Niu et al. (2009)
Aspergillus sp.	LMW-PAHs (2–3 rings)	Silva et al. (2009a)
Trichocladium canadense		
Fusarium oxysporum		
Trichocladium canadense	HMW-PAHs (4–7 rings)	Silva et al. (2009a)
Aspergillus sp.		
Verticillium sp.		
Achremonium sp.		
Consortia		
Rhodococcus sp., *Acinetobacter* sp., *Pseudomonas* sp.	PAHs (fluorene, phenanthrene, pyrene)	Yu et al. (2005)
Bacillus subtilis DM-04, *Pseudomonas aeruginosa* M and NM	Crude petroleum-oil hydrocarbons	Das and Mukherjee (2007)
Mycobacterium fortuitum, *Bacillus cereus*, *Microbacterium* sp., *Gordonia polyisoprenivorans*, *Microbacteriaceae* bacterium, *Fusarium oxysporum*	PAHs (anthracene, phenanthrene, pyrene)	Jacques et al. (2008)
Rhizopus sp., *Penicillium funiculosum*, *Aspergillus sydowii*	Petroleum hydrocarbons	Mancera-López et al. (2008)
Bacillus strains B1F, B5A and B3G, *Chromobacterium* sp. 4015, *Enterobacter agglomerans* sp. B1A	Mixture of PAHs (naphthalene, phenanthrene, anthracene, pyrene, dibenzo[a]anthracene, benzo[a]pyrene)	Silva et al. (2009b)
Achremonium sp., *Aspergillus* sp., *Verticillium* sp.		

Trichoderma is able to grow in a relatively wide range of pH from 5.0 to 7.0, suggesting that this organism could degrade petroleum hydrocarbons under not only acidic but also neutral conditions (Hamzah*et al.*, 2012).*Candida* from soil samples contaminated with acidic oily sludge (pH 1-3) could degrade 73% of the total petroleum hydrocarbons present in the medium at pH 3 in a week. This type of yeast could efficiently degrade the aliphatic and aromatic fractions of the acidic oily sludge at pH 3 and this was confirmed by gas chromatography (Abraham *et al.*, 2011).

1.3.6. Metabolism of petroleum hydrocarbon

Aerobic Alkane Metabolism

Microorganisms are equipped with metabolic machinery to use petroleum as a carbon and energy source. The most rapid and complete degradation of the majority of organic pollutants is brought about under aerobic conditions

The degradation of petroleum hydrocarbons can be mediated by specific enzyme system. Figure 5 shows the initial attack on xenobiotics by oxygenases .Other mechanisms involved are (1) attachment of microbial cells to the substrates and (2) production of biosurfactants (Satpute *et al.*, 2010). The uptake mechanism linked to the attachment of cell to oil droplet is still unknown but production of biosurfactants has been well studied.

The mechanism of *n*-alkane metabolism and the genes involved in the metabolism has been reported by many researchers. From a regulatory genetic standpoint, the most extensively characterized alkane degradation pathway is encoded by the OCT plasmid carried by *Pseudomonas putida* Gpo1 (formerly *Pseudomonas oleovorans*) (Witholt *et al.*, 2001). The membrane-bound monooxygenase and soluble rubredoxin and rubredoxin reductase serve to shunt electrons through NADH to the hydroxylase for conversion of an alkane into an alcohol. The alcohol can be further oxidized to an aldehyde and acid prior to proceeding into the β-oxidation and tricarboxylic acid cycles. Beilen *et al.*, (2001) studied the OCTplasmid, while Canosa *et al.*, (2000) and Panake *et al.*, (1999) examined expression of the AlkS regulator, and Yuste *et al.* (2001) studied the catabolite repression system.

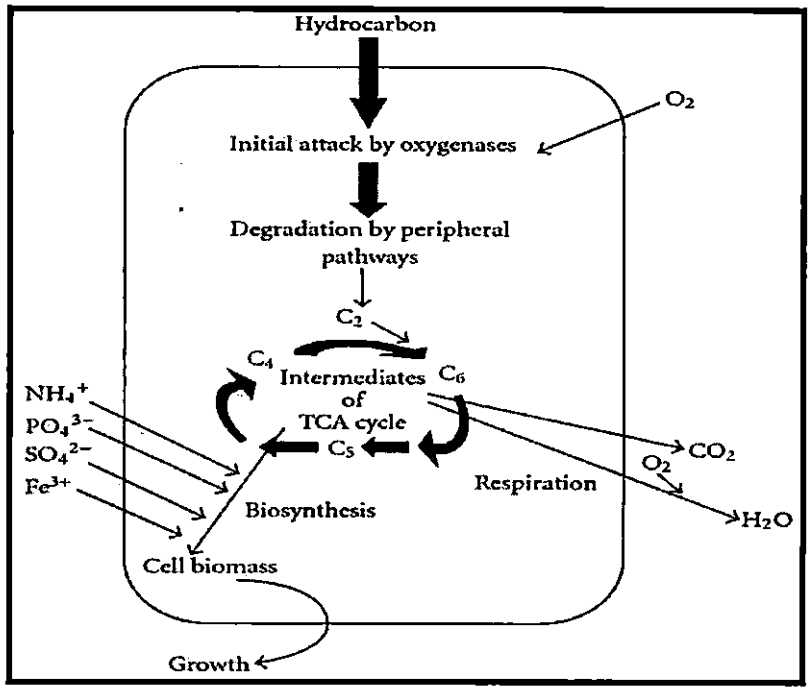


Fig 5 Principle of aerobic degradation of hydrocarbons by microorganisms.(Das and Chandran, 2011)

A model for alkane metabolism, including the locations of the Alk proteins and regulation of the *alk*genes, is shown in Fig. 6. Here, the *alkBFGHJKL* operon encodes the enzymes necessary for converting alkane into acetyl-coenzyme A (CoA), while *alkST* encode a rubredoxin reductase (AlkT) and the positive regulator for the *alkBFGHJKL* operon (AlkS). These two operons are located end to end, separated by 9.7 kb of DNA, within which lies *alkN*, a gene coding for a methyl-accepting transducer protein that may be involved in alkane chemotaxis. The function of *alkL* remains unknown, although it is suspected to be involved in transport. Comparative analysis of insertion sequences in*P. putida* P1 and the previous observation that the G+C content of the *alk* genes is lower than that of both the host strain and the OCT plasmid suggest that the genes are part of an integrated mobile element. Two other plasmid systems have been partially characterized: the OCT plasmid in *Pseudomonas maltophilia* has an *alkA* gene distinct from that of *P. putida*, and the unique pDEC plasmid in *Pseudomonas* sp. strain C12B (Ward *et al.*, 2003).The clustering and regulation of alkane degradation genes varies among the bacteria. *Burkholderia cepacia* has an *alkB* gene that is not linked to other alkane

degradation genes as it is in *P. putida*. The *PalkB* promoter in this organism is down regulated by catabolite repression more strongly than in *P. putida* GPO1 (Rojo *et al.*, 2001). Other differences include the repression of alkane degradation by citrate and the maintenance of repression during stationary phase in *B. cepacia*, two phenomena not observed in *P. putida* GPO1. In *Acinetobacter* sp. strain ADP1, *alkM*, the terminal alkane hydroxylase-encoding gene, is regulated by *alkR*, which shows no similarity to the LuxR-UhpA-like *alkS* regulator in *P. putida*. In addition, the genes in *Acinetobacter* sp.strain ADP1 are not found in a large operon or on a plasmid. Indeed, the genes are 396 kb from *rubA* and *rubB*, which encode rubredoxin and rubredoxin reductase (Ward *et al.*, 2003).

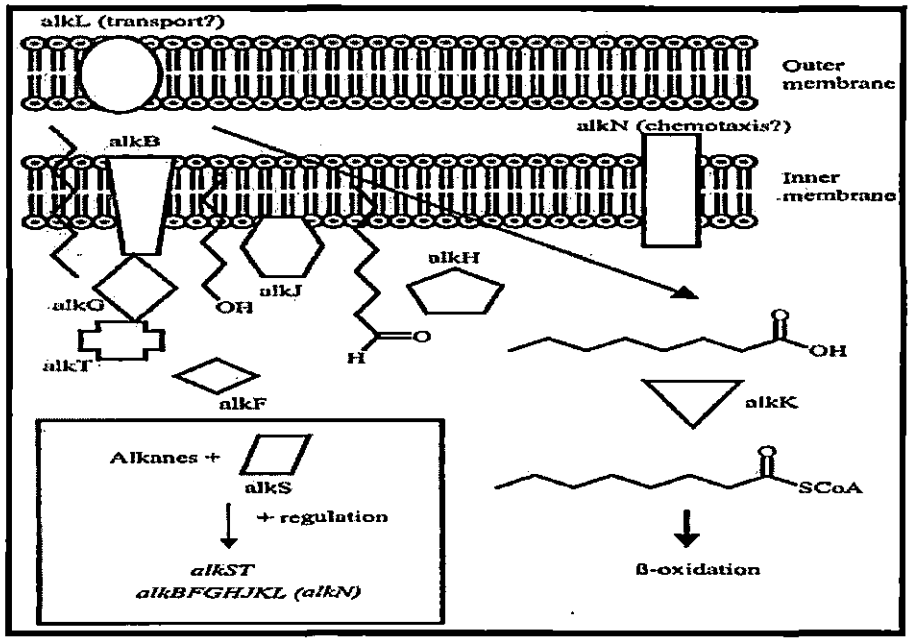


Fig 6. Schematic representation of alkane degradation in gram-negative bacteria, showing the locations and functions of the *alk* gene products(Witholt *et al.*, 2001).The products include AlkB (alkane hydroxylase), AlkF and AlkG (rubredoxins), AlkH (aldehyde dehydrogenase), AlkJ (alcohol dehydrogenase), AlkK (acyl-CoA synthetase), AlkL (outer membrane protein that may be involved in uptake), AlkN (a methyl-accepting transducer protein that may be involved in chemotaxis), AlkT (rubredoxin reductase), and AlkS (positive regulator of the *alkBFGHIJKL* operon and *alkST* genes).

Little information is available for pathways other than the aerobic monooxygenase-mediated pathway for alkane degradation. Evidence where a dioxygenase converts alkanes to aldehydes through *n*-alkyl hydroperoxides without an alcohol intermediate has been described for *Acinetobacter* sp.strain M1 (Koma et *al.*, 2003). The dioxygenase requires molecular oxygen to catalyze

the oxidation of *n*-alkanes (C10 toC30) and alkenes (C12 to C20) without the production of oxygenradicals. A flavin adenine dinucleotide chromophore was detected and the enzyme is thought to contain $Cu2^{+}$. Unlike the case for the 1-monooxygenase in *P. putida*, rubredoxin and NAD(P)H are not required.

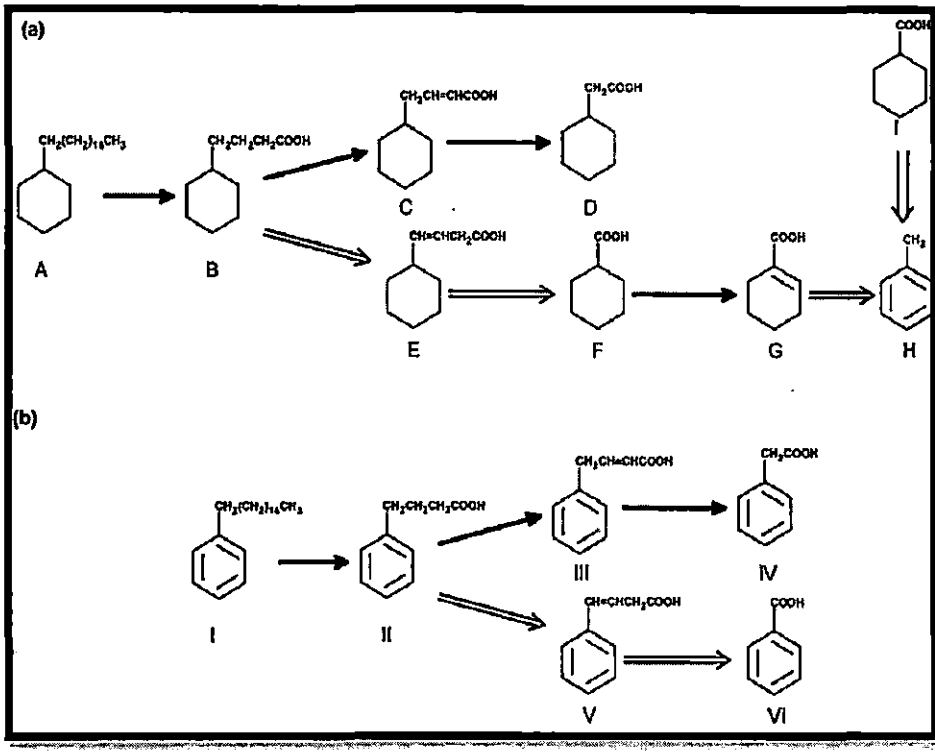


Fig 7. Metabolic pathway illustrating biodegradation of an *n*-alkylcyclohexane (a) and an *n*-alkylbenzene (b) by an *Alcanivorax* sp.strain MBIC4326 (Dutta and Harayama (2001).The major metabolic route of β oxidation is shown with bold arrows, while minor routes are indicated with open arrows and a novel metabolic route by large open arrows. Pathway a: A, *n*-octadecylcyclohexane; B, 4-cyclohexabutanoic acid; C, 4-cyclohexyl-2-butanoic acid; D, cyclohexane acetic acid; E, 4-cyclohexyl-2-butenoic acid; F, cyclohexane carboxylic acid; G, 1-cyclohexene-1- carboxylic acid; H, benzoic acid; I, 3-cyclohexene-1-carboxylic acid. Pathway b: I, *n*-hexadecylbenzene; II, 4-phenylbutanoic acid; III, 4-phenylbutenoic acid; IV, phenylacetic acid; V, 4-phenylbutenoic acid; VI, benzoic acid.

Another novel metabolic pathway has been observed in a *Rhodococcus* mutant. In this case, aliphatics are *cis* desaturated producing products with double bonds mainly atthe ninth carbon from the terminal methyl group. It is postulatedthat a coenzyme A-independent *cis*-desaturase may be involved in this activity. Dutta and Harayama (2001) noted that the degradation of the long side chains of *n*-alkylbenzenesand *n*-alkylcyclohexanes by *Alcanivorax* sp. strain MBIC 4326 proceeds mainly by β oxidation (Fig. 7). However, minor products suggest the possibility of other degradativeroutes.

Aerobic PAH Metabolism

A wide array of microbial species (bacteria, fungi, algae and cyanobacteria) have a diversity of tools to use both low(three rings or fewer) and high (four or more rings) molecular weight PAHs such as naphthalene, acenaphthene, anthracene, fluoranthene, pyrene, and chrysene as sole carbon and energy sources. While no strains have yet been found to utilize PAHs with more than four rings, such as benzo[*a*]pyrene as a sole carbon and energy source, co metabolic transformations have been characterized (Juhasz and Naidu, 2000).The low water solubility and high sorbtion capacity of PAHs are often found to greatly influence biodegradation, but other factors including production of toxic or dead-end metabolites, metabolite repression, the presence of preferred substrates and the lack of cometabolic or inducer substrates must be considered when PAH is persistent (Juhasz *et al.,* 2002). The majority of information on PAH metabolism has come from studying naphthalene catabolic plasmids such as NAH7 from *Pseudomonas putida* strain G7. The first operon (*nahAaAbAcAdBFCED*) encodes the pathway for naphthalene conversion to salicylate (upper pathway), and the second (*nahGTHINLOMKJ*) codes for the conversion of salicylate via catechol*meta*-cleavage to acetaldehyde and pyruvate (lower pathway)(Simmon *et al.,* 1992). The regulator for both operons is encoded by a third operon containing *nahR*, which is induced by salicylate (Schell and Wender, 1986). Molecular and biochemical evidence that the naphthalene plasmid degradative enzymesystem could mineralize other PAHs, such as phenanthrene and anthracene, was first provided by Menn *et al.,* (1993). A variety of new iso functional gene sequences have been also reported in different bacterial species, most notably in *Nocardia*, *Rhodococcus*, and *Mycobacterium* sp., some of which are capable of using high-molecular-weight PAHs such as pyrene as carbon and energy sources. In addition, with respect to PAH metabolism, novel gene sequences and gene orders have been observed in a variety of strains, including *Burkholderia* sp. strain RP007, *phnFECDAcAdB* (Laurie and Lloyd-Jones,1999); *Pseudomonas* sp. strain U2, *nagAaGHAbAcAdBF*(Fuenmayor *et al.,* 1998); *Rhodococcus* sp. strain I24, *nidABCD* (Treadway *et al.,* 1999); *Mycobacterium*sp. PYR1, *nidDBA* (Khan *et al.,* 2001); and *Nocardiodes* sp. strain KP7, *phdABCD* (Sato *et al.,* 1997). Sequence diversity, and the fact that naphthalene catabolic genes have now been found on the chromosome as well as on plasmids indicate that lateral gene

transfer and genetic recombination may have played an important role in the development of these versatile metabolic pathways. A list of microorganisms involved in aerobic degradation of petroleum hydrocarbon is summarized in table 6.

High levels (90%) of homology and a conserved genearrangement are observed in the *nah*, *ndo*, *pah*, and *dox* sequences (Kroutil and Faber, 2000). The first example of two isofunctionalsalicylate hydroxylases in one strain is exhibited by *Sphingomonasyanoikuyae* B1, which has modified andreorganized genes to obtain catabolic pathways for naphthalene,phenanthrene, anthracene, biphenyl, toluene, and *m*- and*p*-xylene. In this case, *nah*, *bph*, and *xyl* genes are present butare not arranged in three distinct operons (Kim and Zylstra, 1999). This gene clustering may be typical of *Sphingomonas* sp. capable of degrading aromatic compounds. Romine et al. (1999) sequenced the pNL1 (184 kb) plasmid of *Sphingomonasaromaticivorans* F199, which is capable of degradingtoluene, xylenes, salicylate, biphenyl, dibenzothiophene,flourene, and benzoate. In this plasmid, at least 13 gene clustersare predicted to encode all of the necessary enzymes.

Table6. Microorganisms involved in aerobic degradation of petroleum hydrocarbon.

Strain	Location	Substrate	Gene	Encoded protein or function
Pseudomonas putida strains	Plasmid	Naphthalene (upper pathway)	*nahAa*	Reductase
			nahAb	Ferredoxin
			nahAc	Iron sulfur protein large subunit
			nahAd	Iron sulfur protein small subunit
			nahB	*cis*-Naphthalene dihydrodiol dehydrogenase
			nahF	Salicyaldehyde dehydrogenase
			nahC	1,2-Dihydroxynaphthalene oxygenase
			nahE	2-Hydroxybenzalpyruvate aldolase
			nahD	2-Hydroxychromene-2-carboxylate isomerase
		Salicylate (lower pathway)	*nahG*	Salicylate hydroxylase
			nahT	Chloroplast-type ferredoxin
			nahH	Catechol oxygenase
			nahI	2-Hydroxymuconic semialdehyde dehydrogenase
			nahN	2-Hydroxymuconic semialdehyde dehydrogenase
			nahL	2-Oxo-4-pentenoate hydratase
			nahO	4-Hydroxy-2-oxovalerate aldolase
			nahM	Acetaldehyde dehydrogenase
			nahK	4-Oxalocrotonate decarboxylase
			nahJ	2-Hydroxymuconate tautomerase
		Regulator for both operons	*nahR*	Induced by salicylate
Pseudomonas putida NCIB9816	Plasmid	Naphthalene	*ndoA*	Naphthalene-dioxygenase genes (these 3 genes correspond to NahAb,-c, and-d listed above)
			ndoB	
			ndoC	
Pseudomonas sp. strain C18	Plasmid	Dibenzothiophene	*dszA*	Naphthalene dioxygenase
		Naphthalene phenanthrene	*dszB*	DoxA, -B, -D correspond to NahAb, -c, and-d listed above
			dszD	
			dszE	*cis*-Naphthalene dihydrodiol dioxygenase
			dszF	Salicylaldehyde dehydrogenase
			dszG	1,2-Dihydroxynaphthalene dioxygenase
			dszH	Isomerase (interchangeable with *dszI*?)
			dszI	Hydratase-aldolase
			dszJ	Isomerase
Pseudomonas sp. strain U2	Plasmid	Naphthalene	*nagAa*	Ferredoxin reductase
			nagG	Subunit of salicylate 5-hydroxylase with Rieske-type iron-sulfur centre
			nagH	Subunit of salicylate 5-hydroxylase
			nagAb	Ferredoxin
			nagAc	Large dioxygenase subunit
			nagAd	Small dioxygenase subunit
			nagB	Naphthalene *cis*-dihydrodiol dehydrogenase
			nagF	Salicylaldehyde dehydrogenase
Burkholderia sp. strain RP007	Plasmid	Naphthalene phenanthrene	*phnR*	Regulatory
			phnS	Regulatory
			phnF	Aldehyde dehydrogenase
			phnE	Hydratase-aldolase
			phnC	Extradiol dioxygenase
			phnD	Isomerase

			phnAc	Large dioxygenase subunit (Rieske-type [2Fe-2S]
			phnAd	Small dioxygenase subunit
			phnB	Dihydrodiol dehydrogenase
Pseudomonas putida OUS82	Chromosome	Naphthalene Phenanthrene A variety of homo-hetero-, and monocyclics converted to phenols	*pahAa*	Ferredoxin reductase
			pahAb	Ferridoxin
			pahAc	Large subunit of iron-sulfur protein
			pahAd	Small subunit of iron-sulfur protein
			pahB	*cis*-Dihydrodiol dehydrogenase
			pahC	Dioxygenase
			pahD	Isomerase
			pahE	Hydratase-aldolase
			pahF	Dehydrogenase
Pseudomonas stutzeri AN10	Chromosome	Naphthalene 2-Methylnaphthalene	*nahG*	Salicylate 1-hydroxylase
			nahH'	Salicylate 1-hydroxylase (outside *meta*-cleavage transcriptional unit)
Nocardioides sp. strain KP7	Chromosome	Phenanthrene	*phdA*	Alpha subunit of dioxygenase
			phdB	Beta subunit of dioxygenase
			phdC	Ferredoxin
			phdD	Ferredoxin reductase
			phdK	2-Carboxybenzaldehyde dehydrogenase
Rhodococcus sp. strain 124	Chromosome	Naphthalene Toluene Indene	*nidA*	Naphthalene-inducible dioxygenase system
			nidB	Dioxygenase small subunit
			nidC	*cis*-Dihydrodiol dehydrogenase
			nidD	Putative aldolase
Mycobacterium sp. strain PYR-1	Chromosome	Anthracene, Phenanthrene	*nidD*	Aldehyde dehydrogenase
		Fluoranthene	*nidB*	Small subunit of dioxygenase
		Pyrene, benzo [a]pyrene, 1-nitropyrene	*nidA*	Large subunit of dioxygenase
Sphingomonas paucimobilis var. EPA505		Phenanthrene	*phtA*	Ring fission dioxygenase
		Anthracene, benzo[b]fluoranthene	*phtB*	Rieske-type ferridoxin subunit of multicomponent dioxygenase
		Naphthalene	*phtC*	Hydratase-aldolase
		Fluroanthene, pyrene	*phtD*	Pyruvate phosphate dikinase
		Intermediate catabolites		

Anaerobic Hydrocarbon Metabolism

Anaerobic metabolism is a vital process with respect to petroleum hydrocarbon biodegradation. A list of microorganisms involved in anaerobic degradation of petroleum hydrocarbon is summarized in table 7. Microbial consortia has been reported to metabolize hydrocarbons such as toluene (Elshahed *et al.,* 2001), alkylbenzenes including *m*-, *o*-, and *p*-xylene and trimethylbenzenes (Phelps and Young, 2001), benzene, naphthalene and phenanthrene (Meckenstock *et al.,* 2000), methylnaphthalene and tetral in (Annweiler *et al.,* 2002), C6 *n*-alkanes (Ehrenrich *et al.,* 2001), branched alkanes (Canosa *et al.,* 2000), and hydrocarbon mixtures under anaerobic conditions. These reactions take place under Fe(III)-reducing, denitrifying, and sulfate-reducing conditions by anoxygenic photosynthetic bacteria or in syntrophic consortia of proton-reducing and methanogenic bacteria. Other terminal electron acceptors used during anaerobic hydrocarbon metabolism include manganese oxides (Langenhoff *et al.,* 1999), soil humic acids and the humic acid model compound anthraquinone-2,6-disulfonate (Cervantes *et al.,* 2001), and fumarate in a fermentative oxidation process (Meckenstock *et al.,* 2001). The diverse set of bacteria including members of α, β, γ and δ subclasses of proteobacteria form an excellent framework to understand the underlying biochemical and molecular mechanisms involved in anaerobic hydrocarbon metabolism. Toluene has been the most studied hydrocarbon with respect to enzymatic and genetic characterizations in the denitrifying bacteria *Azoarcus* sp. strain T, *Thauera aromatica* strain K172,and*Thauera*sp. strain T1 (Achong *et al.,* 2001). In the proposed pathway, fumarate addition totoluene is mediated by benzyl succinate synthase to form benzyl succinate. This unusual addition reaction results in a series of modified β oxidation reactions to convert benzylsuccinate to benzyl-CoA (Beller *et al.,* 2002), which is a central intermediate in the anaerobic degradation of aromatic compounds (Heider *et al.,* 2007). The *bbs* (beta-oxidation of benzylsuccinate) operon contains *bbsDCABE,* with *bbsCAB* encoding the γ, α, and β subunits of benzyl succinate synthase.

Table 7. Microorganisms involved in anaerobic degradation of petroleum hydrocarbon.

Organism	Hydrocarbon(s) used
Anoxygenic photoheterotrophic bacterium	
Blastochloris sulfoviridis ToP1	Toluene
Denitrifying bacteria	
Azoarcus sp. strain EB1	Ethylbenzene
Azoarcus sp. strain T	Toluene, *m*-xylene
Azoarcus tolulyticus Td15	Toluene, *m*-xylene
Azoarcus tolulyticus To14	Toluene
Dechloromonas sp. strain JJ	Benzene, toluene
Dechloromonas sp. strain RCB	Benzene, toluene
Pseudomonas sp. strain NAP-3	Naphthalene
Strain EbN1	Ethylbenzene, toluene
Strain HdN1	C_{13}–C_{20} alkanes
Strain HxN1	C_6–C_8 alkanes
Strain M3	Toluene, *m*-xylene
Strain mXyN1	Toluene, *m*-xylene
Strain OcN1	C_6–C_{12} alkanes
Strain PbN1	Ethylbenzene, propylbenzene
Strain pCyN1	*p*-Cymene, toluene, *p*-ethyltoluene
Strain pCyN2	*p*-Cymene
Strain T3	Toluene
Strain ToN1	Toluene
Thauera aromatica K172	Toluene
Thauera aromatica T1	Toluene
Vibrio sp. strain NAP-4	Naphthalene
Fe(III)-reducing bacteria	
Geobacter grbiciae TACP-2^T	Toluene
Geobacter grbiciae TACP-5	Toluene
Geobacter metallireducens GS15	Toluene
Sulfate-reducing bacteria	
Desulfobacula toluolica To12	Toluene
Desulfobacterium cetonicum	Toluene
Strain AK-01	C_{13}–C_{18} alkanes
Strain Hxd3	C_{12}–C_{20} alkanes, 1-hexadecene
Strain mXyS1	Toluene, *m*-xylene, *m*-ethyltoluene, *m*-cymene
Strain NaphS2	Naphthalene
Strain oXyS1	Toluene *o*-xylene, *o*-ethyltoluene
Strain Pnd3	C_{15}–C_{17} alkanes, 1-hexadecene
Strain PRTOL1	Toluene
Strain TD3	C_6–C_{16} alkanes

1.3.7. **Microbial physiological response to petroleum hydrocarbon**

Microbial physiological responses such as changes in membrane architecture, active uptake and efflux to hydrocarbons have a high degree of impact on bioremediation of petroleum.

The lipophilic molecules such as hydrocarbon partition between the monolayer of the cytoplasmic membrane and the outer membrane (Zahir *et al.*, 2006). Hydrocarbons tend to reside in the hydrophobic area between membrane mono-layers in the acyl chains of phospolipids, with partitioning being related to the octanol-water partition coefficient of the lipophilic compound. Hydrocarbon insertion alters membrane structure by changing fluidity and protein conformations and results in disruption of the barrier and energy transduction functions while affecting membrane-bound and embedded enzyme activity (Heipieper *et al.*, 2007).

In terms of general stress responses, bacteria may form biofilms, alter their cell surface hydrophobicity to regulate their partitioning with respect to

hydrocarbon-water interfaces. In addition, energy-dependent repair mechanisms may be used to compensate for losses in membrane integrity resulting from the partitioning of lipophilic compounds. For example, membrane fluidity can be decreased through increased membrane ordering by affecting *cis/trans* phospolipid isomerizations, by decreasing unsaturated fatty acid content, and by altering phospholipid head groups (Pepi *et al.,* 2009). These changes may be associated with an overall increase in phospholipid content and increased phospholipid biosynthesis in solvent-stressed cells.

These alterations serve to produce a physical barrier to the intercalation of hydrocarbons in membranes, thus offsetting the passive influx of hydrocarbons into the cell. It is generally believed that hydrocarbons interact with microorganisms nonspecifically and move passively into the cells. When microorganisms contact water-solubilized hydrocarbons, decreasing solubility with increasing molecular weight is restrictive. Two additional modes of hydrocarbon accession are direct adherence to large oil droplets and interaction with pseudo solubilized oil (Ward*et al.,* 2003). Hamme and Ward (2001) described a *Rhodococcus* strain that grew directly on crude oil droplets and could be removed with the addition of exogenous chemical surfactant, while a *Pseudomonas* strain required surfactant-solubilized oil to efficiently access hydrocarbons.

Phenanthrene uptake by *Pseudomonas fluorescens* LP6a was reported to be passive via energy dependent phenanthrene efflux (Bugg *et al.*, 2000). With respect to active transport, proton motive force uncouplers have been shown to apparently decrease both *n*-hexadecane (Beal *et al.,* 2000) and naphthalene uptake, which indicate that energy-dependent uptake is important in some strains.

Ramos *et al.* (1995) isolated *P. putida*DOT-T1E, which metabolized toluene and was capable of growing in the presence of 90% (v/v) toluene. DOT-T1E was found to increase membrane rigidity by converting cis-9,10-methylenehexadecanoic acid to 9-cis-hexadecanoic acid and subsequentlyto the corresponding transisomer in less than 1 min upon exposure to toluene.

In the long-term (15 to 20 min) exposure, DOT-T1E decreased the amount of phospatidylethanolamine in the phospolipid polar head groups and increased cardiolipid levels, again increasing membrane rigidity. These changes increase

lipid ordering to restore membrane integrity and reduce organic solvent partitioning in the membrane.

Kabelitz *et al.* (2009) discussed that the adaptive response towards alcohols is related to the physico-chemical properties of short-chain alcohols, which can only penetrate slightly into the hydrophobic center of the phospholipid bilayer, causing a swelling effect on the hydrophilic head groups. To counteract this effect, the insertion of unsaturated fatty acids seems to be a better reaction against those short-chain alcohols. In contrast, long-chain alcohols and aromatic solvents, which are more hydrophobic, penetrate deeply into the membrane, thus causing an increase in the degree of saturation.

1.3.8. Recent advances in petroleum degradation

Role of Plasmids in Adaptation

Genetic factors play important roles in biodegradation potentials on microorganisms. Plasmids probably play leading role in this aspect. The ability to degrade more components of petroleum such, as the aromatic fractions are generally plasmid mediated (Okoh 2006). Exposures of a microbial community to hydrocarbons help to increase in the number of bacterial plasmids types. Catabolic plasmids are non-essential genetic elements but they do provide a metabolic versatility. Such genetic potential of plasmid allow the evolution of integrated and regulated pathways for the degradation of hydrocarbons along with the development in molecular biology, particularly in the application of recombinant DNA technology, gene probes (Mrozik *et al.*, 2010) and polymerase chain reaction (PCR) technology.

Many bacterial catabolic pathways are specified by conjugative plasmids (Table 8). These plasmids are readily transferred laterally into new host bacteria, and enhancing the metabolic potential of other members of an ecosystem. Conjugative plasmids are important agents of genetic changes and evolution in bacteria, and could be picked up from or brought together in different organisms as groups of genes, which through mutations and recombination can specify new metabolic functions (Okoh 2006). The best

characterized of these pathways is encoded by the TOL plasmid (pWW0) of *P. putida* PaW1 (Salam and Obayori, 2014), which converts toluene to benzyl alcohol, benzaldehyde, benzoate, and catechol, which further undergoes *meta* cleavage by an extradiol dioxygenase, or catechol 2,3-dioxygenase (C230). *Pseudomonas putida*F1 metabolizes toluene to 3-methylcatechol, which undergoes *meta*-cleavage by a C230 (Saghafi *et al.*, 2010).

Table 8. Plasmids encoding catabolic functions. (Okoh, 2006)

Plasmid	Host	Components catabolized
TOL	*P. putida*	BTEX
NAH	*P. putida*	Naphthalene
SAL	*P. putida*	Salicylate
pND50	*P. putida*	p-cresol
pWW31	*P. putida*	Phenylacetate
pJP1	*Alcaligenes paradoxa*	2,3- dichlorophenoxyacetic acid
pKF1	*Acinetobacter sp.*	4- chlorobiphenol
pAC21	*Pseudomonas sp.*	3-chlorobenzoate
pRE1	*P. putida*	3-chlorobenzoate

Genetically Modified Bacteria

Applications for genetically engineered microorganisms (GEMs) in bioremediation have received a great deal of attention to improve the degradation of hazardous wastes under laboratory conditions. There are reports on the degradation of environmental pollutants by different bacteria. Table 9 shows some examples of the relevant use of genetic engineering technology to improve bioremediation of hydrocarbon contaminants using bacteria. The genetically engineered bacteria showed higher degradative capacity. However, ecological and environmental concerns and regulatory constraints are major obstacles for testing GEM in the field. These problems must be solved before GEM can provide an effective clean-up process at lower cost. The use of genetically engineered bacteria was applied to bioremediation process monitoring, strain monitoring, stress response, end-point analysis, and toxicity assessment. Examples of these applications are listed in Table 10.

Table 9. Genetic engineering for biodegradation of contaminants.(Das and Chandran, 2011).

Microorganisms	Modification	Contaminants
P. putida	pathway	4- ethylbenzoate
P. putida KT2442	pathway	Toluene/ benzoate
Pseudomonas sp. FRI	pathway	Chloro-, methylbenzoate
Comamonas testosterone VP44	Substrate specificity	o-,p-MCB
P. pseudoalcaligens KF707-D2	Substrate specificity	TCE, BTEX
Pseudomonas sp. LB400	Substrate specificity	PCB

The range of tested contaminants included chlorinated compounds, aromatic hydrocarbons, and non polar toxicants. The combination of microbiological and ecological knowledge, biochemical mechanisms, and field engineering designs are essential elements for successful in situ bioremediation using genetically modified bacteria.

Table10. Application of genetically modified bacteria for assessing the biodegradation process efficiency.(Das and Chandran, 2011).

Microorganisms	Applications	Contaminants
A. eutrophus H850Lr	Process monitoring	PCB
P. putida TVA8	Process monitoring	TCE, BTEX
P. fluorescens HK44	Process monitoring	Naphthalene, anthracene
A. cepacia BRI6001L	Strain monitoring	2, 4D
P. fluorescens 10586s	Strain response	BTEX
Pseudomonas sp. Shk1	Toxicity assessment	2, 4 DNTP
A. eutrophus 2050	End point analysis	Non polar narcotics

Biodegradation of Petroleum Hydrocarbons by Immobilized Cells

Immobilized cells have been used and studied for the bioremediation of numerous toxic chemicals. Immobilization not only simplifies separation and recovery of immobilized cells but also makes the application reusable which reduces the overall cost. Luo *et al.* (2012) used free suspension and immobilized *Pseudomonas sp.* to degrade petrol in an aqueous system. The study indicated that immobilization resulted in a combination of increased contact between cell and hydrocarbon droplets and enhanced level of rhamnolipids production. The rhamnolipids caused greater dispersion of water-insoluble n-alkenes in the

aqueous phase due to their amphipathic properties and the molecules consist of hydrophilic and hydrophobic moieties reduced the interfacial tension of oil-water systems. This resulted in higher interaction of cells with solubilized hydrocarbon droplets much smaller than the cells and rapid uptake of hydrocarbon into the cells. Diaz *et al.* (2002) reported that immobilization of bacterial cells enhanced the biodegradation rate of crude oil compared to free living cells in a wide range of culture salinity. Immobilization can be done in batch mode as well as continuous mode. Packed bed reactors are commonly used in continuous mode to degrade hydrocarbons. Cunningham *et al.* (2004) used polyvinyl alcohol (PVA) cryogelation as an entrapment matrix and microorganisms indigenous to the site. They constructed laboratory biopiles to compare immobilised bioaugmentation with liquid culture bioaugmentation and biostimulation. Immobilised systems were found to be the most successful in terms of percentage removal of diesel after 32 days.

Rahman *et al.* (2006) conducted an experiment to study the capacity of immobilized bacteria in alginate beads to degrade hydrocarbons. The results showed that there was no decline in the biodegradation activity of the microbial consortium on the repeated use. It was concluded that immobilization of cells are a promising application in the bioremediation of hydrocarbon contaminated site.

Commercially available bioremediation agents

Bioremediation agents are classified as bioaugmentation agents and biostimulation agents based on the two main approaches to oil spill bioremediation (Nicholsrate, 2001).In 2002, the U.S. EPA compiled a list of 15 bioremediation agents as a part of the National Oil and Hazardous Substances Pollution Contingency Plan (NCP) Product Schedule, which was required by the Clean Water Act, the Oil Pollution Act of 1990, and the National Contingency Plan (NCP) as shown in Table 11. But the list was modified, and the number of bioremediation agents was reduced to nine.

Studies showed that bioremediation products may be effective in the laboratory but significantly less in the field (Venosa, 2003). This is because laboratory studies cannot always simulate complicated real world conditions such as spatial heterogeneity, biological interactions, climatic effects, and nutrient

mass transport limitations. Therefore, field studies and applications are the ultimate tests for the most convincing demonstration of the effectiveness of bioremediation products.

Compared to microbial products, very few nutrient additives have been developed and marketed specifically as commercial bioremediation agents for oil spill cleanup. It is probably because common fertilizers are inexpensive, readily available, and have been shown effective if used properly. However, due to the limitations of common fertilizers (e.g., being rapidly washed out due to tide and wave action), several organic nutrient products, such as oleophilic nutrient products, have recently been evaluated and marketed as bioremediation agents (Das and Chandran, 2011). Four agents, namely, Inipol EAP22, Oil Spill Eater II (OSE II), BIOREN 1, and BIOREN 2, listed on the NCP Product Schedule have also been put into this category. Inipol EAP22 (Societe, CECA S.A., France) is listed on the NCP Product Schedule as a nutrient additive and probably the most well-known bioremediation agent for oil spill cleanup due to its use in Prince William Sound, Alaska.

Oil Spill Eater II (Oil Spil l Eater International, Corp.) is another nutrient product listed on the NCP Schedule (U.S. EPA, 2002). This product is listed as a nutrient/ enzyme additive and consists of nitrogen, phosphorus, readily available carbon, and vitamins for quick colonization of naturally occurring bacteria. A field demonstration was carried out at a bioventing site in a Marine Corps Air Ground Combat Center (MCAGCC) in California to investigate the efficacy of OSEII for enhancing hydrocarbon biodegradation in a fuel contaminated vadose zone (Das and Chandran, 2011).

Researchers from European EUREKA BIOREN program conducted a field trial in an estuary environment to evaluate the effectiveness of two bioremediation products (BIOREN 1 and 2). The two nutrient products were derived from fish meals in a granular form with urea and super phosphate as nitrogen and phosphorus sources and proteinaceous material as the carbon source.

Table 11. Bioremediation agents in NCP product schedule (Adapted from USEPA, 2002)

Bioremediation agents	Manufacturer
BET BIOPETRO	BioEnviro Tech, TX
BILGEPRO	International Environmental Products, LLC, PA
INIPOL EAP 22	Societe, France
RESTORATION MICRO BLAZE	Verde Environmental Inc, TX
OIL SPILL EATER II	Oil Spill Eater, Dallas, TX
STEP ONE	B & S Research Inc, MN
WMI-2000	WMI International Inc

The major difference between the two formulations was that BIOREN 1 contained a biosurfactant. The results showed that the presence of biosurfactant in BIOREN 1 was the most active ingredient which contributed to the increase in oil degradation rates whereas BIOREN 2 (without biosurfactant) was not effective in that respect. The biosurfactant could have contributed to greater bioavailability of hydrocarbons to microbial attack.

1.3.9. Application of petroleum degrading microorganism for biodiesel production

Biodiesel, an alternative diesel fuel, is defined as monoalkyl ester of fatty acids from vegetable oil and produced by enzyme mediated catalytical transesterification with petrochemically derived methanol. This process is also called alcoholysis (Youssef, 2007). Oleaginous microorganisms like microalgae, bacillus, fungi and yeast are now used for substituting conventional oil in biodiesel production. A potential future fuel completely produced by bacteria is Microdiesel (Kalscheuer et al. 2006). A highly solvent-tolerant *P. putida* DOTT1E, employed for the biotransformation of BTEX, also served as a potential candidate for biodiesel production (Ramos-Gonzalez *et al.*, 2003).

Table12. Comparison of transesterification activities of different lipases on different substarates (Bajaj *et al.*, 2010)

Source of oil	Source of enzyme	Alcohol	Optimum conditions	Immobilization medium	Maximum yield	Other details	Reference
Jatropha	Chromobacterium viscosum	Ethanol	45°C, 10 h, 200 rpm	Celite 545	92%	Water content 0.5%	Shah et al. [2]
	Rhizomucor miehei	Ethanol	45°C, 24 h, 200 rpm	-	79%	Ultrasonic pretreatment, 100 W, 2.3 h	Shah and Gupta [31]
	Pseudomonas aeruginosa	Methanol	30°C, 60 h, 200 rpm	Silica	94%	Used n-hexane as solvent	Kaieda et al. [64]
Tallow	Mucor miehei	Methanol	45°C, 5 h, 200 rpm	-	98.6%	Hexane solvent	Nelson et al. [5]
		Ethanol			98.1%		
		Propanol			98.6%	Used hexane as solvent, 6 mol% water based on triglyceride was added	
		Butanol			96.0%	Used hexane as solvent	
		Isobutanol			98.4%	Used hexane as solvent, 6 mol% water based on triglyceride was added	
Soybean oil	Thermomyces lanuginosus	Methanol (stepwise addition)	40°C, 50 h, 150 rpm	Silica gel	>90%	Molar ratio of methanol to oil 3:1, hexane 10%	Du et al. [4]
	Mucor miehei	Ethanol	45°C, 5 h, 200 rpm	-	91.4%	Hexane solvent	Nelson et al. [5]
	Pseudomonas fluorescens (psychrophilic strain B11)	Methanol (stepwise addition)	20°C, 12 h, 150 rpm	Cellulose fabric	92%	n-Heptane solvent	Lee et al. [12]
	Pseudomonas fluorescens	Ethanol (initial molar ratio to oil 3:1)	30°C, 24 h, 150 rpm	-	71%	Iso-octane solvent, solubilized lipase AY	Zhang et al. [30]
		Methanol (molar ratio to oil 3:1)	40°C, 72 h, 200 rpm	-	60.8%	5 mmol/l Ca^{2+}, 5% water	Yang et al. [58]
	Burkholderia cepacia (lipase PS-30)	Methanol	30.5°C, 6.3 h, 200 rpm	Macroporous weak anionic resin beads	92.2%	n-Hexane solvent, water content 5.8%, molar ratio of methanol to oil 5.4:1	Shimada et al. [11]
Mixture of soybean and rapeseed oils	Candida antarctica	Methanol (stepwise addition)	30°C, 48 h, 150 rpm	5% H2O	95.6%	Water content <400 ppm	Shimada et al. [16]
Sunflower oil	Candida antarctica (Novozyme 435)	Methanol (continuous addition)	50°C, 15 h, 100 rpm	Macroporous resin support, diameter 0.3–0.9 mm	92%	Water content 400 ppm	Belafi-Bako et al. [43]
Waste cooking oil	Rhizopus chinensis CCTCC M201021	Methanol (stepwise addition)	40°C, 72 h, 220 rpm, pH 6.5	Hydrophobic carriers with magnetic particles (Fe3O4)	92%	-	Yan and Chen [61]

Linko *et al.* (1998) reported that lipase was used as the biocatalyst for the production of variety of biodegradable esters and polyesters. 97% conversions of esters were obtained using *Candida rugosa* lipase powder during tranesterification of rapeseed oil with 2-ethyl-I-hexanol. De *et al.* (1999) demonstrated the use of immobilized *Mucor miehei* lipase (Lipozyme IM-20) for the conversion of fatty alcoholesters (C_4-$C_{18:1}$) in a solvent-free system. The lipase from *M. miehei* was the most efficient for conversion of triglycerides to alkyl esters with primary alcohols whereas lipase from *C. antarctica* was most efficient for transesterifying triglycerides with secondary alcohols to give branched alkyl esters. 94.8-98.5% of maximum conversion was noted for the primary alcohols such as methanol,ethanol, propanol, butanol and isobutanol. 61. 2-83.8% conversion was noted for the secondary alcohols such as isopropanol and 2-butanolin the presence of hexane as a solvent (Nelson *et al.*, 1996).Several methods were used to increase the enzyme yield and to decrease the reaction time. Various parameters such as temperature, time duration and agitation speed vary greatly and are optimized with different combinations of lipase source, immobilization media, and alcohol. Table 12 summarizes the yields and acyl-

acceptors on enzymatic transesterification of oil for producing biodiesel using extracellular lipases.

The cost of the lipase is still the main obstacle for exploiting its potential in biodiesel industry. Therefore, reuse of lipase is essential which can be achieved by using immobilized lipases.

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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.

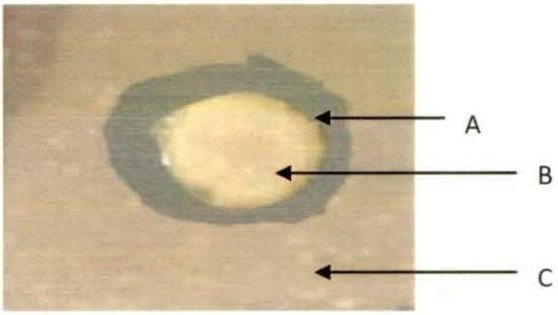


Fig 1. Strain PS11 showing zone of hydrocarbon (crude oil) utilization. (A) Clear zones of hydro-carbon (crude oil) utilization, (B) Single colony of PS11 strain, (C) MS agar plate surface-coated with crude oil (magnification, X 12.5).

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.

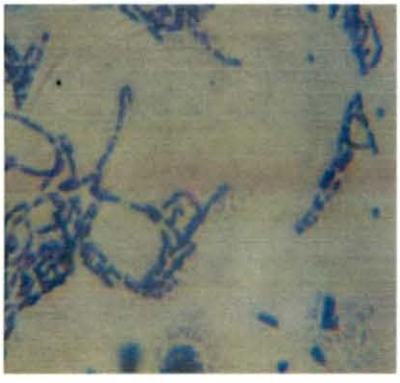


Fig 2: Microscopic (100X) view of gram stainedcells of PS11 strain

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.**

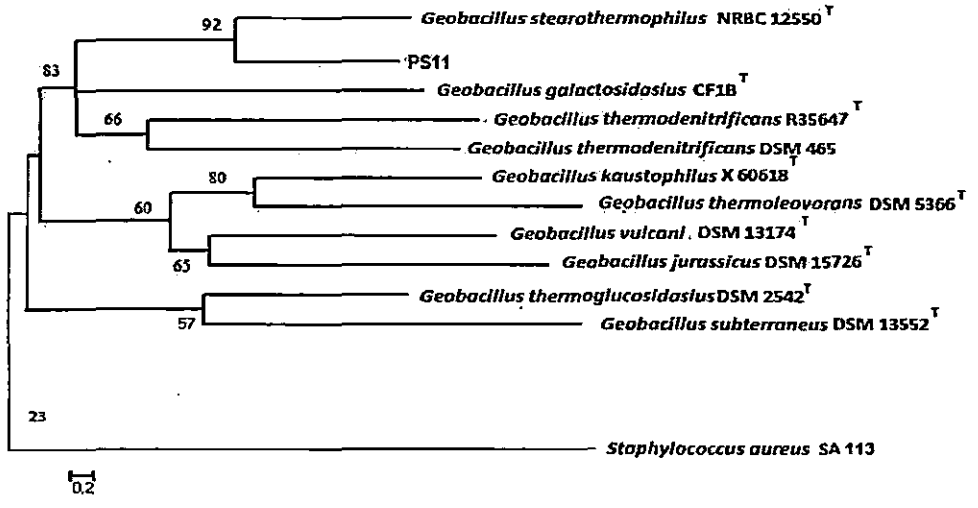


Fig 3. Phylogeneticanalysis of 16S rRNA sequence. (The evolutionary history was inferred using the Neighbor-Joining method. Phylogenetic analyses were conducted in MEGA 4, The numerials are the boot strap values)

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.

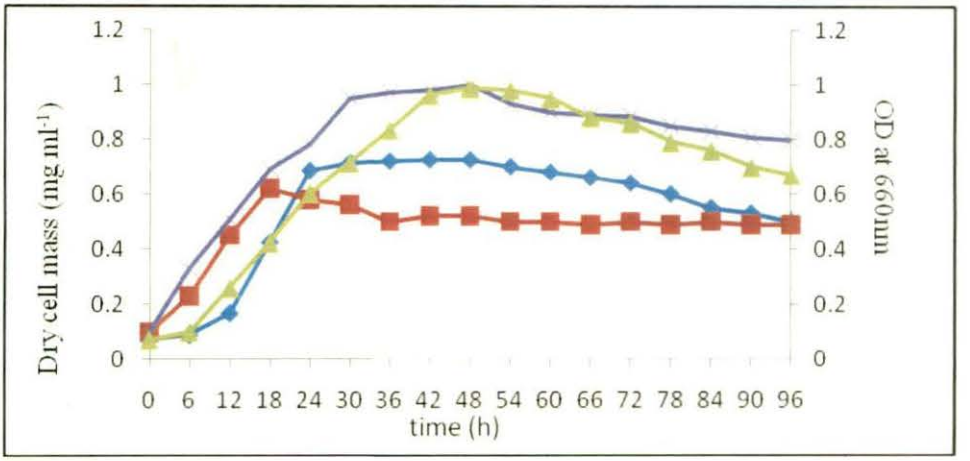


Fig 4. Growth of *Geobacillus stearothermophilus* PS11in the absence and presence of crude oil. Bacterial growth in absence of crude oil: $OD_{660\ nm}$, (▲); dry cell mass, (╲) and growth in presence of crude oil: $OD_{660\ nm}$, (♦); dry cell mass, (■). The experiment was carried out in triplicates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

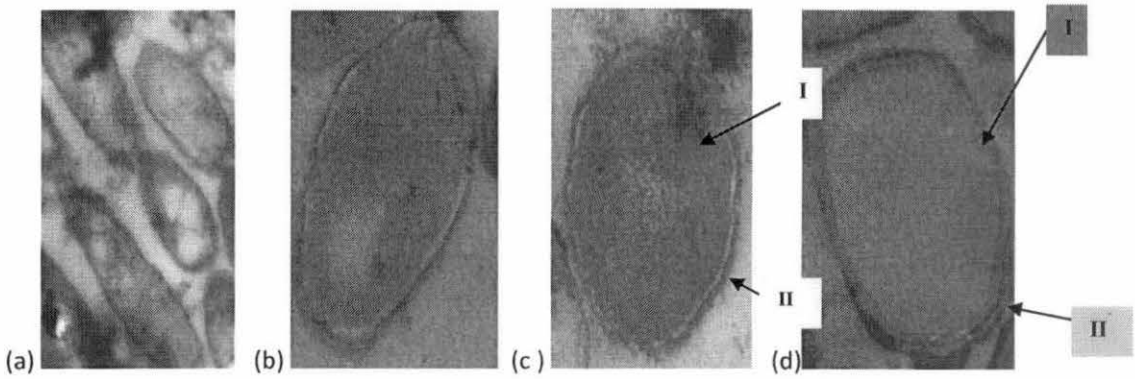


Fig 5. Transmission electron micrograph of *Geobacillus stearothermophilus* PS11cells. **(a)**in the absence of crude oil (exposure 21,000); **(b & c)** in the presence of 10% crude oil (exposure 21,000) after 24 hours incubation - (I) accumulation of solvents and (II) convoluted and disorganized cell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

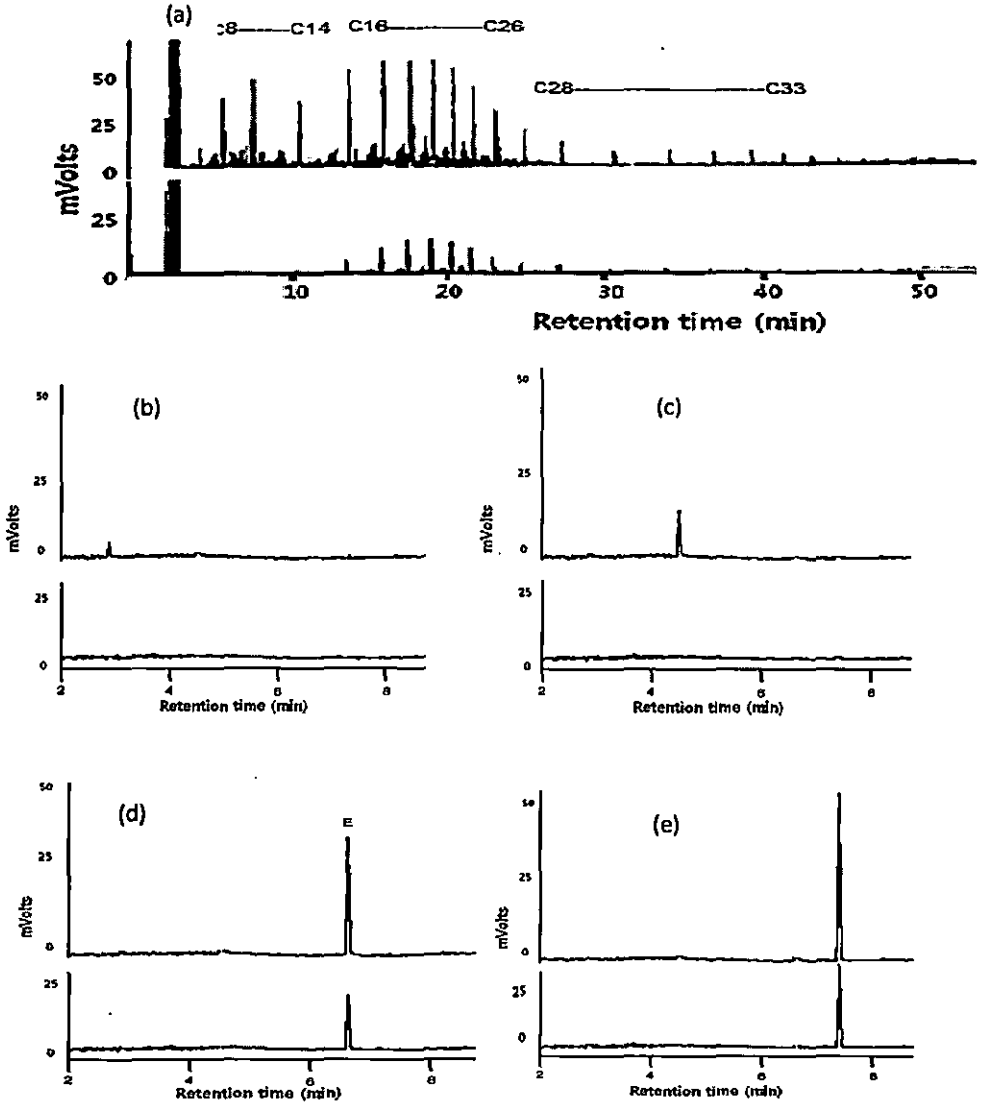


Fig 6: Gas chromatographic pattern of residual total petroleum hydrocarbons (TPH) and BTEX as exhibited by PS11cells after 15 days of incubation.Degradation of 10% v/v TPH (a), 10% v/v benzene(b), 10%v/v toluene(c),5% v/v ethyl benzene (d)and xylene 5% v/v (e) after 15 days incubation.

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.

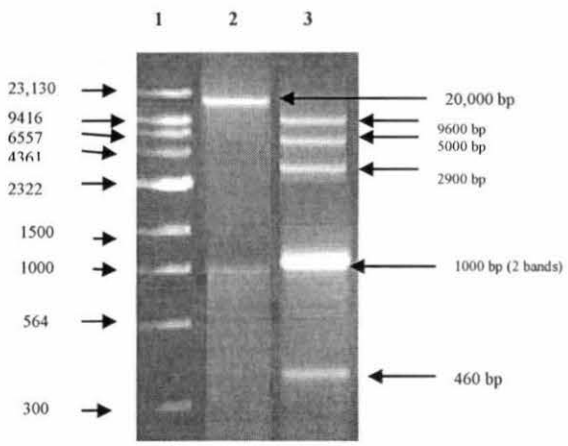


Fig 7. Isolation and restriction digestion pattern of PS11 plasmid DNA. Lane1: Lambda *Hind III* digest DNA marker, Lane 2: Plasmid DNA of PS11, lane 3: BamH1 digest plasmid DNA of PS11

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).

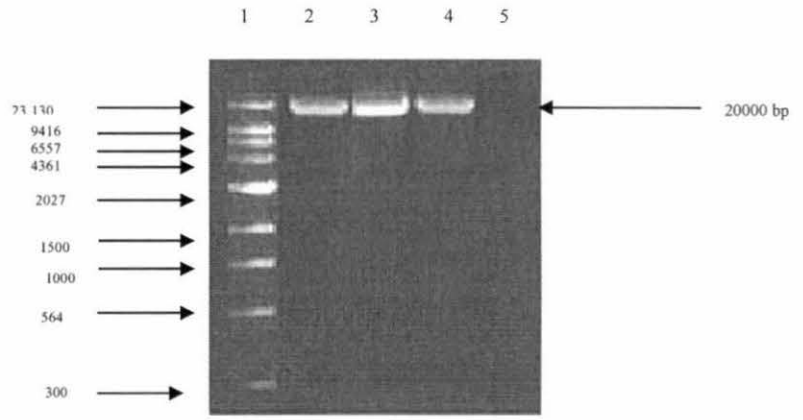


Fig 8. Curing of plasmid with acridine orangeLane1.Lambda*Hind III* digest DNA marker, 2. control plasmid, 3. plasmid with 100 μg/ml acridine orange, 4. plasmid with 150 μg/ml acridine orange, 5. plasmid with 200 μg/ml acridine orange.

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.

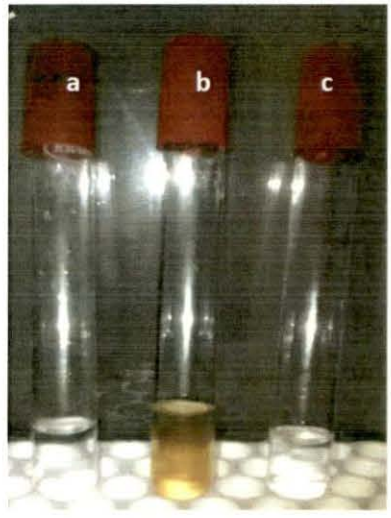


Fig 9. Catechol utilization by transformed JM109 cells. (a) Control w/o inoculam, (b) transformed JM109 cells, (c) non transformed JM109 cells

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.

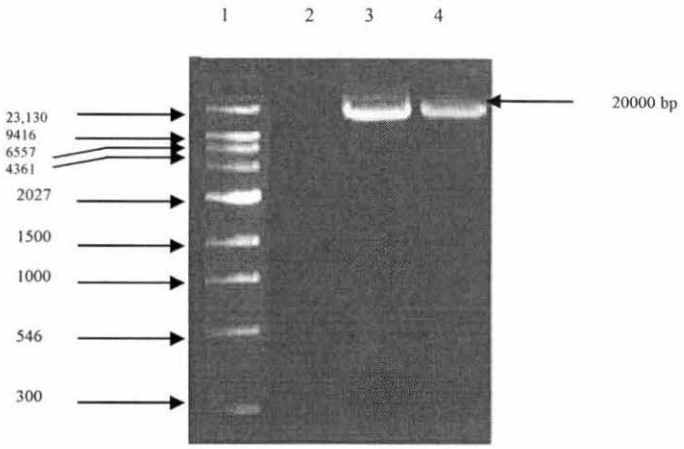
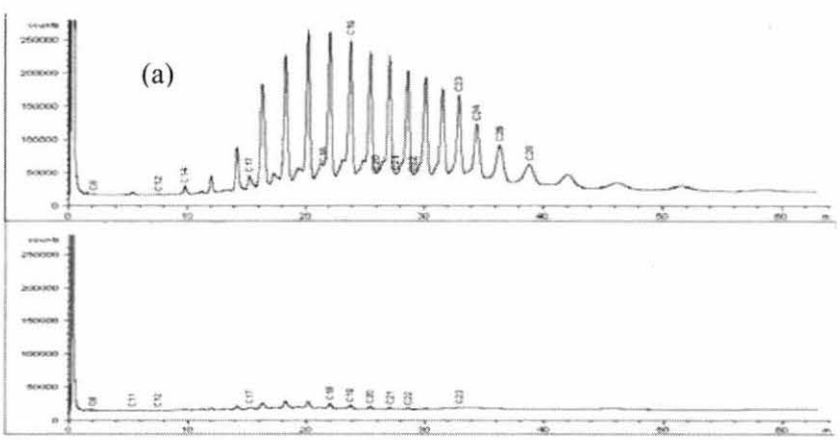


Fig 10. Transformation of *E.coli* JM109 with plasmid from PS11.Lane 1.Lambda *Hind III* digest DNA marker, Lane 2. Wild type JM109, Lane 3.Transformed *E.coli* JM109 cells, Lane 4. Wild type PS11

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.



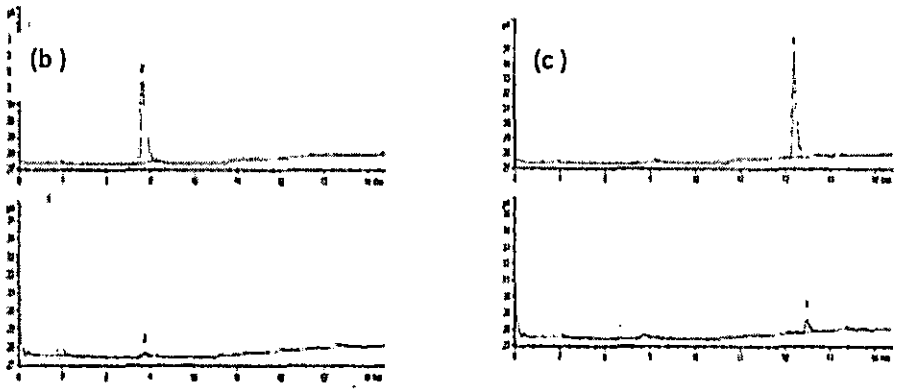


Fig 11. Gas chromatographic pattern of residual total petroleum hydrocarbons (TPH), benzene and ethyl benzene as exhibited by transformed JM109 cells after 15 days of incubation.Degradation of 10% v/v TPH (a), 10% v/v benzene(b) and 5% v/v ethyl benzene (c) after 15 days incubation.

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.

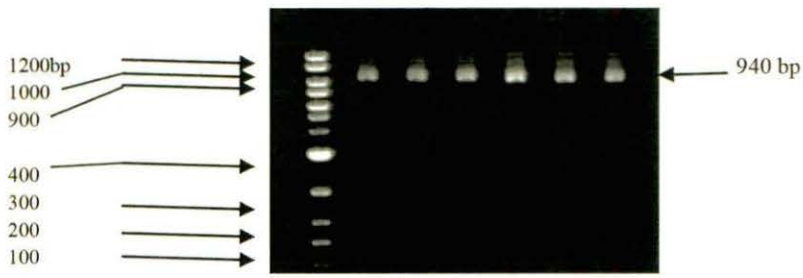


Fig 12.PCR amplification of C23O.Lane 1:100bp ladder, lane 2-4: amplified DNA of C2,3O from transformed cells, lane 5-7: amplified DNA of C2,3O from wild PS11 cells

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.

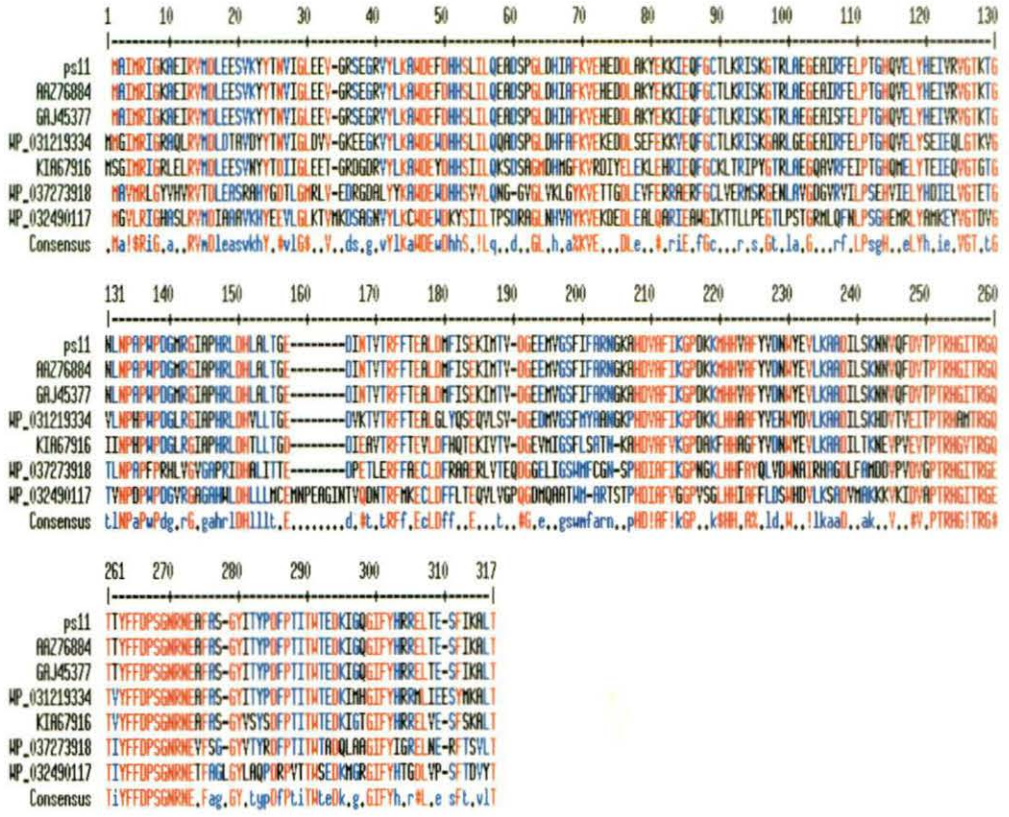


Fig 14. Comparison of deduced amino acid sequence of catechol 2,3-dioxygenase of *G. stearothermophilus* PS11 with other catechol 2,3-dioxygenase. The catechol 2,3-dioxygenase (C23O) of *G. stearothermophilus*PS11was aligned to corresponding enzyme of *G. stearothermophilus* DSMZ6285(AAZ76884), *G. thermoglucosidasius* 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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Chapter 3
Application of PS11 cells for biodiesel production

3.1 Introduction

Lipases produced from solvent degrading bacterial strains have enormous biotechnological potential in the field of biodiesel production due to their stability in organic solvents. With the industrialization bloom in the whole globe, the world is confronted with the twin crises of fossil fuel depletion and environmental degradation (Van, 2005). In order to resolve these problems, researchers are interested to find an alternative environmental friendly reproducible diesel fuel. Biodiesel, a diesel fuel produced from vegetable oils or animal fat is gainingmore importance as an alternative fuel. It has several advantages over diesel fuel, like produces less smoke and particulates, has higher octane numbers, emission of lower carbon monoxide and hydrocarbon, and is biodegradable. Alkali catalysts such as metal alkoxides and hydroxides are widely used for large scale biodiesel production processes (Barnwal and Sharma, 2005). However, the process exhibits certain drawbacks, such as, costly product recovery, generation of environment polluting alkali effluents. Microbial lipases are currently receiving much attention with the rapid development of enzyme technology. They have the ability to perform catalysis at extremes of temperature, pH, and in organic solvents withchemo-, regio-, and enantioselectivity.They can catalyze different reactions including hydrolysis of triglycerides, trans-esterification and chiral synthesis of esters under natural conditions. The lipase catalyzedtrans-esterification of fatty acid is thus an alternate approach to the alkali catalyzed trans-esterification in production of biodiesel fuel. Lipases from *Photobacterium lipolyticum* (Yang *et al.*, 2009), *Pseudomonas aeruginosa* (Ji *et al.*, 2010), *Ralstonia sp.* CS274 (Yoo *et al.*, 2011) have previously been reported to catalyze trans-esterification reactions. However, lipase being costlier than the alkaline catalysts has limited the commercialization of this technique. Moreover, the enzyme can be inactivated during trans-esterification reaction utilizing methanol or ethanol as acyl acceptor. The cost of enzyme catalyzed biodiesel production can be reduced by reusing the enzyme for successive cycles of production (Neilson *et al.*, 2008).Organic solvent tolerant property of lipases, especially tolerating methanol and ethanol, can be exploited for this purpose. Hence, efforts to screen naturally evolved lipases having self-possessed organic solvent tolerance property is highly sought.

As *Geobacillus stearothermophilus* PS11 exhibited different solvent degradation capabilities, it was further employed for the production of solvent tolerant lipase. Therefore, this chapter deals with production, optimization and characterization of lipase produced by petroleum hydrocarbon degrading strain of *Geobacillus stearothermophilus* PS11. The organic solvent tolerance property of PS11 lipase was studied here. Application of solvent tolerant PS11 lipase in enzymatic transesterification of vegetable oil for biodiesel production in presence of high methanol concentration was also studied in this chapter.

3.2 Materials and methods

3.2.1 Determination of lipase production by Geobacillus stearothermophilus PS11

The petroleum hydrocarbon degrading strain of *Geobacillus stearothermophilus* PS11 **(accession no KC311354)** was used to determine its capability in lipase production in this study. The strain was inoculated in nutrient agar supplemented with 1% tributyrin. It was incubated for 48 h at 50 °C. The lipolytic activity was confirmed by the formation of a clear zone around the colonies.

3.2.2 Production media and enzyme preparation and measurement of bacterial growth

Growth and lipase production by *G.stearothermophilus* PS11 was carried out in basal production medium (LPM-1) composed of (g/l): peptone, 5; beef extract, 3; sodium chloride, 2; Tween-80, 5; and olive oil, 10; pH 8. The autoclaved medium was inoculated at 1% (v/v) overnight grown bacterial culture and incubated at 37°C under shaking at 120 rpm for 96 h. After every 6 h cells were removed by centrifugation and the supernatant was desalted and used for measurement of lipase activity.

Growth was followed by recording absorbance at 660 nm. For dry cell mass measurement, 1.0 ml culture broth was centrifuged at 10000 rpm at 4°C for 10 min to pellet down the cell mass. The pellet was washed twice with distilled water and dried at 105°C till constant mass was achieved.

3.2.3 Lipase assay

Lipase activity was measured on the basis of hydrolysis of p-nitro phenyl palmitate (pNPP). A 2.5 ml of the assay mixture consisted of, 2.4 ml of 10μM pNPPin 50 mM Tris HCl (pH 9) and 0.1 ml enzyme preparation. Reaction mixture was incubation at 60°C for 10 minutes followed by addition of 200 μl of 1 M calcium chloride, which precipitates free fatty acids. The precipitate was pelleted by centrifugation at 6000 rpm for 10 mins. Lipase activity was measured spectrophotometrically in the supernatant by increase in absorption at 410 nm due to the release of p-nitrophenol (pNP) from pNPP. One lipase unit (EU) is defined as the

amount of enzyme that liberated 1 µmol p-nitrophenol minute^{-1} under the assay conditions.

3.2.4 Optimization of PS11 lipase production by OVAT method

For optimization of lipase production by *G. stearothermophilus* PS11, the effect of various process variables such as nitrogen source, carbon source, substrate specificity, agitation speed and cultivation time were studied using OVAT method. Each variable was optimized and was incorporated further in the subsequent experiments.

For these, the bacterial cells were grown for 96 h at 37 °C under shaking condition. The cells were removed by centrifugation at 10,000 rpm for 10 min and the supernatants were used for measurement of lipase activity at each step.

Lipase production was studied in presence of different organic and inorganic nitrogen sources such as peptone, yeast extract, tryptophan, sodium nitrate and soyabean meal at a concentration of 1% (w/v). To determine the effect of substrate on enzyme production, olive oil in the LPM-1 was replaced with soybean oil, sunflower oil, ricebran oil and coconut oil, each at 1% (w/v). The production medium was supplemented with different carbon source such as glucose, sucrose, galactose, starch and lactose at 1% (w/v). The agitation speed for enzyme production was further optimized by agitating the culture at variable speed ranging from 100 – 180 rpm for 96 h at 37 °C. Finally, for determining the cultivation time for highest lipase production, the bacterial culture samples were withdrawn at 24 h intervals for a period of 96 h and enzymatic activity was monitored in the culture supernatant.

3.2.5 Purification of PS11 lipase and determination of molecular weight

The bacterial isolate was cultured in LPM-1 for 72 h at 37°C. Bacterial cells were pelleted by centrifugation at 5000 rpm for 10 min. The supernatant obtained after centrifugation was subjected to 0-40% ammonium sulphate precipitation. The precipitate was collected by centrifugation at 10,000 rpm for 40 min and dissolved in 50 mM Tris HCl buffer (pH 9.0). It was then desalted by dialysis with a cellulose dialysis bag in same buffer. The desalted enzyme preparation was loaded on to DEAE-sephacel column pre-equilibrated with 50 mM Tris HCl buffer (pH 9.0) and

eluted with a linear gradient of 0- 0.50 M NaCl in the same buffer. The active fractions were collected, lyophilized and applied on Sephadex G-75 column. The active fractions were pooled and subsequently used for characterization of the enzyme. Molecular weight of PS11 lipase was detected on 15% SDS-polyacrylamide gel electrophoresis (8). Low molecular weight protein standard ranging 20 to 97 KDa was used as marker.

3.2.6 Characterization of purified PS11 lipase

3.2.6.1. Effect of pH on activity and stability of PS11 lipase

The pH optimum was determined by measuring the enzyme activity at pH 4-12 under standard condition. The pH stability of the enzyme was determined by pre-incubating the purified enzyme preparation with different buffers in pH range from 4.0 to 12.0 at 4°Cfor 2 h followed by monitoring the lipase activity. Buffer systems (50 mM) used were acetate-HCl (pH 4.0–5.0), sodium hydrogen phosphate–NaOH (pH 6.0–7.0), Tris-HCl buffer (pH 8.0–9.0) and glycine-NaOH buffer (pH 10.0–12.0).

3.2.6.2. Effect of temperature on activity and stability of PS11 lipase

The effect of temperature on purified lipase was determined by incubating the reaction mixture at a temperature range of 10-80 °C under standard assay conditions. The thermostability of the enzyme was determined by pre-incubating the enzyme at different temperatures from 10 to 80°C for 2 h followed by determination of enzymatic activity.

3.2.6.3. Effect of metal ions and chemical reagents on PS 11 lipase activity

The effect of various metal ions (Ca^{2+}, Na^{+}, Mn^{2+}, Cu^{2+}, Fe^{2+} and Zn^{2+}), enzyme inhibitors (EDTA), reducing agent (mercaptoethanol), and oxidizing agents (hydrogen peroxide) on purified lipase activity was determined at the final concentration of 1 mM and 5 mM. For this the enzyme was pre-incubated with either of these chemicals for 1 h at 4°C. The lipase activity in absence of metal ion or chemical reagent served as control (100% activity).

3.2.6.4. Effect of detergents on lipase stability

The effect of various detergents (1%, v/v), including Triton X-100, Tween-20, Tween-80, Tween 60, N-laurylsarcosine, SDS and 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) (CHAPS) on the lipase activity was investigated by p-NPP method. Lipase activity measured in absence of detergent was considered as 100%.

3.2.6.5 Effect of organic solvent on lipase stability

In order to study the effect of organic solvents on enzyme stability, suitably diluted lipase (250 EU) in 50 mM Tris–HCl (pH 9.0) was mixed with different organic solvents to yield a final concentration of (25%, v/v) and then the mixture was incubated in a shaking incubator (180 rpm) at 4°C. The lipase activity was measured 48h later using p-NPP method. The organic solvents used were hexane, xylene, toluene, benzene, dichloromethane, diethylether, ethylacetate, isopraponal, acetone, ethanol, acetonitrile, methanol and dimethyl sulfoxide (DMSO). The lipase activity without organic solvent was considered as 100%.

Among the organic solvents, methanol is widely used as acyl acceptor in enzymatic trans-esterification of vegetable oils and animal fats. Hence, to determine the stability properties of the enzyme in such media, lipase preparation (250 EU) was incubated with different concentration of methanol ranging from 5 to 50% (v/v) and residual activity was examined after 24 h later.

3.2.7 Application of lipase PS11 in biodiesel production

3.2.7.1. Sample preparation

Enzymatic trans-esterification of commercial sunflower and olive oil in methanol was performed according to the method described by Yang *et al.* (3) with certain modifications. At first, a mixture of sunflower oil or olive oil (4 ml) and methanol (0.50 ml) were prepared. To this mixture, enzyme solution (250 EU) was added. Trans-esterification reaction was conducted for 48 h at 30 °C with rotary shaking (200 rpm).

3.2.7.2. Determination of biodiesel production by TLC method

100 µl sample was withdrawn at 6h intervals and mixed with 200µl of hexane. The mixture is centrifuged and 5 µl of the separated upper layer was applied to TLC plate. The plate was developed in the chamber containing a solvent mixture of hexane, acetone, and acetic acid (95:4:1) and was air dried. The plate was then soaked in potassium permanganate solution (0.5%, w/v) and the biodiesel spot were visualized. Fatty acid methyl ester was used as reference biodiesel.

3.2.7.3. Determination of biodiesel production by gas chromatography

Fatty acid methyl esters (FAMEs) of biodiesel were analyzed using a gas chromatographer (GC-2014,Shimadzu, Kyoto, Japan) which was equipped with a Stabilwaxcolumn and flame ionization detector (FID). The GC was calibratedby methyl oleate, methyl linoleate, and methyl palmitateunder various concentrations. During the analysis, the temperatureof the injector and FID were controlled at 250 °C. Thetemperature of the column was raised programmatically. Thetemperature was initially maintained at 150° C for 2 min, andwas further raised to 250° C by a rate of 10° C min/1. Finally,the temperature of the column was maintained at 250 °C for 5 min.

3.3 Results

3.3.1 Kinetics of lipase production by PS11 cells

Geobacillus stearothermophilus PS11 isolated from soil sample collected from a solvent extraction unit produced extracellular lipase.Time course of cell growth and lipase production by*G. stearothermophilus* PS11 in production medium are shown in Fig 1. The bacteria exhibited a minimal lag phase. The exponential phase lasted up to 18 h and the stationary phase continued till 90 h. Lipase production started during the exponential growth phase but gradually attained its peak at the late stationary phase of growth.

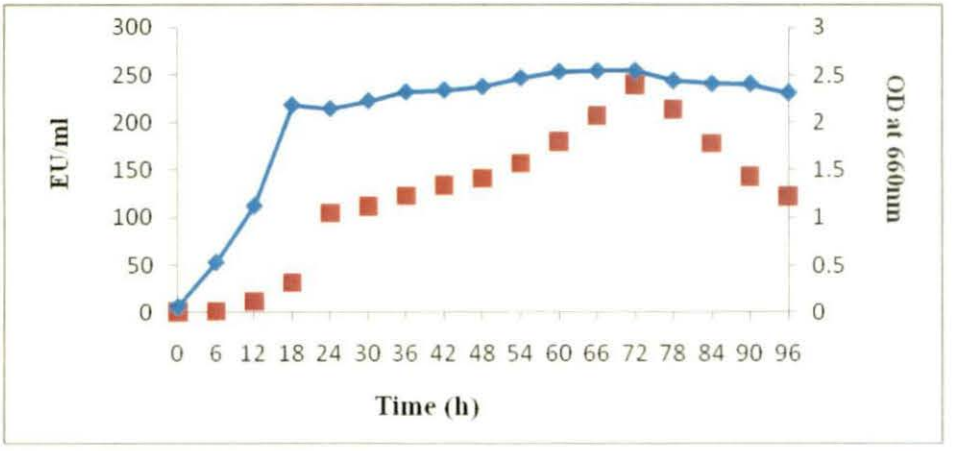


Fig 1.Determination of bacterial growth (◆) and enzyme (■) production.

3.3.2 Optimization of PS11 lipase production by OVAT method

The effective parameters for lipase production was optimized by studying the effect of various process variables, like nitrogen source, carbon source, substrate, agitation speed and cultivation time on enzymatic activity. The results of optimization are summarized in Table-1. Optimization of suitable nitrogen source for enzymeproduction is very important as nitrogen is the precursorfor protein synthesis; besides, it can influence the pH of themedium which in turn may affect the activity and stability ofenzyme. Among various nitrogen sources examined, peptone showed maximum enzyme activity of 310 EU/ml (Table1).

Table 1: Optimization of PS11 lipase production by OVAT method

N source	*EU/ml	C source	*EU/ml	Substrate specificity	EU/ml	Agitation	EU/ml	time	EU/ml
Tryptone	123±2.12	Glucose	367.5±5.19	soyabean oil	226± 2.18	100 rpm	246±1.2	12h	422±1.56
Yeast extract	200.5±3.45	Sucrose	288.12±2.1	sunflower oil	275±2.88	120rpm	312±3.4	24h	567±3.2
Peptone	310±1.23	Galactose	109.45± 5.67	ricebran oil	370±1.56	140rpm	368±2.13	48h	620±2.4
Soybean meal	187±3.12	Starch	187±3.7	coconut oil	312.5±4.1	150rpm	420±4.71	72h	765±2.16
Sodium nitrate	156±4.45	Lactose	276±3.1	olive oil	250±3.86	180rpm	387±2.3	96h	4.1±2.17

*EU means one lipase unit is defined as the amount of enzyme that liberated 1 µmol p-nitrophenol minute^{-1} under the assay conditions.

The effect of various carbon sources such as glucose, sucrose, lactose, galactose and starch, on enzyme production was determined. Glucose supported the highest production of lipase (367.5 EU/ml). Among the various lipid sources tested for lipase production, rice bran oil (1% v/v) was the best inducer (390 EU/ml), followed by coconut, sunflower, olive and soybean oil. Lipase production by *G. stearothermophillus* PS11 was influenced by agitation rates with highest activity (420 EU/ml) at 150 rpm. For determining the incubation time for maximum lipaseproduction all the parameters for highest activity were taken into consideration. PS11 strain exhibited optimum lipase production of 765 EU/ ml at 72 h incubation during stationary phrase.

3.3.3 Purification of PS11 lipase and determination of molecular weight

The PS11lipase was purified from the extracellular medium by $(NH_4)_2SO_4$ precipitation, anion exchange chromatography and gel filtration. The bacterial culture was grown for 72h in LPM-1 at 37°C. The supernatant obtained after centrifugation of culture was subjected to $(NH_4)_2SO_4$ precipitation and about 47% of the enzyme was recovered in 0-40% fraction. The dialysed enzyme preparation was subjected to DEAE-sephacel chromatography. The active fractions were pooled, concentrated and subjected

to gel filtration chromatography, which resulted in increase in specific activity of enzyme to 90.58 and recovery of the enzyme was 22.67% (Table-2).

Table 2: Purification table of lipase produced from PS11 strain

Step	Total EU*	Total Protein (mg)	Specific activity (EU/mg)	Purification fold	Yield (%)
Crude	323.60	28.72	11.26	1	100
Ammonium sulphate	152.60	2.90	52.62	4.67	47.00
DEAE-sephacel	83.60	1.46	56.96	5.05	25.83
Sephadex G-75	73.37	0.81	90.58	8.04	22.67

*EU means one lipase unit is defined as the amount of enzyme that liberated 1 µmol p-nitrophenol minute^{-1} under the assay conditions.

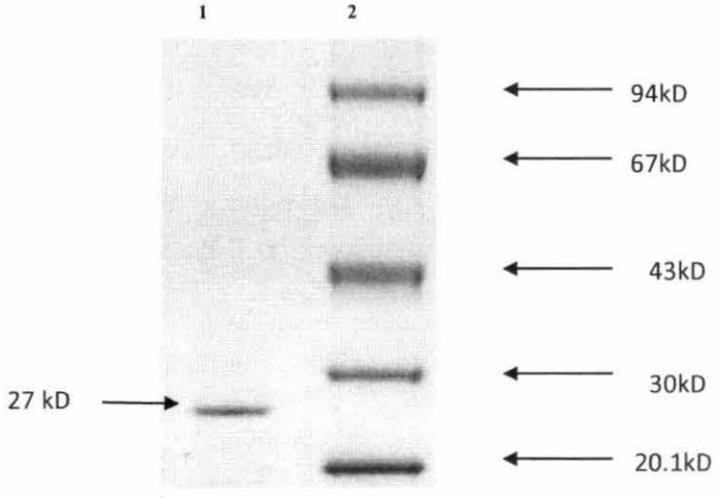


Fig 2.SDS-PAGE of purified lipase from *Geobacillus stearothermophilus* PS 11.Lane a: purified lipase from *Geobacillus stearothermophilus* PS 11. Lane b: protein standard: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and soyabean trypsin inhibitor (20.1 kDa).

The SDS-PAGE analysis of purified enzyme (Fig 2) revealed the presence of a prominent protein band of approximately 27 kD, indicating that the enzyme was probably a single chain protein or a homomultimeric protein of 27 kD subunits.

3.3.4 Effect of pH and temperature on activity and stability of PS11 lipase

The purified enzyme was active in the pH range 4-12 with pH optimum at 9. The effect of pH on the stability of lipase was tested by incubating the enzymes over a range of pH values. The enzyme was alkalostable showing stability over a pH from 8 to 11 (Fig 3). The enzyme could not retain its activity in acidic pH.

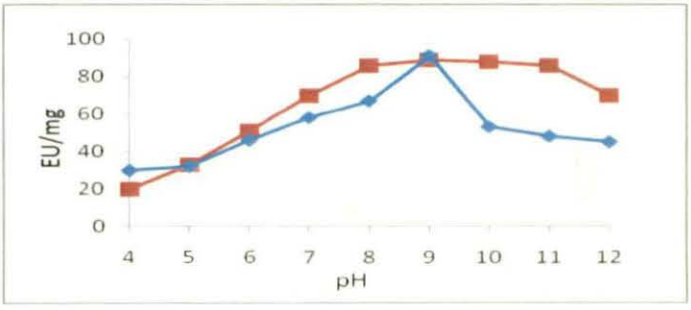


Fig 3. Determination of optimum pH (♦) and pH stability (■) of PS11 lipase

The optimum temperature for enzyme was 50°C and a sharp decline in enzymatic activity was noted with in temperature above the optimum value.For determination of thermal stability, the purified enzyme was pre-incubated at 10° to 80° C for 2h and then assayed for enzymatic activity.

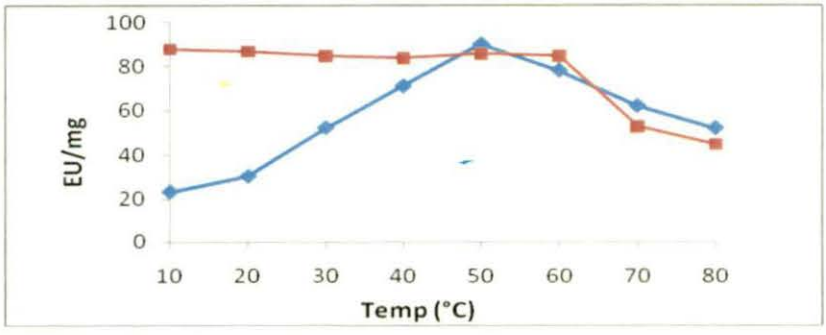


Fig 4. Determination of optimum temperature (◆) and temperature stability (■) of PS11 lipase.

The results in figure 4 indicates that PS11 lipase was highly thermostable as it retained its activity between 10-60°C while the thermal stability profile at higher temperatures showed that the enzyme PS11 is not stable above 60°C.

3.3.5 Effect of metal ions and chemical reagents on PS 11 lipase activity

The impact of various metal ions on the lipolytic activity of *G. stearothermophilus* PS11 is shown in Table 3. Almost 20% lipase activity was inhibited by Fe^{2+}, Mn^{2+}whereas Cu^{2+}, and Zn^{2+} inhibited almost 40%of its activity respectively.

Table 3: Effect of different chemicals on lipase stability

Chemicals	Relative activity (%)	
	1mM	5mM
None	100	100
$FeSO_4$	89 ± 4.1	80 ± 4.1
$MnSO_4$	94 ± 2.7	84 ± 2.7
NaCl	112 ± 1.3	107 ± 1.3
$CuSO_4$	80 ± 2.4	68 ± 2.4
$ZnSO_4$	67 ± 1.2	60 ± 1.2
$CaCl_2$	115 ± 3.5	105 ± 3.5
EDTA	100 ± 1.9	100 ± 1.9
H_2O_2	86 ± 2.1	80 ± 2.1
Mercapthoethanol	105 ± 1.3	105 ± 1.3

The lipase was pre-incubated with the chemicals for 1 h at 50 °Cbefore measuring the enzyme activity.
The presented results are the average of three repeated experiment with±standard deviation from the mean

An increase in activity by 10% was observed in presence of Na^+ and Ca^{2+}. Interestingly, EDTA, a metal ion chelator didn't alter the enzymatic activity indicating that functioning of enzyme did not have absolute requirement of metal ion(s).

3.3.6 Effect of detergents on lipase stability

The influence of various detergents on PS11 lipase was also determined and the results are shown in Table- 4. PS11 lipase activity was increased by Triton-X 100, Tween-80, Tween-60and Tween-20 with maximum enhancement of 168% in presence of Triton-X-100 (Table 4).Decrease in lipase activity was noted in presence of anionic detergents such as N-laurylsarcosine (78%), SDS (69%) with maximum reduction in presence of CHAPS (65%).

Table 4: Effect of different detergentson lipase stability

Detergents	Relative activity (%)
None	100
N-laurylsarcosine	78 ± 2.1
SDS	69 ± 1.8
CHAPS	65 ± 2.1
Tween 20	116 ± 0.7
Tween 60	129 ± 2.7
Tween 80	134 ± 1.9
Triton X 100	168 ± 1.0

3.3.7 Effect of solvents on lipase stability

The organic solvent tolerance capability of the enzyme was investigated to exploit its application in enzymatic biodiesel production. To determine the organic solvent tolerant property, the purified PS11lipase was pre-incubated with organic solvents having log*P* value ranging between -0.76 to 3.60, for 48 h under shaking, followed by determination of enzymatic activity. The lipase was stable and restored almost 90% of its activity when p-xylene, benzene, toluene, hexane and methanol (25% v/v each) were added to the purified enzyme.The residual activity was 76% in presence of ethanol (Table-5).Acetic acid was more harmful than other organic solvents as PS11 lipase could restore only 18% of its activity. This may due to the acidity inactivating the lipase

Table 5: Effect of different organic solvent on lipase stability

Organic solvents	Log *P*	Relative activity (%)
Control	—	100
Methanol	-0.76	90
Acetonitrile	-0.03	30
Ethanol	-0.24	76
Acetic acid	-0.23	18
Benzene	2.0	81
Toluene	2.5	92
p-xylene	3.1	91
n-Hexane	3.1	80

25% (v/v) of organic solvents were added to the enzyme solution andincubated for 48 h in a rotary shaker (180 rpm) at 50° C.

As methanol is one of the widely used media for biodiesel production, stability of PS11 lipase in presence of 25% (v/v) methanol led us to further investigate its tolerance level at different concentration of methanol.

Table 6: Effect of methanol on the stability of PS11lipase

MeOH % (v/v)	Relative activity (%)
None	100
5	123 ± 3.2
10	120 ± 2.8
20	111 ± 1.5
30	87 ± 1.2
40	72 ± 1.2
50	30 ± 0.4

Different concentration of methanol were added to enzymes solution and incubated for 24 h in a rotary shaker (180 rpm) at 50° C

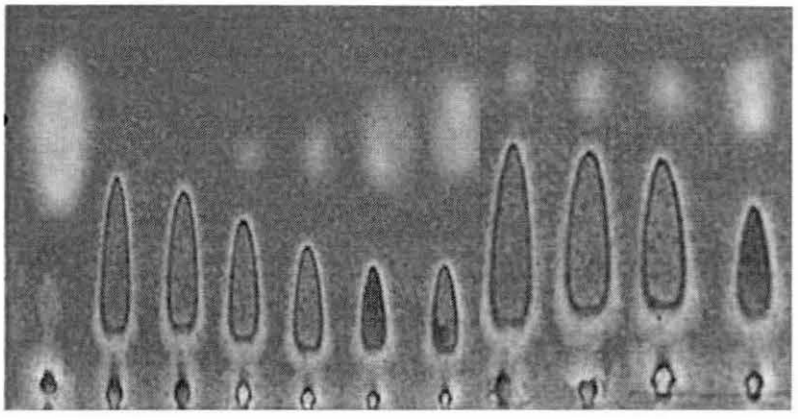
It was observed that PS11 lipase retained 87% and 72% of its activity when grown in presence of 30% and 40% (v/v) methanol respectively (Table 6.).

3.3.8 Application of lipase PS11 in biodiesel production

3.3.8.1. Determination of biodiesel production by TLC method

PS11 catalyzed the transesterification reaction of both sunflower oil and olive oil in methanol (Fig 5), though it preferred olive oil over sunflower oil. Biodiesel production reached its maximum at around 24 h and 36 h in the case of olive oil and sunflower oil respectively.

Olive oil Sunflower oil



BD NC 0h 6h 12h 24h 36h 6h 12h 24h 36h

Fig 5. Application of the purified lipase in biodiesel production. TLC analysis of PS11 lipase catalyzed biodiesel (BD) production using methanol and sunflower oil /olive oil. BD corresponds to reference and produced biodiesel. Negative control (NC) corresponds to the absence of PS11 lipase in the reaction medium.

3.3.8.2. Determination of biodiesel production by gas chromatography

Chromatogram of standard biodiesel (Fig6a) showed that there were three fatty acid methyl esters.The main fatty acid methyl esters of biodiesel include palmitic acid methyl ester, oleic acid methyl ester and linoleic acid methyl ester. These components made up more than 90% of the total biodiesel.Presence of these three main fatty acid methyl esters was also noted in PS11 lipase catalyzed trans esterification of sulflower oil in presence of methanol. GC analysis was a served as a

conclusive proof for biodiesel production by PS11.

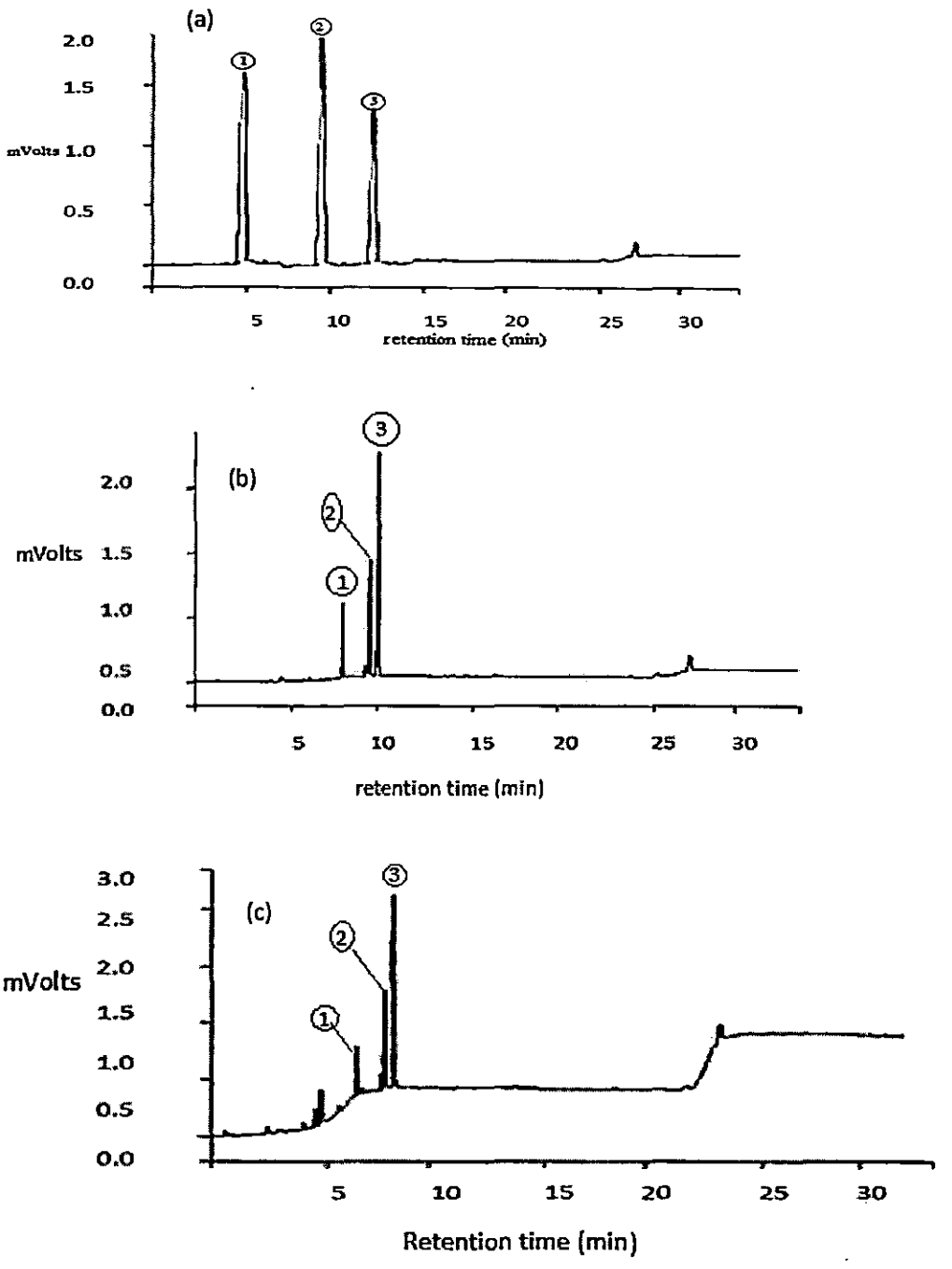


Fig 6: Gas chromatographic pattern of biodiesel from sunflower oil. Standard sunflower oil (a), Peak1. free fatty acid of palmitic acid, 2. free fatty acid of oleic acid and 3. free fatty acid of linoleic acid. Standard biodiesel (b), Biodiesel catalyzed by PS11 lipase mediated transesterification of sunflower oil (c).Peak1. palmitic acid methyl ester, 2. oleic acid methyl ester and 3. linoleic acid methyl ester.

3.4 Discussion

Microbial lipases have enormous biotechnological potential in biodiesel production due to their stability in organic solvents, broad substrate specificity and cofactor independent catalysis. Biodiesel is defined as monoalkyl ester of fatty acids from vegetable oil and produced by enzyme mediated catalytical transesterification with petrochemically derived methanol. Fang *et al.*, (2006) reported the presence of more lipaseproducingisolates in the grease-polluted soilcompared to the soilcollected from other places because there was more lipidic content in those soil samples to be utilized by microorganisms. Similarly, as the isolated strain *G. stearothermophilus* PS11 was isolated from petroleum polluted site hence it exhibited lipase production by producing clear zone of hydrolysis in tributyrin agar plate. Thus, PS11 was further employed for the production of lipase that can mediate the trans esterification of vegetable oil in presence of methanol.

Lipase production by PS11 cells was noticed during its late exponential phase but gradually enzyme production increased in stationary phase of growth. Similar finding was reported by Abada et al. (2008). The possible reason might be related to the recent finding of lipase produced by*Streptomyces rimosus* GDS(L).Vujaklija *et al* (2003) proposed a unique feature among prokaryotes in using triacylglycerols as storage compounds or as possible carbon sources for antibiotic synthesis in stationary phase. Horinouchi *et al.* (2002) proposed that hydrolysing enzymes are required for the stationary phase of bacterial growth.

Production of lipase was optimized using one variable at a time (OVAT) method. Nitrogen sources have varied effect on lipase production and sources like peptone have been reported to augment lipase production (Gunasekaran *et al.*, 2006). Peptone served as the best nitrogen source for PS11 lipase production. Similarly, *Bacillus* sp and *Pseudomonads* produced maximum lipase activity in presence of organic nitrogen sources like peptone and yeast extract (Sirisha *et al.*, 2012). Carbon source is one of the essential constituent of the microbial fermentation medium that affected the overall cellular growth and metabolism. Carbon source are reported as to inducelipases.In this study, glucose supported the highest production of lipase. The result is in agreement with the findings of Lakshmi *et al.* (1999) and Banerjee *et al.*

(1985) who reported maximum production of lipase in medium containing glucose. However, in some studies glucose has been found to cause repression of lipase production (Gupta *et al.,* 2004). Decrease of lipase activity in presence of other carbon source could be due to their catabolic repression in the medium (Kiran *et al.,* 2008). The expression of lipases was markedly influenced by lipid sources due to their substrate inducible nature. Rice bran oil served as the best substrate for PS11 lipase production. Earlier soybean and mustard oils are reported to support maximum lipase production in bacterial isolates (Kumar *et al.,* 2005). The high content of fatty acids specifically PUFA in rice bran oil (38% monounsaturated, 37% polyunsaturated and 25% saturated) might be associated with high level of lipase synthesis.

Microorganisms vary in their oxygen requirements as oxygen acts as a terminal electron acceptor for oxidative reactions to provide energy for cellular activities.The variation in the agitation speed has been found to influence the extent of mixing in the shake flasks or the bioreactor, oxygen transfer rate, surface area of contact with the media components, dispersability of the carbon source and the nutrient availability (Randa *et al.,* 2009). Highest lipase production by PS11 was influenced at the agitation rate of 150 rpm. At this speed, the aeration of the culture medium was increased, and this further led to a sufficient supply of dissolved oxygen in the media. The production of lipase was found to decrease when shaken at 180 rpm. Higher agitation rates could increase the oxygen pressure of the system but did not bring about the increase in production, probably because at a high agitation rate, the structure of enzyme would be altered. However, lowering the aeration rate could cause a reduction in the lipase yields. This indicates that a reduction in oxygen supply is an important limiting factor for lipase synthesis (Abusham *et al.,* 2009).

The present isolated strain PS11 exhibited optimum lipase production in 72 hours. The results of several other studies have shown the optimum incubation period for lipase production to be 12 to 24 h (Dharmsthiti *et al.,* 1998) which was in contrast with our results. However it was noted by Kumar *et al.,* (2012) that a high biomass was obtained at 48 h of incubation and high lipase activity was found in 72 h of incubation time in strains of *Bacillus cereus.* The production of enzyme in the late phase was also reported by others (Joseph *et al.,* 2006; Wang *et al.,* 2009). Most

Bacillus species required more than 2 days for the maximum lipase activity to occur (Kiran *et al.*, 2008).

The PS11lipase was purified from the extracellular medium by ammonium sulfate precipitation, anion exchange chromatography and gel filtration method. 8.04 fold purification of PS11 lipase with a yield of 22.67% was obtained through the mentioned purification processes. Ji *et al.* (2010) reported 4.3 fold purification of an organic solvent tolerant lipase from *P. aeruginosa* LX1 with specific activity of 156 U/mg. SDS-PAGE analysis of purified lipase revealed the presence of a prominent protein band of approximately 27 kD, indicating that the enzyme was probably a single chain protein or a homomultimeric protein of 27 kD subunits. It is almost similar to that of lipase (27.5 kDa) from *S. rimosus* (Abrami *et al.*, 1999) but lower than that of lipase (50 kDa) from *Streptomyces cinnamomeus* (Sommer *et al.*, 1997).

pH acts as an important parameter affecting the activity ofenzymes due to its effect on the structure of proteins. Lipase purified from PS11 was active at an optimum pH 10 and the enzyme was alkalostable showing stability over a pH from 8 to 11. Lipase isolated from *S. rimosus* R6-554W (Abrami *et al.*, 1999) and *S. fradiae* var. k11 (Zhang *et al.*, 2008) also showed activity over a wide range of pH from 4 to10. In contrast, thelipase purified from *Geotrichum* sp. SYBC exhibited stability at acidic pH from 3 to 6 (Cai *et al.*, 2009).

To investigate the effect of temperature, lipase activity was determined at different temperature ranging from 10 to 80°C. PS11 lipase was thermostable as it retained its activity at 60°C. Gradual decrease in the enzyme activity was noted with gradual increase of temperature. It is a well-known fact that the protein conformation is changed or degraded at higher temperatures, and hence decrease in the lipase activity was noted at higher temperatures. An extracellular SCO7513 lipase from *S. coelicolor* A3 (Barnwal and Sharma, 2005) with optimal temperature at 55°C was previously reported by Cote and Shareck (2007). Lipase from *P. aeruginosa* LX1 also showed almost the same thermostable profile retaining 100% activity at 50°C. (Ji *et al.*,2010).

Effects of inhibitors on enzyme activity primarily give an insight into the nature of an enzyme, its cofactor requirements, and the nature of the active center (Sigma and Mooser, 1975). Interestingly, EDTA, a metal ion chelator didn't alter the

enzymatic activity of PS11 lipase indicating that functioning of enzyme did not require metal cofactor. Our results are in agreement with Saeed *et al.* (2005) and Lin and Ko (2005) who also showed that activity of lipases produced from *Bacillus*sp. and *Pseudomonas* sp. were not affected by EDTA. PS11 lipase activity was inhibited by H_2O_2 whereas β mercaptoethanol showed stimulating effect on the enzyme. Dheeman *et al.* (2011) and Soliman et al. (2007) also observed the similar effect of mercaptoethanol on lipase from *Amycolatopis mediterranei* and *Geobacillus thermoleovorans*, respectively.This increase in lipase activity in presence of β mercaptoethanol could be explained as inhibition of oxidation of sulfhydryl groups, in lipase (Gupta *et al.*, 2004).

PS11 lipase activity was increased by Tween-80, Tween-60and Tween-20 with maximum enhancement of 168% in presence of Triton-X-100. Similar report of lipase purified from *Streptomyces* sp. CS133 was activated byTriton X-100, Tween-80 and Tween-20 (Mander *et al.*, 2012). Catalytic activity of lipases is governed by interfacial activation, a property observed when the lipid substrate starts to form an emulsion thereby presenting an interface for the enzyme to act. Addition of a detergent decreases the surface tension between the organic and aqueous phase present in the reaction mixture and enhances the rate of emulsification. The non-ionic detergents weaken hydrophobic interaction within the protein resulting in disaggregation and thus stimulating the enzyme activity. The enhanced PS11lipase activity by tweens could be due to their effectas both inducers because of their chemical similarity to the natural substrates and surfactants, stimulating the enzyme release as suggested by Epsinosa *et al.* (1990). On the other hand, PS11 lipase activity was found to decrease in presence of anionic detergents (SDS, N-lauryl sarcosine) and zwitterionic CHAPS. The anionic detergents may inactivate protein by acting upon the disulphide linkages resulting in decline in activity.

The toxicity of the organic solvents is usually measured by log *P* value (the partition coefficient of the given solvent in an equimolar mixture of octanol and water). The greater is the polarity, the lower the log *P* value and the greater the toxicity of solvent. In this study, various low polarity, water immiscible solvents (high log *P*) such as *p*-xylene, *n*-hexane, toluene and benzene were tested. In addition, high polarity, water-miscible solvents, low log *P* (methanol, ethanol,

acetonitrile and acetic acid) were also investigated. The stability of the PS11 lipase in organic solvents did not follow the log *P* trends. It is well known that water acts as a lubricant that affords a high conformational flexibility to enzyme molecules. If one follows the trends of log *P*, the lower the log *P* values, the less hydrophobic the solvent, so the enzyme is less stable and there may be change in the conformation of the enzyme molecules. However, different organic solvents showed different tolerance profiles to the PS11 lipase. Lipase production was enhanced in presence of solvents having higher log *P*-value. The lipase was stable and restored almost 90% of its activity when p-xylene, benzene, toluene, hexane and methanol (25% v/v each) were added to the purified enzyme. The residual activity was 76% in presence of ethanol (Table-5). Acetic acid was more harmful than otherorganic solvents as PS11 lipase could restore only 18% of its activity. This may due to the acidity inactivating the lipase.It is well-known that the effect of organic solvents on enzyme activity differs from lipase to lipase (Sugihara *et al.*, 1992). There was no clear correlation between the solubility of an organic solvent in water and stability of lipase in its presence (Ogino *et al.*, 2000).

Stability of PS11 lipase in presence of methanol led to explore the tolerance profile of the enzyme in varied concentration of methanol as it is one of the widely used media for biodiesel production. PS11 lipase retained 87% and 72% of its activity when grown in presence of 30% and 40% (v/v) methanol respectively A similar result was shown by the lipase from *Staphylococcus saprophyticus* M36; the residual activity was 32% and 36% respectively, when methanol and ethanol (25% v/v) were added to the enzyme for 15 days at 30°C and 160 rpm (Fang *et al.*, 2006). In contrast, lipase isolated from *Photobacterium lipolyticum* for biodiesel production lost its stability even at very low concentration (10% v/v) of methanol (Yang *et al.*, 2009). High stability PS11 lipase even in presence of higher concentration of methanol (30- 50% v/v) makes it a promising tool in the field of biodiesel production.

Enzymatic transesterification of sunflower oil by PS11 lipase in presence of methanol was carried out to check the ability of lipase for biodiesel production. Commonly, the time period required by various microbial lipases to catalyzetrans-esterification reaction varies in the range 5–72 h (Bajaj *et al.*, 2010). However, the reaction time for PS11 lipase was significantly lesser than the time reported by

Kumari *et al.* (2009) and Yang *et al.* (2009) of 60 h and 72 h, respectively, for transesterification of jatropha and soybean oil in methanol using a lipase extracted from *Enterobacter aerogenes* and *P. fluorescens*, respectively

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Conclusion

Currently, India has 18 oil refineries in the public and private sectors, with a gross refining capacity of over 112 million tones approximately. Every year tones of oil are spilled in marine and terrestrial area. The spilled petroleum hydrocarbons are one of the main environmental pollutants. Their abundance and persistence in several polluted environmental areas have been a matter of concern over years. A number of approaches have been developed for cleaning up the oil spills in marine shorelines and land. Researchers have often preferred bioremediation to clean up the toxic wastes from the environments over the conventional physical and chemical methods. Biodegradation of organic wastes is a useful side effect of microbial metabolism, thus the fundamental principles of biodegradation are integrally linked to microbial physiology. Degradation may be enhanced at some sites because of the adaptation of microorganisms to chronic exposure to chemicals.

Therefore, this study was taken up to isolate a bacterial strain that could efficiently degrade petroleum hydrocarbon along with other organic solvent wastes. The underlying mechanism of degradation and the simultaneous effect of organic wastes on the bacterial cell were also part of the study. Efforts were given to study the application of the isolated strain in biodiesel production.

Thus, it can concluded from the study that

- Among the 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.

- The isolated PS11 strain could utilize crude oil. However, presence of crude oil decreased its growth rate compared to cells grown in absence of crude oil.

- PS11 cells also exhibited growth in presence of various other toxic solvents having a wide range of log *P* (2-4) value. Solvents having log P value less than 1 inhibited its growth.

- Transmission electron microimages showed that presence of organic solvent initially affected membrane system of PS11 cells. Continuous exposure of

solvents resulted in reorganization of cell membrane indicating solvent tolerant characteristic of PS11 cells.

- Gas chromatography analysis showed complete degradation of aromatic compounds and partial metabolic transformation of alkanes in TPH by PS11 strain.

- Complete degradation of benzene and toluene with partial degradation of xylene and ethylbenzene by PS11 cells was also noted in gas chromatograph.

- Membrane adaptation profile of PS11 cells in presence of petroleum hydrocarbon was noted using gas chromatography. Increase in membrane glycolipid and decrease in membrane straight chain fatty acid with simultaneous increase in loosely packed branched chain iso-fatty acid indicated increased membrane stability.

- PS11 cells harbored a mega plasmid of 20 kb as confirmed by agarose gel electrophoresis followed by restriction digestion profile analysis. The role of mega-plasmid in petroleum hydrocarbon degradation was studied by plasmid curing assay. Plasmid cured cells of PS11 could not grow in presence of crude oil, thus, confirming mega plasmid mediated petroleum hydrocarbon degradation.

- *E.coli* JM109 cells were transformed with mega plasmid of PS11. Growth of the transformed JM109 cells in presence of crude oil provided a conclusive proof for the involvement of plasmid DNA in the degradation of petroleum hydrocarbon.

- TPH and BTEX degradation by transformed *E. coli* JM109 generated similar chromatograph as wild PS11 in the same time period.

- PCR amplification of gene encoding catechol 2, 3 dioxygenase, an enzyme involved in catechol metabolism, a common intermediate of aromatic petroleum hydrocarbon meta degradation pathway, using plasmid as template successfully yielded a PCR product of about 900 bp.

- Sequence analysis of the PCR product (916 bp) showed 100% nucleotide homology and amino acid with catechol 2, 3 dioxygenase.

- PS11 cells produced an extracellular, organic solvent tolerant, alkaline lipase. Optimization of production conditions by OVAT approach enhanced the enzyme production by 2.46 folds.

- Lipase was purified from the extracellular medium by ammonium sulfate precipitation followed by anion exchange chromatography and gel filtration. PS11 lipase was purified by 8.04 fold with 22.6% yield.

- SDS-PAGE analysis of purified lipase indicated that the enzyme was probably a single chain protein or a homomultimeric protein of 27 kD subunits.

- Optimum pH and temperature for lipase was 10 and 50 °C, respectively. It showed 100% stability in the pH range 8 to 11 for 2h. The enzyme retained 50 % activity at 70 °C for 2h.

- Organic solvent tolerance property of lipase from PS11 cells was confirmed when the enzyme restored 90% of its activity in presence of p-xylene, benzene, toluene, hexane and methanol.

- High stability of PS11 lipase even in presence of higher concentration of methanol (30- 50% v/v) makes it a promising tool in the field of biodiesel production.

- Gas chromatography analysis of the enzymatic trans-esterification of sunflower oil in presence of methanol confirmed the catalytic activity of lipase from PS11 cells in biodiesel production

Thus, the isolated strain *Geobacillus stearothermophilus* PS11 could serve a dual purpose towards "GREEN ECOSYSTEM". Firstly, it can be employed for cleaning up of petroleum hydrocarbon wastes and secondly, for ecofriendly biofuel production.

Abbreviations

NH_3	Ammonia
A	Anteiso
Bp	Base pair
BTEX	Benzene, Toluene, Ethylbenzene, Xylene
BLAST	Basic Logarithm Alignment Search Tool
CO_2	Carbon dioxide
C 2,3 O	Catechol 2, 3 dioxygenase
cfu	Colony forming unit
$CaCl_2$	Calcium chloride
°C	Degree Celsius
DDBJ	DNA Data Bank of Japan
DMSO	Dimethyl sulfoxide
DPG	Diphosphatidyl glycerol
EPS	Extracellular Poly Saccharide
EPA	Environmental Protection Agency
FAME	Fatty Acid Methyl Esters
GC	Gas Chromatography
G+C	Guanine+Cytosin
GC-MS	Gas Chromatography-Mass Spectroscopy
GEM	Genetically Engineered Microorganism
h	Hour
H_2O_2	Hydrogen peroxide
i	Iso
IARC	Indian Agricultural Research Council
kD	Kilo dalton
kb	Kilo basepair
km	Kilometer
kV	Kilo volts
l	Litre
LB broth	Luria berteni broth
log P	Partition coefficient of solvent in equimoler concentration of n –octanol and water
ml	Mililitre
min	Minutes
mM	Milimolar
µg	microgram
MULTALIN	Multiple alignment
nm	Nanometer
NTP	Nicotinamide tri phosphate
NADPH	Nicotinamide adenosine di phosphate hydrogen
NCP	National control of pollution
O_2	Oxygen
OD	Optical density
ORF	Open reading frame

PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PGL	Phosphoglycolipid
PCR	Polymerase chain reaction
PAH	Poly aromatic hydrocarbon
ppm	Parts per million
PVA	Poly vinyl alcohol
rpm	Rotation per minute
$NaHCO_3$	Sodium bi carbonate
NaCl	Sodium chloride
sq	Square
SDS	Sodium dodecyl sulfate
TPH	Total petroleum hydrocarbon
TAE	tris
TLC	Thin layer chromatography
UV	Ultra violet
v/v	Volume/volume
w/v	Weight/volume

Thesis related publications

Manuscript accepted in peer reviewed Journal of Microbiology, Biotechnology and Food Sciences. Vol 4 (Feb 2015 issue)

REGULAR ARTICLE

Purification and characterization of solvent stable lipase from a solvent tolerant strain of *Geobacillus stearothermophilus* PS 11

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ABSTRACT

An extracellular organic solvent stable lipase produced by solvent tolerant strain of *Geobacillus stearothermophilus* PS11 was purified and characterised. The overall purification was 8.04 fold with a yield of 22.6%. The molecular weight of purified lipase was approximately 27.5 kDa. The purified lipase activity was stable (745 EU/ml at 72h incubation) in presence of toluene, benzene, propanol, methanol etc. The enzyme activity was maximum (764 EU/ml) when assayed under optimum temperature and pH of 50°C and 10.0, respectively. The enzyme showed stability at a wide range of temperature from 10°C to 60°C. This solvent stable lipase can be a novel tool for biodiesel industry.

Keywords: Solvent stable, lipases, *Geobacillus stearothermophilus*

INTRODUCTION

Diesel fuels are the key to the industrial economy of a developing country. With the industrialization bloom in the whole globe, the world is confronted with the twin crises of fossil fuel depletion and environmental degradation **Van. (2005)**. In order to resolve these problems, researchers are interested to find an alternative environmental friendly reproducible diesel fuel. Biodiesel is an alternative diesel fuel that is produced from vegetable oils or animal fats. It have the following advantages over diesel fuel: produce less smoke and particulates, have higher cetane numbers, produce lower carbon monoxide and hydrocarbon emissions, are biodegradable, and nontoxic engine lubricity to low sulfur diesel fuels. With this huge range of advantages, it is gaining more and more importance as an alternative fuel. Lipase is the key enzyme used to produce biodiesel **(Marchetti *et al.* 2005, Barnwal and Sharma 2005)**. Only organic solvent tolerant lipase, especially tolerating methanol and ethanol, can distinctly improve the production of biodiesel **(Kaieda *et al.* 2001)**. Till date, the number of bacteria producing organic solvent tolerant lipase is limited, and most of the isolated organic solvent-tolerant strains belong to the genera are *Pseudomonas* isolated from soil or marine samples [5]. We have previously isolated a solvent tolerant strain of *Bacillus thermophilus* PS11 (now known as *Geobacillus stearthermophilus*) that could grow in presence of wide range of solvents. In this work, we report that the bacterial strain is capable of producing lipase that is stable in presence of various solvents such as toluene, n-octanol, propanol, methanol, benzene etc. This will open novel and simpler routes for the synthetic processes and in turn pave a route to alternative biodiesel production.

MATERIAL AND METHODS

Isolation of lipase producing solvent tolerant bacterial strains

Solvent tolerant strain of *Geobacillus stearothermophilus* PS11 (accession no KC311354), previously isolated from soil is used in this study **Sarkar and Ghosh (2012)**. It was plated on nutrient agar supplemented with 1% tributyrin. After 48 h of incubation at 37°C, the lipolytic activity was confirmed by the formation of a clear zone around the colonies.

Measurement of bacterial growth

Overnight grown culture of PS11 strain was inoculated in nutrient broth overlaid with 5% tributyrin and incubated at 37 °C under shaking condition at 140 rpm. Growth and dry cell mass of the isolate was determined according to the process described by **Sarkar and Ghosh (2012)**.

Production media and enzyme preparation

A 1% (v/v) bacterial suspension was transferred from an overnight nutrient broth seed culture to the basal production medium (LPM-1) composed of (g l^{-1}): peptone 5, beef extract 3, sodium chloride 2, tween 80 5 and olive oil 10, pH 8. Bacterial cells were grown at 37 °C under shaking condition at 140 rpm for 96 h. After every 24 h cells were removed by centrifugation and the supernatant was desalted and used for measurement of lipase activity.

Lipase assay and protein content determination

Lipase activity was measured on the basis of hydrolysis of p-nitro phenyl palmitate (pNPP). A 2.5 ml of the assay mixture consisted of, 2.4 ml of 50 mM Tris HCl (pH 9) with pNPP and 0.1 ml concentrated enzyme preparation. Incubation of the reaction mixture at 60°C for 10 minutes was followed by addition of 200 µl of 1 M calcium chloride. The precipitation of

free fatty acids with calcium chloride was used to detect lipase activity. Release of p-nitrophenol (pNP) from pNPP was measured as the increase in absorption at 410 nm. One lipase unit is defined as the amount of enzyme that liberated 1 µM p-nitro phenol per minute under the assay conditions.

Optimization of production media

Optimization of process parameters and manipulation of media composition are the most important techniques used for the overproduction of lipase to meet industrial demands. Optimization was carried out through modification of several growth parameters. For these, the bacterial cells were grown for 96 h at 37 °C under shaking condition. The cells were removed by centrifugation and the supernatants were used for measurement of lipase activity. The various parameters optimized for obtaining maximal lipase yield were, incubation time (24, 48, 72 and 96 h), agitation speed (100 – 200 rpm). Substrate specificity was determined using 1% (v/v) rice bran oil, sunflower oil, soybean oil, olive oil, and mustard oil. Further the effect of various carbon sources (1% w/v) such as glucose, sucrose, lactose, maltose, and nitrogen sources (1% w/v), that is, soybean meal, sodium nitrate, peptone, beef extract, yeast extract were also examined for production of lipase. For each step lipase activity was assayed under standard condition to know the optimal yield.

Purification and Molecular weight of crude enzyme

The bacterial isolate was cultured in LPM-1 for 72 h at 37°C. Bacterial cells were removed by using cooling centrifuge (4°C) at 5000 rpm for 10 min. The enzyme was precipitated by adding solid ammonium sulphate (0-40%) at 4°C for 24 h. The precipitate was collected by centrifugation at 10,000 rpm for 40 min and dissolved in 50 mM Tris HCl buffer (pH 9.0). It was then desalted by dialysis with a cellulose dialysis bag (3500 Da pore-size) in same buffer. The desalted enzyme preparation was loaded on to DEAE-sephacel column. The active fractions were collected, lyophilized and applied on sephadex G75 column. The active fractions were used to determine the molecular weight. Protein content (mg ml -1) was determined by Lowry method **Lowry *et al.* (1951).**
Molecular weight of lipase was detected on 15% polyacrylamide slab gel using mini gel system Bio-Rad. Low molecular weight protein standard ranged from 20-97 KD was used to determine the molecular weight of the purified lipase.

Effect of organic solvent on lipase stability

In order to study the effect of organic solvents on enzyme stability, suitably diluted purified lipase in 50 mM Tris–HCl (pH 9.0) was mixed with different organic solvents to yield a final concentration of (25%, v/v) and then the mixture was incubated on a shaking incubator (180 rpm) at 4 °C for 48 h. The residual lipase activity was measured using p-NPP method. The used organic solvents were hexane, xylene, toluene, benzene, dichloromethane, diethylether, ethylacetate, isopraponal, acetone, acetonitrile, methanol and dimethyl sulfoxide (DMSO). The initial lipase activity (without containing organic solvents) was considered to be 100%.

Effect of pH on activity and pH stability of PS11 lipase

The optimum pH of purified lipase was determined by incubating it with various buffers at a pH range of (4.0–12.0) and lipase activity was determined under standard assay conditions. The pH stability of the enzyme was characterized by preincubating the purified enzyme with different buffers at pH range from 4.0 to 12.0 at 4 °C for 2 h and assayed for lipase activity. Buffer systems were used at a concentration of 50 mM: acetate–HCl buffer (pH 4.0–5.0), sodium hydrogen phosphate–NaOH buffer (pH 6.0–7.0), Tris–HCl buffer (pH 7.0–9.0), disodium hydrogen orthophosphate–NaOH buffer (pH 9.0–11.0), and glycine–NaOH buffer (pH 10.0–12.0).

Effect of temperature on activity and temperature stability of PS11 lipase

The effect of temperature on purified lipase was determined by incubating the reaction mixture at a temperature range of 10°-80° C under standard assay conditions. The thermostability of the enzyme was determined by incubating the enzyme at different temperatures from 10° to 80° C for 2 h and lipase activity was determined.

RESULTS AND DISCUSSION

Measurement of bacterial growth and lipase production

Time course of cell growth and lipase production by PS11 in production medium are shown in figure 1. The bacteria exhibited a minimal lag phrase. The exponential phase lasted up to 18 h and the stationary phase continued till 90 h. A slight decline in biomass was noted after 96 h probably due to nutrient depletion. The findings are similar with results reported by **Abada (2008).**

Lipase production was noted from the early stationary phase (24h) but the production attained maximum level (760 EU/ml) during the late stationary phase (72h) of the microorganism suggesting that the extracellular lipase is a secondary metabolite. Furthermore, the enzyme activity gradually decreased after 72h. Therefore, the optimum incubation periods was maintained throughout the studies.

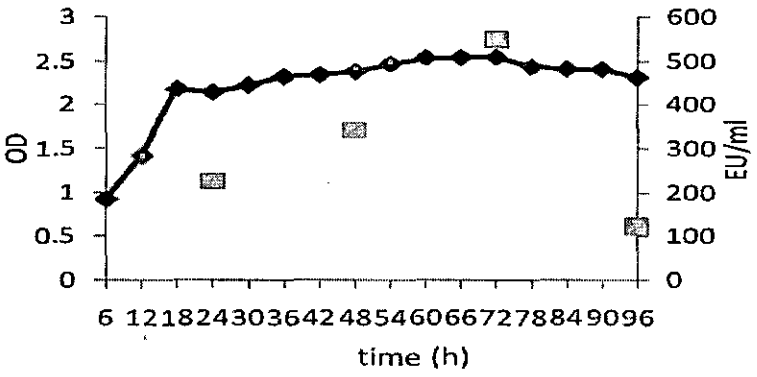

Figure 1 Determination of bacterial growth (◆) and enzyme (□) production.

Optimization of lipase production media

Different sources of nitrogen were tested in order to determine their influence on the synthesis of lipase. Peptone showed maximum enzyme activity (310 EU/ml) as shown in figure 2a. **Gunasekaran and Das (2004)** has also reported about peptone to augment lipase production

Since each microorganism requires a different carbon source to produce lipase at its maximum level, the influence of different carbon sources was tested. Glucose (367.5 EU/ml) was the best carbon source (figure 2b). In some cases glucose has been found to cause repression of lipase production but the case was opposite for PS11 where glucose acted as an inducer. The present study is in agreement with the findings of (**Lakshmi *et al.* 1999** and **Banerjee *et al.* 1985**) who reported maximum production of lipase in medium containing glucose.

Lipidic sources seem to be essential for obtaining a high lipase yield. Among the various lipidic carbon source tested rice bran oil (370 EU/ml) best supported lipase production by PS11 (figure 2c.). The high content of fatty acids specifically PUFA in rice bran oil (38% monounsaturated, 37% polyunsaturated, and 25% saturated) might support high level of lipase synthesis.

The production of lipase was observed till 96h of incubation and it was highest at 72h (765 EU/ml) during stationary phrase. The results of several other studies have shown the optimum lipase production at varying time period between 12 to 24 h **Dharmsthiti *et al.* (1998)** which was in contrast with our results. However it was noted by **Kumar *et al.* (2005)** that a high biomass was obtained at 48 h of incubation and high lipase activity was found in 72 h of incubation time in strains of *Bacillus cereus.* Most *Bacillus* species required more than 2 days for the maximum lipase activity to occur (**Joseph *et al.* 2006, Wang *et al.* 2009**).

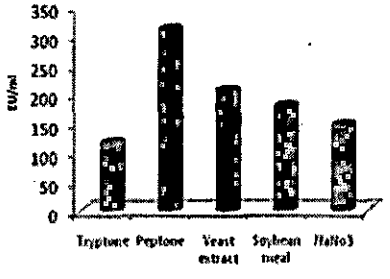

Figure 2(a)

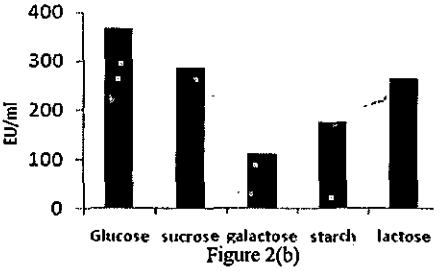

Figure 2(b)

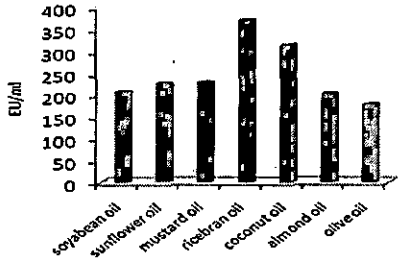

Figure 2(c)

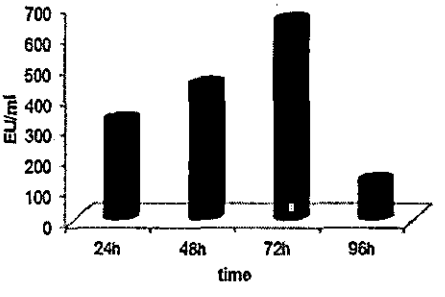

Figure 2(d)

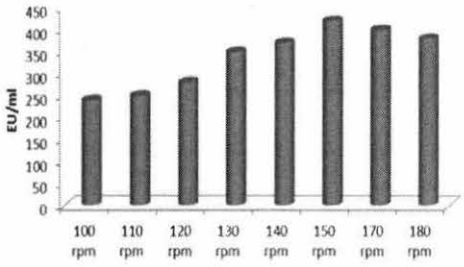

Figure 2(e)

Figure 2 Optimization of lipase production. a. nitrogen source, b. carbon source, c. substrate, d. incubation time, e. agitation rate

Agitation rates influenced the lipase yield and cell growth. Lipase production increased with increase in agitation speed and reached a maximum at 150 rpm (420 EU/ml) due to sufficient supply of dissolved oxygen in the media. Further increase in agitation lowered the production of lipase (figure 2e) probably because at a high agitation rate, the structure of enzyme would be altered Micro-organisms vary in their oxygen requirements as oxygen acts as a terminal electron acceptor for oxidative reactions to provide energy for cellular activities. The variation in the agitation speed influence the extent of mixing in the shake flasks or the bioreactor, oxygen transfer rate, surface area of contact with the media components, dispersability of the carbon source and the nutrient availability.

Purification and molecular weight determination

Lipase was purified from the extracellular medium by ammonium sulfate precipitation followed by different column chromatography. A large amount of lipase was lost after ammonium sulfate precipitation; less than 5O% of the precipitated lipase was resolubilized. Our three step purification protocol to purify the lipase enzyme from PS11 involving ammonium sulfate precipitation, ion exchange chromatography, and gel filtration chromatography resulted in 8.04 fold purified lipase with 22.6% recovery rate (Tab 1) and the characterizations of the purified enzyme revealed a molecular mass of 27.5 kDa in SDS-PAGE (Figure 3).

Table 1 Purification of lipase from PS11 strain

Step	Total EU	Total Protein(mg)	Specific activity (EU/mg)	Purification fold	Yield (%)
Crude	323.60	28.72	11.26	1	100
Ammonium sulphate	152.60	2.90	52.62	4.67	47.00
DEAE-sephacel	83.60	1.46	56.96	5.05	25.83
Sephadex G-75	73.37	0.81	90.58	8.04	22.67

Figure 3 SDS-PAGE of purified lipase from *Geobacillus stearothermophilus* PS 11. Lane a: purified lipase from *Geobacillus stearothermophilus* PS 11. Lane b: protein standard : phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and soyabean trypsin inhibitor (20.1 kDa).

Organic solvent stability of lipase

To date, some organic solvent stable lipases have been reported. The toxicity of the organic solvents is usually measured by log P value (the partition coefficient of the given solvent in an equimolar mixture of octanol and water). The effect of different organic solvents on purified lipase is shown in Table 2. In this study, organic solvents with various log P value ranging from low to high were selected. The lipase was stable and restored almost 90% of its activity when p-xylene, benzene, toluene, and hexane (25% v/v each) were added to the purified enzyme for 2h at C and 1 0 rpm. In presence of methanol and ethanol, the residual activity was 42% and 46%, respectively.

Table 2 Effect of different organic solvent on lipase stability

Organic solvents	Log P	Relative activity (%) at concentration of 25% (v/v)
Control	—	100
Methanol	-0.76	90
Acetonitrile	-0.33	30
Ethanol	-0.24	76
Acetic acid	-0.23	18
Benzene	2.0	81
Toluene	2.5	92
P-xylene	3.1	91
n-Hexane	3.1	80
Hexane	3.6	86

25% (v/v) of organic solvents were added to the enzyme solution and incubated for 48 h in a rotary shaker (180 rpm) at 4 °C.

A similar result was shown by the lipase from *Staphylococcus saprophyticus* M36; the residual activity was 32% and 36%, respectively, when methanol and ethanol (25% v/v) were added to the enzyme up to for 15 days at 30 C and 160 rpm **Fang *et al.* (2006)**.

The stability of the PS11 lipase in organic solvents did not follow the log *P* trends. The greater is the polarity, the lower the log P value and the greater the toxicity of solvent. It is well known that water acts as a lubricant that affords a high conformational fexibility to enzyme molecules. If one follows the trends of log*P*, the lower the log *P* values, the less hydrophobic the solvent, so the enzyme is less stable and there may be change in the conformation of the enzyme molecules. However, different organic solvents showed different tolerance profiles to the PS11 lipase. It is well-known that the effect of organic solvents on enzyme activity differs from lipase to lipase **Sugihara *et al.* (1992)**. There was no clear correlation between the solubility of an organic solvent in water and stability of lipase in its presence **Ogino *et al.* (2000)**.

Effect of pH on activity and pH stability of PS11 lipase

PS11 was inoculated in the lipase production medium and incubated at a wide range of pH (4- 12). At pH 10, maximum lipase activity of 417.5 EU/ml was observed (Figure 4.).The enzyme activity gradually increased from pH 5 to its maximum at pH 10.

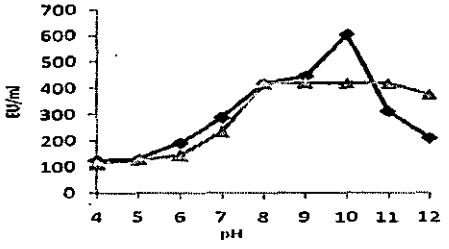

Figure 4 Determination of optimum pH (◈) and pH stability (△) of PS11 lipase

The pH of the production medium plays a critical role for the optimal physiological performances of the bacterial cell and the transport of various nutrient components across the cell membrane aiming at maximizing the enzyme production. **Moon and Parulekar (1991)** reported that the pH of culture has been shown to strongly affect many enzymatic processes and transportation of various components across the cell membrane. The effect of pH on the stability of lipase was tested by incubating the enzymes over a range of pH values. The enzyme also exhibited stability in high alkaline pH which is an important industrial attribute.

Effect of temperature on activity and temperature stability of PS11 lipase

Temperature is a critical parameter which needs to be controlled and this usually varies from organism to another. The optimum temperature for the lipase production was 50°C, although at 40°C the enzyme activity was good. The lipase activity gradually decreased and reached a minimum at 70^0 C (Figure 5).

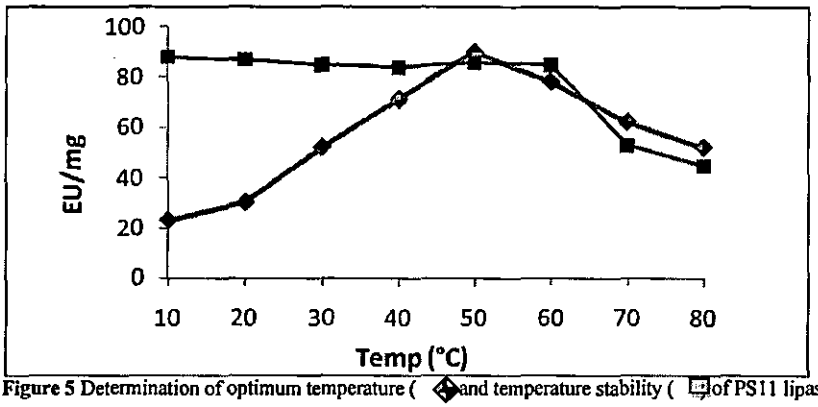

Figure 5 Determination of optimum temperature (◆) and temperature stability (□) of PS11 lipase

The enzyme stability was tested by exposing the enzyme to different temperatures for 2 h. PS 11 showed highest activity at 50°C and its activity remained stable from 10°C to 60°C. The activity further declined as the temperature increased to 70°C showing that the enzyme was not stable at high temperatures. The studies by **Frankena *et al.* (1986)** showed that there was a link between enzyme synthesis and energy metabolism in bacteria, and this was controlled by the temperature and oxygen uptake. As for the extra-cellular enzymes, temperature was found to influence their secretion, possibly by changing the physical properties of the cell membrane. It is a well-known fact that the protein conformation is changed or degraded at higher temperatures, and hence a decrease in the lipase activity was noted at higher temperatures.

CONCLUSION

The major challenge of biodiesel catalyzed by lipase is that its activity significantly reduces in presence of organic solvents, especially methanol and ethanol. Therefore, in this study the solvent tolerant lipase of 27.5kDa was purified from *Geobacillus stearothermophilus* PS11 that could be very useful for biodiesel production as it is stable in presence of methanol and ethanol.

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DEGRADATION OF AROMATIC PETROLEUM HYDROCARBONS (BTEX) BY A SOLVENT TOLERANT BACTERIAL CONSORTIUM

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Abstract: Petroleum aromatic hydrocarbons like benzene, toluene, ethyl benzene and xylene, together known as BTEX, has almost the same chemical structure. These aromatic hydrocarbons are released as pollutants in the environment. This work was taken up to develop a solvent tolerant bacterial consortium that could degrade BTEX compounds as they all share a common chemical structure. We have isolated almost 60 different types of bacterial strains from different petroleum contaminated sites. Of these 60 bacterial strains almost 20 microorganisms were screened on the basis of capability to tolerate high concentration of BTEX. Ten different consortia were prepared and the compatibility of the bacterial strains within the consortia was checked by gram staining and BTEX tolerance level. Four successful microbial consortia were selected in which all the bacterial strains concomitantly grew in presence of high concentration of BTEX (10% of toluene, 10% of benzene 5% ethyl benzene and 1% xylene). Consortium #2 showed the highest growth rate in presence of BTEX. Degradation of BTEX by consortium #2 was monitored for 5 days by gradual decrease in the volume of the solvents. The maximum reduction observed was 85% in 5 days. Gas chromatography results also reveal that could completely degrade benzene and ethyl benzene within 48 hours. Almost 90% degradation of toluene and xylene in 48 hours was exhibited by consortium #2. It could also tolerate and degrade many industrial solvents such as chloroform, DMSO, acetonitrile having a wide range of log P values (0.03–3.1). Degradation of aromatic hydrocarbon like BTEX by a solvent tolerant bacterial consortium is greatly significant as it could degrade high concentration of pollutants compared to a bacterium and also reduces the time span of degradation.

Keywords: BTEX degradation; solvent tolerant; microbial consortium

INTRODUCTION

Release of petroleum hydrocarbons in the environment has of late attracted the researchers. One particular concern is the contamination of drinking water sources by the toxic, water soluble and mobile petroleum components like, benzene, toluene, ethyl benzene and xylene (BTEX). Some BTEX compounds persist in the environment at levels exceeding regulatory thresholds (Anneser *et al.*, 2008).

They are widely used chemical substances in several industrial processes (Lin *et al.*, 2010), besides being present in high amounts in fossil fuels (ASTDR, 2004), what determines contamination of atmosphere, soil and waters. The motility rate of such hydrocarbons in soil-water systems is related to their low octanol–water partition coefficient that leads to slow soil absorption and, consequently, a preferential water transport, thereby favoring the contamination of water.

These compounds usually occur at trace levels in superficial waters as a result of their volatility. However, they can be found in high concentrations in groundwater and are considered as the priority contaminants of such resources (Falcó & Moya, 2007). The frequency of groundwater contamination with hydrocarbons, including BTEX, has been increasing (Reusser *et al.*, 2002), demanding the development of more efficient methods to remove or minimize the damages caused by these compounds. Several factors, such as pollutant concentration, active biomass concentration, temperature, pH, availability of inorganic nutrients and electron acceptors, and microbial adaptation, influence the rate and extent of biodegradation of BTEX.

Several studies have been carried out in order to find out efficient microorganisms for BTEX degradation, so they could be used in environmental remediation for this mixture. Affinity of a bacterial strain towards a particular hydrocarbon ensures a wide choice of degradation. As most of the aromatic hydrocarbon pollutants have a similar structure it is possible that the bacterial strain showing affinity towards one hydrocarbon shows affinity towards other related aromatic hydrocarbon. Degradation of benzene in the presence of other aromatic compounds was found to be stimulated by the presence of either toluene or o-xylene (Arvin *et al.*, 1989). Several researchers have reported that toluene is degraded more readily than benzene in aquifer systems (Wilson *et al.*, 1990). However, the opposite trend was observed in other field studies (Russer *et al.*, 2002).

Recent work shows that a single bacterium cannot degrade a wide range of organic solvents. In 2000 Harwood *et al.* reported that bacteria that can tolerate and degrade a particular organic solvent shows affinity towards other organic solvents having almost similar structure. Emphasis has been given on development of microbial consortia capable of degrading a wide variety of organic solvents (Mac Carthy *et al.*, 2001; Singh *et al.*, 2003).

Therefore, this study was taken up to develop a bacterial consortium that can effectively degrade high concentration of BTEX compounds in short time span. Further, solvent tolerance property of the consortium was determined in presence of various industrial solvents having a wide log *P* value.

MATERIALS AND METHODS

Microbial isolates

Soil samples were collected from different hydrocarbon rich areas in Siliguri, W.B., India. The soil samples were suitably diluted using standard serial dilution procedure and inoculated into 20 ml nutrient broth over laid with 1% v/v benzene in 100 ml sealed serum bottles and incubated at 37°C with constant shaking at 120 rpm orbital shaker for 5 days. 0.1% of these 5 days old inoculum were then plated in nutrient agar plates and incubated overnight at 37°C. Growing colonies were purified by repeated streaking on agar plates. The isolated strains were maintained on nutrient agar slants at 4°C.

Culture conditions

Inoculam of the strains were prepared by inoculating them in nutrient broth overlaid (v/v) with benzene (1–10%) in 100 ml sealed serum bottles and incubated at 37°C with constant shaking at 120 rpm orbital shaker. The cultures in absence of the solvent and uninoculated media enriched with the solvent were used as control under similar condition.

Screening of BTEX tolerance strain

BTEX tolerance by bacterial strains was determined by inoculating 20 ml nutrient broth supplemented separately with benzene (1–10%), toluene (1–10%), ethylene benzene (0.1–5%) and xylene (0.5–1%) in 100 ml screw cap flask with 1% overnight grown enriched culture and incubated at 37°C at 180 rpm. All the experiments were carried in duplicate.

Preparation of Bacterial Consortia

To prepare successful microbial consortium, bacterial cultures must be compatible with each other in order to concomitantly degrade BTEX. Ten different consortia were prepared and incubated overnight at 37°C in 120 rpm. The compatibility of the bacterial strains within the consortia was checked by gram staining (Sarkar *et al.*, 2011). Microbial consortium was prepared by inoculating 5ml of overnight grown bacterial strains in 20ml of nutrient broth overlaid with 1% (v/v) benzene.

Growth of consortia in presence of BTEX

One percent of the overnight grown enriched consortia, that showed tolerance towards high concentration of BTEX, were inoculated in 20 ml nutrient broth supplemented separately with benzene (10%), toluene (10%), ethylene benzene (5%) and xylene (1%) in 100ml screw cap flask and incubated at 37°C under shaking (180 rpm) for 48 h. Growth of the bacterial consortia in presence of BTEX was determined by increase in O.D. at 660nm at a constant time interval of 8 h till 48 h. The cultures in absence of the solvent and uninoculated media enriched with the solvent were used as control under similar condition. All the experiments were carried in duplicate.

DETERMINATION OF BTEX DEGRADATION

Gas chromatography analysis of BTEX Degradation

The degradation of the BTEX was analyzed using 1% of the enriched bacterial consortium. It was inoculated in four different 100ml capacity serum bottles filled with 20 ml of nutrient broth overlaid with 10% v/v of toluene & benzene, 5% xylene and 1% ethyl benzene separately. The bottles were then closed with Teflon-coated septa and aluminum caps and were incubated for 48h at 37°C under 180 rpm. Degradation of BTEX was monitored in Perkin–Elmer 900 gas chromatograph provided with a flame ionization detector. Separation was carried out on 181m X 0.76mm 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 and the concentration of the volatile compound was determined 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 the solvent were used as control under similar condition. All the experiments were carried in duplicate.

Degradation of other organic solvents

The solvent tolerance property was determined by inoculating the bacterial consortia in nutrient broth overlaid with different organic solvents (10% v/v) with log P_{ow} values ranging 0.28–4.5, such as isooctane, dimethylsulphoxide (DMSO), xylene, acetonitrile, toluene, benzene, chloroform, 1-butanol, 2-propanol and ethanol, incubated at 37°C with shaking at 140 rpm (Sarkar & Ghosh, 2012). Evaporation of solvent was prevented by plugging the flasks with butyl-rubber stoppers. Degradation was determined by measuring the volumetric reduction of solvent in the media after the bacterial growth. The bacterial culture growing in absence of organic solvent under similar conditions served as control. Growth and dry cell mass were monitored similarly as Sarkar & Ghosh (2012).

RESULTS AND DISCUSSION

Isolation and screening of BTEX tolerant strain

About 60 different bacterial cultures were isolated from the above mentioned sites in presence of 1% (v/v) benzene. Most of the isolated strains were gram positive rods. The isolated strains were further characterized on the basis of the BTEX tolerance level. 20 bacterial strains were screened that could tolerate high concentration of BTEX (**Table 1**). Similar result was also reported by Singh et al. in 2010. An environmental contaminant acts on the microflora of the ecosystem, eliminating or selecting microorganisms in accordance to sensitivity in the presence of the toxic agent.

Among the biota present in the contaminated site, microorganisms capable of using contaminants or just resisting their toxicity can be found (Mcnaughton *et al.*, 1999). These microorganisms are able to break down compounds to be used as energy source, thereby eliminating them from contaminated environments (Pedrozo *et al.*, 2002). According to Kataoka (2001), the biodegradation of organic compounds is more efficient when the microorganisms in the inoculum are pre-selected and thus become potentially more adapted to target pollutants. As BTEX is a very toxic mixture, selection of microorganisms through enrichment were carried out in this work. This initial screening step was important for successful biodegradation because the selected microorganisms were adapted to BTEX mixture. Shokrollahzadeh *et al.* (2008) also used activated sludge microflora from a petrochemical industry treatment system to biodegrade hydrocarbon contaminated wastewater.

Table 1. Morphological characteristics and BTEX tolerance level by different bacterial strains

Bacterial strains	Gram Character	Benzene (%)	Toluene (%)	Ethyl Benzene (%)	Xylene (%)
Ps1	(+) rods	7	5	0.5	0.1
Ps2	(-) rods	10	10	5	0.1
Ps3	(+) rods	1	5	1	0.2
Ps4	(+) rods	1	2	0.2	0.1
Ps5	(+) cocci	7	10	2	0.2
Ps6	(-) rods	2	2	0.2	0.1
Ps7	(+) rods	5	7	5	0.1
Ps8	(-) rods	5	7	5	0.2
Ps9	(+) cocci	7	5	2	0.1
Ps10	(-) rods	7	5	2	0.1
Ps11	(+) rods	5	2	1	0.2
Ps12	(+) cocci	2	5	1	0.2
Ps13	(+) rods	2	5	1	0.1
Ps14	(+) rods	7	7	2	0.2
Ps15	(-) rods	7	5	2	0.1
Ps16	(-) rods	5	2	1	0.1
Ps17	(+) rods	10	10	5	0.5
Ps18	(-) rods	10	7	2	0.2
Ps19	(+) cocci	5	10	2	0.5
Ps20	(+) rods	7	7	1	0.2

Preparation of bacterial consortium

The 20 BTEX tolerant bacterial strains were combined with each other by permutation combination in order to make different microbial consortia. 10 different bacterial consortia (**Table 2**) were prepared of which 4 consortia showed the best compatibility when gram staining was performed. Utilization of a consortium rather than a single microorganism has always exhibited increased rate of degradation. Many mesophilic, sulfate-reducing bacterial consortia and individual isolates have been reported to be capable of degrading BTEX-type compounds. Most of these studies were carried out with sediments containing the BTEX degraders (Lovley *et al.*, 1995).

Determination of growth

All 4 bacterial consortia exhibited almost the same growth pattern in presence of 10% (v/v) benzene and 5% (v/v) ethyl benzene (**Figs 1and 2**). In the initial 8 hours all 4 consortia were in their lag phase as there was no gradual increase in the absorbance. All the 4 consortia attained their log phase between 8 to 24 hours of incubation and gradually entered the stationary phrase from 24 hours.

In presence of 10% (v/v) toluene consortium #7 and 9 showed the highest absorbance in initial (8h) hours of incubation but gradually in the later phase of incubation there was no constant increase in the absorbance (**Fig. 3**).

These indicated that they had a very short log phase and a prolonged stationary phase in their growth cycle. While consortium #2 and 6 had a prolonged lag phase but gradually attained their log phase from 24 hours of incubation with a sharp increase in their absorbance that continues till 48 hours.

Table 2. Different composition of the bacterial consortia

Consortia	Composition
1	Ps1, Ps6, Ps9,Ps11
2	**Ps2, Ps4, Ps7, Ps12**
3	Ps3, Ps15, Ps16, Ps9
4	Ps4, Ps17, Ps8, Ps14
5	Ps5, Ps19, Ps11, Ps1
6	**Ps6, Ps11, Ps13, Ps10**
7	**Ps7, Ps12, Ps20, Ps5**
8	Ps8, Ps18, Ps 17, Ps10
9	**Ps9, Ps3, Ps16, Ps14**
10	Ps10, Ps 3, Ps 17, Ps 1

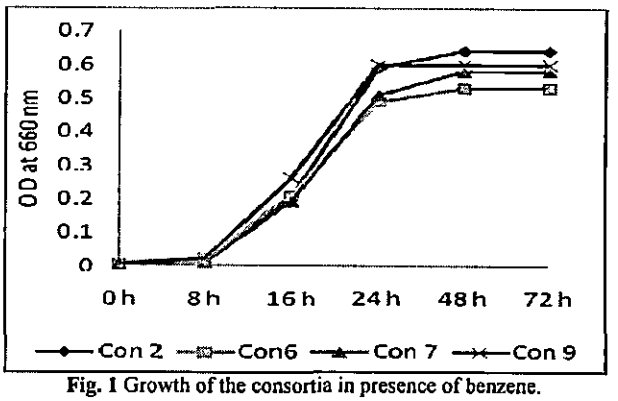

Fig. 1 Growth of the consortia in presence of benzene.

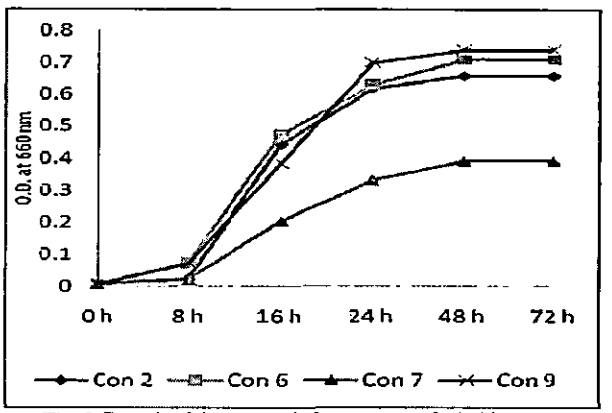

Fig. 2 Growth of the consortia in presence of ethyl benzene.

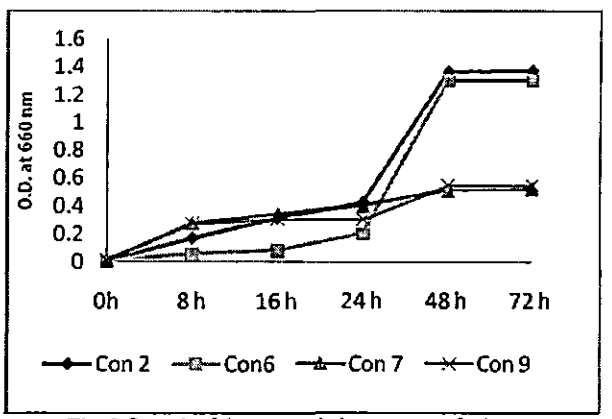

Fig. 3 Growth of the consortia in presence of toluene.

A very short lag phase was noted in all 4 consortia in presence of 1% xylene. There was a sharp increase in growth from 8 hours and it lasted till 48 hours. All the consortia entered their stationary phase after 48 hours (**Fig. 4**).

In all the cases the consortia could utilize benzene, toluene, ethyl benzene and xylene as sole source of carbon and energy. The results are in agreement with Chen and Taylor (1997). They developed two thermophilic bacterial consortia that could utilize BTEX as sole carbon and energy source.

Gas chromatography analysis of BTEX degradation

As consortium #2 was the best degrader of BTEX among the four consortia so it was selected for further analysis of BTEX degradation by Gas Chromatography. GC analysis revealed consortium #2 degrades different BTEX compounds .at different rate in the same time span. The selected consortium could degrade 100% benzene (10%) and ethyl benzene (5%) present in the growth medium. 100% degradation was confirmed by the absence of the benzene (**Fig. 5**) and ethyl benzene peak in the GC chromatogram. while the toluene (10%) and xylene (1%) was degraded almost up to 90% (**Fig. 6**) as the peak was significantly shorter compared to both the controls without the inoculam.

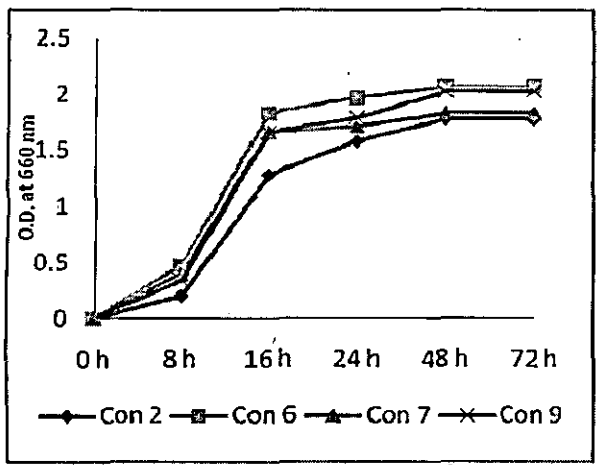

Fig. 4 Growth of the consortia in presence of o-xylene.

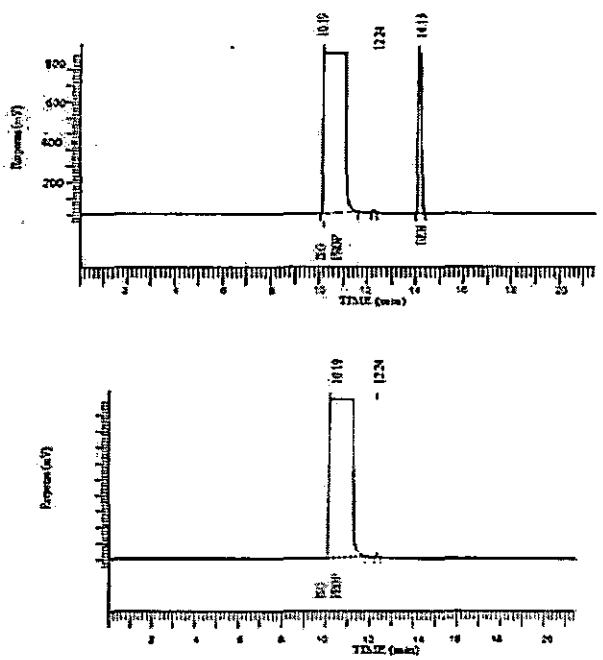

Fig. 5 GC analysis of control media having 10% (v/v) benzene, GC analysis of media having 10 (v/v) benzene inoculated with consortium #2.

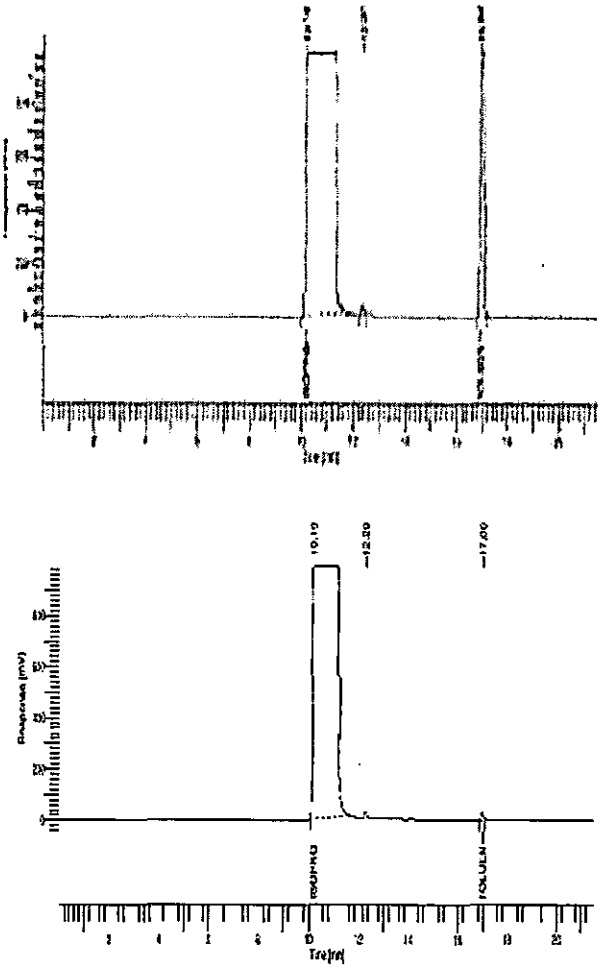

Fig. 6 GC analysis of control media having 10%(v/v) toluene, GC analysis of media having 10 (v/v) toluene inoculated with consortium #2.

Degradation of other organic solvents

The response of consortium #2 towards other solvents was studied by monitoring its growth and degradation capabilities in medium broth overlaid with solvents of varying log *Pow* values. The log *P*-value is defined as the index for measuring toxicity of solvents. Solvents with log *P*ow values between two and four, are highly toxic for microorganisms (Torres *et al.*, 2009). Degradation was determined by measuring the volumetric reduction of solvent in the media after the bacterial growth. The selected consortium could grow in solvents having higher log *P*-value, but surprisingly the alcohols having very low log *P*-value inhibited the growth (**Table 3**).

CONCLUSION

Biodegradation time tested was insufficient for the total elimination of solvents other than BTEX, implying the need for periods exceeding 5 days in

Table 3. Growth of consortium #2 in presence of organic solvents and its degradation capabilities

Solvent	log *P*	O D [a]$_{660}$	% degradation
Control[b]		1.99	–
Isooctane	4.5	*	–
DMSO	-1.35	1.8	57
Xylene	3.1	1.24	90
Acetonitrile	0.03	0.208	42
Cyclohexane	3.2	1.57	83
Toluene	2.5	1.05	90
Benzene	2	1.84	100
Chloroform	2	0.97	70
1-Butanol	0.8	*	–
2-Propanol	0.28	–	–
Ethanol	-0.24	–	–

* O D $_{660}$ value < 0.1 after 5days of growth, [b]without solvent

order to achieve this process' effectiveness. Effective bioremediation of highly recalcitrant compounds like BTEX, is most likely to rely on a consortia of microorganism rather than on the action of a single microorganism (Sarkar *et al.*, 2011). The selected consortium could grow and degrade a wide range of solvents. From the application point of view, this consortium could be a promising tool for aromatic monohydrocarbon degradation and solvent waste management.

This indicated that the selected consortium could grow and degrade hydrophobic solvents rather than hydrophilic. Similar finding of growth pattern in presence of various solvents was also reported in case of B. thermophilus PS11 strain (Sarkar & Ghosh, 2012).

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Bioremediation potential of a newly isolate solvent tolerant strain *Bacillus thermophilus* PS11

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ABSTRACT

The increased generation of solvent waste has been stated as one of the most critical environmental problems. Though microbial bioremediation has been widely used for waste treatment but their application in solvent waste treatment is limited since the solvents have toxic effects on the microbial cells. A solvent tolerant strain of *Bacillus thermophilus* PS11 was isolated from soil by cyclohexane enrichment. Transmission electron micrograph of PS11 showed convoluted cell membrane and accumulation of solvents in the cytoplasm, indicating the adaptation of the bacterial strain to the solvent after 48h of incubation. The strain was also capable of growing in presence of wide range of other hydrophobic solvents with log P-values below 3.5. The isolate could uptake 50 ng/ml of uranium in its initial 12h of growth, exhibiting both solvent tolerance and metal resistance property. This combination of solvent tolerance and metal resistance will make the isolated *Bacillus thermophilus* PS11 a potential tool for metal bioremediation in solvent rich wastewaters.

Key words: *Bacillus thermophilus,* solvent tolerance, uranium, bioremediation

Introduction

Organic solvent wastes are an interesting topic of research due to its increasing release in the industrial effluent thus polluting the water and soil ecology (Li et al., 1998). Organic solvents are used as permeabilization agents, disinfectants, food preservatives, and industrial solvents. Though they are widely used in various industries, their uncontrolled dumping in waters, sediments and the disposal sites near rivers, oceans make them a potential environmental hazard (Bustard et al., 2002).

Microbial bioremediation, which has been used for waste treatment in many industrial processes, is less feasible for solvent wastes because of toxic effect of solvents on the microbial cells (Isken & deBont, 1998). The extreme toxicity of organic solvents toward living microorganisms is because of their accumulation in the hydrophobic biological membranes. The toxicity of a solvent to bacteria depends upon its concentration in the membrane, which relates to its water solubility and its ability to partition from the water phase to the membrane (Zahir et al., 2006). The intrinsic toxicity of a specific organic solvent can be expressed as logarithm of its partition coefficient in n-octanol and water and termed as log P_{ow}. Solvents with log P_{ow}[s] below two are generally too hydrophilic to partition into membranes well, and solvents with log P_{ow}[s] above four are too hydrophobic to have high water solubility (Kieboom et al., 1998). It has been established that solvents with log P_{ow} values between two and four are highly toxic for microorganisms (Zahir et al., 2006)

The solvent tolerant microbes with unique ability to sustain under non-aqueous system have drawn considerable attention. Such organisms are attractive for applications in solvent bioremediation and biotransformation in non-aqueous media (Isken & deBont, 1998; Pieper & Reineke, 2000; Sardessai & Bhosle, 2004) Solvent tolerant bacteria have been isolated from ecological niche such as soil or deep sea and identified to belong to genera *Pseudomonas* (Ramos et al., 1995; Ikura et al., 1997; Tao et al., 2011), *Bacillus* (Bustard et al., 2002), *Flavobacterium* (Moriya & Horikoshi, 1993) and *Rhodococcus* (Paje et al., 1997). Recently, *Pseudomonas putida* Idaho (Tao et al., 2011), an organic-solvent-tolerant bacterium was isolated that can grow in the

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presence of more than 50% toluene, *m*-xylene, *p*-xylene 1, 2, 4-trimethylbenzene, and 3-ethyltoluene. Many of these bacteria have developed mechanisms to resist the lethal effect of organic solvents either by alterations in the composition of cytoplasmic and outer membrane in presence of organic solvents or by metabolic transformation of toxic compound into non-toxic products. Furthermore, an efflux system actively decreasing the amount of solvent in the cell has been described.

Solvent tolerant microbes often possess metal resistance. A wide range of microbes are well known for metal accumulation and detoxification but without solvent tolerance trait. The combination of the two properties has better potential in microbial metal bioremediation in solvent rich wastewaters (especially in chemical and hospital wastes), which is otherwise not possible due to microbicidal nature of solvents. The biosorption of uranium and the growth of several bacterial communities, in environments that are generally poor in nutrients, with various chemical and physical properties, is not easy to stimulate. In addition, once the uranium is immobilized, it is important to impeach its reoxidation or desorption. For these reasons, a good bioremediation strategy will always depend on a good knowledge of the microbiological, geochemical and geological properties of the site to decontaminate. Bacteria, including *Citrobacter freudii* and cell components from members of the *Firmicutes* have also been described as U(VI) biosorbents (N'Guessan et al., 2008). The biosorption efficiency seems to be positively related to temperature and can occur sometimes within hours, which is considerably faster than direct bioreduction (that can take months or years).

The present work describes isolation of solvent tolerant strain of *Bacillus thermophilus* PS11 from soil by cyclohexane enrichment. The adaptation to solvent by the bacterial strain was studied at the membrane level by transmission electron microscopy. This is the first report of solvent tolerance in *B. thermophilus* to the best of our knowledge. Growth in presence of uranium indicated uranium resistance property of the bacterial strain.

Materials and Methods

Isolation of solvent tolerant bacterial strains

Soil samples were collected from the proximity of a solvent extraction unit in Siliguri, India. A known amount of soil was suspended in sterilized distilled water and 250 µl of suspension was transferred to a test tube having 5.0 ml of solvent tolerance medium (STM) containing (g l^{-1}): yeast extract, 4.0; peptone, 2.5; glucose, 1; starch, 1; olive oil, 10; KH_2PO_4, 0.5; $MgSO_47H_20$, 0.5; NaCl, 0.25; cyclohexane (10%, v/v) and pH adjusted to 7.5 (Gupta et al., 2006). The test tube was incubated for 5days at 37°C with constant shaking at 220 rpm in an orbital shaker. Resultant culture fluid was spread on STM agar plate overlaid with cyclohexane. Growing colonies were further purified by repeated streaking. Finally, a solvent-tolerant strain B5 was chosen for further studies because of its highest growth rate in presence of solvent. Morphological and biochemical characteristics of the isolate were determined. The 16S rDNA of B5 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 sequenced. The identity of isolate was confirmed by phylogenetic analysis of 16S rDNA sequence using the software package SeaView. It was maintained on nutrient agar at 4°C.

Growth of the isolated strain

For bacterial growth, the inoculum was prepared by inoculating a loopful of isolated cells from slant into STM followed by incubation at 37°C and 140 rpm. One millilitre of overnight grown culture having 10^6 cfu.ml^{-1} was used to inoculate 100 ml of STM overlaid with 20% v/v cyclohexane. The incubation was carried out at 37°C with constant shaking at 140 rpm in an orbital shaker. To prevent the evaporation of solvent, flasks were sealed with butyl rubber stoppers. The bacterial culture growing in absence of organic solvent under similar conditions served as control. Growth was followed by recording absorbance at 660 nm. For dry cell mass measurement, 1.0 ml culture broth was centrifuged at 10000*g* at 4°C for 10 min to pellet down the cell mass. The pellet was washed twice with distilled water and dried at 105°C till constant mass was achieved.

Organic solvent tolerance of the isolated strain

The solvent tolerance of the microorganism was checked in Erlenmeyer flasks containing STM overlaid with organic solvents (20% v/v) with log P_{ow} values ranging 0.28-4.5, such as isooctane, dimethylsulphoxide (DMSO), xylene, acetonitrile, toluene, benzene, chloroform, 1-butanol, 2-propanol and ethanol, incubated at 37°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

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conditions served as control. Growth and dry cell mass were monitored similarly as described in the previous section.

Sample preparation for transmission electron microscopy (TEM)

To prepare specimens for transmission electron microscopy, cells were grown for 24h in culture medium in absence or presence of cyclohexane (20% v/v) and harvested by centrifugation at 5000 rpm for 10 min. Then cells were fixed overnight in a solution containing 2.5% (w/v) glutaraldehyde in 0.1M Na_2HPO_4/KH_2PO_4 (pH7.2) buffer at 4°C and post fixed with 1% osmium tetroxide (OsO_4). The cells were then dehydrated with ethanol and embedded in Spurr. The sections were stained with 1% (w/v) uranyl acetate and 1% (w/v) citrate and examined with a Philips model CM10 electron microscope at an accelerating voltage of 80 kV.

Uranium bioremediation by the isolated strain

Culture media containing varying amount of uranium (5-50 $ng.ml^{-1}$) was inoculated with 1% (v/v) freshly prepared inoculum of isolated strain. The inoculum preparation and culture conditions were kept same as described above for the growth of the isolate. Samples for estimating residual uranium in the media were periodically withdrawn. Total uranium contents were measured by cold vapor atomic absorption spectrometry (Hatch & Ott, 1986).

Results and Discussion

Isolation and screening of bacterial strains with solvent tolerance property

Tolerance to grow in the presence of solvents is often observed among the microbes inhabitating the soil exposed to solvents. In the present work, soil samples from the sites near to the solvent extraction unit were screened for solvent tolerant microbes. A solvent tolerant isolate B5 was chosen for further study because of its highest growth rate in presence of solvent. Similar results, mostly in case of *Pseudomonas* sp., *Flavobacterium* and *Rhodococcus,* have been also reported (Inoue & Horikoshi, 1989; Paje et al., 1997; Bustard et al., 2002).

The bacterial isolate was characterized morphologically and biochemically as aerobic, gram-positive, motile rod with very simple nutritional requirements that grow best at neutral pH and temperatures in the mesophilic range. Molecular characterization by 16S rDNA based phylogenetic analysis (accession numbers awaited) identified the bacterial isolate as *Bacillus thermophilus* and hence, tentatively named as *Bacillus thermophilus* PS11.

Growth characteristics of B. thermophilus in presence of cyclohexane

The growth curve of *Bacillus thermophilus* in the absence and presence of cyclohexane (20%, v/v) is shown in Figure 1.

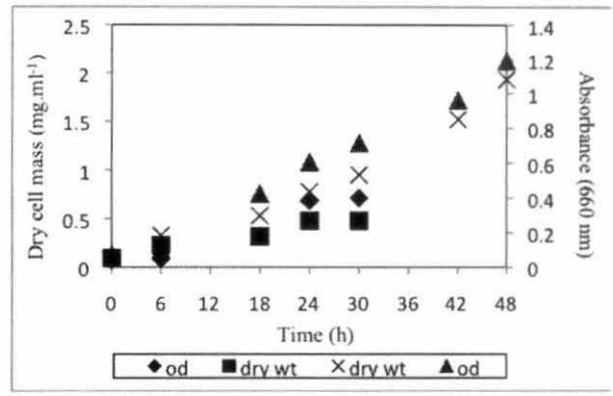

Figure 1. *Growth of B. thermophilus in the absence and presence of cyclohexane. B. thermophilus was grown in culture media in the absence and presence of cyclohexane (20% v/v). The growth (A_{660}) and dry cell mass were recorded at various time intervals. Bacterial growth in absence of cyclohexane: $OD_{660\ nm}$ (▲), dry cell mass (✕) and growth in presence of cyclohexane: $OD_{660\ nm}$ (♦), dry cell mass (■). The experiment was carried out in triplicates and the difference in the individual results was less than 3%.*

Incorporation of cyclohexane into the media served as a factor for screening of solvent tolerant strain. The isolated strain PS11 showed delayed growth pattern in presence of cyclohexane and log phase started after 18h, as compared to 6h in its absence. 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 cyclohexane on cells. The dry cell mass of the culture in the presence of cyclohexane 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).

Growth characteristics of B. thermophilus in presence of other organic solvents

The response of *Bacillus thermophilus* towards other

solvents was studied by monitoring its growth in medium broth overlaid with solvents of varying log P_{ow} values. The log *P*-value is the index for measuring the toxicity of solvents. Solvents with log P_{ow} values between two and four, are highly toxic for microorganisms (Torres et al., 2009). However, the results summarized in Table 1 show that the isolated strain PS11 could grow in solvents having higher log *P*-value, but surprisingly the alcohols having very low log *P*-value inhibited the growth. Similar finding of growth pattern in presence of various solvents was also reported in case of *B. cereus* R1 strain (Matsumoto et al., 2002). Bacterium grew well on the medium plates overlaid with cyclohexane, DMSO, toluene, chloroform, benzene, hexane, xylene but did not grow in the presence of isopropanol, 1-butanol and ethanol thus indicating its tolerance for hydrophobic solvents rather than hydrophilic. The cell mass in presence of DMSO was comparable to that in cyclohexane overlaid medium, whereas cell growth was least in presence of acetonitrile.

Table 1. *Growth of B. thermophilus in presence of organic solvents.*

Solvent	Log *P*	OD_{660} [a]	Dry cell mass (mg.ml^{-1})[a]
Control [b]		2.69	1.47
Isooctane	4.50	*	*
DMSO	-1.35	1.78	1.19
Xylene	3.10	0.41	0.54
Acetonitrile	0.03	0.30	0.13
Cyclohexane	3.20	1.67	1.05
Toluene	2.50	0.89	0.76
Benzene	2.00	0.74	0.68
Chloroform	2.00	0.76	0.68
1-Butanol	0.80	*	*
2-Propanol	0.28	-	-
Ethanol	-0.24	-	-

Legend: * OD_{660} value < 0.1 and dry cell mass (mg.ml^{-1}) < 0.05 after 24h of growth; [a] After 24h of growth; [b] without solvent.

Transmission electron micrograph of B. thermophilus

Transmission electron micrographs of the *Bacillus* cells growing in the absence and presence of cyclohexane are shown in Figure 2. Transmission electron micrograph of *B. thermophilus* cells in the presence of 20% cyclohexane showed accumulation of solvents and convoluted, disorganized cell membrane. Similar cellular changes have been reported for *Pseudomonas* sp. cells grown in *p*-xylene and *Enterobacter sp.* grown in the presence of cyclohexane (Gupta et al., 2005, 2006).

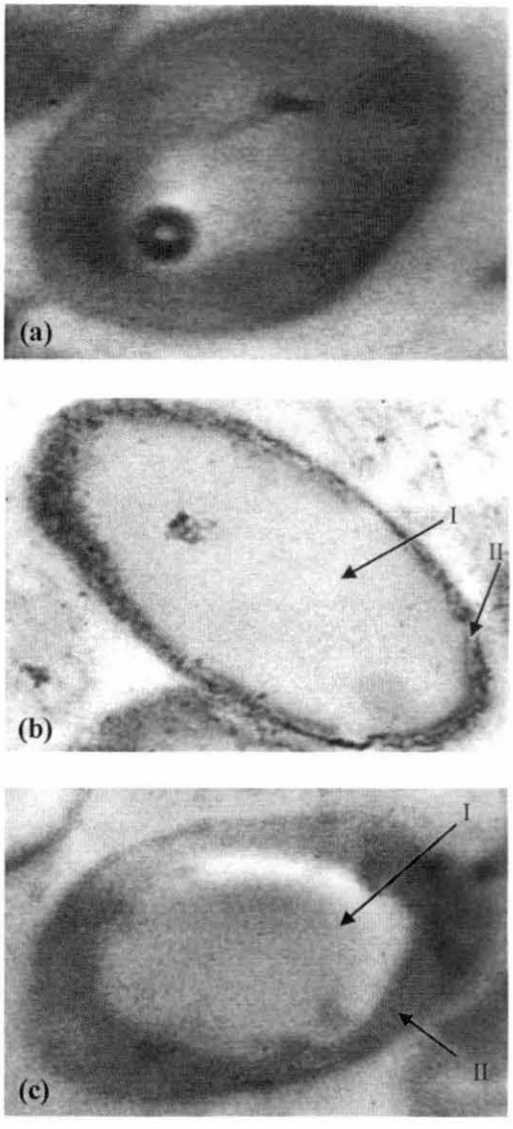

Figure 2. *Transmission electron micrograph of B. thermophilus cells:* **(a)** *in the absence of cyclohexane (exposure 21,000);* **(b)** *in the presence of 20% cyclohexane (exposure 21,000) after 48 hours incubation - (I) accumulation of solvents and (II) convoluted and disorganized cell membrane;* **(c)** *in the presence of 20% cyclohexane (exposure 21,000) after 96 hours incubation. (I) and (II) regeneration of cytoplasm and cell wall.*

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Solvents are reported to damage the integrity of cell membrane structure resulting in loss of permeability regulations. In extreme cases leakage of cell RNA, phospholipid and protein also take place (Sikkema et al., 1995). Moreover, it is clearly visible that the solvent accumulation inside the cell was initially increased occupying the entire cytosolic region at 48h of incubation (Figure 2b). The decline in solvent accumulation and reorganization of cell membrane were observed on further incubation till 96 hours (Figure 2c).

Structural changes seen in TEM of *Bacillus* cells suggest that organic solvent affected the membrane system. The solvent tolerant nature of the bacterial strain is evident from the reorganization of cell membrane and decline in cytosolic accumulation of solvent during prolonged exposure to solvent. Hence, solvent adaptation property of *Bacillus thermophilus* PS11 seems to be related to both restoration of membrane fluidity and metabolic transformation of hydrophobic solvent to less toxic products. Cell morphology alterations and filamentous growth were observed in solvent resistant bacteria in response to environmental stress, including organic solvents (Toress et al, 2009). 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. Efflux systems in case of gram positive bacteria are either secondary transporters or ATP binding cassette (ABC) type transporters (Bolhuis et al., 1997). Solvent tolerant cells 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. However, the variety of mechanisms that could confer adaptation to organic solvents implies that bacterial solvent tolerance cannot only possibly be provided by a single one type mechanism (Heipieper et al., 2007). It is very likely that the combination of different metabolic strategies leads to cellular solvent tolerance.

Uranium bioremediation by B. thermophilus

Solvent tolerant strains are often endowed with heavy metal resistance (Chang & Hong, 1995; Wagner-Döbler, 2003). To check the simultaneous effect of these two traits, this solvent tolerant isolate of *Bacillus thermophilus* was grown up to 50 ng/ml of U in media (Figure 3). The bacterium could uptake uranium in its initial 12h of growth. During this time the growth rate is less compared to the growth rate when uranium is up taken by the cells. The lag phase prolonged with increasing uranium concentration, which is a common pattern in microbial metal resistance (Wagner-Döbler, 2003).

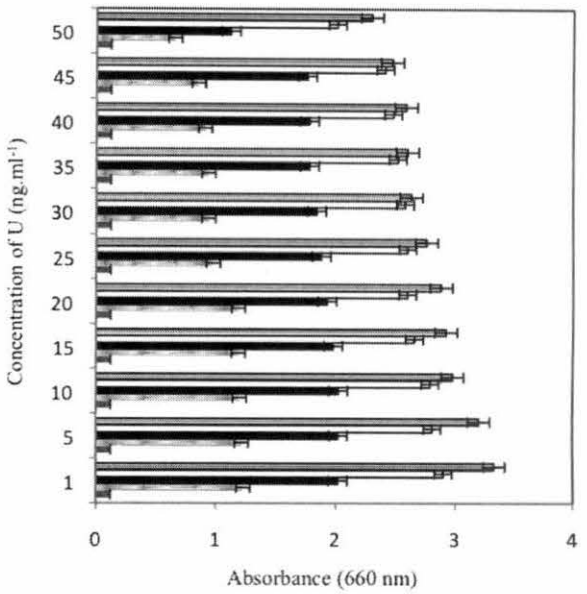

Figure 3. *Growth of B. thermophilus in varying concentration of uranium. The isolate was grown in media containing varying concentration of U (5-50 ng/ml). OD_{660nm} were recorded after 0 h (▤), 12 h (□), 24 h (■), 48 h (▥) and 72 h (▩) of growth. Each experiment was done in duplicate and the difference between two sets of experiments was less than 3%.*

The level of uranium in the medium decreased with bacterial growth, thus confirming its uptake by the microorganism (Figure 4). The observed U resistance by the test isolates may be explained by uranium-bacteria interaction mechanisms resulting in cell surface or intracellular sequestration of uranium as a means to limit U toxicity, which may be followed by bioprecipitation and biomineralization (Merroun et al., 2008). Similar uranium biomineralization by a metal-resistant *Pseudomonas aeruginosa* strain was recently reported (Choudhary et al., 2011). Mechanism of uranium detoxification is presumed to be by the metallic reductase enzyme or due to metal resistant genes encoding efflux pumps present in the microbes. Further works regarding the reasons behind heavy metal tolerance is under process.

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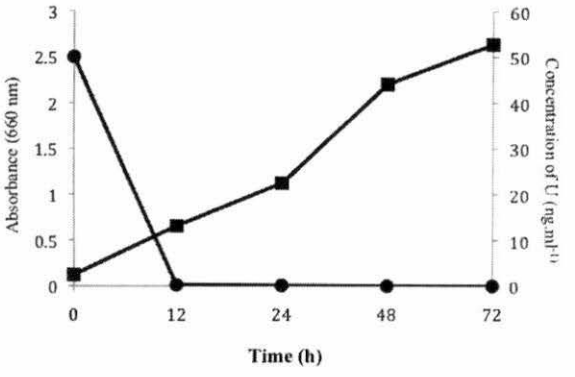

Figure 4. *Growth and uptake of uranium by B. thermophilus cells. The bacterium was grown in culture containing 50 ng/ml U. The growth (A_{660}) and concentration of the U were meadured at various time intervals. $OD_{660\ nm}$ (■), U – ng/mL (●). The experiment was carried out in triplicates and the difference in the individual results was less than 3%.*

Conclusion

The isolated gram positive solvent tolerant strain of *Bacillus thermophilus* PS11 could grow in presence of various solvents having varying log P value. Change in the membrane structure was observed as an adaptive feature when grown in presence of solvents. The strain could also uptake uranium from the media. Thus, this strain could be promising for treatment of solvent wastes and detoxification of uranium.

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