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Enhanced Degradation of Carcinogenic PAHs Benzo (a) Pyrene and Benzo (k) Fluoranthene by a Microbial Consortia

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Abstract

Background/Objectives: Bioremediation of High Molecular Weight PAHs (HMW PAHs) with a combination of microorganisms was investigated. In the present study, the PAHs degradation capacity of a microbial consortium consisting of fungal, bacterial cultures was evaluated. Within 7 days, consortium showed 64% and 69% degradation of Benzo (a) pyrene (BaP) and Benzo (k) fluoranthene (BkF) each PAH of 30mg/L concentration respectively as the sole carbon source. The culture broth was analyzed for the presence of metabolites by gas Chromatography-Mass Spectrometry (GC-MS) and the activities of enzymes produced by the consortium were also assayed during the degradation process. Coexistence of three metabolic pathways, cytochrome P450 monooxygenase with the detection of epoxy derivatives leading to phenols, trans-dihydrodiols, cis-dihyrodiols leading to salycyclicacid and catechol by bacterial dioxygenases and via quinones of ligninolytic pathway were identified. Besides, Microbial consortia also demonstrated good biosurfactant production capacity, achieving a maximum emulsification activity of 61% (BaP and BkF) in the presence of PAHs as sole carbon source. These results indicate that this consortium can be used in the bioremediation of PAHs mixtures cometabolically in the contaminated environments.

Keywords: Biosurfactant, Degradation Pathways, GC-MS Metabolites, PAHs, Microbial Consortium

1. Introduction

Polycyclic aromatic hydrocarbons are a class of organic compounds, which, when present at higher concentrations, pollute the environment. By virtue of their higher hydrophobic structure, they are completely insoluble in water. In addition, their large negative resonance energy enables them to be chemically stable[1]. Generally PAHs are comprised of two or more fused aromatic rings arranged in different structural configurations. They are broadly divided into two types Low Molecular Weight (LMW) and High Molecular Weight (HMW) PAHs. PAHs are widespread and are generated from natural and anthropogenic sources. The latter being the cause of environmental hazards. Their affinity towards fatty acids enable them to react with fatty acids, thereby accumulate in food[2] and tend to stay in the environment for longer periods. Furthermore, their biological toxicity increase the concern for environment[3]. Improper management of PAHs poses severe health hazards.

Therefore a significant effort is being put in to remove these pollutants from the environment. Among different methods, one of the methods which is effective, economical and eco- friendly and yields sustainable result is bioremediation using different microbes. Nevertheless, many works reported efficient bioremediation using inoculation of microbes[4,5]. Besides, biological degradation of PAHs renders less soil imbalance[6]. Bacterial degradation of PAHs is found to be a promising technique[7] and many reports documented their usage as growth substrates[8,9].

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Bioremediation using bacterial community takes both aerobic and anaerobic pathways, but degradation of HMW PAHs is slower when compared to LMW PAHs. This is due to the fact that the initial energy required do not meet the demands of the initial growth of bacteria and hence additional carbon source is prerequisite for the degradation to take place.

This facilitates HMW PAHs more persistent in the environment, making them more toxic. Several research reports affirmed PAHs biodegradation using Fungi[10–13]. Both lignonolytic and non-ligninolytic fungi engage in degradation of PAHs. Whatsoever, bacteria or fungi as biodegrading sources, in vitro studies ordinarily include isolation of microbes as pure cultures, followed by elicitation of the metabolic pathways through which microbes degrade PAHs. On the other hand degradation of PAHs in the natural environment is quite complex as both high molecular weight and low molecular weight PAHs are present. This further becomes more complex as a broad range of microbial population is present in the environment.

This situation can be better addressed by employing consortium instead of pure culture, facilitating, degradation of both high and low molecular weight PAHs where, both bacteria and fungi work in synergy to achieve better results. This increased performance of microbes is due to the fact that some microbes degrade primary PAHs while some react with intermediate metabolites there by resulting in complete degradation[14]. Works by[15-17] emphasized usage of consortium for complex PAHs degradation.

Though biodegradion using microbes as entity is proved to be advantageous for soil contaminants, there exists several limitations for the same to remove contaminants in water. Factors like solubility, dissolution rate, partition coefficient, visocity hampers usage of cell as a whole for biodegradation of PAHs in water. These viabilities can be conquered by using biosurfactants which favors biodegradaion by enhancing oil dispersion into aqueous phase, which further decreases the volatalisation of low carbon hydrocarbons[16]. Being ecologically accepted is one of the important and convincing advantages of biosurfactants over chemical surfactants. Hence the present work aim at analyzing degradation capability of consortium and its ability to produce biosurfactants. Also, work targets at identifying the metabolites produced and enzymes involved during degradation.

2. Method

2.1 Chemicals and Culture Media

Aromatic hydrocarbons Benzo (a) pyrene (BaP) and Benz (k) fluoranthene (BkF) were purchased from Sigma-Aldrich (India). All other chemicals were of analytical grade (Merck India Ltd., India). Nutrient Broth (NB) and Potato Dextrose Agar (PDA) were purchased from Himedia- India. MSM (Mineral Salts Medium) contained M9 salts 5x (g/L):

$Na_2HPO_4{\cdot}7H_2O$, 64; KH_2PO_4, 15.0; (NH_4)cl, 5.0; Nacl, 2.5; 2 mL of 1M $MgSO_4{\cdot}7H_2O$, 0.1 mL $Cacl_2$ and 1 mL Trace elements solution (g/L); $FeSO_4.7H_2O$, 1; $(NH_4)6Mo_7O_{24}.4H_2O$, 0.22; ZnSO4·$7H_2O$, 4.4; $CuSO_4{\cdot}5H_2O$, 0.32; H_3BO_3, 2.2; $CoCl_2{\cdot}6H_2O$, 0.32; in 1000 mL deionized water; pH 7.2–7.5.

2.2 Microorganisms and Cultivation

2.2.1 PAHs Degrading Consortia

Organisms isolated from different hydrocarbon contaminated soil and waste environments were obtained from MTCC, Chandigarh and were used for development of consortium which includes mixtures of pure cultures viz., Acinetobactercalcoaceticus (MTCC 2409, 2289), Serratiamorcescens (MTCC 2645), Pseudomonas species (MTCC 2445), Stenotrophomonasmaltophilia (MTCC 2446) and Aspergillusterricolavar Americanus (MTCC 2739). These isolates were maintained on nutrient agar for further studies.

2.2.2 Inoculum Preparation

The inoculum was prepared by growing each culture individually in 50 mL sterile MSM with 2% dextrose for 24-48 hours at 300C, 150 rpm in a rotary shaker followed by the centrifugation of cells at 40C, 9000 rpm for 15 minutes and the obtained pellet was washed in sterile MSM and the suspension was used as inoculum. Fungal inocula was prepared by collecting the small mycelia pellets grown in Potato Dextrose Broth (PDB) through Whatmann no. 1 paper filtration, washed with MSM and these suspensions were used as inocula in the degradation experiments.

2.3 Biodegradation of PAHs in Liquid Medium

The degradation capacity of the consortium was evaluated by the shake flask method. All experiments were per-

formed in a 100 mL Erlenmeyer flask containing 20 mL of the MSM and the sacrifice technique was used for degradation analysis. PAHs dissolved in Dichloromethane (DCM) were added to the autoclaved flask and evaporated. Autoclaved MSM was transferred into the flask to a final concentration of 30 mg/L for each PAH compound (BaP and BkF). MSM-PAH was inoculated with mycelial pellets to obtain an initial fungal biomass of (dry weight) 1.5 mg/ml. The effect of pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0) on the degradation of PAHs was studied. All experiments were conducted in triplicate and kept in an orbital shaker at 150 rpm, 30°C for 7 days. Non-inoculated bottles were set as controls and the samples were sacrificed at every 2 days interval for analysis. Two other flasks were used to determine the metabolite analysis.

2.4 Analysis

2.4.1 Evaluation of Growth

The growth of consortium utilizing the PAHs was determined by measuring the dry biomass and the residual PAH in the culture medium. After extracting the residual PAH from the culture medium, pellet was collected by filtration and with subsequent drying, the biomass was determined by gravimetric method[18].

2.4.2 HPLC Analysis of PAHs Degradation

The entire content of the flask 20 mL was extracted with DCM and analysis was performed using HPLC (Schimazdu) with a Phenomenex Luna C18 Reverse phase column (4.6mm x 250) and a SPD-20A UV-VIS detector using Methanol-water (1 mL/min) as mobile phase and PAHs elution were detected at 254 nm. The percentage reduction of BaP and BkF was evaluated by comparing with the peak area of the control flaks.

The degradation of PAH was calculated as:

$Rd = (C0 - Cn)/C0 \times 100\%$

where, Rd (%) represents degradation rate, C0 (mg/L) represents the initial concentration of HMW-PAH and Cn (mg/L) represents the remained concentration of HMW-PAH after incubation for n days[19].

2.4.3 Analysis of PAHs Metabolites

The cultures were sampled on 5th day and the isolation of metabolites was performed using the method of [20]. Ethyl acetate-extracted metabolites present in the neutral fraction and acidic fraction were analyzed by using a Thermo Scientific chromatograph with a model TSQ Quantum XLS mass spectrometer equipped with a HP-5MS column. The inlet and the interface temperature were kept at 300°C and 280°C with a split ratio of 10 for 2 min.

Helium was used as the carrier gas with a flow rate of 1 mL/min and the temperature program was set as follows: 80°C for 1 min, and then 7°C/min up to 300°C for 5 min hold. The mass spectrometer was operated at electron ionization energy of 70 eV with a scan range from 35-500 m/z (mass to charge ratio). Metabolite identification was based on mass spectra comparison with the NIST-011 library.

2.5 Screening of Enzyme Activities during Degradation

The consortium was screened for its enzyme producing potential in Mineral Salts Medium (MSM) broth to study their involvement at different stages of degradation. Enzymatic assays were assayed using a UV-Visible spectrophotometer (Thermo scientific). Cultures were subjected to centrifugation at 8000 rpm for 20 min for separation of extracellular and intra cellular products. The supernatant was directly utilized for extracellular enzyme activities, whereas, intracellular enzymes were extracted through sonication and centrifugation at 8000 rpm for 15 min to avoid cell debris. Total protein content for intra and extra cellular extracts was performed by using Lowry's method[21]. Activities of bacterial enzymes like 1 hydroxy 2-Naphthoicacid dehyrogenase (1-H-2NAD), 2-carboxybenzaldehyde dehydrogenase (2CABL), catechol 1,2 dioxygenase (C1,2O), protocatechuate 3,4 dioxygenase (P3,4O) and protocatechuate 4,5 dioxygenase (P4,5O) were assayed according to[22]. Activities of fungal enzymes like laccase was measured by monitoring the oxidation of ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) at 405 nm (ε405 nm = 36.8 mM/cm) and Lignin peroxidase (LiP) by the oxidation of veratryl alcohol to form veratrylaldehyde at 405 nm (ε405 nm = 9.3 mM/cm)[23]. All the experiments were carried out at 30°C and the enzyme activity was expressed as µmoles product formed/min/mg protein.

2.6 Emulsification Index (E24)

The emulsifying capacity of the cultures was evaluated by E24 method[24]. 2 mL of supernatant was added to 2 mL of kerosene, petrol, diesel in a screw cap test tube, vor-

texed for 2 minutes and mixture was made to stand for 24 hours. The percentage of Emulsification index was calculated using the formula:

E24 = Height of emulsion formed (cm) X 100/Total height of solution

2.7 Biosurfactant Production and Extraction

The biosurfactant produced by the consortium was extracted on 5th day as described by[25]. In brief, the entire content of the flasks (250ml) containing PAHs (BaP and BkF) as the sole carbon source was centrifuged at 8000rpm for 15min at 4oC and the collected supernatant was acidified with 6N HCl at pH2 and left for overnight at 4^0C in the refrigerator for precipitation, while the remaining supernatant was used for estimating different properties of biosurfactant.

The isolated biosurfactant was characterized by Silica gel 60 F254 TLC plates (Merck). The running solvent was a mixture of chloroform, methanol and water in the ratio of 65:25:4 (v/v). Plates were air dried and sprayed with p-anisaldehyde reagent to detect glycolipids[22]. Carbohydrate content in the biosurfactant was determined by Phenol sulphuric acid[26] and the protein by lowry method[21] at 620 nm and 660 nm respectively by UV-visible spectrophotometer.

3. Results and Discussion

3.1 PAHs Biodegradation

A consortium of bacterial, fungal cocultures was developed and its degradation efficiency and growth were evaluated every two days with HMW PAHs (BaP and BkF) as the sole carbon source. Biomass showed a linear relationship with the degradation rate in the first 5 days, except for the decline during the 7th day of incubation, which could be attributed to the depletion of the carbon source (Figure 1(a)).

The biodegradation of PAHs in liquid medium by consortium was evaluated by HPLC over the period of 7 days. The effect of pH on PAHs biodegradation by consortium was illustrated in Figure 1(c). It was observed that the pH of the MSM could influence the PAHs degradation. The maximum degradation of BaP was attained at pH 7.0 (64%), while for BkF at pH 6.5 (69%) was observed respectively. A minimum degradation of 50% was observed for all PAHs within the pH range 6.5-7.5 and clearly the maximum degradation at pH 7.0 and 6.5 as mentioned earlier concluding that the bioremediation by consortiumcan suitably adapt to the environmental conditions.

The degradation rate of Benzo (a) pyrene and Benz (k) fluoranthene in liquid medium after 1, 3, 5 and 7 days of incubation was shown in Figure 1(c), reporting the degradation of 60% within 7 days of incubation for BaP and BkF. Also consortium when tested without fungus showed maximum degradation of 44% and 48.4%, whereas only fungus *Aspergillusterricola* 55.8% and 42% and together as fungal bacterial consortium degraded 64% (BaP) and 69% (BkF) respectively, an enhanced degradation rate when compared to consortium of only bacteria and fungal strain indicating that bacterial fungal cocultures degrade PAHs effectively compared to pure cultures[27,28]. (Figure 1(b)).

The present work correlated with the studies of[15] who has shown the mineralization of BaP to CO_2 by 28% in 49 days by defined fungal bacterial cocultures and[29] showed 44.07% degradation of BaP (10 mg/L) by bacterial consortium in 14 days and also reported the high degradation rate with assemblage of organisms over individual organisms indicating synergism among the consortial members.

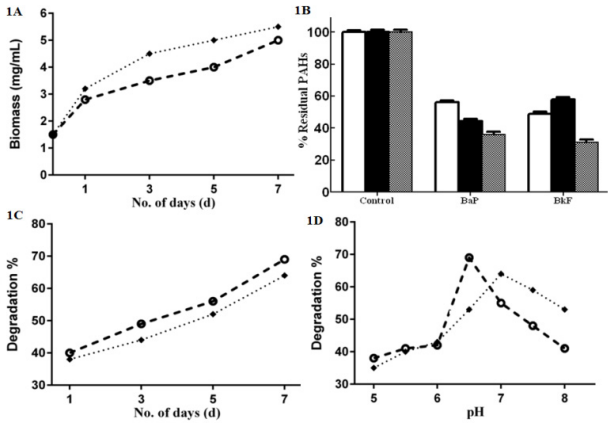

Figure 1. Biomass and degradation percentage of PAH's.

Figure 1. Degradation of PAHs at different pH and time intervals. **Legends: (a)** Biomass of fungal and bacterial consortium over a period of 7 days with PAHs (BaP: ·◆·, BkF: -⊖-) as sole carbon source. **(b)** Residual percentage of PAHs remained after 7 days of incubation with only bacteria consortia (▭), fungus (■) and fungal-bacterial cocultures (▩). **(c)** and **(d)** Rate of degradation

of PAHs (BaP: ◆, BkF: ⊖) and degradation percentage of PAHs with respect to pH by consortium.

Based on previous studies[30], showed only 34-37 % removal of 50 mg/L BaP by *Stenotrophomonasmaltophilia strain VUN10,003* in 42 days. Also[31,32] reported the degradation of BaP and BkF with metabolites identification by *Sphingomonas species*[33] HMW-PAHs degradation by *Mycobacterium species*. In the current study, the individual degradation of BaP and BkF above 60% in 7 days with PAH as their sole carbon source by the microbial consortium has been reported.

Although the other consortium members *Acinetobactercalcoaceticus* and *Serratiamorcescens* did not show high degradation rate individually, their presence played an important role in production of biosurfactant for enhanced degradation as consortium in their absence showed a decreased degradation rate (data not shown).

Hence, it was hypothesized that fungus commences the initial steps of the oxidation of PAHs by producing extracellular enzymes, facilitating the remaining bacterial microorganisms in synthesizing the biosurfactants thereby enhancing the PAHs bioavailability and utilizing them as carbon source with increased PAHs degradation rate. Also by selecting consortial members that are in turn capable of producing biosurfactants, PAHs biodegradation can be enhanced. It was also worth further exploring the metabolites of PAHs by GC-MS analysis in understanding the pathways employed in biodegradationthe consortium.

3.2 GC-MS Analysis: Degradation Pathway Elucidation

The metabolites formed during the degradation of PAHs were analyzed on the 5th day of incubation using GC-MS. Amongst the detected compounds, a mixture of alkanes, alkenes, amines, aromatic compounds, ketones, alcohols, fatty/carboxylic acids and epoxy derivatives of PAHs were noted. The presence of epoxy derivative metabolites in the medium, Oxirane [(hexadecyloxy)methyl]-(Propane, 1,2epoxy3(Hexadecyloxy), (BaP and BkF) elucidates the initiation of the PAHs oxidation to epoxide (arene oxide) mediated by the cytochrome P450 monooxygenase of the consortium[34].

Also, the presence of phenols, Phenol, 2,4 bis (1,1 dimethyl ethyl) (Rt-7.03) and Phenol, 4,4'-(1methylethylidene) bis (2,6-dimethyl) (Rt- 14.42) (BaP and BkF) as metabolites (Table 1.) during PAHs degradation indicates the successive conversion of epoxides nonenzymatically. In[35,36] also observed Phenol, 2,4bis (1,1-dimethylethyl) as a metabolite during the degradation of naphthalene by *Streptomyces* and PAHs by *Acinetobacter sp.* Further the phenols were converted to conjugates of sulfates 1-Hexadecane sulfonyl chloride (Rt- 10.73) (BkF) and 1Docosanethiol (Rt-25.63) (BaP) and methoxyls 2',3',4 Trimethoxyacetophenone (Rt-13.99), 3,4,5 Trimethoxybenzoicacid hydrazide (Rt-15.10) (BaP) and Cedryl propyl ether (Rt-8.97) and Benzyl alcohol, 4,5dibenzyloxy (Rt- 23.06) (BkF) infers the transformation of phenols. In[34,37] reported the oxidation of Benzo(a) pyrene to benzo[a]pyrenyl sulfate via cytochrome P450 monooxygenation and sulfate conjugation. Diols like 1,2-Benzenediol, 3,5-bis (1,1-dimethylethyl) (Rt- 16.97) (BaP and BkF) and (n)-Hydroxy metabolites Naphthalene,2-decyldecahydro (Rt- 19.32) (BaP) were observed during degradation on the 5th day that are successively converted to diones and to quinones (Table 1).

Curidone(6,10-dimethyl-3-(1methylethyl)-6cyclododecene-1,4dione) of Rt-8.97, 1,3-butanedione, 1-(2,6,6-trimethyl-1-cyclohexen-1-yl) (Rt- 9.58) (BaP) and 2Propanone,1(1,3dioxolan2yl) (Rt- 5.04), Ethanone, 1-(4methyl-2-thienyl) (Rt-4.84) (BkF) and Acetophenone (Rt- 3.58) (BaP and BkF) was observed during the PAHs degradation. Other major metabolites identified were quinones which confirms the involvement of fungal extracellular ligninolytic enzymes specifically lignin peroxidase and laccases which oxidizes poly aromatic hydrocarbons to quinones, with subsequent ring cleavage to produce phthalic acids and eventually to CO_2[38]. Previous studies reported 1,6 and 3,6 quinones as GC-MS metabolites during the degradation of BaP and BkF by fungal species like *Cunninghamellaelegans, Pencilliumchrysogenum and Fusarium*[34]. In the present study, 2,5-di-tert-butyl-1,4 benzoquinone (Rt- 6.64) (BaP and BkF), p-benzoquinone 2,5-bis (1,1,3,3 tetramethyl butyl) (Rt- 15.47) (BaP) were observed as quinone derivatives during the PAHs degradation.

On the other hand, bacterial dioxygenases can convert PAHs to cis-dihyrodiols by bacterial dioxygenases and diols to catechol by dehydrogenases[31]. Phenols can also be oxidized aerobically by bacteria to catechol by monooxygenase and catechol subsequently undergoes otho or meta ring cleavage and finally produces succinate, Acetyl CoA or Acetaldehyde and Pyruvate[39]. Salicylic acid, 3-fluoro (Rt- 4.97) (BkF) and p-tert-butyl catechol (Rt-4.52) was observed during the degradation of BaP and BkF.

PAHs are converted into products like simple hydrocarbon alkanes, alkenes, cycloalkanes (Table 2.) which

undergoes terminal and subterminal oxidation to produce alcohols, fatty acids which are further mineralized by β oxidation pathway to form intermediary compounds. In[40] reported alkanes, CO_2 and water as PAHs degradation products. The accumulation of alkanes (decane-Heneicosane), alkenes- 3-dodecene (z)-(Rt- 4.38) and 17-Pentatricontene (Rt-41.38) (Table 2.) and cycloalkanes in our culture medium indicates the breakdown of PAHs into simple hydrocarbons as there were none present in the controls. Also, the presence of fatty/dicarboxylic acids, alcohols reported earlier as intermediates in alkane degradation indicates utilization of the PAH compounds[41,42].

Fattyacids like dodecanoic acid (Rt-7.63), n-hexadecanoic acid

(Rt-17.26), tetradecanoic acid (Rt- 11.56), octadecanoic acid (Rt-24.37) fatty alcohols (n-pentadecanol-n-Heneicosonal) were also detected during degradation.

Table 1. GC-MS metabolites of each PAH in 5th day of the experimentation

Type of Metabolite	BaP	RT	BkF	RT
Epoxy derivatives	Oxirane [(hexadecyloxy)methyl]-(Propane, 1,2epoxy3(Hexadecyloxy)	13.36	Oxirane,[(dodecyloxy)methyl] (Propane,1(dodecyloxy)2,3epoxy-)	13.11
Aromatic organic compounds	t-butyl hydroquinone/ p-tert-butyl catechol	4.52	t-butyl hydroquinone/ p-tert-butyl catechol	4.52
	2,5-di-tert-butyl-1,4-benzoquinone	6.64	Salicylic acid, 3-fluoro (Benzoic acid, 3fluoro-2-hydroxy)	4.97
	Phenol,2,4-bis (1,1-dimethylethyl)-	7.03	2,5-di-tert-butyl-1,4-benzoquinone	6.64
	Phenol, 4,4'-(1methylethylidene) bis (2,6-dimethyl)	14.42	Phenol,2,4-bis (1,1 dimethylethyl)-	7.03
	P-Benzoquinone, 2,5-bis (1,1,3,3-tetra methyl butyl)-	15.47	Phenol, 4,4'-(1methylethylidene) bis (2,6-dimethyl)	14.40
	3,9-ditert-butyl Phenanthrene	17.42		
Ketone deivatives	Acetophenone	3.58	Acetophenone	3.58
	Curidone (6,10-dimethyl-3-(1methylethyl)-6cyclododecene-1,4dione)	8.97	Ethanone, 1-(4methyl-2-thienyl)	4.84
	1,3-butanedione, 1-(2,6,6-trimethyl-1-cyclohexen-1-yl)	9.58	2Propanone,1(1,3dioxolan2yl) (1(1,3Dioxolan2yl) acetone)	5.04
			2-propanone	22.79
Methoxyls/Ethers	2',3',4 Trimethoxyacetophenone	13.99	Cedryl propyl ether	8.97
	3,4,5Trimethoxybenzoicacid hydrazide	15.10	Benzyl alcohol, 4,5dibenzyloxy- / Benzene methanol, 3,4-bis (phenyl methoxy)-	23.06
			Heptane, 1,1'-oxybis- (bis (1-heptyl) ether)	12.81
Diol/Triol derivatives	1,2-decanediol	6.08	1,2-Benzenediol, 3,5-bis(1,1-dimethylethyl)-	38.98
	1,2-octadecanediol	12.38		
	Naphthalene, 2-decyldecahydro	19.32		
	1,2-Benzenediol, 3,5-bis(1,1 dimethylethyl)-	16.97		
Sulfates	1Docosanethiol	25.63	1-Hexadecane sulfonyl chloride	10.73
TCA cycle intermediates	Formic acid, isopropyl ester	5.04	Aceticacid, trifluoro-dodecyl ester	4.38
	Acetoaceticacid, sec-butyl ester	5.24	2Hydroxy2methylsuccinic acid	5.25
	Formic acid, chloro-isopropyl ester	33.16	Acetic acid, n-octadecyl ester	15.64

Table 2. GC-MS metabolites of each PAH in 5th day of the experimentation

Type of Metabolite	BaP	RT	BkF	RT
Alkane derivatives	Decane, 5ethyl-5methyl-	4.98	Tetradecane	5.92
	1-Tetradecene	5.86 5.92	Hexadecane	8.35
	n-Hexadecane	8.35	Eicosane, 7hexyl	11.96
	Dodecane,1cyclopentyl4(3cyclopentyl propyl)	10.73	n-Heptadecane	12.68
	Heptadecane , 9-hexyl	11.48	n-Eicosane	18.66
	Heneicosane,11-(1ethylpropyl)	11.97	Octadecane-3ethyl-5-(2ethylbutyl)-	24.93
	n-Eicosane	12.69	Cyclohexane, 1,1'-pentylidenebis (1,1-Dicyclohexyl pentane)	25.60
	Cyclopentane, 1,2,4-trimethyl	12.82		
	Cyclohexane, 1,3,5-trimethyl-2-octadecyl	16.08		
	Octadecane, 5,14-dibutyl	16.22		
	Cyclopentane,1,1'[3(2cyclopentylethyl) 1,5pentanediyl]bis	18.28		
	Eicosane, 2-cyclohexyl	19.65		
	Pentadecane, 8hexyl	26.04		
	Dodecane,5,8-diethyl	26.28		
	Hexadecane, 1-iodo	31.59		
	Octadecane, 3ethyl5(2ethylbutyl)	30.94		
Alkene Derivatives	3-Dodecene (z)-	4.38	3Dodecene,(Z)-	4.38
	17Pentatriacontene	41.88	1 Heptene, 4methyl	31.73
Fatty acid Derivatives	Dodecanoic acid	7.63	Dodecanoic acid	7.63
	Tetradecanoic acid	11.57	Tetradecanoic acid	11.56
	Propanoicacid, 2-methyl, 3hexenyl ester	22.40	n-Hexadecanoic acid	17.26
	n-Hexadecanoic acid	17.23		
	Octadecanoicacid,phenylmethyl ester	23.10		
	Octadecanoic acid	24.37		
Alcohol Derivatives	1-Heptadecanol	8.23	n-Pentadecanol	5.86
	n-Nonadecanol	12.51	1-Hexadecanol	8.23
	1dodecanol,3,7,11-trimethyl	13.12	n-Heptadecanol	12.50
	n-Tetracosanol-1	25.81	1-Heneicosanol	18.43
	1-Heptacosanol	35.49	n-Tetracosanol	25.78
	1-Heptadecanol acetate	15.66	n-Heptacosanol	41.83

	Cyclooctanemethanol	31.35	1-Hexadecanol, acetate	15.64
			1Cyclopentyl2propen1ol	38.36
Amine Derivatives	Trans2,5Dimethylpiperazine	30.83	1,2 Ethanediamine, N-methyl	4.09
Aldehydes	Propanal,3(dimethylamino)2,2dimethyl	42.23	Hexanal, 2ethyl	16.96
	Acetaldehyde	4.09		

Moreover, the detection of aldehydes like Acetaldehyde, Propanal,3(dimethylamino)2,2dimethyl (Rt's-4.09 and 42.23) (BaP) and Hexanal, 2ethyl (Rt-16.96) (BkF) and the intermediate of TCA cycle Aceticacid, trifluoro-dodecyl ester, 2Hydroxy2methylsuccinic acid, Formicacid, isopropylester and Aceticacid, n-octadecyl ester (Rt's-4.38, 5.25, 5.04 and 15.64), indicates the complete utilization of PAHs.

From results, the microbial consortium degrades PAHs through three different ways 1. Conversion of PAHs to epoxides by fungal, bacterial moxygenase and to phenols and trans-dihyrodiolsnonenzymatically and by hdrolases which are further mineralized to hyroxy PAHs, diones and quinones. 2. Direct oxidation of PAHs to quinones by fungal extracellular enzymes laccase and ligninpeoxidase to phthalicacids. 3. Oxidation of PAHs to cis-dihydrodiols by bacterial dioxygenases and subsequently to catechol and intermediary compounds. The altogether metabolites identified by GC-MS are in agreement with the degradation pathways proposed by[43].

The existence of complementary degradation mechanisms, metabolic cooperation between microorganisms may result in enhanced PAHs degradation and also co-metabolism of PAHs which analogize with the studies of [44] where an efficient PAHs degradation was observed with the combined action of bacterial monooxygenase and fungal laccase. This was attributed to the advantage of using fungal, bacterial consortium signifying the synergistic degradation of PAHs.

3.3 Screening of Enzyme and Biosurfactant Production during PAHs Degradation

Different degradative enzymes involved in the various stages of PAHs degradation and their differential expression were studied for 7days. In this study, activities of eight degradative enzymes, such as 1 hydroxy 2-Naphthoicacid dehyrogenase, catechol 1,2 dioxygenase, 2-carboxybenzaldehyde dehydrogenase, protocatechuate 3,4 dioxygenase, protocatechuate 4,5 dioxygenase, laccase and lignin peroxidase, were monitored during the PAHs metabolism (Figure 2).

The total protein concentration and the activities of these enzymes during degradation (1, 3, 5, 7 days) were studied.

The total protein concentration was observed to be in accordance with the growth rate with maximum on day 5 and 7 for BaP and BkF. Induction of all enzymes was found during the degradation of all PAHs by the consortium.

For BaP, the activity of 1-H-2NAD was maximum at day 1, showing activities of 0.497 µmoles/min, while LiP (1.138 µmoles/min), P3,4O (1.443 µmoles/min), P4,5O (1.565 µmoles/min) and C1,2O (0.802 µmoles/min) was maximum on day 3, lacccase (0.714 µmoles/min) and 2-CBAL (1.443 µmoles/min) on day 5.

From the results, it was inferred that the dissolved and available BaP in the medium was initially degraded by bacterial 1-H-2NAD and later on, day 3and 5 the unavailable PAH in the medium acted upon by fungal LiP and laccase transforming them to quinones and phenols followed by the bacterial enzymes P4,5O, C1,2O and P3,4O converting protocatechuate and catechol into cis-muconate. This correlated with the observance of GC-MS metabolites of phenols and quinones constitutes about 10% of the total metabolites (Figure 2(a) and 2(b)).

For BkF, the aqueous solubility is less (0.76 µg/L) when comp ared to BaP (3.8 µg /L)[45] on day 1 1-H-2NAD and 2CBAL activity was maximum (0.461 and 3.214 µmoles/min), P3,4O activity on day 3 (2.166 µmoles/min), fungal enzymes laccase and LiP activity on day 5 (2.194 and 1.912 µmoles/min) and P4,5O and C1,2O on day 7 (0.781 and 1.241 µmoles/min) (Figure 2(c) and 2(d)) indicating the initiation of oxidation of BkF by fungus facilitating the bacteria to utilize the compound. Induction of these enzymes during degradation correlated with the studies of[22,44] where differential expression of catabolic enzymes were observed during FLU degradation by the bacterial consortium and enhanced degradation rate with combined action of bacterial, fungal enzyme systems.

From results, it can be deduced that in fungal, bacterial consortium both lignolytic and nonlignolytic enzyme activities was observed, although differential expression was observed with respect to type of PAH compound and its bioavailability. In order to determine the role of microorganisms in the consortium during the degradation of PAHs consortium was screened for biosurfactant production.

The biosurfactant production ability of the consortium with PAHs as sole carbon source was measured every 24hrs using E_{24} method. Consortium showed highest biosurfactant production showing an emulsifying activity of maximum 61% (BaPandBkF) in 7 days of cultivation (Figure 2). The maximum production was observed on day 5 where the biosurfactant was isolated and characterized with TLC and FTIR.

Figure 2. Enzymatic analysis of bacterial and fungal enzymes**. Legends:** Bacterial enzymes 1-H-2NAD: ; 2CBD: ; P3,4DO: ; P4,5DO: and C1,2O: estimated over a period of 7 days with different PAHs including. **(a)** BaP **(c)** BkF. Fungal enzymes Laccase: and Lip: assayed in presence of. **(b)** BaP and **(d)** BkF over a period of 7 days.

Also, the carbohydrate and protein content was quantified and it was observed that on an average the protein content was 2.4 and 2.8 µg/ml and carbohydrate content 26 and 25 µg/ml with PAHs BaP and BkFas sole carbon source in the medium. The presence of blue spots on the silica plates indicates the presence of carbohydrate moiety in the biosurfactant when sprayed with p-anisaldehyde.

The IR spectra of the biosurfactant produced by the consortium with different PAHs as a sole carbon source showed strong absorption bands at 3370 cm^{-1} are found due to stretching vibration of –O–H group and the asymmetrical stretching (Vas CH2) of methylene occur at 2945cm-1(BkF) and 2963 cm^{-1} (BaP). The fatty alcohols plays an important role in the biosynthesis of biosurfactants[46]. Also, fattyalcohols were observed as a GC-MS metabolite during the degradation of degradation of PAHs to simple hydrocarbons and to intermediate compounds. The absorption at at 1028 cm^{-1} might be due to C-O of glucose in pyranose form and at 1218 cm^{-1} due to the presence of a glycosidic bond (C-O-C) of glycolipid and the appearance of N-CH_3 at 720-790 cm^{-1} confirms the phospholipid choline functional groups.

Also, from previous studies in[47] reported the production of biosurfactant by *Acinetobactercalcoaceticus* BU03 enhancing PAHs degradation and bioavailabilty. In[48] described the positive effect of biosurfactant in enhancement of PAHs degradation. Form results, it can be inferred that enhanced PAHs degradation rate was observed in the presence of biosurfactant producing microorganisms as members of the consortium with PAHs as the sole carbon source.

4. Conclusion

HMW-PAHs degrading microbial consortium consisting of bacterial, fungal cocultures could effectively degrade PAHs (BaP and BkF) as a sole carbon source signifying the synergistic action of microbes. Biosurfactant producing microorganisms selection in consortium formulation enhances PAHs bioavailability and bioremediation. Also, the degradation of PAHs with combined bacterial fun-

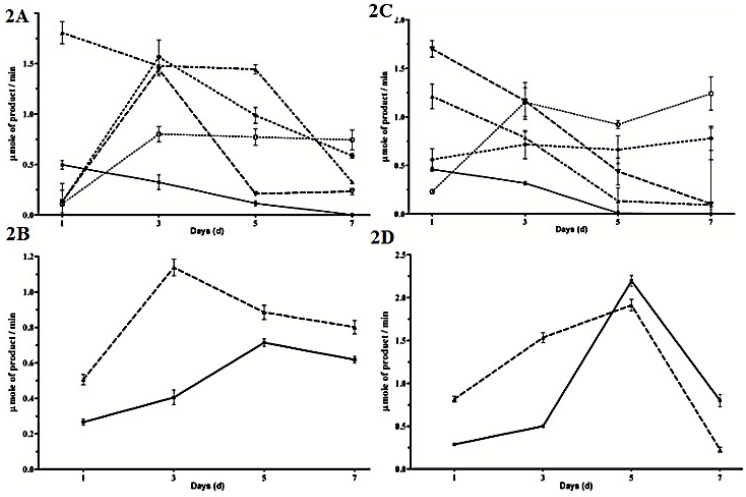

Figure 2. Activity profiles of intra and extra cellular enzymes.

gal enzyme systems was discussed. This emphasizes the potential application of this consortium in the treatment of treatment of PAHs contaminated sites.

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