Available online at: www.mbai.org.in doi:10.6024/jmbai.2021.63.2.2248-11

Isolation and screening of multiple polycyclic aromatic hydrocarbons (PAHs) degrading bacteria from historically contaminated coastal sites of Gujarat, India

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Received: 25 Oct 2020 Accepted: 25 Nov 2021 Published: 15 Dec 2021

Original Article

Abstract

Major aim of the present study was isolation and screening of multiple polycyclic aromatic hydrocarbons (PAHs) degrading bacteria from the historically contaminated coastal sites i.e. Alang-Sosiya Ship Breaking and Recycling Yard (ASSBRY) and Navlakhi Port (NAV), Gujarat, north-west coast of India. Individual bacterial candidate from consortia can be used as lead PAHs degrader for the biological remediation of contaminated sites. For that, significant 14 components of ONR7a Medium were examined using multifactorial statistical design for PAHs degradation. Selected multiple PAHs were [phenanthrene, anthracene (low molecular weight) and fluoranthene (high molecular weight)] frequently found at ASSBRY. A rapid search of carbon, nitrogen, phosphorus, and potassium along with trace elements from a multivariable system was achieved using statistical approach, minimizing the error in determining the effect of medium components. The significant variables identified by this procedure will allow one to rank them in order to decide which to scrutinize for a more detailed study to determine the optimum value to use them further in more precise experiments like Response Surface Methodology.

Keywords: *Polycyclic Aromatic Hydrocarbons (PAHs), natural consortia, ONR7a medium, multiple PAHs degradation*

Introduction

Marine ecosystem is most diverse and productive ecosystems in the globe (Panseriya *et al.*, 2021). The complex microbial communities residing in marine ecosystem are widespread consisting of cultivable and uncultivable members participating in diverse biogeochemical cycles (Tortell *et al.*, 1999; Arrigo, 2004; Falkowski *et al.*, 2008). Marine ecosystem is also one of the most polluted ecosystems due to natural and anthropogenic activities which affect aquatic lives posing deleterious effect on them. Marine ecosystem receive various hazardous compounds due to improper handling of coastal activities which is a serious concern. Hazardous compounds *i.e.* heavy metals (Panseriya *et al.*, 2019, 2020), PAHs (Sachaniya *et al.*, 2019, 2020), PHC, nutrients etc. Among them, PAHs is a diverse group of hydrocarbons consisting two or more fused aromatic rings. PAHs are one of the major components of oil and petroleum products which enter in to the marine environment by various natural and anthropogenic sources. PAHs and their metabolites have proved to be carcinogenic (Sutherland, 1995), mutagenic (Mersch-Sundermann *et al.*, 1992) and teratogenic in environment as well as human health. Hence, United States Environmental Protection Agency (USEPA) has listed 16 PAHs as priority pollutants (Dudhagara *et al.*, 2016). PAHs persist in the environment due to some

of their exceptional physico-chemical properties such as high molecular weight (HMW), tough atomic bonds, extensive half-lives etc. In addition, hydrophobic nature of PAHs is one of the potential reasons for their bio-magnification through trophic transfers in the marine environment. Photo-oxidation, chemical oxidation, evaporation and volatilization are some of the physical and chemical fates of PAHs in nature. In context to the same, biodegradation and bioremediation has gained wide acceptance in scientific communities for restoration of PAHs contaminated sites (Hadibarata *et al.*, 2009; Damare *et al.*, 2012). Many previous studies have successfully isolated PAHs degrading bacteria (Verrhiest *et al.*, 2002; Ghevariya *et al.*, 2011; Rajpara *et al.*, 2015) and mixed culture (Boonchan *et al.*, 2000; Yu *et al.*, 2005; Patel *et al.*, 2013) from contaminated sediments for the bioremediation of marine environment.

Depending on the source of PAHs contamination, sites selected for the study were Alang-Sosiya Ship Breaking and Recycling Yard (ASSBRY) and Navlakhi Port (NAV). ASSBRY is situated in the Gulf of Khambhat, Bhavnagar District in Gujarat, India. This site is the largest ship grave yard in Indian subcontinent with respect to ship breaking and recycling in the globe, operated by beaching method having around 167 ship-breaking and recycling plots. The site has received great environmental concern due to contamination of petroleum hydrocarbons (PHCs), PAHs and heavy metals in the coastal province caused by ship breaking activities (Reddy *et al.*, 2003; Basha *et al.*, 2007). Whereas, Navlakhi Port is located in the interior of Gulf of Kachchh in Morbi District, Gujarat, India. The major commodity of Navlakhi Port is coal imported by various agencies and its supply to thermal power stations and other electricity generating companies across India. The port facility comprises about a total of 43 hectors of area. Coal transportation leads to widespread PAHs contamination at the site (Gosai *et al.*, 2018a, b, c).

The assessment and source distribution of PAHs present at the selected sites were carried out as part of our previous study (Gosai *et al.*, 2018a; Sachaniya *et al.*, 2020). A recent study (Dudhagara *et al.*, 2016) also showed excessive concentration of Phenanthrene (Phe) and Anthracene (Ant) in ASSBRY and hence were considered for the present study. Phe and Ant are PAHs, comprising three condensed rings with same molecular weight (Mw=178) and are generally used as model PAHs in biodegradation studies (Lakshmi and Velan, 2011; Swaathy *et al.*, 2014). In addition to these two PAHs, a HMW PAH viz., Fluoranthene (Flt) has also been selected for the present study to isolate the potential multiple LMW and HMW PAHs degraders. The degradation study was conducted considering individual isolates, natural consortia and mixed cultures that were multiple PAHs degraders (Rehmann *et al.*, 2001). The growth of PAHs degraders mainly depends on optimum nutrient components which accelerate rate of PAHs degradation. With respect to this, screening of medium components significantly affected PAHs biodegradation is important to reduce the complexity of the study. Therefore, the present study was envisioned for 1) isolation of potential PAHs degraders from contaminated sites, 2) to check PAHs degradation (%) by potential degraders and 3) to enhance the degradation rate by selection of medium component using PB design.

Material and methods

In this study, the mixed bacterial culture was developed from contaminated sites (ASSBRY and NAV) through enrichment technique and investigated potential model for the phenantherene, anthracene and fluoranthrene. Among them natural consortia and mixed cultures were also developed to examine their degradation capability.

Sampling and screening strategy of PAHs degraders

Contaminated sediment samples were collected from ASSBRY and NAV during pre-monsoon, post-monsoon and winter seasons of 2015-2016. Sampling procedure was followed as described by Gosai *et al.* (2018a,b,c). Surface sediment samples (10-15 cm depths) were collected in triplicate in case of wastage or shortage of sample. Samples were immediately transferred to MKBU laboratory under 4°C then preserved at -20ºC untill further analysis. Enrichment and isolation of PAHs degraders, ONR7a known as artificial seawater medium was used Dyksterhouse *et al.* (1995). Isolation and enrichment of PAHs degraders, 1g of sediment suspended in 9 ml sterile Ringer's solution (7.2 g/L NaCl, 0.17 g/L $CaCl_2 \cdot 2H_2O$, 0.37 g/L KCl and pH 7.3). Enhancement for growth of PAHs degraders suspension was mixed (150 rpm for 10 min) and inoculated (5ml) in 50 mL of sterile ONR7a medium having defined concentration of various PAHs (Geiselbrecht *et al.*, 1998; Hedlund and Staley, 2006; Ghevariya *et al.*, 2011). Each PAHs concentration was 40 ppm and gradually increased up to 100 ppm. The flasks were kept on a rotary shaker (New Brunswick, USA) at 150 rpm at 30°C. After sufficient bacterial growth serial dilution was (10^{-3} to 10^{-4}) performed and 100 μL bacterial suspension spread on solid ONR7a medium plates. The help of 10% ethereal solutions of Phe/Ant/Flt, plate was coated (Kiyohara *et al.*, 1982) and incubated at 30°C until visible growth observed. Colonies showing clear zones were selected and purified for further studies. From each sampling site and season, 3 different multiple PAHs degrading natural consortia were obtained with prolonged enrichment. For the secondary screening ONR7a liquid medium was used with 300 ppm Phe, Ant and Flt (100 ppm of each PAH). Total 9 natural consortia was observed and screened for PAHs degradation as mentioned above.

Multiple PAHs degraders identification using polyphasic approach

Morphological and biochemical characterizations were carried out for primary identification of 3 potential PAHs degraders. The observations were confirmed using the Bergey *et al*., 1939. Genotypic identification of these isolates was carried out using 16S rDNA sequencing. The obtained 16S rDNA sequences were aligned using Basic Local Alignment Search Tool (BLAST) to determine similar sequences for the identification of PAHs degraders. Phylogenetic trees were constructed using MEGA 7.0 with neighbor joining algorithm. The obtained sequences have been submitted to NCBI.

Multifactorial statistical Plackett- Burman (PB) design-screening of medium components

Statistical design has proved to be easy and more efficient to screen significant components affecting the process from a pool of multiple components. A statistical tool Plackett-Burman (PB) design is used to identify the most important factors in the early phase of experimentation when complete knowledge about the system is unavailable (Plackett Burman, 1946).

Significant constituents of growth medium were examined in the degradation process. ONR7a medium were considered for analysis by statistical multi factorial "Plackett-Burman" design generated using MINITAB®version17.0 statistical software. Table 1 displayed "Low" and "High" values for medium components taken into consideration due to 14 medium components, the PB design consisted of 20 trials. Table 2 illustrates PB design experiment matrix along with run number and coded values of variables. After incubation,

Table 1. Levels of variables (medium components) considered in Plackett- Burman design

Medium components	Symbol	Experimental values (g/L)	
		Low (-)	High (+)
NaCl	A	20	24
Na_2SO_4	B	3.5	4.5
KCl	C	0.5	0.9
NaBr	D	0.07	0.09
$NaHCO_3$	E	0.025	0.035
H_3BO_3	F	0.02	0.035
NaF	G	0.2	0.3
NH_4Cl	H	0.24	0.30
$Na_2HPO_4.7H_2O$	I	0.085	0.094
TAPSO	J	1.0	1.6
$MgCl_2.\ 6H_2O$	K	8.0	15.0
$CaCl_2.\ 2H_2O$	L	1.0	2.0
$SrCl_2.\ 6H_2O$	M	0.02	0.03
$FeCl_3.\ 4H_2O$	N	0.001	0.003

Table 2. Plackett-Burman design matrix of variable in coded level with response for each individual isolate, natural consortia and mix culture obtained from both sites

	Plackett-Burman Design matrix														ΣPAHs degradation (%)					
															ASSBRY			NAV		
Run Order	A	B	C	D	E	F	G	H	I	J	K	L	M	N	BS 8	7A	BS178	GH 8	5N	GH148
1	24	4.5	0.5	0.07	0.025	0.02	0.3	0.24	0.094	1	15	2	0.03	0.003	74.14	78.16	82.25	87.25	56.91	81.54
2	24	4.5	0.9	0.09	0.025	0.02	0.3	0.3	0.085	1.6	15	1	0.02	0.001	60.19	88.9	61.9	70.12	42.46	51
3	20	3.5	0.5	0.07	0.025	0.02	0.2	0.24	0.085	1	8	1	0.02	0.001	81.4	78.93	30.87	36.85	76.13	61.46
4	24	4.5	0.5	0.07	0.035	0.035	0.2	0.3	0.094	1	8	1	0.02	0.003	81.63	57.16	81.08	91.29	30.83	70.5
5	20	3.5	0.9	0.09	0.025	0.035	0.3	0.24	0.085	1	8	2	0.02	0.003	83.46	84.4	15.52	42.89	54.67	60.3
6	20	3.5	0.5	0.07	0.035	0.02	0.3	0.24	0.094	1.6	15	2	0.02	0.001	72.38	78.23	63.98	63.48	78.14	91.55
7	20	3.5	0.9	0.07	0.035	0.02	0.3	0.3	0.094	1.6	8	1	0.03	0.003	85.6	78.7	87.1	83.12	59.3	60.44
8	24	4.5	0.9	0.07	0.025	0.035	0.3	0.24	0.094	1.6	8	1	0.02	0.001	82.1	65.62	69.9	82.37	10.36	51.43
9	20	4.5	0.9	0.09	0.035	0.02	0.2	0.3	0.094	1	15	2	0.02	0.001	79.52	86.52	87.1	67.6	66.25	72.58
10	20	4.5	0.9	0.07	0.035	0.035	0.2	0.24	0.085	1	15	1	0.03	0.001	80.37	81.4	5.92	37.33	36	21.55
11	24	3.5	0.9	0.09	0.025	0.02	0.2	0.24	0.094	1	15	1	0.03	0.003	56.85	77.29	91.93	82.8	54.8	81.05
12	24	3.5	0.9	0.09	0.035	0.035	0.2	0.24	0.094	1.6	8	2	0.03	0.001	69.78	77.46	73.33	87.54	43.93	82.58
13	24	3.5	0.9	0.07	0.035	0.035	0.3	0.3	0.085	1	15	2	0.02	0.003	43.76	88.33	55.92	73.09	12.61	50.37
14	20	4.5	0.5	0.09	0.035	0.035	0.3	0.24	0.085	1.6	15	1	0.03	0.003	83.2	72.03	5.09	46.32	66.42	71.43
15	20	3.5	0.5	0.09	0.025	0.035	0.2	0.3	0.094	1.6	15	1	0.02	0.003	69.19	61.43	79.92	79.91	65.82	90.39
16	24	3.5	0.5	0.09	0.035	0.02	0.3	0.3	0.085	1	8	1	0.03	0.001	61.35	78.21	70.31	80.99	76.3	71.43
17	20	4.5	0.5	0.09	0.025	0.035	0.3	0.3	0.094	1	8	2	0.03	0.001	90.24	68	46.82	69.05	90.23	90.42
18	24	4.5	0.5	0.09	0.035	0.02	0.2	0.24	0.085	1.6	8	2	0.02	0.003	76.05	83.56	47.46	76.24	72.53	71.16
19	20	4.5	0.9	0.07	0.025	0.02	0.2	0.3	0.085	1.6	8	2	0.03	0.003	85.24	90.36	55.72	61.31	77.23	40.52
20	24	3.5	0.5	0.07	0.025	0.035	0.2	0.3	0.085	1.6	15	2	0.03	0.001	52.45	78.64	57.79	78.13	60.17	70.48

residual PAHs concentration was estimated using GC-MS as mentioned previously. Response i.e., ΣPAHs degradation (%) obtained was analyzed using MINITAB®version17.0

PAHs degradation (%) analysis

The residual PAHs were extracted using solvent extraction method by Ghevariya *et al.* (2011), which were concentrated at room temperature and re-suspended in 10 mLdichloromethane (DCM) then passed through silica gel/sodium sulphate column for removal of moisture. The residual PAHs were quantified using GC-MS (Shimadzu QP2010+, Japan) described by Rajpara *et al.* (2015) and ΣPAHs their degradation (%) was estimated by Gosai *et al.* (2018a).

Results

Multiple PAHs degraders and their identification

From ASSBRY, a total of 206 morphologically distinct marine bacterial isolates were obtained. Among these, 57 isolates found to be PAHs degraders, as confirmed by visible reduction in the PAHs concentration. Amongst 57 isolates, 10 isolates exhibiting clear zone surrounding the colonies on PAHs coated plates were considered for secondary screening. These 10 isolates were designated as BS1-BS10. In NAV, 178 morphologically distinct marine bacterial isolates were obtained. 53 out of 178 isolates were found to be PAHs degraders based on visible zone of reduction in PAHs. Amongst these, 10 isolates that exhibited clear zones were considered for further studies and designated as GH1-GH10. In ASSBRY (Fig. 1a), secondary screening revealed that 5th day maximum PAHs degradation of Phe, Ant and Flt was 97, 70 and 47% by BS8, followed by 77, 72 and 55% by BS7 and 70, 71 and 67% by BS1, respectively. Whereas in NAV (Fig. 1b), maximum PAHs degradation of Phe, Ant and Flt was 70, 66 and 59% by GH8, followed by 61, 55 and 48% by GH4 and 58, 61 and 53% by GH1, respectively. Combinations of above 3 isolates from each site were considered for mixed culture studies on PAHs degradation and were designated as BS178 and GH148 for ASSBRY and NAV, respectively.

The most promising multiple PAHs degraders from ASSBRY and NAV were identified based on morphological and biochemical characteristics along with 16S rDNA sequencing. For molecular identification, 16S rDNA sequences were subjected to analysis using BLAST. Based on BLAST, the isolates were identified and partial 16S RNA gene sequences were submitted to GenBank. The results revealed that, BS1 identified as *Bacillus oceanisediminis* strain BS1 (accession number KY933448), BS7 as *Bacillus circulans* strain BS7 (accession number KY082686) and BS8 as *Lelliottia amnigena* strain BS8 (accession number KY082685). Similarly, GH1 identified as *Bacillus campisalis* strain GH1 (accession number KY780510), GH4 as *Achromobacter mucicolens* strain GH4 (accession number KY780509) and GH8 as *Stenotrophomonas maltophilia* (accession number KY933478).

Potential degraders and Plackett-Burman (PB) design for screening of medium components -ASSBRY and NAV

Natural consortia obtained from each site for PAHs degradation were 9 (each season, 3 consortia) consisting of inhabitant microbial population. Samples for natural consortia consisted of a composite sample of 3 sub-samples taken at a distance of 100 meter each. The multiple PAHs degrading bacteria were enhanced by enriching them with PAHs as sole source of carbon for prolonged time period. The natural consortia from ASSBRY were designated as 1A-9A and from NAV as 1N-9N. Based on the maximum PAHs degrading ability, natural consortium selected from ASSBRY was 7A and from

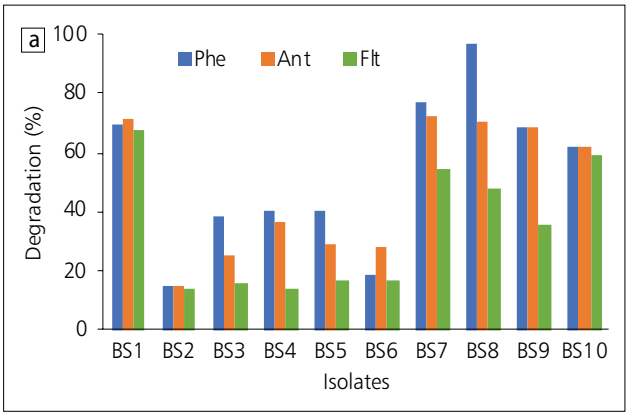

Fig. 1 (a). Degradation (%) of Phe, Ant and Flt by isolates from ASSBRY

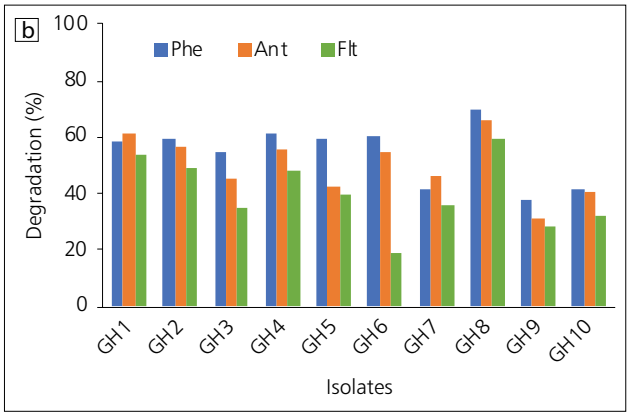

Fig. 1 (b). Degradation (%) of Phe, Ant and Flt by isolates from NAV

NAV was 5N. ONR7a medium components depends on significant *p* value and their effects. The confidence level of screening experiment was set to 95% i.e., $p=0.05$. The component showing negative effect ($p\leq0.05$) indicated that the component is affecting significantly on PAHs degradation but less concentration than its selected negative value is required. While positive effect ($p\leq0.05$) indicated that the concentration of that particular component required is higher than its set positive value (Chauhan *et al.*, 2007). ASSBRY, PB design revealed that 14 medium components gave significant effect on PAHs degradation. Their effects along with the coefficients for each system (isolate BS8, natural consortium (7A) and mixed culture (BS178)) are depicted in Tables 3-5. ΣPAHs degradation was mostly affected by $Na_2HPO_4{\cdot}7H_2O$. Result indicates effect to be positive for BS8 and BS178 while negative for 7A. The second most common components were NaCl, Na_2SO_4 and NH_4Cl that showed contradictory effect on ΣPAHs degradation in BS8 and BS178. $MgCl_2{\cdot}6H_2O$ showed significant effect on PAHs degradation by BS8 and 7A with negative and positive effects, respectively. H_3BO_3 showed negative effect for 7A and BS178. KCl and $CaCl_2{\cdot}2H_2O$ showed significant effect only in 7A. The overall *p* value for all the three systems was between 0.000-0.014 which was under the selected level (0.05).

Tables 6-8 summarizes the results for screened medium components for multiple PAHs degradation using isolate GH8, natural consortium 5N and mixed culture GH148 obtained from NAV. KCl, NaBr and $CaCl_2{\cdot}2H_2O$ were found to be significant in PAHs degradation by 5N and GH148. The effect of all the three components was found to be similar for both, but the *p* value for these three components was found more significant in GH148 as compared to 5N. NaCl was found significant with high *p* value for GH8 and 5N but the effect was contradictory. $Na_2HPO_4{\cdot}7H_2O$ was found highly significant with positive effects in both GH8 and GH148. NH_4Cl, TAPSO, $FeCl_2$, and H_3BO_3, $SrCl_2{\cdot}6H_2O$ were found to be significant components only for GH8 and 5N, respectively. Na_2SO_4 was significant for GH148 only. The overall *p* value for all the three systems was between 0.000-0.018 which was under the selected level (0.05).

Table 3. Estimated effects and coefficients of degradation (%) for BS 8 (ASSBRY)

Term	Effect	Coefficient	SE Coefficient	t-value	p - value	R^2 (%)	R^2 [adj] (%)
Constant		73	0.665	110.50	0.000		
NaCl	-15.23	-7.61	0.665	-11.46	0.000		
Na_2SO_4	11.64	5.82	0.665	8.76	0.000		
NH_4Cl	-5.05	-2.52	0.665	-3.80	0.002	95.90	94.43
Na_2HPO_4	5.39	2.69	0.665	4.06	0.001		
$MgCl_2$	-12.48	-6.24	0.665	-9.39	0.000		

Table 4. Estimated effects and coefficients of degradation (%) for natural consortium 7A (ASSBRY)

Term	Effect	Coefficient	SE Coefficient	t-value	p - value	R^2 (%)	R^2 [adj] (%)
Constant		77.66	0.384	202.22	0.000		
KCl	8.46	4.23	0.384	11.02	0.000		
H_3BO_3	-8.43	-4.22	0.384	-10.99	0.000	97.31	96.34
Na_2HPO_4	-9.61	-4.80	0.384	-12.52	0.000		
$MgCl_2$	2.85	1.42	0.384	3.71	0.002		
$CaCl_2$	7.39	3.69	0.384	9.63	0.000		

Table 5. Estimated effects and coefficients of degradation (%) for mix culture BS178 (ASSBRY)

Term	Effect	Coefficient	SE Coefficient	t-value	p - value	R^2 (%)	R^2 [adj] (%)
Constant		58.50	1.48	39.57	0.000		
NaCl	21.38	10.69	1.48	7.23	0.000		
Na_2SO_4	-8.34	-4.17	1.48	-2.82	0.014	95.41	93.77
H_3BO_3	-18.73	-9.37	1.48	-6.34	0.000		
NH_4Cl	19.74	9.87	1.48	6.68	0.000		
Na_2HPO_4	35.69	17.85	1.48	12.07	0.000		

Table 6. Estimated effects and coefficients of degradation (%) for isolate GH8 (NAV)

Term	Effect	Coefficient	SE Coefficient	t-value	p - value	R^2 (%)	R^2 [adj] (%)
Constant		69.88	0.94	73.95	0.000		
NaCl	22.19	11.09	0.94	11.74	0.000		
NH_4Cl	11.15	5.57	0.94	5.90	0.000	95.43	93.79
Na_2HPO_4	19.11	9.55	0.94	10.11	0.000		
TAPSO	5.94	2.97	0.94	3.14	0.007		
$FeCl_3$	5.07	2.53	0.94	2.69	0.018		

Table 7. Estimated effects and coefficients of degradation (%) for Natural Consortium 5N (NAV)

Term	Effect	Coefficient	SE Coefficient	t-value	p - value	R^2 (%)	R^2 [adj] (%)
Constant		56.65	1.51	37.41	0.000		
	90.32						
NaCl	-21.13	-10.56	1.51	-6.98	0.000		
KCl	-21.79	-10.89	1.51	-7.19	0.000	93.38	90.32
NaBr	13.77	6.89	1.51	4.55	0.001		
H_3BO_3	-18.70	-9.35	1.51	-6.17	0.000		
$CaCl_2$	9.63	4.81	1.51	3.18	0.007		
$SrCl_2$	11.35	5.67	1.51	3.75	0.002		

Table 8. Estimated effects and coefficients of degradation (%) for mix culture GH148 (NAV)

Term	Effect	Coefficient	SE Coefficient	t-value	p-value	R^2 (%)	R^2 [adj] (%)
Constant		67.10	0.98	67.85	0.000		
Na_2SO_4	-9.79	-4.89	0.98	-4.95	0.000		
KCl	-19.85	-9.92	0.98	-10.04	0.000	95.53	93.93
NaBr	14.25	7.12	0.98	7.20	0.000		
Na_2HPO_4	20.27	10.13	0.98	10.25	0.000		
$CaCl_2$	8.08	4.04	0.98	4.09	0.001		

Discussion

Present study focused on isolation of potential PAHs degrading strains from historically contaminated site. Enrichment of the native bacterial population was capable of utilizing PAHs as sole carbon source. Results suggested that native bacterial communities were enhanced multiple PAHs biodegradation by utilizing ONR7a. In natural consortium, various bacterial spp. indirectly played an important role by secreting biosurfactants and bioemulsifiers, that enhance solubility of PAHs and making them available to microbes. Along with that, intermediate products of PAHs degradation also consumed by microbes for their growth. Similar reports suggest that ellochthonous microbial population plays a vital role in bioremediation process (Varjani *et al*., 2015). Multiple PAHs degraders were isolated from the enriched consortia. Thus these bacteria are said to have acquired the degradation characteristics during enrichment by various means like genetic mutations (McDonald *et al*., 2006). ASSBRY, morphological, biochemical and genetic characteristics of the isolates revealed that isolates belonged to the phylum *Firmicutes*, i.e., *Bacillus circulans* and *Bacillus oceanisediminis* and phylum Proteobacteria, i.e., *Lelliottia amnigena.* Similarly NAV, isolate *Bacillus campisalis* belonging to phylum *Firmicutes*, whereas isolates *Achromobacter mucicolens* and *Stenotrophomonas maltophilia* belong to the phylum proteobacteria. This indicates both phyla were leading candidates for biodegradation of PAHs.

The isolate, *B. oceanisediminis* has been reported previously as a hexane degrader (Zhang *et al*., 2010; Helgadóttir, 2016) whereas, *B. circulans* from marine origin has not been reported yet for PAHs degradation. *Lelliottia amnigena* was previously classified as *Enterobacter amnigenus* but recently reclassified as a novel genus *Lelliottia* as *Lelliottia amnigena* (Brady *et al*., 2013). To the authors' best knowledge *L. amnigena* reported for naphthalene and fluoranthene biodegradtion, in this study has been observed to be a multiple PAHs degrader. Recently *B. campisalis* has been identified as a new species (Kumar *et al*., 2015) and *Achromobacter mucicolens* has not been studied for biodegradation of PAHs. *Stenotrophomonas maltophilia* has been reported for the degradation of HMW PAHs like fluoranthene, benz(a)anthracene, pyrene, etc and surfactant enhanced biodegradation of PAHs (Boonchan *et al*., 1998; Juhasz *et al*., 2000). Majority of the obtained strains belong to genera that are well known for biodegradation, but their related species have not been studied well regarding the degradation of PAHs.

ONR7a medium components (14) were analysed using PB design by isolates, natural consortia and mixed cultures for 2PAHs degradation. Despite being common, all the significant components have shown different effects (positive/negative) for each system. The component shared by two or all three systems having different effects indicating common components may be required but their required concentration differ. The *p* values lower than the selected point (0.05 indicating 95% confidence level) confirmed the high significant nature of the screened components and R^2 value indicates the reproducibility of the experimental design. The obtained R^2 (adjusted) values were nearer to the R^2 value computed statistically by the software. Na_2HPO_4, TAPSO (3-[N-Tris (hydroxymethyl) methylamino]-2-hydroxypropanesulfonic acid) are known buffering agents that maintain the pH of the medium during growth and degradation process. BO and Ca as trace elements might play an important role in PAHs biodegradation. Divalent ions such as Mg^{2+} and Ca^{2+} are found to be involved in various cellular processes in microorganisms. Ribosome, cell membrane and nucleic acids are reported to be stabilized by Mg^{2+} (McNeil and Harvey, 2008). Ca^{2+}has been found to be essential for maintenance of cell wall rigidity. It is also found that oligomeric proteins and covalently bound protein-peptidoglycan complexes present in the outer membrane of bacterial cell gets stabilized by Ca^{2+} ions (Macció *et al*., 2002). In liquid cultures, for biodegradation of fluoranthene Ca^{2+}in particular and $Mg^{2+}$to a lesser extent were found to be essential (Willumsen and Karlson, 1998). K^+ is particularly required by enzymes involved in protein synthesis. The components found to be significant for enhancement of PAHs biodegradation will now further be considered for more precise study on optimization of various growth conditions using more accurate and efficient experimental designs like Response Surface Methodology (RSM) and Artificial Neural Network (ANN).

Conclusion

In the present study PAHs biodegraders were isolated from historically contaminated sites situated along the coast line of Gujarat, India. Positive results of mixed culture indicate co-metabolism helps in PAHs biodegradation. The metabolic product of one isolate could be used by other isolates of mixed culture. PB design revealed that nutritional value of microbes depends on their native environments. Isolates from both the sites showed difference in nutritional requirements. These changes might be due to different type of PAHs contamination i.e. ASSBRY, an oil contaminated site while Navlakhi Port, a coal contaminated site. Thus, abundance and variety of microorganisms inhabiting both the sites have shown a discrepancy based on the type of contamination. Individual PAHs biodegrader of natural consortia and mix culture used as lead candidates for the remediation of PAHs contaminated sites. Thus, by amending the growth medium with selected concentrations of various components, efficiency of all isolates, mixed cultures and natural consortia to degrade PAHs was enhanced from 65 to 90% within 5 days.

Marine bacteria have been reported as leading candidates for PAHs biodegradation (Atlas, 1981). Most of the studies

related to PAHs biodegradation have involved individual PAHs degradation. Natural environment is mixture of PAHs, these studies however, do not reveal the actual complexity of the biodegradation process but potential bacterial strain to be used for *in situ* bioremediation. The study suggests PAHs degraders (bacterial strains and natural consortia) must be proficient to utilize either multiple PAHs or selective PAH in presence of other PAHs and contaminants. This study along with the previous reports may provide a significant knowledge of PAHs biodegradation. To the author's best knowledge potential candidates for PAHs biodegradation have been reported first time at ASSBRY and NAV. Thus, this study is baseline research for microbial degradation of multiple PAHs simultaneously. This research may also provide primary knowledge about nutritional requirements for indigenous bacterial communities for enhancement of PAHs biodegradation at the selected sites. Indigenous bacterial communities were more suitable at their optimum conditions. The present piece of work would provide a more sophisticated way of manipulating the indigenous microbiota to bring rapid removal of toxic organic contaminants from polluted habitat.

Aknowledgements

Authors are thankful to Earth Science and Technology Cell (ESTC), Ministry of Earth Sciences (MoES), Government of India (GoI), New Delhi for financial assistance (MoES/16/06/2013-RDEAS) to carry out this research work.

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