Isolation and Molecular Characterization of Polycyclic Aromatic Hydrocarbon Degrading Bacteria from Effluent Water from Weras River Park, Sri Lanka

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ABSTRACT

The present study records the detection of PAHs such as naphthalene and anthracene and isolation of PAHs degrading bacteria from a restaurant site, Weras River Park, in Boralesgamuwa, Sri Lanka. Water samples were collected in seven locations of the study area. Water temperature (°C), pH, and electric conductivity (EC) were measured at the site itself using standard meters. Nitrogen nitrate ($N\text{-}NO_3^-$) and total phosphate (TP) were measured at the laboratory following the standard methods. Following the extraction of PAHs in collected water samples, detection was carried out using the PDA-HPLC diode array method. PAH degrading bacteria were identified using microplate assay. The selected bacteria strains were subjected to degradation kinetic studies following molecular identification by 16S rRNA analysis. Phylogenetic analysis identified *Achromobacter spanius* as potential naphthalene degrading bacteria, where *Alcaligens faecalis* was recorded as an anthracene degrader. Degradation study confirmed that *A. spanius* efficiently degraded naphthalene at the rate of 0.145±0.002 ppm/day, whereas *A. faecalis* degraded anthracene at the rate of 0.181±0.036 ppm/day, respectively. Degradation of structures of the Naphthalene and Anthracene by *A. spanius* and *A. faecalis* was further analyzed by Fourier Transform Infrared Spectroscopy (FTIR). This is the first record on naphthalene degradation by the bacterium *A. spanius.*

1. INTRODUCTION

Polycyclic Aromatic Hydrocarbons (PAHs) are non-polar organic compounds that are comprised of 2 to 7 fused rings and are arranged in linear, angular or clustered structures (Guruge et al., 2008; Kim et al., 2013). Natural and anthropogenic activities such as incomplete combustion of fossil fuels, forest fires, open burning, and the incomplete burning of fuel oils give rise PAHs in the environment (Nkansah, 2012; Witt, 2002). Long term persistence of PAHs in the environment will lead to biomagnification through several fold, leading to adverse aquatic fauna and human health impacts (Guruge et al., 2008; Guruge et al., 2007; Partila, 2013). The bioaccumulation of PAHs in aquatic animals is dependent on several factors, such as the octanol/water partition coefficient (K_{OW}) of each PAH congener, concentration in environmental media, bioavailability, and depuration/excretion of PAHs. PAHs are hydrophobic chemicals that have a high affinity with organic matter in water and sediment compared to the water phase. This trend is more predominant in high-molecular-weight PAHs (more than five-ring) than in low-molecular-weight PAHs because of high K_{OW} values. Typical persistent organic pollutants, such as polychlorinated biphenyls, have the same trend, and high K_{OW} values generally suggest a high bioaccumulation factor. For example, fish are considered to have a higher metabolism capacity and can metabolize/depure PAHs quickly; therefore, a generally positive correlation between the concentration of PAHs in the body and the K_{OW} value is not observed in higher trophic-level fish (Collins et al., 1998; Devi et al., 2016).

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PAHs enter into the aquatic environment through the direct deposition of atmosphere while light PAHs comes into the water via rain washout and surface water runoff (Stogiannidis and Laane, 2015; Guruge et al., 2007). A recent study showed the PAHs derived from pyrogenic activities are highly concentrated in urban areas (Abdel-Shafy and Mansour, 2016). Crude oil, fuels and lubricants (Stogiannidis and Laane, 2015; Jiang et al., 2015), oceanic and freshwater oil spills, transportation vessels, boats, storage and use of crude oil (Abdel-Shafy and Mansour, 2016; Liyanage and Manage 2015; Ohura et al., 2015) are the other main identified sources of petrogenic PAHs to the environment. Further, low molecular weight PAHs commonly contained in petrogenic substances have also been recorded (Stogiannidis and Laane, 2015).

PAHs are considered as carcinogens, mutagens and toxic due to their high octanol water (K_{OW}) and organic carbon adsorption (K_{OC}) partition coefficients (Witt, 2002). According to the International Agency for Research on Cancer (IARC), 400 carcinogens out of 900 agents are PAHs (Ohura et al., 2015). Due to low water solubility and electro-chemical stability, PAHs are persist in the environment for a long time and available to bioaccumulate through the food chain (Guruge et al., 2008) and reach high concentrations in soil and many freshwater bodies of the world (Ohura et al., 2015).

Comprehensive studies have been carried out on the carcinogenicity of PAHs to mammals, including humans (Menzie et al., 1992). Briefly, PAHs are transported into cells because of their hydrophobicity and induce gene expression of the cytochrome P450 (CYP) enzyme group. Expressed CYP enzymes metabolize PAHs into additional metabolites. It is important to note that several intermediates in this metabolic pathway can bind to DNA and become mutagenic/carcinogenic. Because of their carcinogenicity, the International Agency for Research on Cancer (IARC) classified three PAHs: benzo(a)anthracene (BaA), benzo(a)pyrene (BaP), and dibenz(a,h)anthracene, as being probably carcinogenic chemicals (Poliakova et al., 2000). Eight PAHs-BaA, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, BaP, dibenz(a,h)anthracene, indeno (1,2,3-c,d) pyrene, and benzo(g,h,i)perylene are considered possible carcinogens (Menzie et al., 1992). In aquatic animals, such as fish, epizootic neoplasia is strongly associated with environmental chemical pollution, which has increased exponentially since the 1940s with the growth of synthetic organic chemical-producing industries (Bailey et al., 1984). Certain fish species (e.g., rainbow trout and medaka) are well-established sensitive models for evaluating the effects of exogenous and endogenous factors of chemical carcinogenesis (Bailey et al., 1984; Varanasi et al., 1987). In feral fish, carcinogenic properties of PAHs have also been examined in English sole (*Parophrys vetulus*) and flounder (*Platichthys stellatus*) (Varanasi et al., 1987).

To date many studies have focused on the isolation of PAH degradation by bacteria. Idroos et al. (2015) recorded the degradation of naphthalene by *Stenetrophomonas maltophilia* by 37.5%. Further, Oyehan and Thukair (2017) studied the degradation of the polycyclic aromatic hydrocarbons (pyrene- and phenanthrene) by *Pseudomonas aeruginosa*, *P. citronellolis*, *Ochrobactrum intermedium*, and *Cupriavidus taiwanensis*. Further, Dharmawan et al. (2015) have also reported a similar study on PAH (Fluoranthene and pyrene) degradation by *Burkholderia fungorum* and *Mycobacterium gilvum*.

In Sri Lankan context, most of the recorded studies on PAHs are limited to petrochemical stations (Hamid et al., 2017; Undugoda et al., 2016) and water (Guruge et al., 2007). However, no studies on wastewater released from restaurants and eateries have been addressed. Thus, the present study focuses on quantifying PAHs like naphthalene and anthracene released from effluent waters from a restaurant site. In addition this study also emphasizes the isolation and characterization of PAHs (naphthalene and anthracene) degrading bacteria from the site of effluent water release. Hence, this study, which was carried in 2017, presents the first record on isolation of PAHs (naphthalene and anthracene) degrading bacteria from effluent water released from a restaurant. Consequently, the isolated bacterial strains could be effectively used as a biological agent to treat PAH contaminated sites in the future.

2. METHODOLOGY

2.1 Chemicals

Chemicals used for analysis of water quality parameters were analytical grade (Sigma, Aldrich). HPLC-graded methanol, acetone, dichloromethane, naphthalene and anthracene were obtained from the Department of Zoology and Department of Chemistry, University of Sri Jayewardenepura, Sri Lanka respectively.

Bacteriology grade peptone, yeast extract, sodium chloride, bacteriological agar, and 0.9% saline water needed for bacteriological studies were obtained from Centre for Water Quality and Algae Research, Department of Zoology, University of Sri Jayewardenepura.

2.2 Study area and sampling

Surface water samples were collected from seven sites of Weras River Park (6°50'28.9"N 79°53'37.0"E) representing effluent water releasing sites. The control site was selected from a location which was 200 m away from the effluent water releasing site. Water samples were collected using 1 L sterilized glass bottles. Sampling was carried out from June to September 2017 at seven locations. The sixth and seventh locations were selected as reference sampling points. Water temperature (°C), pH, and conductivity (μS/cm) were measured at the sites using thermometer immersion (Philip Haris, England), pH meter (HACH-HQ 40D) and conductivity meter (HACH-HQ 40D), respectively. Total nitrate and total phosphate concentrations in water were measured according to standard spectrophotometric methods (Total nitrate 420 nm and total phosphate 465 nm) described by American Public Health Association (APHA, 2017).

2.3 Quantification of PAHs

Naphthalene and anthracene extraction was carried out using the modified protocol given by Princewill-Ogbonna and Adikwu (2015). Quantification of naphthalene and anthracene were done using the HPLC system consisting of Agilent 1200 series. A sample volume of 25 μL was injected into a 250×4.6 mm, C18 column at a flow rate of 1 mL/min. Two mobile phases were used for the gradient run (65% Milli-Q water and 35% Methanol). Concentrations of naphthalene and anthracene were determined with a pre-prepared calibration curve of the peak areas at 290 nm for naphthalene and at 375 nm for anthracene, respectively, with an external standard. The HPLC method had a detection limit of 0.5 μg/mL and recoveries for naphthalene and anthracene were obtained greater than 95% with a relative precision of 5%.

2.4 Preparation of calibration curve for naphthalene and anthracene for colorimetric assay

Methylene blue was used as the redox indicator. A concentration series of naphthalene and anthracene was prepared ranging from 0.5 μg/mL to 4 μg/mL and 0.1 mL methylene blue was added for each concentration following measurement of absorption at 609 nm. Calibration plots were developed using the absorbance values and the corresponding concentrations.

2.5 Isolation of PAHs degradation bacteria

Isolation of PAH degrading bacteria was carried out according to Manage et al. (2009).

2.5.1 Enrichment studies

Enrichment of naphthalene and anthracene degrading bacteria was carried out in triplicates for each water and sediment samples in vitro. In the enrichment process, 1 mL of 0.1% naphthalene and anthracene were added separately into each collected 99 mL of effluent water samples.

2.5.2 Isolation of potential PAH degrading bacteria from water sample

Following 14 days of enrichment, 1 mL of sample was removed from each flask and serial dilutions were prepared up to 10^{-3} using sterilized saline solution (0.9% NaCl). Exactly 1 mL of subsample was removed from 10^{-1} to 10^{-3} dilutions and inoculated into sterilized, labeled Petri dishes and then the pour plate method was followed using 1.5% Luria-Bertani (LB) agar medium (peptone 13.1 g/L; yeast extract 4.6 g/L; sodium chloride 4.6 g/L; agar 9.1 g/L) to isolate bacteria. Following two days of incubation, the colonies that appeared on the agar plates were recorded as Colony Forming Unit (CFU/mL) for each plate. Colonies with different morphological features were picked up and inoculated into sterilized liquid LB medium. The liquid cultures were incubated at 28°C overnight, subcultured and repeated streaking on LB agar was followed to prepare pure cultures of bacteria. A portion of each bacteria was stored in LB-glycerol at -20°C for future studies.

2.5.3 Colorimetric screening of PAHs degrading bacteria

This method was modified according to Manage et al. (2009). A loop of morphologically different bacterial strains was transferred into 5 mL of liquid LB medium and incubated overnight at 28°C. The exponentially growing cultures were centrifuged at 10,000 rpm for 20 min and the supernatant was discarded. Bacterial pellets were re-suspended in 0.9% saline solution and incubated at 28°C in a shaking incubator at 100 rpm overnight to exhaust residual

carbon content. Then the bacteria were centrifuged at 100 rpm, the supernatant was discarded, and the remaining pellets were washed three times using filter sterilized saline solution following centrifugation. Finally, the turbidity of bacterial suspensions was equalized (A_{590}=0.35) using a spectrophotometer (SPECTRO UV-VIS double beam PC). Microplates (96 wells) were used to screen naphthalene and anthracene degrading bacteria. Different concentrations of naphthalene (100, 200, and 300 µg/mL) and anthracene (1.0, 1.5, and 2.0 µg/mL) were used for degradation study. Exactly 9 µL of bacterial inoculum in saline solution was added to each well and control wells were treated with 9 µL of filter sterilized saline solution. Subsequently 1 µL of Methylene Blue solution was added to each well and triplicate wells were maintained for each concentration of naphthalene and anthracene. The treated plates were wrapped with wet paper towels and stored in the dark at 28°C. The absorbance of the plates was read at 0, 3, 6, 9, 12, 18, 24, and 48 h intervals using an Elisa Plate Reader (MULTISKAN EX, Thermo Scientific, USA) at 609 nm. PAH degradation by each bacteria strain was measured using the equation given below (Undugoda et al., 2016).

$$\text{PAH degradation percentage} = 1 - [(\text{Absorbance of sample}) / (\text{Absorbance of control})]\ 100$$

2.5.4 Degradation kinetics

To study the degradation kinetics of selected PAHs degrading bacteria stains, overnight starved bacteria suspensions were equalized to A590=0.350 and added into 100 mL of sterile lake water, containing either naphthalene (200 µg/mL) or anthracene (1 µg/mL) with 0.1 mL of Methylene blue. The PAHs degradation rates (h) of the bacteria were calculated using the equation below.

$$h = -[(C/C_0)]\ t$$

Where; "C_0" and "C" are the concentration of PAHs at the beginning and at the end of the time interval "t", respectively. Half-life time was calculated as the duration for removal of 50% of the PAHs from the start of each experiment.

2.6 Fourier Transform Infrared Spectroscopy (FTIR)

Depending on the observations of degradation study, an FTIR (Fourier transform infrared spectroscopy) was carried out to study the degradation of naphthalene and anthracene by selected bacteria strains. Overnight starved bacteria suspensions were equalized to A_{590}=0.350 and added into sterile water containing either naphthalene (200 µg/mL) or anthracene (1.0 µg/mL). Flasks were maintained at 28°C at 100 rpm with continuous shaking. Sample aliquots (1 mL) were removed at 0th and 14th day of incubation and subjected to FTIR analysis.

2.7 Molecular identification of bacteria

Genotypic identification of naphthalene and anthracene degrading bacteria was carried out by amplifying and partial sequencing of the 16S rRNA region of PH1 and PH7 bacterial strains. 16F27 (5'-AGAGTTTGATCMTGGCTCAG-3') and 16R1541 (5'-AAGGAGGT GATCCAGCCGCA-3') primers were employed for the amplification of 16S rRNA region. The PCR reaction mixture (50 µL) contained 20 ng genomic DNA, 0.5 µM of each primer (IDT), 1×Go Taq Green Master Mix (Promega, USA), and 1 mM $MgCl_2$ (Promega, USA). Thermal cycling was performed using a Peltier thermal cycler (MG 25+, 001-31085). The initial denaturation step at 94°C for 2 min was followed by 30 cycles of DNA denaturation at 94°C for 10 sec, primer annealing at 55°C for 20 sec, strand extension at 72°C for 1 min and final extension at 72°C for 7 min. DNA sequencing was performed through commercially available service by Macrogen, Korea.

2.8 Statistical Analysis

2.8.1 Cluster analysis

A cluster analysis was carried out using Minitab 17 software to study whether there is a significance difference in Naphthalene and Anthracene concentrations in sampling locations.

2.8.2 Principle Component Analysis (PCA)

Principle Component Analysis (PCA) was carried out using Minitab 14 software to categorize the studied sampling locations depending on analyzed physico-chemical parameters. Reading was as considered as significant when p was $\leq$0.05.

3. RESULTS

Physico-chemical parameters such as water temperature (°C), pH, electric conductivity (EC), nitrogen nitrate (N-NO_3^-), total phosphate (TP) which were measured in water samples from seven study locations are given in Table 1.

Table 1. Physico-chemical parameters of water samples in seven locations in Weras Ganga Park

Locations	Temperature (°C)	Conductivity (µS/cm)	pH	NO_3^- (mg/L)	PO_4^- (mg/L)
Location 1	33.0±0.1	399.00±2.65	6.91±0.01	0.72±0.01	0.81±2.20
Location 2	32.4±0.2	478.00±0.58	6.92±0.01	0.04±0.02	0.75±6.72
Location 3	32.2±0.3	403.00±1.53	6.66±0.01	4.53±0.06	0.75±4.58
Location 4	32.5±0.3	330.00±2.00	6.39±0.04	5.28±0.04	0.79±1.27
Location 5	32.1±0.1	332.00±1.00	7.00±0.02	1.91±0.03	0.76±3.36
Location 6	31.2±0.2	264.00±2.52	7.28±0.03	0.45±0.02	0.72±8.33
Location 7	30.7±0.3	249.00±2.08	7.12±0.01	0.11±0.09	0.77±2.54

The highest water temperature of 33.0±0.1°C was recorded in location 1, whereas the lowest was at 30.7±0.3°C in location 7. The conductivity ranged between 249±2 to 478±1 µS/cm, whereas pH ranged between 6.39±0.04 to 7.28±0.03 during the study period. The highest nitrate was recorded at location 4 (5.28±0.04 mg/L) and the lowest recorded at location 2 (0.04±0.02 mg/L). The highest phosphate concentration was recorded in location 1 (0.81±2.20 mg/L) while the lowest was in location 6 (0.72±8.33 mg/L).

Table 2 shows the naphthalene and anthracene concentrations in water samples collected from seven sampling locations of the study. The highest naphthalene and anthracene concentrations were detected from effluent water sampling location 1 whereas the lowest values were detected in the locations 6 and 7 which were the reference points.

Naphthalene degradation potential of PH1-PH5 bacterial strains and anthracene degradation potential of PH6-PH10 bacterial strains were assessed using a colorimetric detection method (Figure 1).

Table 2. Naphthalene and anthracene concentrations in sampling locations

Sampling locations	Naphthalene concentration (µg/mL)	Anthracene concentration (µg/mL)
Location 1	235.21±0.06	1.88±0.05
Location 2	33.36±0.46	1.76±0.01
Location 3	31.09±0.46	1.74±0.01
Location 4	30.64±0.26	1.70±0.01
Location 5	11.14±0.10	1.65±0.01
Location 6	7.32±0.11	1.10±0.02
Location 7	2.75±0.03	0.99±0.02

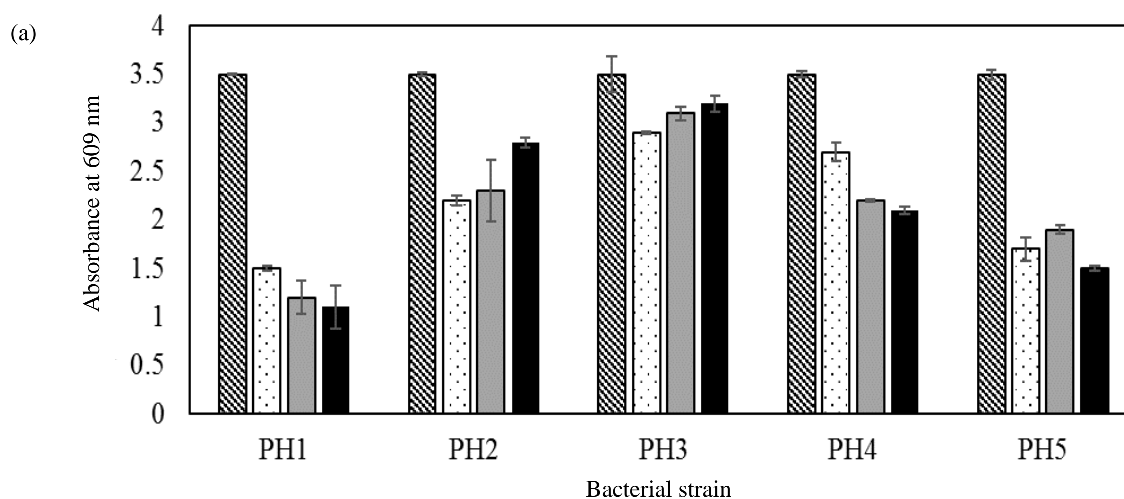

Figure 1. (a) Screening for naphthalene degradation by PH1-PH5 bacterial strains at 24 h of incubation (hatched bars-control, dotted bars-100 µg/mL, ash bars-200 µg/mL, black bars-300 µg/mL). (b) Screening anthracene degradation of by PH6-PH10 bacterial strains at 24 h of incubation (hatched bars-control, dotted bars-100 µg/mL, ash bars-200 µg/mL, black bars-300 µg/mL).

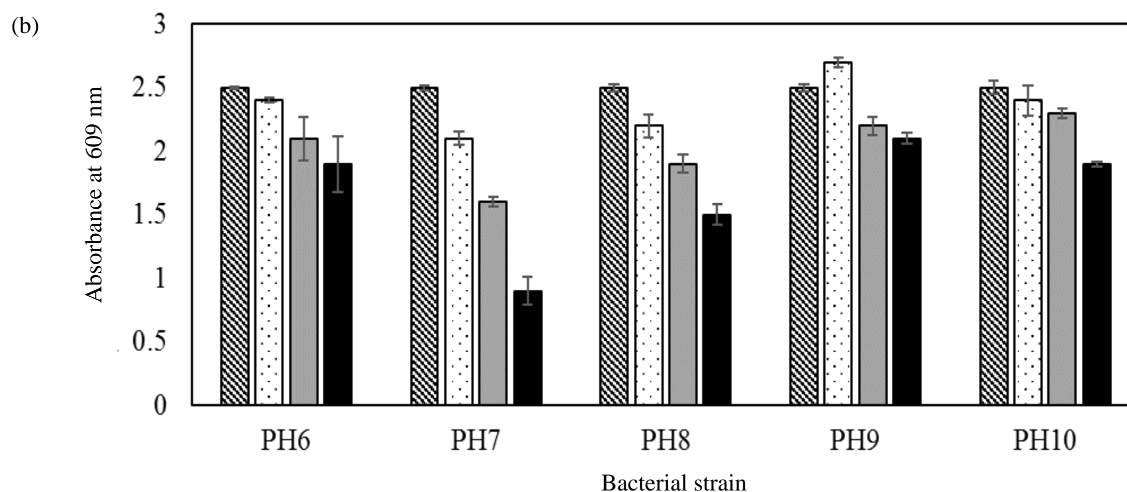

Figure 1. (a) Screening for naphthalene degradation by PH1-PH5 bacterial strains at 24 h of incubation (hatched bars-control, dotted bars-100 µg/mL, ash bars-200 µg/mL, black bars-300 µg/mL). (b) Screening anthracene degradation of by PH6-PH10 bacterial strains at 24 h of incubation (hatched bars-control, dotted bars-100 µg/mL, ash bars-200 µg/mL, black bars-300 µg/mL) (cont.).

According to the colorimetric assay, reduction of absorbance values of wells corresponds to bacterial degradation activity. In the case of naphthalene degradation, PH1 showed the lowest absorption confirming the highest degradation rates. Furthermore, the absorbance value for PH1 was the lowest (1.10±0.01) at 300 µg/mL of naphthalene corresponding to highest degradation. The screening for anthracene degradation confirmed that PH7 as the efficient degrader. Interestingly, PH7 also indicated the lowest absorbance (0.90±0.02) for 2 µg/mL of anthracene proving anthracene degradation ability by the bacterium PH7 is inversely proportional to anthracene concentration (Figure 2).

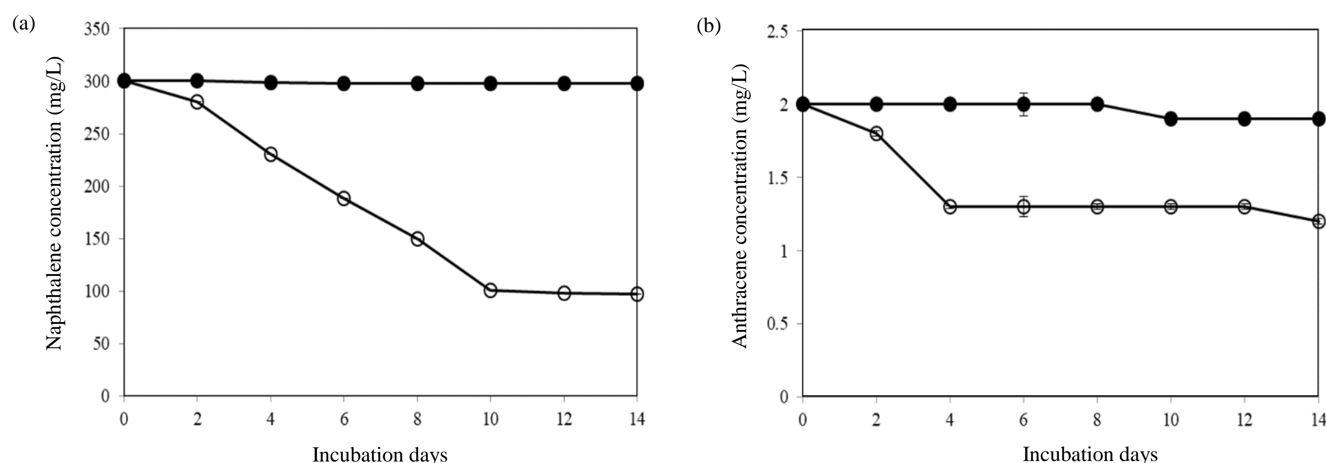

Figure 2. (a) Degradation of naphthalene (300 mg/L) by PH1 (control-closed circles, experiment-open circles). (b) Degradation of anthracene (2 µg/mL is suggested) by PH1 (control-closed circles, experiment-open circles).

The degradation kinetic study confirmed that PH1 (Figure 2(a)) degraded 300 µg/mL of naphthalene up to 99.2 µg/mL within 14 days of incubation whereas PH7 strain degraded 2.00±0.04 µg/mL of anthracene up to 1.02±0.04 µg/mL at the end of 14 days of incubation.

An FTIR analysis further confirmed the naphthalene and anthracene degradation by PH1 and PH7 respectively. According to the FTIR results, the percentage transmission for naphthalene (Figure 3(a) and Figure 3(b)) and anthracene (Figure 4(a) and Figure 4(b)) was reduced after 14 days of incubation.

According to FTIR results the wavelength obtained for anthracene as 2,924 shows complete removal at the 14th day of incubation.

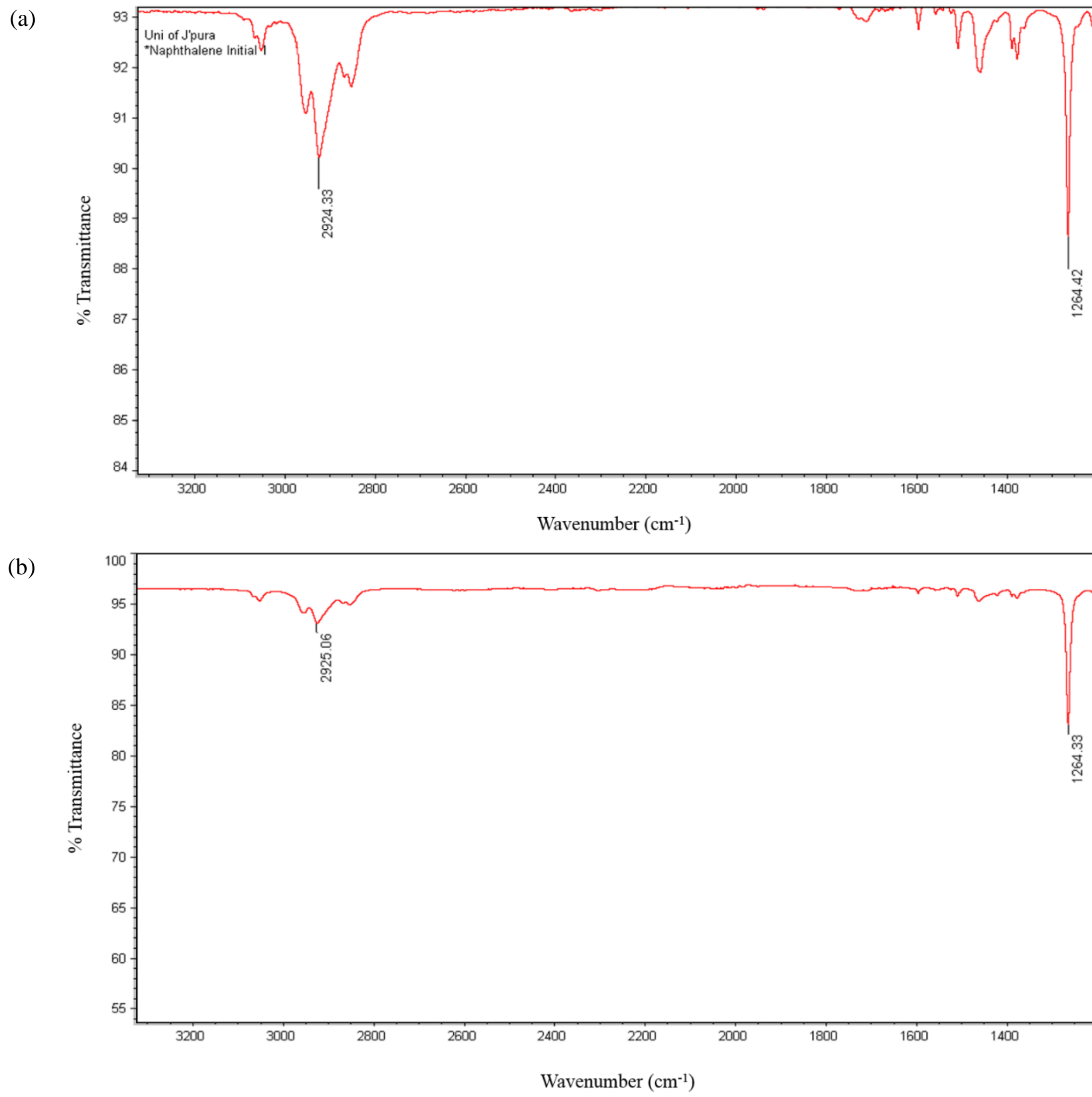

Figure 3. Fourier Transform Infrared Spectroscopy (FTIR) for naphthalene degrading PH1. [(a) 200 µg/mL initial concentration; (b) after 14 days naphthalene concentration]

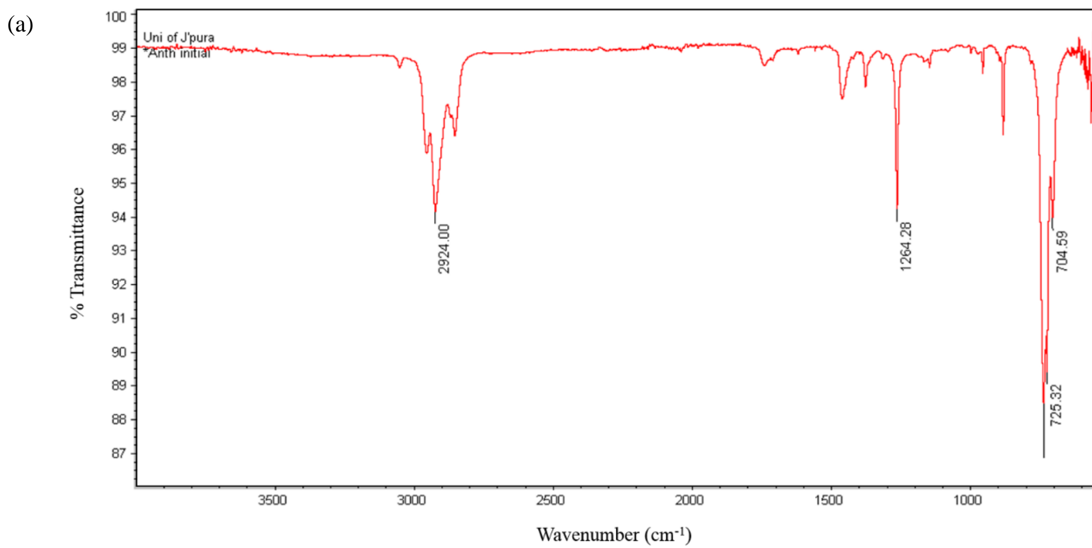

Figure 4. Fourier Transform Infrared Spectroscopy (FTIR) for anthracene degrading PH7. [(a) 200 µg/mL initial concentration; (b) after 14 days anthracene concentration]

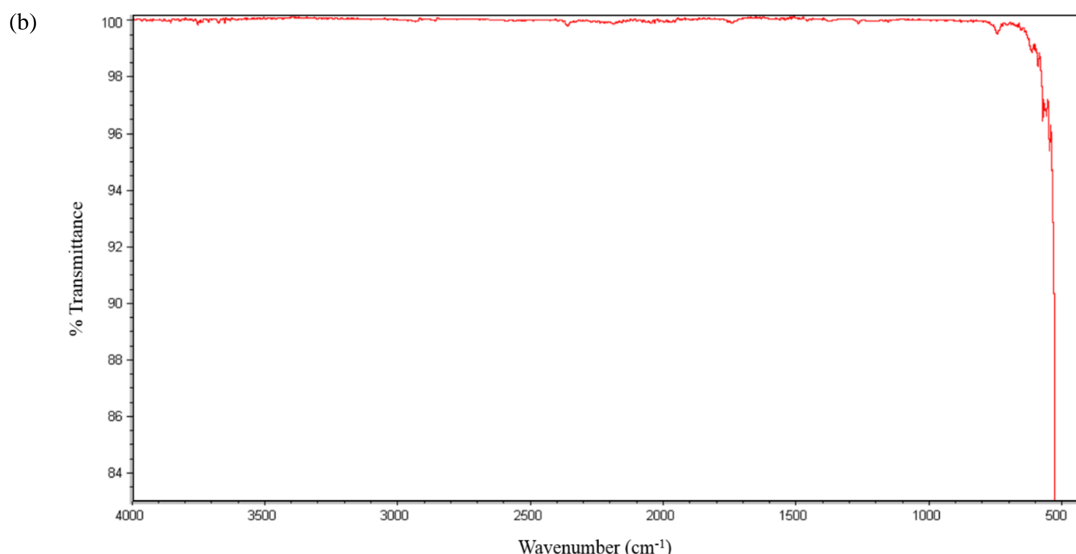

Figure 4. Fourier Transform Infrared Spectroscopy (FTIR) for anthracene degrading PH7. [(a) 200 µg/mL initial concentration; (b) after 14 days anthracene concentration] (cont.)

Morphological characterization of PH1 showed that it was a gram negative, rod shaped and forms light brown colored, circular shaped colonies with irregular margins, whereas PH7 strain was gram negative, rod shaped and forms yellow colored circular shaped colonies with circular margins. The 16S rRNA sequence confirmed that PH1 strain was 98% similar to that of *Achromobacter spanius* and that PH7 strain was 99% similar that of *Alcaligens faecalis* (Figure 5).

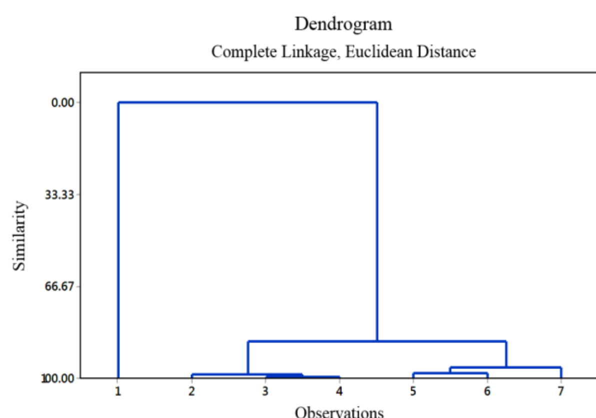

Figure 5. Dendogram showing the clustering of sampling locations based on naphthalene and anthracene concentrations.

The cluster analysis confirmed that location 1, which is the immediate PAH releasing point, occupied a separate cluster denoting high concentrations of naphthalene (235.23±0.06 µg/mL) and anthracene (1.88±0.05 µg/mL) while locations 2, 3, and 4 clustered together with moderate concentrations of naphthalene and anthracene (Figure 6). Locations 5 and 6 showed a different cluster indicating lower low levels of naphthalene and anthracene than locations 2, 3, and 4. Interestingly, location 7 which was away from the immediate PAH releasing site had the lowest concentrations of naphthalene and anthracene (Figure 6).

Furthermore, the PCA categorized the studied sampling sites into three groups based on analyzed physico-chemical properties of collected water samples (Figure 6). The highest water temperature, pH, conductivity, phosphate, naphthalene and anthracene concentration were recorded at location 1 and 2. The maximum concentration for nitrate was recorded at location 3 (4.53±0.06 mg/L) and 4 (5.28±0.04 mg/L). In the case of naphthalene and anthracene contamination, the highest concentrations for naphthalene (235.23±0.06 µg/mL) and anthracene (1.88±0.05 µg/mL) was recorded in location 1 whereas the lowest naphthalene (2.75±0.03 µg/mL) and anthracene (0.995±0.019 µg/mL) concentrations were recorded in location 7. Thus, location 1 and 2 clustered in group A, location 3 and 4 in group B, location 5 in group C and location 6 and 7 in group D (Figure 6).

4. DISCUSSION

This study records the presence of PAH (naphthalene and anthracene) in a restaurant site in Sri Lanka. The water samples obtained from the study area were subjected regular exposure of cooking oil contaminated effluent waters. Water quality parameters revealed that, location 1 has the highest value of temperature (33°C), phosphate (0.81 mg/L),

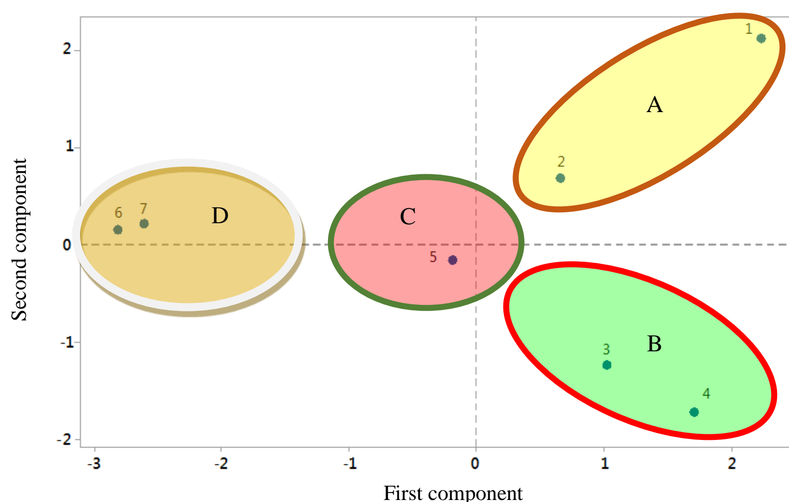

Figure 6. Results of PCA analysis

naphthalene (235.23±0.06 μg/mL) and anthracene concentration (1.78±0.05 μg/mL). This is because location 1 is the immediate wastewater releasing site of the restaurant. However, the nitrate and phosphate concentrations were higher in location 3 (4.53±0.06 mg/L) and 4 (5.28±0.04 mg/L) and this may due to soil, geochemical properties and vegetation type of these sites. During the sampling period it was observed that the Location 3 and 4 cater drinking water needs of domestic animals such as cattle and dogs. Hence, animal droppings to the water in these sites may have resulted in high nitrate concentrations.

According to the industrial wastewater quality standards (BOI, 2011), the temperature of waste water should be at or below 40°C. The highest value of temperature was recorded as 33°C at location 1. The temperature of the rest of sampling locations remained below the maximum tolerance limit (40°C). According to the guide to industrial waste water quality of Sri Lanka (BOI, 2011) water pH should be in the range of 6.0-8.5. The results of the study showed all the sampling locations water pH ranged from 6.39 to 7.12. As stipulated in the wastewater quality guide, nitrate of the industrial and domestic wastewater should be below 50 mg/L. In the study the nitrate concentrations were from 4.53 to 5.28 mg/L. The highest concentration of phosphate (0.080±0.002 mg/L) was recorded at location 1 and the recorded concentrations were below the maximum tolerance limit (1.0 mg/L) given for industrial wastewater quality of Sri Lanka.

In the present study, a colorimetric assay was employed with the methylene blue as the redox indicator to screen naphthalene and anthracene degrading bacteria. When PAH degrading bacteria is present in a microplate well along with PAH and methylene blue, a colour change from blue to colorless would be visualized. This is due to H^+ generated by bacterial metabolism of naphthalene and anthracene. This H^+ together with 2 electrons reduces methylene blue into leucomethylene blue (Hallock et al., 2003). Hence, two bacterial strains, namely PH1 and PH7, were identified as effective naphthalene and anthracene degraders respectively. The 16SrRNA analysis of PH1 and PH7 confirmed the degraders as *Achromobacter spanius* and *Alcaligens faecalis*. It is established that selection of PAH-degrading microorganisms, as with other xenobiotic chemicals, occurs as a result of their previous exposure to this substances in the environment (Lewis et al., 1987). However, these adaptations occur slowly and usually depend on the recalcitrance or biodegradability of the particular substance involved (Tao et al., 2004). PAHs usually have low aqueous solubility, thus are poorly bioavailable for microbial utilization as sole carbon source (Johnsen et al., 2005).

In the present study, naphthalene degradation by *A. spanius* was 66.8±0.02% and the recorded degradation percentage was less than values recorded by Karimi et al. (2015). The results of the studies suggested that the degradation potential is species specific Moreover, Bisht et al. (2010) has recorded 85.3% and 95.8% of naphthalene degradation by the bacterium *B. circulans* (SBA12) and *Kurthia* sp. (SBA4), respectively. Sadighbayan et al. (2016) recorded 57.1% naphthalene degradation by a mixed bacteria consortium from PAH contaminated soil.

In the case of anthracene, *A. faecalis* showed 50.5% degradation. However, less degradation compare to anthracene degradation (55.1%). More or less similar results were recorded when employed

mixed bacterial consortia isolated from soil contaminated with petrochemical by Tao et al. (2004). Barranaco et al. (2004) has recorded two bacterial strains, *B. circulans* (SBA12) and *Kurthia* sp. (SBA4) that degraded anthracene significantly achieving 87.5% and 86.6%, respectively.

The difference for degradation rates of both naphthalene and anthracene by isolated bacteria is specific and is contributed through their unique enzymes secreted during biodegradation (Bisht et al., 2010). Hence the isolated *A. spanius* and *A. faecalis* show different degradation rates for naphthalene and anthracene respectively.

However, it is noteworthy that almost all the studies have been done for the isolation of PAH degradation bacteria in petrochemical sites where high concentrations of PAHs were detected (Undugoda et al., 2016). However, the present study records the isolation of naphthalene and anthracene degrading bacteria from a restaurant site contaminated with cooking oil. Thus, *A. spanius* and *A. faecalis* strains were isolated as potential degraders for naphthalene and anthracene in the present study. However, the recorded degradation percentages of the bacteria *A. spanius* and *A. faecalis* are comparatively low compared to the bacteria recorded by the other studies suggesting previous continuous exposure to particular chemical may enhance the degradation potential.

Interestingly, PAH degrading strains isolated by Dharmawan et al. (2015) showed 100 % removal of PAHs. Gran-Scheuch et al. (2017) also recorded the PAH (Phenanthrene) degradation by *Sphingobium xenophagum* isolated from a diesel oil contaminated soil. According to Gran-Scheuch et al. (2017), *Sphingobium xenophagum* showed 95% removal of Phenanthrene. Hence, it is noteworthy that all recorded studies have isolated PAH degrading bacteria from petroleum oil contaminated sites. However, the present study have deviated from these traditional sites and focused on restaurant effluent water releasing sites. Thus, bacterial strains isolated from the present study shows lower degradation rates as environmental PAH concentration in effluent waters released from restaurant sites have comparatively lesser concentrations of PAHs in comparison to petrochemical stations. The results of this study supports the perspective of the green solution for further development of biotechnological solutions to eliminate of pollutants like naphthalene and anthracene from the aquatic environment.

5. CONCLUSION

This study presents the first record of isolation of naphthalene degrading *Achromobacter spanius* and anthracene degrading *Alcaligens faecalis* strains isolated from a restaurant site. Degradation studies confirmed that *A. spanius* efficiently degrade naphthalene at the rate of 0.145±0.002 ppm/day, whereas *A. faecalis* degraded anthracene at the rate of 0.181±0.036 ppm/day. This is the first study recording an isolation of naphthalene and anthracene degrading bacteria from a restaurant site. Hence, these bacterial strains show comparatively lower naphthalene and anthracene degradations than bacterial strains isolated from petrochemical station sites. Therefore, these isolated naphthalene degrading *Achromobacter spanius* and anthracene degrading *Alcaligens faecalis* strains could be effectively used to treat cooking oil PAH contaminated sites.

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