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Isolation and Characterization of Hydrocarbon utilizing Microbes from Petrochemical Contaminated Site

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Abstract

Hydrocarbons are hazardous class of organic compounds which enter the ecosystem through anthropogenic and natural sources. Microbial degradation is a valuable aspect for ecological recovery of hydrocarbons contaminated sites. The present study aims screening and characterization of hydrocarbon utilizing bacteria and fungi isolated from a petrochemical site. Screening and characterization of indigenous microbes is vital step to get information about their metabolic potential to degrade the target pollutant. Thus, physicochemical characterization of the collected sample was done. Identification of isolated microbes was done by 16S rRNA and 18S rRNA sequencing. Twelve bacterial and five fungal hydrocarbon utilizing strain were isolated and screened out for paraffin oil, mixture of PAHs (Naphthalene, Anthracene and Phenanthrene). Results showed that *Bacillus sp (LOR)*, *Acinetobacter sp. (SL1)*, *Microbacterium sp. (SOY)*, *Acinetobacter sp. (SOD)*, *Fusarium sp.* (WC) followed by *Aspergillus sp.* (WB) were more efficient hydrocarbon utilizers. The growth rate constants and doubling time of bacterial isolates were measured at the range 0.085hr^{-1}, 8.10hr; 0.081 hr^{-1}, 8.49 hr^{-1} ; 0.082 hr^{-1} , 8.38 hr^{-1}; 0.084 hr^{-1}, 8.24 hr^{-1} respectively. Significant results were observed during assessment of biosurfactant production capacity of isolates.

Introduction

Consumption of petroleum products all over the world has led to rise in crude oil extraction and processing. This has resulted in an increased generation of large amounts of oily waste and other harmful organic compounds (Bhattacharyya and Shekdar, 2003; Obi *et al*., 2016). The constituents of crude oil sludge and effluent includes various saturated aliphatic and aromatic hydrocarbons like PAHs which are toxic, mutagenic and carcinogenic and may persist in the environment for longer duration and posing a major risk to ecosystems as well as on human health (Enabulele and Obayagbona 2013; Obi *et*

al., 2016). Hydrocarbons are gaining more attention due to their possible toxicity for higher organisms and recalcitrance to microbial degradation. Aromatic hydrocarbons are the most common pollutant found in the soil and ground water (Gupta *et al*., 2014). Therefore, the presence of these aromatic compounds in aquatic as well as in terrestrial environments is very toxic and harmful for human health also. Along with hydrocarbon pollution, elevated concentration of heavy metals generally found at petrochemical contaminated site. So, there is a need of a treatment technology which can efficiently cope up with co-contamination and there removal. Remediation of these hydrocarbons has resulted in the development of certain technologies viz. physical and chemical, which stimulate the destruction, relocation, immobilization and detention of the oil sludge. Bioremediation is a biological approach that involves the use of microorganism's metabolic potential to degrade pollutants into harmless compounds (Milic *et al*., 2009; Hara *et al*., 2013; Singh and Chandra, 2014). Several species of microorganisms have been successfully utilized in major hazardous waste clean-up processes (Levinson *et al*., 1994). Implementation of a successful bioremediation strategy requires a detailed evaluation of the roles of the indigenous microbial isolates (bacteria, fungi, yeast, algae) (Piehler *et al*., 1999; Abarian *et al*., 2016). Several species of microorganisms like *Pseudomonas sp., Vibrio sp., Mycobacterium sp., Marinobacter sp.*, and *Sphingomonas sp.* have been successfully utilized in major hazardous waste clean-up processes (Levinson *et al*., 1994; Hedlund *et al*., 1999). Recently, Abarian *et al* (2016) reported that *Sphingobacterium multivorum* AHB38N isolated from mine showed best growth at 600 ppm of naphthalene. Lahkar and Deka (2016) also reported the anthracene (25 mg/L) degradation potential of *Aspergillus* sp. strain HJ1 for isolated from Oil-Contaminated Soil. Colorimetric method for the identification of hydrocarbon degrading isolates is rapid detection method in which oxygen is substituted by a synthetic mediator i.e 2,6-dichlorophenolindophenol (DCPIP). The basic concept of this redox indicator relies on the oxidation of the hydrocarbon as substrate, in which electrons are transferred to the electron acceptors. The utilization of hydrocarbons can be detected based on the change of indicators blue colour to colourless. 2,6-DCPIP and piodonitrotetrazolium indicators used to assess microbial strain to degrade crude oil and found that *Rhodococcus sp., Trichoderma tomentosum*, and *Fusarium oxysporum*, significantly degraded PAH compounds in the mixture (Marchand *et al*., 2017).

Development of effective techniques for bioremediation of hydrocarbon contaminated sites is a need for present environmental condition. Isolation and screening of microorganisms for their efficiency in utilization of hydrocarbons before field trials is important in bioremediation process (Varjani *et al*., 2013). This study describes the characterization and isolation, screening of various isolates of hydrocarbon degrading bacteria and fungi isolated from contaminated site. Simultaneously, isolated cultures were screened out for, capability to produce biosurfactant, ligninolytic enzymes and heavy metal tolerance.

Material and Methods

Sampling Site

Sampling was done from Oil and Natural Gas Corporation (ONGC) refinery plant, Koyali Terminal, Vadodara, Gujarat. Soil samples were collected aseptically from a layer 0–10 cm deep site a few meters away from the refinery plant. During separation of heavy and light oil they tend to form suspension within a immobile fluid, as well as in storage tanks, where it accumulate at the bottom as viscous substance commonly known as sludge, so raw sludge was collected from few meters away from the refinery plant and 5 L of effluent discharge from outlet of refinery industry were collected aseptically. All collected samples were stored at 4°C up to 7 days for complete analysis.

Physico chemical and biological characterization

Physical and chemical parameters were analyzed by standard methods (APHA, 2012). Heavy metals estimation was done by acid digestion of samples and analyzed by ICP-MS (APHA, 2012). GC MS analysis (USEPA Method 8310) was done to know the presence of PAH compounds and other organic compounds by solvent extraction method (results not shown here). For microbial isolation colony forming unit (CFU) count was done. Isolated bacterial and fungal cultures were submitted to Gujarat State Biotechnology Mission (GSBTM), 16S rRNA & 18S rRNA sequencing respectively for molecular identification of microbial isolates.

Heterotrophic Bacterial and fungal Counts

Serial dilution and spread plate, four ways streaking method was used for isolation of bacterial and fungal colonies. For serial dilution 1 g of the mixed soil and sludge, 1ml of effluent was added separately to 9 mL of deionized water, and 0.1 mL of this diluted sample was spread by plating on Nutrient Agar medium from the appropriate dilution tubes and then incubated at the room temperature for 24 h. The fungal colonies were counted after 3-5 days of incubation (Janbandhu and Fulekar, 2009). Total Heterotrophic Bacterial Counts was carried out on nutrient agar medium and fungal colony count on czapek dox agar medium as described by Rahman *et al.* (2002).

Hydrocarbon Utilizing Bacterial and fungal Counts

Total Hydrocarbon Utilizing Bacterial and fungal Counts was carried out on Bushnell Hass (BH) medium by using soil, sludge and effluent sample. PAH mixture (Naphthalene, anthracene, phenanthrene 50 µl of each by dissolving in acetone) was used as the carbon source and 2% agar was added to solidify the medium. The bacterial and fungal plates were incubated at 37°C and 27^{o}C respectively for 5 days. Fluconazole was used as antifungal agent in bacterial plates. The colonies were counted and sub-cultured

to obtain pure colonies. Only hydrocarbon utilizing bacterial and fungal colonies were streaked and isolated for molecular identification.

Fungi culture conditions & Dry Biomass production

Fungal cultures were maintained in petri plates on Czapek Dox agar medium. Streptomycin was used as an antibacterial agent. All stock cultures were maintained on Czapek Dox agar and broth medium. The plates and broth medium were incubated at 27°C for 5 days and stored at 4°C. Growth and tolerance of fungal cultures to different test experiments used in the present study were measured in terms of dry biomass and radial growth extension on plate (in cm^{-1}). For dry biomass estimation of the fungal mycelium was harvested at 7^{th} and 15^{th} day of growth, separated from the culture liquid by filtration through a Whatman No. 1 filter paper. The mycelia pellet was repeatedly washed with distilled water and dried at 70°C overnight (Srivastava *et al*., 2011).

Screening of hydrocarbon utilizing microbes

Redox indicator DCPIP was used to screen for efficient hydrocarbon degradation by bacteria and fungi. It is a preliminary step to screen hydrocarbon degrading microbes. Bacterial and fungal isolates were tested for their potential to utilize hydrocarbon substrate mixture (naphthalene, anthracene, and phenanthrene) as carbon sources. Bacterial and fungal isolates were inoculated into 7.5 ml of BH medium incorporated with 50 µl of each PAH compound along with 40 µl of DCPIP (to be utilized as PAH degradation indicator) was added and incubated at 35^0C. The DCPIP was prepared by dissolving 50 mg of the DCPIP powder in 500 ml of sterile distilled water (Mariano *et al*., 2008). Absorbance of the medium at 600 nm was measured at intervals of 24 h for 6 days using a spectrophotometer (Bidoia *et al*., 2010).

Assessment of Biosurfactant Production

Hydrocarbon utilizing bacterial and fungal cultures was assessed for production of biosurfactant. Based on four methods; oil displacement test, emulsification index, CTAB assay, cell surface hydrophobicity, glycolipid estimation. Following Nasr et al., 2009; Techaoei et al., 2007; Fatemeh et al., 2015 method, did oil displacement test. Crude oil (20 µl) was poured into the petri dish containing 50ml of double distilled water. Bacterial and fungal suspension (10 µl) was added on the crude oil. The halo formation was the indication of biosurfactant production. The emulsification index was investigated by adding two different carbon sources (paraffin oil, PAH mixture) to BH + NB medium consisting of microbial culture in 2:4 ratios (v/v). The solution was vortexed at high speed for 2 min and incubated for 24 h & 48 h. After the specified time, emulsification percentage was calculated through the height of emulsion layer.

BH agar medium supplemented with glucose as carbon source (2%) and cetyltrimethyl ammonium bromide (CTAB: 0.5 mg/mL) and methylene blue (MB: 0.2 mg/mL) were used for the detection of anionic biosurfactant (Satpute *et al*., 2008). Microbial adhesion to the hydrocarbon method was carried out using the procedure described by Rosenberg *et al*., (1980). The culture was grown on 50 µl hexadecane. Cells in the exponential phase were centrifuged at 8000g for 5 min, washed twice with a PUM buffer (19.7 g /1 K_2HPO_4, 7.26 g /1 KH_2PO_4, 1.8 g /l H_2NCONH_2 and 0.2 g /1 $MgSO_4$ $7H_2O$). The optical density was measured at 600 nm using a UV–Visible Spectrophotometer. 100 µl of hexadecane was added to 5 ml of the microbial suspension and vortexed for 2 min. After 10 min the optical density of the aqueous phase was measured (Hanna *et al*., 2011).

Glycolipid concentration in the cell-free culture broth was estimated by a colorimetric method by anthrone sulphuric acid method with slight modification (George and Jayachandran, 2013). The reaction mixture constitutes 0.5 ml of pre-diluted sample (individual culture grown on mixture of naphthalene, anthracene, phenathrene as carbon source, 5 mg/L each), 4.5 ml of anthrone reagent (200 mg of anthrone in 100 ml of 75% sulphuric acid). These contents in tube were mixed well and incubated in water bath at 100°C for 10 mins followed by cooling at room temperature, absorbance was measured at 625 nm, and the glycolipid concentration was calculated using a standard curve prepared using different concentrations of L-rhamnose.

Determination of maximum heavy metal tolerable concentration (MTC)

The highest concentration that allowed visible bacterial growth after 48 h of incubation was used to select MTC of heavy metal. The increasing concentration of heavy metals (Pb, Cd, Cr, Ni and V) i.e.10, 20, 30, 40, 50, 60, 70 and 80 ppm were filter and then added into nutrient agar and czapek dox for testing the MTCs of bacteria and fungi respectively (Afzal *et al*. 2017).

Qualitative analysis of ligninolytic enzymes

Isolates were screened for ligninolytic activity through rapid plate detection experiments for different enzyme assay. Phenol-red plate assay according to Singh *et al*., 2006 for lipase was performed. Czapek dox and nutrient agar medium was employed for the screening of ligninolytic enzymes activities. 0.0025% azure B (w/v) or 0.0025% phenol red (w/v) were used to detect lignin peroxidase (Archibald and Roy, 1992) or manganese peroxidase (Kuwahara *et al*., 1984), respectively. Laccase was tested by adding 0.05% (w/v) guaiacol, modified from Kiiskinen and Saloheimo, 2004 (Ali *et al*., 2012). The peroxidase activity was determined qualitatively using the method proposed by Rayner and Boddy, 1988. Phenoloxidase was detected by inoculation of czapek dox or nutrient agar supplemented with 0.5% (w/v) tannic acid. After 5–7 days culture brown

color around growth area indicated positive result (Rayner and Boddy, 1988; Lopez *et al*., 2006).

Results

Characterization of environmental samples collected from sampling site

The following physical-chemical descriptions of contaminated site highlight important sample characteristic (table 1). The pH value was observed 6.4 in all collected samples. TDS was found high in comparison with other chemical parameters in effluent. High concentration of TDS, TSS, COD, DO, BOD, PO_4^{3-}, SO_4^{2-}, Cl^- and NO^-_3 were also observed. These parameters were interpreted on the basis of central pollution control board (CPCB) standards laid down for petrochemical industry. Analysis of aromatic hydrocarbons was done by GC MS, which confirmed the presence of polycyclic aromatic hydrocarbons (PAHs) like naphthalene and anthracene related compounds in sludge (2,4a,8,8-tetramethyl decahydro cyclopropa[d]naphthalene, 9 butyltetradecahydro anthracene, soil (oct-5-en-2-ol,8-(1,4,4a,5,6,7,8,8a-octahydro-2,55,8a tetramethyl naphthalene, 1-naphthalene propanol, alpha-ethenyl decahydro-2-hydroxy-alpha). Mostly naphthalene associated organic compound were found in all matrix. The concentrations of nine heavy metals in samples were analyzed (table 2). V, Zn, Fe, Ni, Cu concentration is elevated relative to other metals in sludge and soil samples. The Fe concentration is very high in all the samples.

Table 1 Physicochemical characterization of petrochemical site

Parameters	**Effluent**	**Soil**	**Sludge**
DO (mg/L)	4.4 ± 1.67		-
Particle size distribution		Clay % - 6.566 ± 0.25 Silt %- 23.4 ± 0.47 Sand %- 66.42 ± 0.68	
BOD (mg/L)	14.62 ± 3.29	-	-
COD (mg/L)	234.8 ± 12	-	-
TDS (ppt)	2.03 ± 0.57	-	-
Ph	6.7 ± 0.15	6.424 ± 0.1	6.44 ± 0.12
EC (µS)	3.54 ± 1.81	854.2 ± 51.9	722.8 ± 5.5
Chloride (mg/L)	709.9 ± 104.23	2.04 ± 0.35 %	3.342 ± 0.43
Total Alkalinity (mg/L)	270 ± 21	-	-
Hardness (mg/L)	178 ± 8.3	47.68 ±2.26 ($CaCO_3$ %)	42.24 ± 1.14 ($CaCO_3$ %)
Ammonical Nitrogen (mg/L)	22.9 ± 12.3	5.9 ± 0.4	35.51 ± 0.2
Sulphate (mg/L)	59.79 ± 28.9	127.6 ± 1.7	131.4 ± 1.2
Inorg. P (mg/L)	36.6 ± 1.9	207.5 ± 1.6	241.42 ± 1.9
Org. P (mg/L)	7.518 ± 0.9	19.1 ± 1.63	35.22 ± 0.2
Nitrite-N (mg/L)	44.9 ± 36.06	17.19 ± 3.2	27.51 ± 2.5
Phenol (mg/L)	8.11 ± 5.4	2.19 ± 0.5	1.692 ± 0.02
Nitrate-N (mg/L)	34.8 ± 26.9	77.3 ±17.4	64.12 ± 11.3
Organic carbon %	-	16.3 ±4.5	18.4 ± 6.9
*Value indicate the average of triplicate samples and standard deviation			

Molecular identification

The bacterial isolate were further identified by 16S rRNA sequencing and showed 99% similarity with the genera, similarity accession number and evolutionary relationship of these isolates coding (table 3) such as *Agromyces sp. SOB, Microbacterium sp. SOY, Acinetobacter sp. SOD, Beijerinckia sp. SOE, Microbacterium sp. SL1Y, Streptomyces sp. SOC, Acinetobacter sp. SL1, Bacillus sp. LOR, Acinetobacter sp. SL, Bacillus sp. P, Xanthobacter sp. SL4, Exigubacterium sp. WOR,* fungal isolates were identified by 18S rRNA sequencing and showed 99% similarity with *Fusarium sp. WC, Aspergillus sp. G, Aspergillus sp. WB, Penicillium sp. WY, Penicillium sp. MG.*

Table 2 Heavy Metal Analysis

HEAVY METALS	Effluent (µg/L)	Soil (µg/Kg)	Sludge (µg/Kg)
VANADIUM (V)	57.8 ± 14.95	23.43 ± 12.7	33.81 ± 20.08
CHROMIUM (Cr)	5.85 ± 5.89	10.4 ±17.5	15.04 ± 17.7
IRON (Fe)	156.2 ± 0.60	354.8 ± 12.9	485.9 ± 19.17
NICKEL (Ni)	22.10 ± 7.06	54.15 ± 13.12	77.25 ± 18.4
COPPER (Cu)	18.13 ± 5.82	37.41 ± 13.5	86.51 ± 19.09
ZINC (Zn)	22.88 ± 41.51	263.3 ± 13.10	404.23 ± 19.04
ARSENIC (As)	18.57 ± 4.8	2.72 ± 35.03	4.59 ± 17.7
CADMIUM (Cd)	0.04 ± 75	0.07 ± 16.6	0.25 ± 17.2
LEAD (Pb)	0.13 ± 27.05	3.42 ± 15.6	7.25 ± 21.26

Table 3 Molecular identification of hydrocarbon utilizing microbes

16S rRNA (Bacterial strains)	18S rRNA (Fungal strains)
Agromyces sp. SOB	*Fusarium sp.* WC
Microbacterium sp. SOY	*Aspergillus sp.* G
Acinetobacter sp. SOD	*Aspergillus sp.* WB
Beijerinckia sp. SOE	*Penicillium sp.* WY
Microbacterium sp. SL1Y	*Penicillium sp.* MG
Streptomyces sp. SOC	
Acinetobacter sp. SL1	
Bacillus sp. LOR	
Acinetobacter sp. SL	
Bacillus sp. P	
Xanthobacter sp. SL4	
Exigubacterium sp. WOR	

Screening and identification of hydrocarbon utilizing microbial count

Screening of hydrocarbon utilization capacity for microbial isolates may indicate that isolates capable of utilizing aromatic or aliphatic hydrocarbon compounds. It was found that total heterotrophic microbial count of samples was higher compared to hydrocarbon utilizing microbial count (figure 1). Screening of hydrocarbon utilization and degradation by bacterial and fungal cultures was performed by using DCPIP redox

indicator as primary stage in the present work. It provides information on hydrocarbon degrading microorganisms which can utilize hydrocarbon as carbon source. In this assay *Microbacterium sp*. SOY, *Acinetobacter sp.* SL1, *Agromyces sp.* SOB, *Xanthobacter sp.* SL4, *Acinetobacter sp*. SOD were found with the capability to degrade both classes of compounds (figure 2). Among fungi *Aspergillus sp.* WB and *Penicillium sp.* MG showed the fastest rate of dye removal (figure 3). Twelve hydrocarbon utilizing bacteria and five fungi were isolated and characterized. All twelve bacterial isolated showed luxuriant growth in terms of turbidity in the presence of PAH mixture and paraffin oil (saturated hydrocarbons). The growth rate constant (K) and doubling time ($t_{2/1}$) for each culture was computed (figures 4 a,b). For screening out of PAH tolerant fungal isolates radial growth was measured on 7 day incubated plates tested with 500µL of naphthalene, anthracene and phenanthrene set up with negative control (without PAH). It was found that *Fusarium sp.* WC, *Aspergillus sp.* WB, *Penicillium sp.* MG, showed maximum growth 2, 1.7, 1.8 cm^{-1} respectively (figure 5).

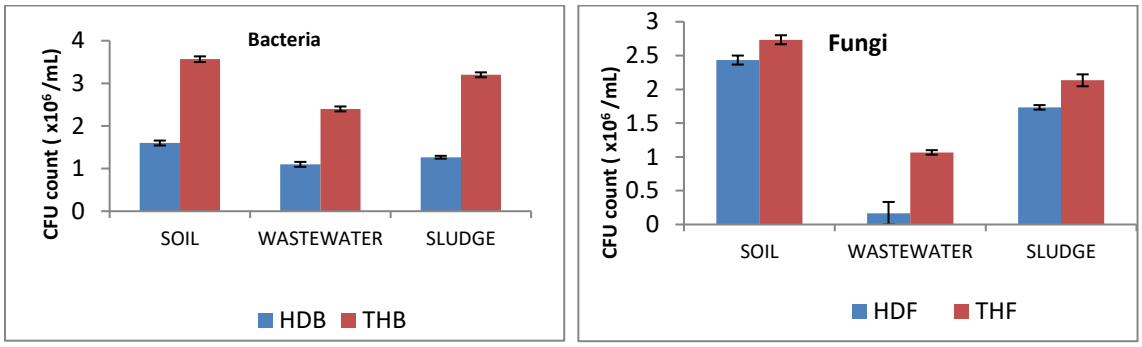

Figure 1 CFU count of total heterotrophic bacteria (THB) and fungi (THF) compare with hydrocarbon utilizing bacteria(HDB) and fungi (HDF) (**Data presented are the mean of triplicates with standard error (5%))

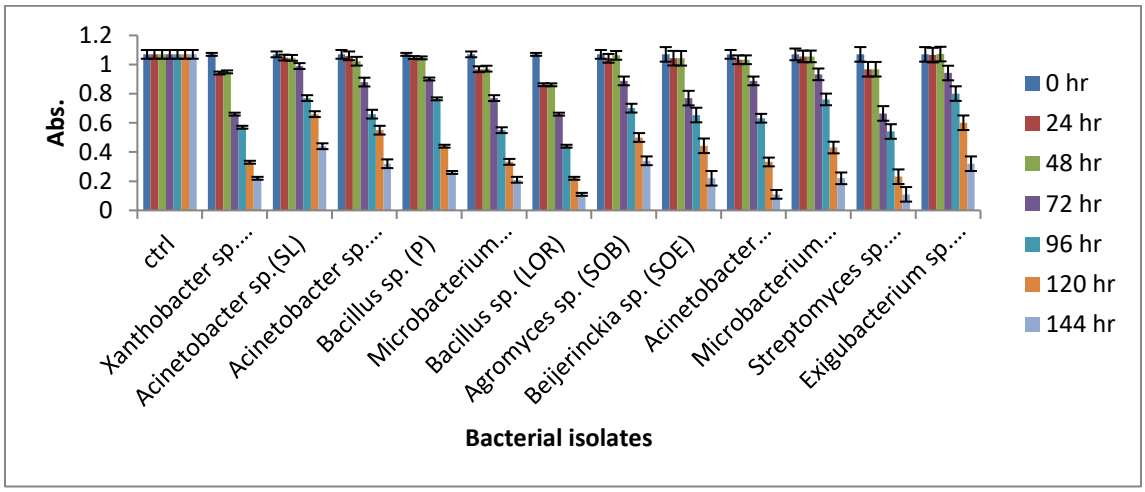

Figure 2 DCPIP Assay for bacterial strains in presence of PAH mixture (*Data presented are the mean of triplicates with standard error)

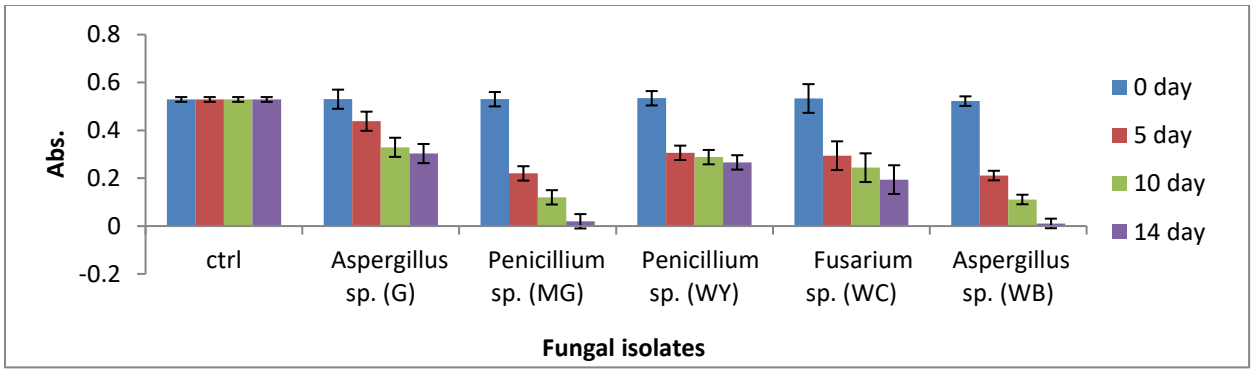

Figure 3 DCPIP Assay for fungal isolates in presence of PAH mixture (*Data presented are the mean of triplicates with standard error)

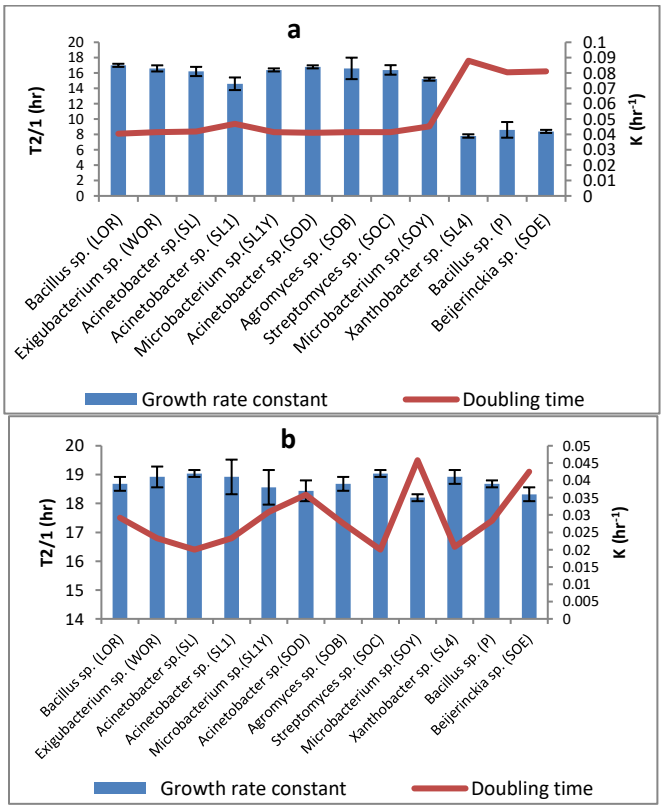

Figure 4. Growth rate constant and doubling time of bacterial isolates in presence of a) paraffin oil and b) PAH mixture (*Data presented are the mean of triplicates with standard error)

The selected bacterial and fungal isolates were cultivated individually on culture medium with PAH mixture (figure 6) as carbon source to evaluate their ability to produce glycolipid biosurfactant. Among fungi *Fusarium sp*. WC and *Penicillium sp. MG* showed

highest glycolipid concentration upto 200 mg/L. Whereas bacterial isolates; *Acinetobacter sp*. SOD, *Agromyces sp.* SOB, *Microbacterium sp*. SL1Y has shown considerably maximum glycolipid (59.3, 56.7, 56.2 mg/L) production. For screening of biosurfactant production Oil displacement test (ODT) all the isolates showed positive results accept *Exigubacterium sp.* WOR & *Agromyces sp.* SOB and among fungal isolates only *Fusarium sp.* WC showed positive result. Fungal isolates *Fusarium sp.* WC & *Penicillium sp.* WY showed maximum dry biomass values i.e. 2 and 1.8 g/L followed by *Penicillium sp.* MG, *Aspergillus sp.* WB, *Aspergillus sp*. G (table 4) in an experimental set up of 15 days on PAH mixture and paraffin oil (table 4). In CTAB assay only *Exigubacterium sp.* WOR bacterial strain was found positive indicating production of anionic biosurfactant. In case of fungi *Fusarium sp.* WC showed positive response.

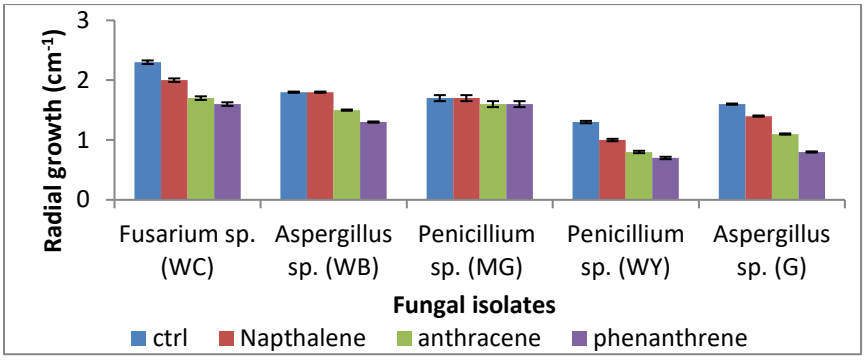

Figure 5. Measurement of Radial Growth tested with PAH Compounds (500µL each) on 7th Day (*Data presented are the mean of triplicates with standard error)

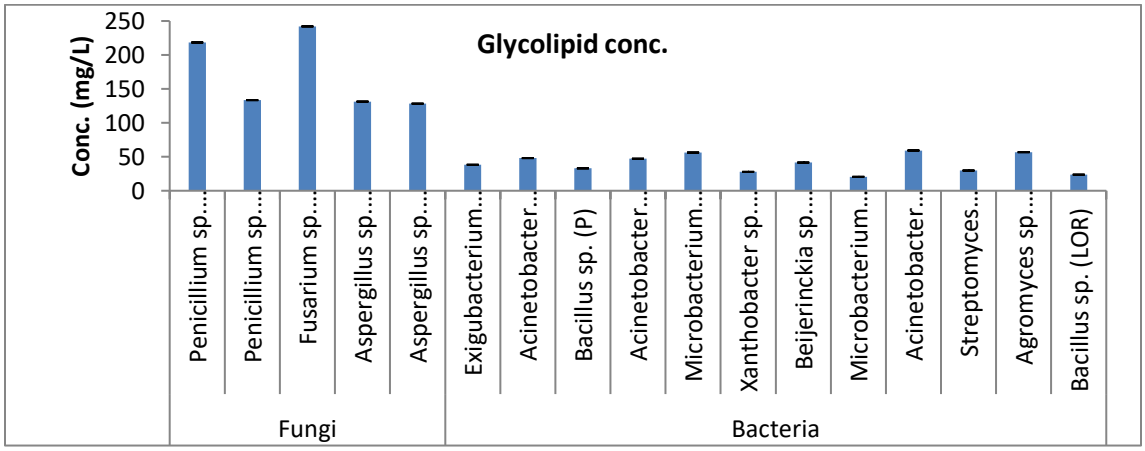

Figure 6 Glycolipid concentration of isolated bacterial and fungal isolates (*Data presented are the mean of triplicates with standard error)

Table 4. Screening of biosurfactant producing bacterial and fungal isolates by qualitative test

Bacterial isolates	**Oil displacement test**	**CTAB assay**
Exigubacterium sp. WOR	-	+
*Acinetobacter sp.*SL	+	-
Bacillus sp. P	+	-
Acinetobacter sp. SL1	+	-
Microbacterium sp. SL1Y	+	-
Xanthobacter sp. SL4	+	-
Beijerinckia sp. SOE	+	-
Microbacterium sp. SOY	+	-
Acinetobacter sp. SOD	+	-
Streptomyces sp. SOC	+	-
Agromyces sp. SOB	+	-
Bacillus sp. LOR	+	-
Fungal isolates		
Penicillium sp. MG	-	-
Penicillium sp. WY	-	-
Fusarium sp. WC	+	+
Aspergillus sp. G	-	-
Aspergillus sp. WB	-	-

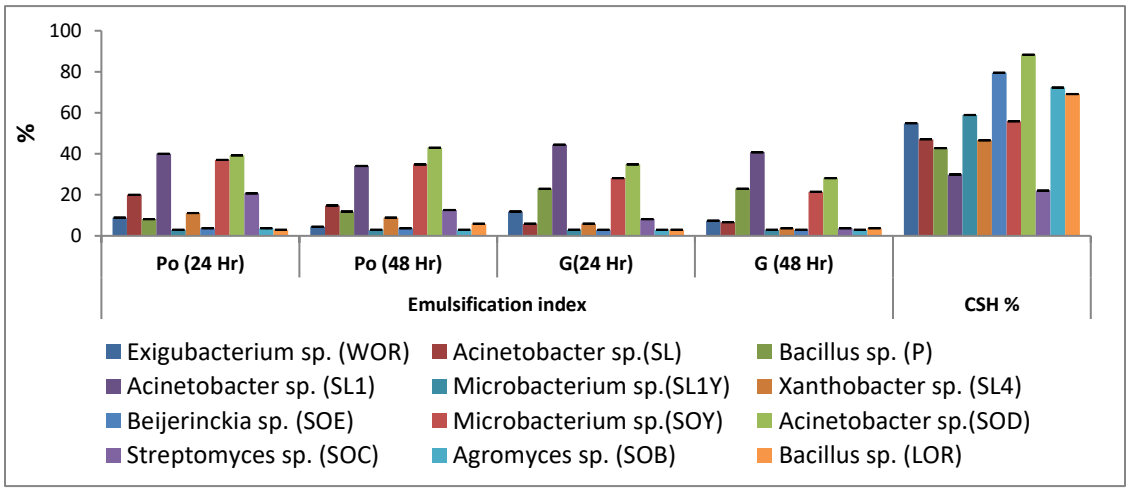

Figure 7. Emulsification index and cell surface hydrophobicity percentage of bacterial isolates (*Data presented are the mean of triplicates with standard error)

The emulsification of the both hydrocarbon compounds (PAH mixture and paraffin oil) by isolated species was calculated after 24 and 48 h. Emulsification index of *Acinetobacter sp.* SL1, *Microbacterium sp.* SOY, and *Acinetobacter sp.* SOD showed a higher capability to make emulsion with both hydrocarbons i.e. 40, 37.03, 39.25 % respectively (figure 8). All bacterial isolates only *Streptomyces sp.* SOC and *Acinetobacter sp.* SL1 showed lowest range of hydrophobicity percentage whereas all isolates showed CSH% above 40. In case of fungi *Penicillium sp.* MG showed highest

percentage of CSH followed by *Aspergillus sp.* G and *Penicillium sp.* WY (58.8> 53.9> 52.14 % respectively). *Fusarium sp.* WC & *Aspergillus sp.* G (45.1 , 47 respectively) exhibited highest emulsification activity followed by *Aspergillus sp.* WB, *Penicillium sp.* WY, *Penicillium sp*. MG (42.9 > 29.6 > 22.9) (figure 9). Heavy metal tolerance among all the isolates *Penicillium sp* WY and *Bacillus sp.* P showed highest tolerance against Pb> V> Cd> Ni> Cr. The lowest lead tolerance was observed in *Microbacterium sp*.(SOY), *Fusarium sp.* (WC), *Xanthobacter sp.* (SL4) tolerated lead upto 60> 50> 40 ppm (respectively). Microbial cultures showed highest sensitivity to Cr since proliferation was inhibited at higher concentration except *Bacillus sp.* (P) (figure 9). Qualitative analysis of ligninolytic enzymes; lipase and non-specific peroxidases was observed 24% (6) bacterial isolates. Among fungi isolates 29% (5) isolates showed manganese peroxidase activity and 23% (4) lipase activity. However laccase, lignin peroxidase, phenol oxidase were less often found in isolates (table 6).

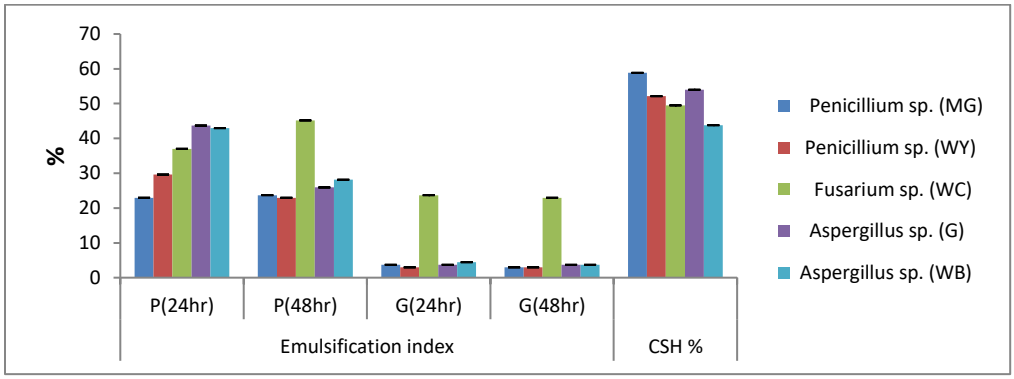

Figure 8 Emulsification index and cell surface hydrophobicity percentage of fungal isolates (*Data presented are the mean of triplicates with standard error)

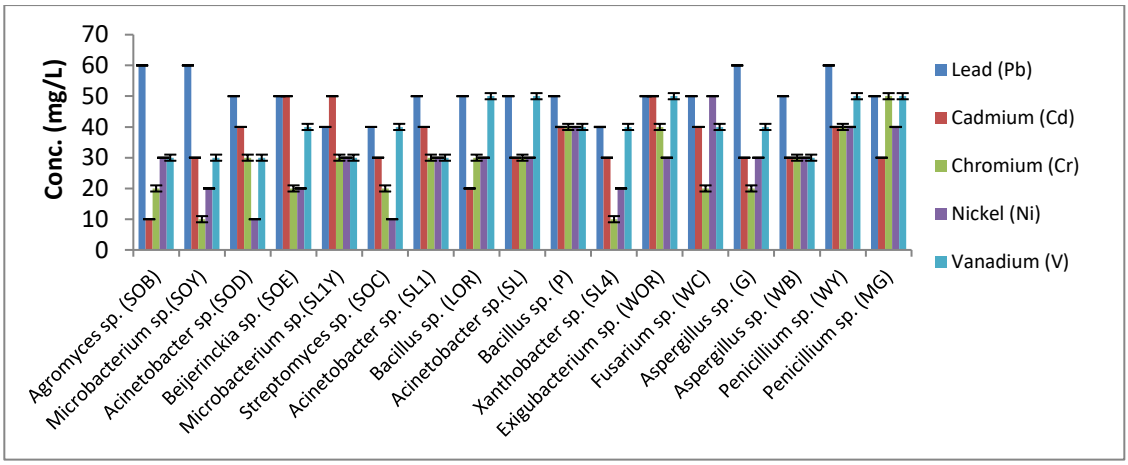

Figure 9 Metal tolerance concentration (ppm) of different heavy metals with bacterial and fungal isolates (*Data presented are the mean of triplicates with standard error)

Table 5 Qualitative analysis of ligninolytic enzymes

Isolates	**Lignin peroxidase**	**Manganese peroxidase**	**Laccase**	**Non-specific peroxidase**	**Lipase**	**Phenol oxidase**
Agromyces sp. SOB	-	-	-	-	-	-
Microbacterium sp. SOY	+	-	-	+	+	+
Acinetobacter sp. SOD	-	+	-	+	+	-
Beijerinckia sp. SOE	-	-	-	-	-	+
Microbacterium sp. SL1Y	+	-	-	+	+	-
Streptomyces sp. SOC	-	-	-	-	-	-
Acinetobacter sp. SL1	-	-	-	-	-	-
Bacillus sp. LOR	-	+	+	+	+	-
Acinetobacter sp. SL	-	+	-	+	+	+
Bacillus sp. P	+	+	-	+	-	-
Xanthobacter sp. SL4	-	-		-	-	-
Exigubacterium sp. WOR	-	+	+	-	+	-
Fungi						
Fusarium sp. WC	-	+	+	-	+	-
Aspergillus sp. G	-	+	-	-	+	-
Aspergillus sp. WB	-	+	-	+	+	-
Penicillium sp. WY	+	+	-	+	-	+
Penicillium sp. MG	+	+	+	+	+	-

(+ present, - absent)

Discussion

High concentration of TDS, TSS, COD, DO, BOD, PO_4^{3-}, SO_4^{2-}, Cl^- and NO_3^- were also observed in previous research study on petrochemical effluent (Gupta *et al*., 2015). Higher values of electrical conductivity in all the samples were observed. In case of effluent high EC may results into high turbidity and presence of dissolved suspended solids, which can cause problem in water purification processes such as flocculation and filtration, hence increases the treatment cost. In soil and sludge samples the higher conductivity indicates excessive clay and CEC high clay content may limit crop production, thus reducing inputs in agricultural field. High amount of sulphate, phosphate and nitrate was found in all the samples that may lead to high salt content resulting in nutritional imbalance and mineral toxicity in plants as well as to human health. Varied concentrations of these metals were found due to discharge during transportation, extraction and refining process of crude oil.

The release of biosurfactant decreases the surface tension, the applied hydrocarbons and available to microorganism for absorption, metabolism and ultimately the emulsification of hydrophobic compounds (Anyanwu and Chukwudi, 2010; Calvo *et*

al., 2004; Fatemeh *et al*., 2015). The formation of a stable and constant emulsion, while the hydrophobic compounds mixed with bacterial colonies and fungal cultures, is the sign of surfactant existence (Makkar and Cameotra, 2002). Oil displacement test (ODT) is another qualitative method applied for biosurfactant production, when the activity and quantity of biosurfactant is low (Walter *et al*., 2013). Cell surface hydrophobicity (CSH) is a rapid identification of biosurfactant producing strains can be achieved by assaying this trait (Liu *et al*., 2013). Glycolipid consisiting of L-rhamnose at the stationary phase indicating that, microorganisms utilizes most of the carbon sources available first and then formation of product take place (Pathaka and Nakhate, 2015). From the previous research studies it was found that only *Pseudomonas* is known for the production of glycolipid but recent studies reported that some strains of *Acinetobacter* and *Enterobacter* also possess the glycolipid production capacity (Dong *et al*., 2016). DCPIP assay presented a decrease in absorbance, which indicates a positive interaction of the bacteria in oil degradation. This observation is supported by the findings of Selvakumar *et al*. 2014 according to which the isolates are potential hydrocarbon oxidizers. Although Foght *et al*.(1990) postulated that bacteria having multi-degradative capacity might exist. In previous studies *Pseudomonas* and *Bacillus* have been reported to be among the most frequently isolated bacteria from hydrocarbon-polluted sites (Atlas, 1992; Okoh and Trejo-Hernandez, 2006). Species of *Pseudomonas, Bacillus, Micrococcus* and *Proteus* isolated from hydrocarbon-contaminated site utilize hydrocarbon through oxidation of DCPIP (Roy *et al*., 2002; Joshi and Pandey, 2011; Patil *et al*., 2013; Yoshida *et al*., 2001; Adegbola *et al*., 2014; Balogun *et al*., 2015). Growth substrate could either be due to the consecutive nature of hydrocarbon assimilation capabilities in the isolates or reflect the adaptation of the isolates due to previous exposure of exogenous hydrocarbons. It may also indicate the ability of the bacterial and fungal strains to emulsify hydrocarbon, which is a major factor for hydrocarbon uptake and assimilation (Ekundayo and Obuekwe, 2000). Singh *et al*., 2010 reported that moderately tolerated tested metal were good hydrocarbon degrading organism. These results are in conformity with the observation of Thavamnai (2012) that co-occurance of heavy metal and hydrocarbons might be the source of the selective evolution of few distinctive species able to cope up with both type of contamination at the same time. Accept these metabolic characteristic features role of different enzymes are very important aspect. Here, Ligninolytic enzyme system has been focused which enables microbes to degrade organic compounds including aromatic hydrocarbons. Ali *et al*. (2012) reported that α- protobacteria, ϒ-protobacteria, actinomycetes possess lignin degrading ability.

Conclusion

This study revealed a qualitative evaluation of hydrocarbon tolerant bacteria and fungi. The methodology adopted for screening of hydrocarbon utilizing microbes showed that all the isolated bacterial and fungal strains consist characteristic growth in presence of different hydrocarbons (as carbon source) and hydrocarbon utilizing trait. In experiments like emulsification index, ODT and CTAB Assay *Acinetobacter sp.* SL1,

Microbacterium sp. SOY, *Acinetobacter sp.* SOD, *Fusarium sp.* WC showed maximum emulsification index and positive response for other test. During estimation of glycolipid, CSH% was observed above 40% among all the isolated strains. All the twelve bacterial shown biodegradation capability against different hydrocarbons used in DCPIP assay. Fungal strains such as *Penicillium sp.* MG**,** *Aspergillus sp.* WB showed high rate of dye removal and biodegradability followed by *Aspergillus sp.* G, *Penicillium sp.* WY in presence different hydrocarbon sources. Results obtained can be used for comparative potential of bacterial and fungal strains for the hydrocarbon degradation. Thereby giving a measureable ability of these groups of bacterial and fungi possible use in hydrocarbon impacted soil or wastewater remediation with their ability to produce valuable enzyme as well as biosurfactant. Study also provides a strategic criterion for selecting potential bacteria and fungi, which may play major role in the degradation of hydrocarbons specifically. Moreover, these strains can be used for the preparation of potential bacterial or fungal consortium for the degradation of selected hydrocarbon because of their multi-degradative capacity and strain specific characteristics.

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