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MICROBIAL CONSORTIA OF FUNGAL AND BACTERIAL COCULTURES INCREASES BIODEGRADATION RATE OF POLYCYCLIC AROMATIC HYDROCARBONS

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ABSTRACT

Isolation and characterization of polycyclic aromatic hydrocarbons (PAHs) degrading microorganisms from contaminated sites is well studied. A single organism having the potential to degrade different PAHs is exceptional and hence in the present study, the PAHs degradation capacity of a microbial consortium consisting of fungal, bacterial cocultures was evaluated. Within 7 days, consortium showed 99%, 57.4%, 60% and 82% degradation of Phenanthrene (PHE), Pyrene (PYR), Fluoranthene (FLU) and Chrysene (CHY), each PAH of 100mg/L concentration respectively. The culture broth was analyzed for the presence of degradation metabolites by GC-MS and the enzymes produced by the consortium. Coexistence of three metabolic pathways, cytochrome P450 monooxygenase with the detection of epoxy derivatives, catechol by bacterial dioxygenases and via Quinones of ligninolytic pathway were identified. Concurrently, microbial consortia also demonstrated good biosurfactant production capacity in the presence of PAHs. These results highlight that the microbial consortium can be of potential use with increased rate of bioremediation of PAHs cometabolically.

KEYWORDS: **PAHs, Microbial consortium, Biosurfactant, GC-MS metabolites, Degradation pathways**.

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INTRODUCTION

PAHs contamination is of environmental concern because of their carcinogenic, teratogenic and persistent nature. Biological treatment of PAHs has gained importance and recommended for practice as it has provided an economical and environmentally friendly solution compared to physical and chemical methods.[1,2] The inability of an individual organism to degrade combination of low and high molecular weight PAHs resulted in the employment of assemblage of mixed communities. PAHs because of their complex and recalcitrance nature cannot serve as growth substrate for a single microbe, but they are oxidized in a series of steps by a consortium of microorganisms.[3] In a consortium, some microbes can degrade a broad range of hydrocarbons while others can use partially degraded metabolites that could be toxic or inhibitory for some of the primary degraders.[4,5] This was evident with the isolation and identification of microbial populations containing different genera from various oil and PAHs contaminated sites.[6-8] Moreover, synergism among consortia members was apparent with sequential change in the bacterial groups during PAHs utilization.[9,10] Lately, to enhance the degradation rate usage of microbial consortia containing bacterial-fungal cultures has gained importance due to their capacity to use broad range of PAHs and to produce various degradative enzymes in complementary degradation pathways by different strains.[6,11] As a remediation strategy, bacteria and fungi basically releases biosurfactants into the medium to increase the bioavailabilty and utilization of the PAH compound.[12] The biosurfactants produced by a consortium member degraded 91% of the hydrocarbon content of soil contaminated with 1% (v/v) crude oil sludge in 5 weeks and it was identified as a mixture of 11 rhamnolipid congeners.[13] Organisms belonging to the genera *Pseudomonas, Acinetobacter calcoaceticus* and *Serratia morcescens* were already known for biosurfactant production. In the present study, an attempt was made to understand the role of individual microorganisms in the consortium and their impact on the association by checking (1) its ability to degrade and utilize PAHs (PHE, PYR, FLU and CHY) as a sole carbon source in the liquid medium through synergism, (2) biosurfactant production capacity, (3) to investigate the metabolites and (4) to identify the enzymes produced during the degradation process.

MATERIALS AND METHODS

i. Chemicals and Culture Media

Aromatic hydrocarbons Pyrene, Phenanthrene, Fluoranthene and Chrysene, were purchased from Sigma-Aldrich. All other chemicals were of analytical grade (Merck India Ltd.,). Nutrient broth (NB), Tryptic Soy broth (TSB) and Blood agar base were purchased from Himedia. 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; $ZnSO4 \cdot 7H_2O$, 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.

ii. Microorganisms and Cultivation

a. PAH degrading consortia

Organisms isolated from different hydrocarbon contaminated soil and waste environments were obtained from MTCC, Chandigarh and were used for development of a consortium which includes *Pseudomonas* species (MTCC 2445), *Acinetobacter calcoaceticus* (MTCC 2409, 2289), *Serratia morcescens* (MTCC 2645) and *Aspergillus terricola var americanus* (MTCC 2739). These isolates were maintained on nutrient agar for further studies.

iii. Inoculum Preparation

The inoculum was prepared by growing each culture individually in 50 mL sterile MSM with 2% dextrose for 24-48 hours at 30°C, 150 RPM in a rotary shaker followed by the centrifugation of cells at 4°C, 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..

iv. Biodegradation of PAHs in liquid Medium

The degradation capacity of the consortium was evaluated by the shake flask method. All experiments were performed 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 100 mg/L for each PAH compound (PHE, PYR, FLU and CHY). The effect of pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0) on the degradation of PAHs was also studied. All experiments were conducted in triplicate and kept in an orbital shaker at 150 RPM, 30°C for 10 days. Non-inoculated bottles were set as controls and the samples were sacrificed at every 2 days interval for analysis.

v. Analysis

a. Evaluation of consortium growth

The growth of the consortium utilizing 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, cells were pelleted down and was further dried to estimate the biomass by gravimetric method.[14]

a. 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 was detected at 254 nm. The percentage reduction of PAH was evaluated by comparing with the peak area of the control sample. The degradation of PAH was calculated as: $Rd = (C0 - Cn)/C0 \times 100\%$ Where, Rd (%) represents the degradation rate, $C0$

(mg/L) represents the initial concentration of PAH and *Cn* (mg/L) represents the remained concentration of PAH after incubation for *n* days.[9]

b. Analysis of PAHs Metabolites

The cultures were sampled on 5th day and the isolation of metabolites was performed using the method of Roy M 2012.[15] Ethyl acetate extracted metabolites present in the neutral fraction and acidic fraction were analyzed by using a Thermo Scientific 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.

vi. Screening of enzyme activities during degradation

The consortium was screened for its enzyme producing potential in the mineral salts medium (MSM) broth to study their involvement at different stages of degradation. Enzymatic assays were performed using a UV-Visible spectrophotometer (Thermo scientific). Cultures were subjected to centrifugation at 8000rpm for 20min for separation of extracellular and intracellular products. The supernatant was directly utilized for extracellular enzyme activities, whereas, intracellular enzymes were extracted through sonication of cell pellet and centrifugation at 8000rpm for 15min to avoid cell debris. Total protein content for intra and extra cellular extracts was performed by using Lowry's method.[16] 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 Mishra S 2014.[17] Activities of fungal enzymes like laccase was measured by monitoring the oxidation of ABTS (2,2'-azino-bis (3-ethyl benzothiazoline-6-sulphonic acid) at 405nm (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).[18] All the experiments were carried out at 30°C and the enzyme activity was expressed as µmole product formed/min/mg protein.

vii. Emulsification Index (E_{24})

The emulsifying capacity of the cultures was evaluated by E_{24} method.[19] 2 mL of supernatant was added to 2 mL of kerosene, petrol, diesel in a screw cap test tube, vortexed for 2 minutes and the mixture was made to stand for 24 hours. The percentage of Emulsification index was calculated using the formula:

E_{24} = (Height of emulsion formed in cm × 100) / (Total height of solution in cm)

viii. Biosurfactant production and extraction

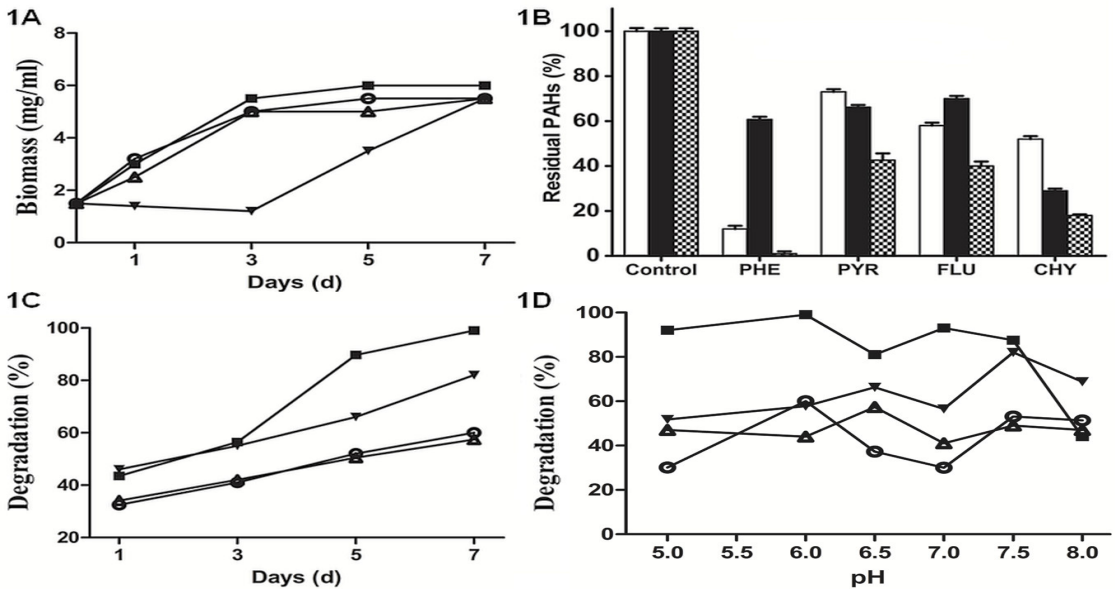
The biosurfactant produced by the consortium was extracted on the 5th day as described by Rahman K 2003.[20] In brief, the entire content of the flasks (250ml) containing PAHs as the sole carbon source was centrifuged at 8000 rpm for 15min at 4°C and the collected supernatant was acidified with 6N HCl at pH 2 and left for overnight at 4°C 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. Carbohydrate content in the biosurfactant was determined by Phenol sulphuric acid [21] and protein by Lowry's method.[16]

RESULTS AND DISCUSSION

i. PAHs biodegradation

Microorganisms isolated from hydrocarbon contaminated sites (MTCC, Chandigarh) were screened based on their ability to degrade PAHs as a sole carbon source and a consortium of bacterial, fungal cocultures was developed and its degradation efficiency was evaluated with different PAHs (PHE, PYR, FLU and CHY) as the sole carbon source. The growth of the consortium was evaluated every two days and the biomass growth till 5th day with PHE, PYR and FLU showed a linear relationship with the degradation rate while biomass with CHY sustained till 3rd day and then started growing (Figure 1A). The biodegradation of individual PAHs in liquid medium by the consortium was evaluated by HPLC over the period of 7 days (Figure 1C). The effect of pH on biodegradation of PAHs by the consortium was studied (Figure 1D). It was observed that the pH of the MS medium influences the PAH degradation. The maximum degradation of PHE and FLU was attained at pH 6.0 (99% and 60%), while for PYR at pH 6.5 (57.4%) and CHY at pH 7.5 (82%) respectively. A minimum degradation of 50% was observed for all PAHs within the pH range 6.0-7.5 indicating that the bioremediation by consortium can suitably adapt to the environmental conditions. Consortium members when tested individually showed maximum degradation of 81% of PHE (*Pseudomonas putida*), CHY 71%, PYR 34% (*Aspergillus terricola var americanus*) and FLU 30% (*Acinetobacter calcoaceticus*) while only bacterial consortium degraded 88% (PHE), 48% (CHY), 42% (FLU) and 27% (PYR) respectively. An enhanced degradation rate of 99% (PHE), 82% (CHY), 60% (FLU) and 57.4% (PYR) was observed with bacterial fungal cocultures (Table.1). Although the other consortium members *Acinetobacter calcoaceticus* and *Serratia morcescens* were not efficient PAH degraders individually, their presence played an important role in production of biosurfactant for enhanced degradation. The degradation rate was decreased in the absence of theses cultures in the consortium (data not shown). The effect of PAHs mixtures at the concentration of 100 mg/L (individual PAH concentration of 25mg/L) on the biodegradation efficiency of the consortium was studied. The degradation followed the order of PHE (96.5%), CHY (84%), FLU (78.4%) and PYR (74.4%) respectively.

Figure 1
Biomass and Biodegradation of PAHs



Degradation of PAHs at different pH and time intervals : (1A) Biomass of fungal and bacterial consortium over a period of 7 days with PAHs (PHE: , PYR: , FLU: and CHY:) as sole carbon source. (1B) Residual percentage of PAHs remained after 7 days of incubation with only bacterila consortia( ), fungus ( ) and fungal-bacterial cocultures ( ). (1C) and (1D) Rate of degradation of PAHs (PHE: , PYR: , FLU: and CHY:) and degradation percentage of PAHs with respect to pH by consortium.

In the present study, an assemblage of cultures degraded PAHs effectively compared to pure cultures indicating synergism among the consortium members. This correlated with the previous studies[22] where the consortium showed 80% degradation of PHE compared to individual cultures, *Acinetobacter baumannii* (48%), *K. oxytoca* (11%) and *Stenotrophomonas maltophilia* (9%) respectively after 360hrs of incubation. The capacity of microbial consortium containing fungal bacterial cocultures showed an average degradation of 78% of PAHs (Anthracene, PHE and PYR) over a period of 30days in soil, while the fungal and bacterial isolates when inoculated separately showed less mineralization when compared to the consortium.[23] 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 leading to increased PAHs degradation rate.

Table 1
Percentage degradation of PAHs by individual microorganisms and consortium.

S.NO.	Bacterial strain	PHE	PYR	FLU	CHY
1	*Acinetobacter calcoaceticus* (MTCC 2409)	75	24	31	39
2	*Pseudomonas putida* (MTCC 2445)	81	18	18.5	21
3	*Serratia morcescens* (MTCC 2645)	52	9	18	26
4	*Acinetobacter calcoaceticus* (MTCC 2289)	82	12	27	27
5	Bacterial consortium	88	27	42	48
6	*Aspergillus terricola var americanus* (MTCC 2739)	39	34	30	71
7	Consortium (Bacteria and fungus)	99	57.4	60	82

ii. 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. The presence of epoxy derivatives in the medium, 2,6 Tridecadienoicacid, 10,11 epoxy 7ethyl 3,11, dimethyl, methyl ester (E,E) cis- (PHE), and Trichothec9en8one, 12, 13 epoxy 3a, 4a, 7a, 15 tetrahydroxy, (PYR, FLU) elucidates the initiation of the PAHs oxidation to epoxide (arene oxide) mediated by the cytochrome P450 monooxygenase of the consortium (Suppl. Table 1 and 2). The oxidation of PAHs to epoxides, Phenanthrene to Phenanthrene 1,2-epoxide, Phenanthrene 3,4-epoxide and Phenanthrene 9,10-epoxide, Pyrene to Pyrene 1,2 and 4,5- epoxide by various fungi is reported.[24] Also the presence of phenols, Phenol, 2,4 bis (1,1 dimethyl ethyl) (Rt-7.08) (PHE,PYR, FLU and CHY) and Phenol (Rt-2.9 with CHY) (Suppl.Table 1) during PAHs degradation indicates the successive conversion of epoxides nonenzymatically. Phenol, 2,4 bis(1,1-dimethylethyl) was also observed as a metabolite during the degradation of naphthalene by *Streptomyces* and PAHs by *Acinetobacter species.* [25,26] Further the phenols were converted to conjugates of sulfates di-tert-dodecyldisulfide (Rt-6.86), ditert-hexadecane thiol (Rt-7.31) (PYR) Dimethyl Sulfone (Rt-2.62), Thiophene, 3(1,1dimethylethyl) (Rt-4.87) and methoxyls 2^1,6^1-

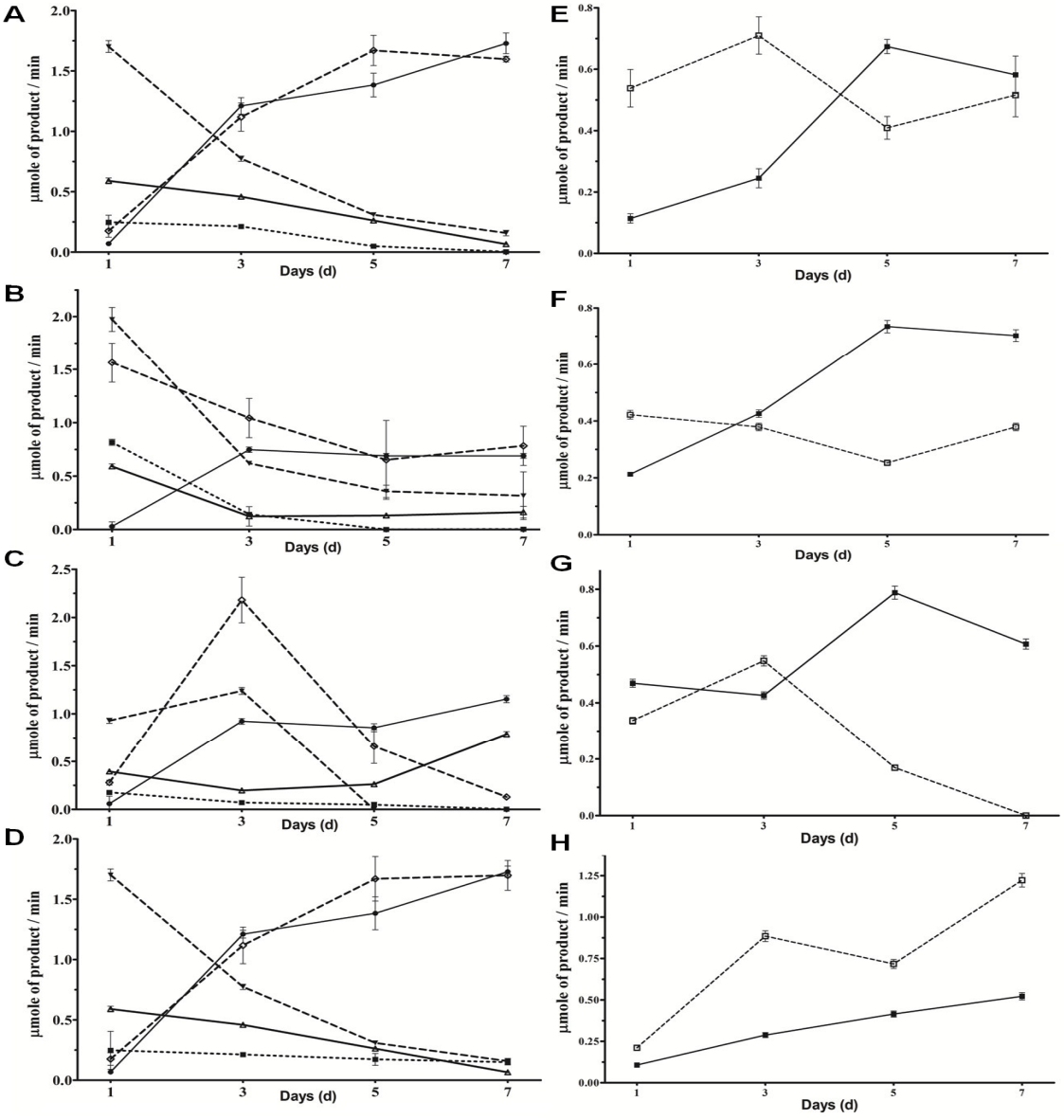
dimethoxy Acetophenone (Rt-3.58), Phenol, 3,5dimethoxy (Rt-15.86) infers the transformation of phenols. Even though, trans-dihydrodiols were absent (n)-Hydroxy metabolites were observed during degradation on the 5th day that are successively converted to diones and to quinones (Suppl.Table 1). 9,10 Anthracenedione 1(methyl amino) 4[(4 methyl phenyl) amino] of Rt-5.25 (Suppl.Table 1) was observed during the degradation of PHE and PYR which correlated with the previous studies [27,28] in the degradation complex hydrocarbons in spent engine oil by bacterial consortium isolated from deep sea sediments. Other major metabolites identified were quinones which confirms the involvement of fungal extracellular ligninolytic enzymes specifically lignin peroxidase and laccases which oxidizes PAHs to quinones, with subsequent ring cleavage to produce phthalic acids and eventually to CO_2.[29] Previous studies reported 9,10-phenanthrene quinone, anthraquinone and chrysene quinone as GC-MS metabolites during the degradation of PHE, Anthracene (ANT), CHY by fungal species like *Polyporus, Aspergillus fumigatus, Armillaria and Fusarium*.[28,30-32] In the present study, tert-butyl-p-benzoquinone (Rt-4.55), hyroquinone, 2,5-di-tert-butyl p-quinone (Rt-6.63), p-benzoquinone 2,5-bis (1,1,3,3 tetramethyl butyl) (Rt-15.43) and 2,6-di-tert-butyl (Rt-16.87) were noticed (Suppl.Table 1). Presence of carboxylic acids 2-Naphthalene carboxylicacid 4,4'methylenebis[3methoxy]- (Rt-8.66) and Phenanthro [3,4d]1,3dioxole-5carboxylicacid, 8methoxy-6nitro (Rt-17.37) (Suppl.Table 1) during the degradation of PYR and FLU correlated with earlier reports[33,34] where Naphthalene 1,8 dicarboxylic acid, 2-(2-carboxy-vinyl)-naphthalene-1-carboxylic acid, naphthalene-1,2-dicarboxylic acids were reported during the degradation of FLU and PHE. The occurrence of naphthalene carboxylic derivatives denotes the degradation and utilization of these PAHs. On the other hand, bacterial dioxygenases can convert PAHs to cis-dihyrodiols and further to catechol by dehrogenases. Phenols can also be oxidized aerobically by bacteria to catechol by monooxygenase and catechol subsequently undergoes ortho or meta ring cleavage and finally produces succinate, Acetyl CoA or Acetaldehyde and Pyruvate.[35] Salicylic acid, 3-fluoro (Rt-4.96) and catechol (Rt-4.51) was observed (Suppl.Table 1) during the degradation of CHY which is in correlation with the studies of Nayak AS et.al., 2011.[36] PAHs are converted into products like simple hydrocarbon alkanes, alkenes, cycloalkanes (Suppl. Table 2) which undergoes terminal and subterminal oxidation to produce alcohols, fatty acids which are further mineralized by β oxidation pathway.[37] The accumulation of alkanes (propane-Tetracoane), alkenes- 1-decene (Rt-3.01), 2-decene (Rt-3.02) and 17-Pentatricontene (Rt-10.46) (Suppl. Table 2) in 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.[38,39] Fattyacids like bromotetradecanoic acid (Rt-16.07), octanoic acid (Rt-4.13), dodecanoic acid (Rt- 7.62), hexadecanoic acid (Rt-15.69), oleicacid methyl ester (Rt-21.28), 9,12,15-octadecanoic acid (Rt-23.73 and 3.38) and 5,8,11,14-Icosatetraynoic acid (Rt-15.48) simple ketone derivatives 4chlorobutyrophenone (Rt-3.52), Valerophenone (Rt-3.62) and Ethanone, 1(4methyl2thienyl)- (Rt-4.83) fatty alcohols (n-pentadecanol- n-Heneicosonal) were also detected during degradation (Suppl.Table 2). Moreover, the detection of acetic acid, trifluoro-3,7-dimethyloctyl ester, phenyl-benzyl ester and L-ascorbic acid, 6-stearate (Rt's-10.71, 23.03 and 37.20) the intermediate of TCA cycle, indicates the complete utilization of PAHs. Altogether, the microbial consortium degrades PAHs through three different ways (1) conversion of PAHs to epoxides by fungal, bacterial moxygenase and to phenols and trans-dihyrodiols nonenzymatically and by hydrolases which are further mineralized to hydroxy 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 overall metabolites identified by GC-MS are in agreement with the degradation pathways proposed by Cerniglia.[40] The existence of complementary degradation mechanisms, metabolic cooperation between consortium microorganisms resulted in enhanced PAHs degradation and also co-metabolism of PAHs.

iii. Screening of the enzymes produced during PAHs degradation

Different degradative enzymes involved in the various stages of PAHs degradation and their differential expression were studied for 7days. Activities of eight degradative enzymes, such as 1Hydroxy, 2-Naphthoicacid dehyrogenase (1-H-2NAD), Catechol 1,2 dioxygenase (C1,2DO), 2-Carboxybenzaldehyde Dehydrogenase (2CBD), Protocatechuate 3,4 dioxygenase (P3,4DO), Protocatechuate 4,5 dioxygenase (P4,5DO), Laccase and Lignin peroxidase (LiP) were monitored during the PAHs metabolism (Figure 2). The total protein concentration and the activity of these enzymes during degradation (1,3,5 and 7 days) was studied. The total protein concentration was observed to decline initially for 3 days followed with an increase on day 5 and 7 for PYR and CHY whereas for PHE and FLU it showed a linear relationship except for a decline on the 7th day (data not shown). This might be due to the difference in the induction of enzymes among the consortium members with respect to type of PAH compound. For PHE, the activity of 1-H-2NAD, 2CBD and P3,4DO was maximum on day 1, showing activity of 0.248, 0.59 and 1.702 µmoles/min, respectively, while LiP was maximum on day 3 (0.710 µmoles/min), laccase (0.674 µmoles/min) and P4,5DO (1.67 µmoles/min) on day 5 and C1,2DO (1.729 µmoles/min) on day 7. From the results, it was inferred that the dissolved and available PHE in the medium was initially degraded by bacterial 1-H-2NAD and 2CBD to phthalate. Later, on day 3 and 5 the unavailable PHE in the medium was acted upon by fungal LiP and laccase transforming them to quinones and phenols. On day 5 and 7 protocatechuate and catechol was acted upon by the bacterial enzymes P4,5DO and C1,2DO converting them into cis-muconate. This correlated with the presence of GC-MS metabolites, phenols and quinones which constituted about 10% of

the total metabolites identified (Figure 2A and 2E). For PYR and FLU, as the aqueous solubility is less (135µg/L and 260µg/L) when compared to PHE (1.29 mg/L)[41] very little is available for degradation. The bacterial enzymes 1-H-2NAD, 2CBD, P3,4DO and P4,5DO activity was maximum on day1 (0.816, 0.590, 1.974 and 1.565 µmoles/min) with PYR. However, LiP activity was observed maximum on day 1 (0.422 µmoles/min) and laccase on day 5 (0.735 µmoles/min) to make the PYR (Figure 2B and 2F) bioavailable whereas for FLU, laccase and LiP activity was maximum on day 5 and 3 respectively (0.788 and 0.548 µmoles/min) and 2CBD and C1,2DO on day7 (0.787 and 1.153 µmoles/min) indicating the initiation of oxidation of FLU by fungus facilitating the bacteria to utilize the compound. Induction of these enzymes during degradation correlated with the earlier studies[17] where differential expression of catabolic enzymes was observed during FLU degradation by the bacterial consortium (Figure 2C and 2G). For CHY, on day 1; 1-H-2NAD, 2CBD and P3,4DO activity was maximum (0.248, 0.590 and 1.702 µmoles/min) like PHE. LiP, laccase and C1,2DO activity was maximum on day 7 (1.222, 0.522 and 1.729 µmoles/min) (Figure 2D and 2H). The activity of nonlignolytic enzymes 1,2 and 2,3-dioxygenases by *Fusarium species* while both nonlignolytic and lignolytic enzymes by *Armillaria sp.F022* during CHY degradation, which correlated with the present study. [32,42] From results, it can be deduced that in fungal, bacterial consortium both lignolytic and nonlignolytic enzymes activity was observed, although differential expression was seen with respect to type of PAH compound and its bioavailability.

Figure 2
Activity profiles of intra and extra cellular enzymes



Enzymatic analysis of bacterial and fungal enzymes: Bacterial enzymes 1-H-2NAD: ; 2CBD: ; P3,4DO: ; P4,5DO: and C1,2O: estimated over a period of 7 days with different PAHs including 2A (PHE), 2B(PYR), 2C (FLU) and 2D (CHY). Fungal enzymes Laccase: and Lip: assayed in the presence of 2E (PHE), 2F (PYR), 2G (FLU) and 2H (CHY) over a period of 7 days.

iv. Biosurfactant production and characterization

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 an emulsifying activity of maximum 66% (CHY) and 64% (PHE, PYR) and 58% (FLU) in 7 days. The biosurfactant was isolated and characterized for the protein and the carbohydrate content. Protein content was found to be 2.5 µg/ml with all the PAHs tested. The carbohydrate content was high in PHE (26 µg/ml) while the rest of three PAHs showed only 20 µg/ml. The presence of carbohydrate moiety in the biosurfactant was also evident with p-anisaldehyde staining of TLC plates. The IR spectra of the biosurfactant produced by the consortium with different PAHs as a sole carbon source showed strong absorption bands at 3344cm^{-1} which can be attributed to stretching vibration of –O–H group. This fatty alcohols plays an important role in the biosynthesis of biosurfactants.[43] Concurrently, fatty alcohols were observed as GC-MS metabolites during the degradation of PAHs. The absorption at 1734cm^{-1} could be endorsed to the stretching of C=O of carboxylate anion and at 1032cm^{-1} due to C-O of glucose in pyranose form. The asymmetrical stretching (Vas CH2) of methylene is known to occur at 2951 cm^{-1} (PHE), 2046cm^{-1} (PYR). The absorption at 1218 cm^{-1} might be due to the presence of a glycosidic bond (C-O-C) of glycolipid and the appearance of N-CH_3 at 720-790cm^{-1} confirms the phospholipid choline functional groups. Production of biosurfactants by microbial consortia was higher in the presence of PAHs as the sole carbon source when compared to dextrose which correlates with the previos reports [44] where hydrocarbon based grown cells are more hydrophobic and showed high affinity towards aromatic and aliphatic hydrocarbons compared to dextrose grown cells. Also, increased laacase production was reported in the presence of biosurfactant[45] which is correlated in the present study where both laccase and biosurfactant activity was observed maximum on day 5. Also, from previous studies,[46-48] the production of biosurfactant by *pseudomonas species* during PAHs degradation and Lawniczak L et.al., 2013[49] described the positive effect of biosurfactant in enhancement of PAHs degradation. Alike, *Acinetobacter calcoaceticus* and *Serratia morcescens* were reported for their biosurfactant production. [50-53] Based on these results, it can be deduced that (1) Production of biosurfactant was the inherent property of the microorganisms and consortium showed more biosurfactant production with PAHs as the sole carbon source compared to dextrose. (2) The enhanced PAHs degradation rate was observed in the presence of biosurfactant producing microorganisms as members of the consortium.

CONCLUSION

Microbial consortium consisting of bacterial, fungal cocultures could effectively degrade PAHs (PHE, PYR, FLU and CHY) individually and in mixtures as a sole carbon source. Biosurfactant production by consortium members and their selection in consortium formulation enhances PAHs bioavailability and bioremediation. Also, the degradation of PAHs utilizing lignolytic and nonlignolytic enzyme pathways and the role of individual microorganisms in the consortium were discussed. This emphasizes the potential application of this consortium in the treatment of PAHs contaminated sites with increased rate of biodergradation.

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CONFLICT OF INTEREST

All authors mentioned are not having any conflict of interest to submit this article.

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