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Research article

Screening of polycyclic aromatic hydrocarbon degrading bacterial isolates from oil refinery wastewater and detection of conjugative plasmids in polycyclic aromatic hydrocarbon tolerant and multi-metal resistant bacteria

Khalida Khatoon *, Abdul Malik

Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh, 202002, Uttar Pradesh, India

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ABSTRACT

Wastewater were collected from the effluent channel in the vicinity of Mathura oil refinery, U.P. (India) and analysed for physicochemical characteristics, heavy metals as well as organic compounds including PAHs. The interaction of PAHs and heavy metals with various group of microorganisms revealed the viable count of aerobic heterotrophs, asymbiotic nitrogen fixers, actinomycetes and fungi were found to be 2.38×10^6, 1.89×10^4, 2.20×10^4 CFU/mL and 8.76×10^3 CFU/mL respectively. We have selected and screened 50 bacterial isolates for their resistance/tolerance to heavy metal and PAHs. Out of 25 multi-metal resistant isolates, 6 were able to tolerate PAHs at the concentration of 5000 μg/mL (50μg/disc) to naphthalene, anthracene, phenanthrene and pyrene. The PAH degradation efficiency of the isolates was assessed using spectrophotometer with 100 μg/mL of phenanthrene and observed different degree of degradation ranging from 34-66% after 96 h of incubation. One of the bacterial isolates KWB3 (identified as *Enterobacter ludwigii* by 16S rDNA sequencing) exhibited maximum degradation efficiency (66%) was further tested for phenanthrene degrading ability in the presence and absence of a co-substrate (glucose) in a mineral salt medium; and a number of metabolites were produced and detected by GC-MS which revealed the presence of benzocoumarin, phthalic acid, catechol and several low molecular weight compounds. The DNA derived from multi-metal and PAHs tolerant bacteria were PCR amplified using Inc specific primers and positive PCR products were obtained with *oriT* and *trfA2* of the IncP group; indicates that these bacteria have gene-mobilizing capacity.

1. Introduction

Environmental pollution caused by xenobiotics has now become a major issue of concern. Industrialization is a critical factor for the development of the economy of a country. Most of the industrial activities generate huge amounts of gaseous, liquid, or solid hazardous wastes. During the process of refining crude oil, large volumes of fresh water is used by refineries (Shpiner et al., 2009) and generate huge amount of wastewater (Mustapha et al., 2015).

Oil refinery being an important industrial sector produce wastes that contains various chemicals in a significant concentrations including oil and greases, phenols (creosols and xylenols), sulphides, ammonia, suspended solids, cyanides, nitrogenous compounds, heavy metals, mono and polycyclic aromatic hydrocarbons (Hardik et al., 2010; Dhananjayan et al., 2012; Hara and Marin-Morales, 2017; Bahri et al., 2018).

The indigenous microbes which are present in wastewater and soil have been found to degrade refinery pollutants such as PAHs either aerobically or anaerobically (Dhaker and Jain, 2011; Jain et al., 2011; Zhao et al., 2017) using different enzymes like mono- and dioxygenases, laccase, and peroxidase etc. which involves the oxidation of PAH rings (Haritash and Kaushik, 2009). Gram negative bacterial community has been reported to be more efficient PAHs degraders (Ahmad et al., 2019). Several bacteria including *Acinetobacter calcoaceticus, Alcaligenes denitrificans, Alcaligenes odorans, Arthrobacter polychromogenes, Bacillus thuringiensis, Burkholderia cepacia, Mycobacterium vanbaalenii, Mycobacterium flavescens, Pseudomonas aeruginosa, Pseudomonas putida, Sphingomonas paucimobilis, Stenotrophomonas maltophilia* etc have been reported to efficiently degrade the PAHs (Liu et al., 2017).

It is reported that the abundance of plasmids is more in polluted sites than unpolluted zone; however experimental data are limited (Smalla et al., 2006; Heuer et al., 2009; Dealtry et al., 2016). Genes coding for enzymes that enable bacteria to resist antibiotics or heavy metals or to

* Corresponding author.
E-mail address: khalidasiraj826@gmail.com (K. Khatoon).

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degrade xenobiotics are often found on plasmids (Top and Springael, 2003; Broszat et al., 2014; Wolters et al., 2016) and that plasmid harbouring strains have been isolated from polluted sites (Nour et al., 2017).

Several authors have reported the impact of long-term application of untreated/partially treated wastewater on agricultural soil which results in accumulation of antibiotics, heavy metals, polychlorinated substances, antibiotic and heavy metal resistance genes (Ansari et al., 2008; Broszat et al., 2014; Jechalke et al., 2015). So far little information is available about effects of treated/partially treated oil refinery wastewater mixed with domestic sewage, applied for several decades on agricultural soils on the abundance of IncP-1 plasmids and class1 integrons, which may contribute to the accumulation and spread of resistance genes in the environment, and their correlation with heavy metal and PAHs concentrations.

The present paper focuses on screening, isolation and characterization of indigenous bacteria for their PAHs degrading ability and the metabolites were identified by GC-MS. The most promising isolates were tested for their resistance to metal ions. The physicochemical characterization of refinery wastewater and their impact on microbial flora was also assessed. An additional aim of the study was to test the multi-resistant/tolerant bacteria for the presence of plasmids using *Inc*-specific PCR; demonstrated the gene mobilizing capabilities of the isolates, which help in spreading the resistance/tolerance genes to the native bacterial population in agricultural soil by wastewater irrigation.

2. Materials and methods

2.1. Collection of samples

Wastewater samples were collected from the effluent channel in the vicinity of Mathura oil refinery, U.P, India. A total of 12 composited wastewater samples were collected from March 2015 to March 2018 (usually at three months' interval) and transported to the laboratory as described in standard methods (APHA, 2005). The treated oil refinery effluent is discharged into Yamuna River at the downstream of Mathura City whose water is used for irrigation of food crops by the local farmers. Mathura Oil Refinery, a constituent of the Indian Oil Corporation Ltd., processes indigenous Bombay high crude and various imported crudes is situated adjacent to the Agra-Delhi National highway in the outskirts of Mathura city which is located at the latitude 27.28°N and longitude 77.41°E.

2.2. Physicochemical analysis

Physico-chemical parameters such as pH, TDS, bicarbonate, carbonates and chloride of the wastewater sample was carried out according to the procedure of Gupta (2004).

2.3. Heavy metal analysis

For the metal analysis, 25 mL of the wastewater was taken in a 100mL beaker and digested with HNO_3: $HClO_4$ (10mL each) as described in Standard Methods (APHA, 2005). The digested samples were filtered with 0.45 μm syringe filter. The filtrate was transferred to a volumetric flask, and make up the volume upto 100 ml and analysed for the presence of heavy metals by atomic absorption spectrophotometer (Model: GBC 932 Plus, Australia). The wavelength for the metal analysis by AAS were: Pb (283.31 nm), Cd (228.80 nm), Cu (324.75 nm), Ni (232.0 nm), Cr (375.9nm) and Zn (213.86 nm). The flame consisted of Air and Acetylene having flow of 10 L/min and 2.50 L/min respectively.

2.4. Extraction of wastewater and GC-MS analysis

Wastewater (500 mL) samples were extracted with 20 ml n-hexane (HPLC grade, SRL, India) and Dichloromethane (DCM) (HPLC grade, SRL, India) using liquid-liquid extraction procedure (APHA, 2005). Homogenized wastewater samples were shaken vigorously in separatory funnel thrice, each time using 20 ml n-hexane (HPLC-grade). When the solvent and water layers were separated, the solvent layer was collected in 100 mL amber coloured bottle after separation from the water phase. In case of DCM, acidic and basic fractions were collected by extracting the water samples at pH $>$ 2 and pH $<$ 11 respectively. The extracts were evaporated to dryness and re-constituted in 2 ml of respective solvents and transferred to GC vials and analysed by GC-MS (VARIAN GC-MS-4000), a VARIAN CP-8410 auto sampler and an ion trap mass spectrometer. The system was controlled by a 0 varian star MS work station v6.9.1. The chromatographic column was a Zebron ZB-1701 (30 m 0.25 mm i.d.; 0.15 mm film thickness). The head pressure of the helium carrier gas was at a pressure of 8.7 psi. The sample injection (injection volume 1μL) was made in split mode (having split ratio 10) using a bruker-glass liner. The compounds were identified on the basis of mass spectra using the NIST MS search v2.0 library (National Institute of Standards and Technology).

2.5. Enumeration of microbial flora and screening of PAHs degrading bacteria

Enumeration of microbial population of wastewater i.e. aerobic heterotrophic bacteria, fungi, actinomycetes and asymbiotic nitrogen fixers were determined by plating on nutrient agar, Rose Bengal Agar medium, Kenknight and Munaier's medium and Jensen's medium respectively. Aerobic heterotrophic bacteria were counted after 24 h of incubation at 37 °C. Fungi were grown at 28 °C for 3–5 days, actinomycetes and asymbiotic nitrogen fixers were grown at 30 °C for 2–4 days (Khan and Malik, 2018). Moreover, the microbial population were also determined on phenanthrene supplemented nutrient agar plates in a concentration range of 25–100 μg mL^{-1}.

Enrichment culture technique (Darmawan et al., 2015) with some modifications was used for the isolation of PAHs utilizing bacteria using minimal salt medium (MSM) containing di-potassium phosphate 7gm/L, Mono-potassium phosphate 2 g/L, Sodium citrate 0.50 g/L, Magnesium sulphate 0.10 g/L, Ammonium sulphate 1.00 g/L. Wastewater (10 mL) was transferred to the minimal salt broth (90 mL) amended with 100 μg $mL^{-1}$of and incubated under shaking condition (200 rpm) at 37 °C for 7 days. The cycle was repeated three times with 1% (v/v) inoculum. After three weeks of incubation, 100 μl of the inoculum was plated on the MSA plates containing 100 μg mL^{-1} of phenanthrene. After incubation the colonies with different size and morphology were picked up and purified by repeated sub-culturing on the same minimal agar plates amended with 100 μg mL^{-1} of phenanthrene. These bacterial isolates were further screened for their ability to tolerate different PAHs (i.e. Naphthalene, Phenanthrene, Anthracene and Pyrene) in the concentration range of 100–2000 μg mL^{-1} on minimal agar plates.

2.6. Determination of minimum inhibitory concentrations (MICs) of heavy metals

Metal MIC of each PAHs tolerant bacterial isolate was determined using plate dilution method as described by Ansari et al. (2008) in the concentrations ranging from 3.12-2200 μg/mL with Ni^{+2}, Cu^{+2}, Cr^{+3}, Cd^{+2}, Cr^{+6} and Pb^{+2} using $NiCl_2$, $CuSO_4$, $CrCl_3$, $CdCl_2$, $K_2Cr_2O_7$ and $Pb(CH_3COO)_2$ salts respectively. Sterile double distilled water was used to prepare the stock solutions of metals. 10μl of freshly grown culture of 0.14 OD (3×10^8 viable cells per mL) was spot inoculated and incubated at 37 °C for 24–48 h. The maximum concentration at which the growth was inhibited; considered as MIC of the bacterial isolates tested. C600 strain of *E. coli* (*thr-1, leuB6, fhuA21, lacY1, glnV44, rfbD1, glpR200, thi-1*) was used as control (*E. coli* Genetic Stock Centre, Department of Biology, Yale University, New Haven, USA).

2.7. Tolerance of bacteria to polycyclic aromatic hydrocarbons

Bacterial isolates were tested for their tolerance to PAHs i.e. naphthalene (Nap), anthracene (Ant), phenanthrene (Phe), and pyrene (Pyr) by surface plate assay as described by Zafra et al. (2014). Sterile filter discs (Hi-media, India) were impregnated with PAHs mixture consist of Nap, Ant, Phe and Pyr (1:1:1:1) having concentrations of 5, 10, 20, 30, 40, and 50 μg/disc used in the test. 100μl of each bacterial culture (OD 0.14) were spread onto minimal salt agar plates with 1% glucose and discs of each PAHs concentration were placed onto the plate. Plates were incubated at 37 °C for 24–48 h and the radii of growth inhibition zone were measured. Discs with solvent (without PAHs) were used as control.

2.8. Analysis of phenanthrene biodegradation

All the multi-metal resistant and PAHs tolerant bacterial isolates were tested for their PAHs degrading ability in minimal medium amended with 100 μg-mL^{-1} of phenanthrene and incubated at 37 °C under shaking condition at different time intervals (0, 24, 48, 72 and 96 h). After every 24 h, the culture was withdrawn (upto 96 h) and centrifuged at 8000×g for 20 min. The supernatant was extracted with the equal volume of n-hexane and the extract was estimated for the residual phenanthrene by recording the absorbance at 251 nm (λmax of phenanthrene). The percent degradation was calculated spectrophotometrically by determining the initial absorbance (before degradation) and final absorbance (after degradation).

$$\%\ \text{Degradation} = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100$$

Half-life ($t_{1/2}$) of the Phenanthrene degradation was calculated as follows:

$$N_t = N_0 \times {}^1\!/_2{}^n$$

N_0 = initial concentration; N_t = remaining concentration; T = Time of incubation (96 h)

$$T = t_{1/2} \times n$$

Mean life time is simply the arithmetic average of the lifetimes of individual atom of phenanthrene was calculated as:

$$\textit{Mean life time}\ (\tau) = \frac{\textit{half life}}{\ln(2)}$$

The degradation rate or degradation constant is the fraction of the total mass that degrades in one-unit time was calculated using the following formula:

$$\textit{Degradation rate} = \frac{1}{\textit{Mean life time}}$$

Bacterial isolate (KWB3) exhibited the highest phenanthrene degradation spectrophotometrically was selected further for the GC-MS analysis of metabolites under similar experimental condition according to Nie et al. (2016). The minimal salt medium amended with 100 μg mL^{-1} of the PAH (Phenanthrene) in the presence (1%) and absence of glucose was incubated with bacterial isolate KWB3 under shaking condition (200 rpm) at 37 °C. After 96 h of incubation, the culture supernatant was initially acidified to pH 2.0 using 1 N HCl and extracted thrice with ethyl acetate (HPLC grade). The extracted organic phase containing residual phenanthrene was filtered through 0.22 μm filter then reduced to 5 mL and analyzed by GC-MS (Shimadzu QP2010 Plus). Samples were analyzed for degradation products by GC-MS and the metabolites were identified on the basis of mass spectra using the NIST library (National Institute of Standards and Technology). Control samples extracted from minimal salt broth amended with phenanthrene only (without bacterial isolates) were also analyzed simultaneously.

2.9. Plasmid specific PCR

Oligonucleotide primers to amplify plasmid backbone regions related to replication of IncP, IncN, IncW and IncQ published by Gotz et al. (1996) and pMV158 rolling circle type by Alexandrino (2006) were used in this study. PCR was performed in a 50 μL reaction mixture containing 5μL 10X reaction buffer (Fermentas), 1 μL of dNTPs mix (25mM each), 3.75 mmol/L $MgCl_2$ (4.75 mmol/L in case of IncQ oriT), 1 μL of each primer (0.20 μm) and Taq DNA polymerase (2.5 U/μL) according to the amplification program mentioned in Ansari et al. (2008). PCR was carried out in a Primus 96 Thermocycler (BIOER XP Cycler, Germany). For product size confirmation and yield estimation, 5μl of the PCR products were loaded onto 1.5% agarose gel was subjected to electrophoresis for 2.5 h at 40V.

3. Results and discussion

3.1. Physicochemical analysis of wastewater

The physicochemical characteristics and heavy metals analysis of oil refinery wastewater are presented in Supplementary Table1. Wastewater samples were collected from the effluent channel showed pH of 8.0 and yellowish in colour. The concentration of total dissolved solids was found to be 254 ± 19.43 mg L^{-1} while inorganic minerals such as bicarbonates, carbonates and chloride were found to be 14.6 ± 1.63 mg L^{-1}, 79 ± 7.0 mg L^{-1} and 173.67 ± 11.59 mg L^{-1} respectively.

AAS analysis revealed that the refinery wastewater is contaminated with several heavy metals. The concentration of Ni, Cu, Cr, Cd and Zn were found to be in the range of 0.952–2.592 mgL^{-1}, 0.02–0.052 mgL^{-1}, 1.488–1.624 mgL^{-1}, 0.012–0.156 mgL^{-1} and 0.504–0.66 mgL^{-1} respectively. Some of the detected metals are toxic to the biological system. Rasheed and Saleh (2016) investigated physicochemical properties of the well water, wastewater and soil in the vicinity of Bazian oil refinery (Iraq) and found heavy metals chromium, lead, cadmium, copper, zinc and nickel. They found chromium in the range of 1.27–1.34 mg l^{-1}, mean lead concentration was 1.64 mg l^{-1} while cadmium ranged from 0.71 to 0.88 mg l^{-1} for wastewater samples. The concentration of Cu, Ni, and Zn were found to be 0.188 mg l^{-1}, 0.76 mg l^{-1} and 1.44 mg l^{-1} respectively. Wokoma and Edori (2017) studied the heavy metal content of the oily wastewater effluent from an oil firm at the point of discharge. They reported Zn in the range of 0.206–0.330 mg/L, Fe (0.231–0.275 mg/L) and Pb (0.018–0.135 mg/L). Their results showed that the samples were contaminated in the order of Zn > Fe > Pb > Cd. However, in the present study, we found the concentration of heavy metals in the following order: Ni > Cr > Zn > Cd > Cu. Pb was not detected in any of the samples tested. The concentrations of Cr and Cd in refinery wastewater were found to be higher than the permissible limits as assigned by WHO and US Environmental Protection Agency. Our results are corroborated with those of previous findings, where excessive metal concentrations were detected in wastewaters contaminated with industrial discharges (Oyetibo et al., 2017; Rasheed and Saleh, 2016). Wastewater irrigation to the agricultural land/food crops is a common practice in India, which results in the accumulation of the metals and xenobiotics in crops and causes toxicity to humans and animals (Ahmad et al., 2011).

3.2. GC-MS analysis of wastewater

GC-MS is a powerful analytical tool for identification of organic pollutants in the environmental samples (Haleyur et al., 2016). The mass spectra of the major peaks in the gas chromatograms of refinery wastewater at particular retention time were compared with the NIST library (National Institute of Standards and Technology) showed the presence of a number of aliphatic and aromatic organic compounds viz. long chain alkanes (hexadecane, nonadecane, undecane etc), acids (Acetyl benzoic acid, Cis-10-Nonadecenoic Acid, Cis-13-Eicosenoic acid, Methoxyacetic

Table 1
Compounds identified in oil refinery wastewater using Gas chromatography and mass spectrophotometry.

Extraction Solvent	Identified Compounds
n-hexane	Acetyl benzoic acid Cis-10-Nonadecenoic Acid Cis-13-Eicosenoic acid E-8-Methyl-9-tetradecen-1-ol acetate Dibutyl phthalate Phthalic acid, 2,4-dichlorophenyl ester 1,2 Benzenedicarboxylic acid Phenol, 2,6-bis dimethylethyl Cyclononasiloxane Methoxyacetic acid Tert-hexadecanethiol
Dichloromethane (Acidic fraction)	(Z)-13-Docosenamide, 1,3 cyclohexane diol Benzene methanol Biphenyl esters of 4-fluoro-2,methylbenzoic acid Butyl benzoate Phenol 2,4 bisdimethylethyl
Dichloromethane (Basic fraction)	Benzene enoylamine Bicyclohexane 1-Bromoeicosane 1,54-dibromo-Tetrapentacontane, N-(4-bromo-n-butyl)2-Piperidinone, 1-chloro-Octadecane, Hexadecane 1-Iodo-2-methylnonane 7-Methyl-Z-tetradecen-1-ol acetate Nonadecane Phthalate Tert-Hexadecanethiol Undecane

acid etc), esters and phthalate etc (Table 1). Saien and Shahrezaei (2012) reported aliphatic and aromatic petroleum hydrocarbons at different concentrations viz. methyl-tetrabutyl ether, phenol, 2,3,5,6-tetramethylphenol, naphthalene, xylene, tetradecane, 4-chloro-3-methylphenol and 3-tert-butylphenol in the pre-treated refinery wastewater from a biological treatment unit in Kermanshah refinery plant (Iran) by GC-MS. Boczkaj et al. (2016) reported 2,3-Dihydropyran, 2-Butanol, Ethyl acrylate, 2-Pentanone, 2-Hexanone,1-Hexanol,Cyclohexanol, Cyclohexanone, 3-Methylcyclohexanone, Furfural in raw refinery wastewater, while *o*-Cresol, Phenol and *m*-Cresol were also found in treated refinery effluents.

3.3. Microbiological characteristics of oil refinery wastewater

The impact of wastewater on microbial community of receiving water bodies can provide valuable information on the ecosystem health. The microbial diversity of wastewater is presented in supplementary table 3. Various group of microorganisms and their interaction with PAHs and heavy metals showed the viable count of aerobic heterotrophs, asymbiotic nitrogen fixers, actinomycetes and fungi were 2.38×10^6, 1.89×10^4, 2.20×10^4 and 8.76×10^3 CFU/mL respectively. The microbial counts of heterotrophic bacteria, fungi, actinomycetes and N_2-fixers were also determined on phenanthrene-amended agar plates in the concentration from 25 to 100 μg mL^{-1}. It was found that microbial populations were declined gradually with increasing concentration of phenanthrene as compared to control. The total aerobic heterotrophs decreased from 2.38×10^6 (without phenanthrene) to 1.03×10^6 CFU/mL at 100 μg mL^{-1} of the phenanthrene, whereas CFU/mL of fungi and asymbiotic nitrogen fixers were $0.29 \times 10^4$and 3.23×10^3 respectively on Rose Bengal and Jensen's agar plates containing 100 μg mL^{-1} of the phenanthrene at 28 °C after 3–5 days of incubation. Actinomycetes were completely inhibited at the dose of 50 and 100 μg mL^{-1} of the phenanthrene containing Kenknight's agar medium (Suppl. Table 2). This might be due to the detrimental effect of the toxic contaminants present in the wastewater. Obiukwu and Otokunefor (2014) found decrease in microbial population density and disappearance of organisms during the study of microbial community of refinery effluent and sediments of Okrika sector of the Bonny estuary (Nigeria).

Table 2
Minimum inhibitory concentration (MIC) of bacterial isolates from wastewater against heavy metals.

S. No.	Isolates	Heavy Metals					
		Ni^{+2}	Cu^{+2}	Cr^{+6}	Cd^{+2}	Pb^{+2}	Cr^{+3}
1	KWB-1	400	800	200	200	1200	1800
2	KWB-2	200	400	200	12.5	800	1000
3	KWB-3	600	1000	600	150	1200	1200
4	KWB-4	200	400	200	25	1000	1000
5	KWB-5	600	800	200	100	1200	1200
6	KWB-6	400	600	400	25	800	1000
7	KWB-7	800	600	1000	25	1400	1000
8	KWB-8	200	800	600	150	1200	1200
9	KWB-9	200	600	400	12.5	1000	1000
10	KWB-10	100	400	200	50	800	1000
11	KWB-11	100	400	400	12.5	800	1000
12	KWB-12	800	800	400	100	1800	1200
13	KWB-13	400	800	400	50	1000	1400
14	KWB-14	200	600	200	25	1000	1200
15	KWB-15	1000	1000	400	200	1800	1400
16	KWB-16	100	600	600	25	1200	1000
17	KWB-17	100	400	200	12.5	1000	1000
18	KWB-18	1000	600	600	200	2000	1400
19	KWB-19	200	800	600	300	1200	1800
20	KWB-20	800	600	600	12.5	1600	1200
21	KWB-21	400	800	600	300	1200	1400
22	KWB-22	800	800	400	100	1600	1600
23	KWB-23	100	800	200	150	1200	1200
24	KWB-24	200	800	800	300	1200	1400
25	KWB-25	200	800	600	100	1200	1200

3.4. Determination of minimum inhibitory concentration (MIC) of heavy metals

Enrichment technique is a method of choice for isolating bacteria

Table 3
Bacterial growth Inhibition (Zone radius in mm) in the presence of PAHs mixtures of naphthalene, anthracene, phenanthrene and pyrene (having conc. of 1:1:1:1).

S.no	Isolates	PAHs concentration (μg/disc)						
		Control	5	10	20	30	40	50
1.	KWB-1	0	0.0	0.0	1.0	1.5	2.0	3.0
2.	KWB-2	0	0.0	1.0	1.5	1.5	1.5	2.5
3.	KWB-3	0	0.0	0.0	0.0	0.0	0.0	0.0
4.	KWB-4	0	1.0	1.0	1.0	1.0	1.0	1.0
5.	KWB-5	0	0.5	0.5	1.0	1.0	1.5	1.5
6.	KWB-6	0	0.5	1.0	1.0	1.5	1.5	2.0
7.	KWB-7	0	0.0	0.0	0.0	0.0	0.0	0.0
8.	KWB-8	0	0.0	0.5	0.5	1.0	1.0	1.5
9.	KWB-9	0	0.0	0.0	0.0	0.5	0.5	1.0
10.	KWB10	0	0.0	0.0	0.0	0.5	1.0	1.0
11.	KWB-11	0	1.0	1.5	1.5	2.0	2.0	2.0
12.	KWB-12	0	0.0	0.0	0.0	0.0	0.0	0.0
13.	KWB-13	0	0.5	0.5	0.5	1.0	1.0	1.5
14.	KWB-14	0	0.0	0.0	0.5	0.5	1.0	1.5
15.	KWB-15	0	0.0	0.0	0.0	0.0	0.0	0.0
16.	KWB-16	0	0.0	1.0	1.0	1.0	1.5	1.5
17.	KWB-17	0	1.0	1.5	2.0	2.0	2.0	2.5
18.	KWB-18	0	0.0	0.0	0.0	0.0	0.0	0.0
19.	KWB-19	0	0.0	0.0	0.0	0.5	1.0	1.5
20.	KWB-20	0	0.5	1.0	2.0	2.0	2.5	3.0
21.	KWB-21	0	0.0	0.0	1.0	1.0	1.5	2.0
22.	KWB-22	0	0.0	0.0	0.0	0.0	0.0	1.0
23.	KWB-23	0	0.0	1.0	1.0	2.0	2.0	2.5
24.	KWB-24	0	1.0	1.0	1.5	1.5	2.0	2.0
25.	KWB-25	0	0.5	0.5	1.0	1.0	1.5	2.0

0.0 indicates no growth inhibition.

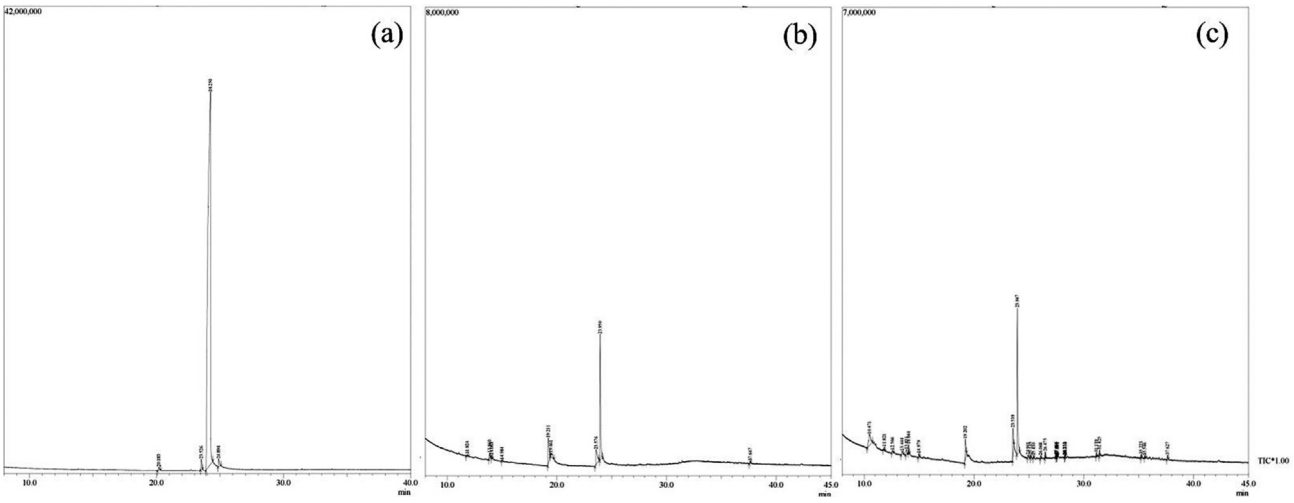

Fig. 1. Gas Chromatogram of (a) Phenanthrene; (b) Degradation of phenanthrene by *Enterobacter ludwigii* KWB3; (c) Degradation of phenanthrene by *Enterobacter ludwigii* KWB3 with glucose (1%).

capable of degrading complex compounds like PAHs. A total of 25 bacteria from oil refinery wastewater were isolated on minimal agar plates and tested for their tolerance to PAHs viz. naphthalene, anthracene, phenanthrene and pyrene and resistance to metal ions (Ni^{+2}, Cu^{+2}, Cr^{+3}, Cd^{+2}, Pb^{+2} and Cr^{+6}). Present study indicated that 36% of the wastewater isolates were resistant to Cd, 80% to Ni whereas 100% isolates were resistant to Cu^{+2}, Cr^{+3}, Cr^{+6} and Pb^{+2}. Maximum MIC of 2000 μg mL^{-1} for Pb and $Cr^{+3}$was displayed by these bacterial isolates (Table 2). Majority of the isolates showed resistance to multiple metal ions. 48% of the isolates exhibited resistance to five metals at a time while 52% were resistant to six heavy metals. All the multi-metal resistant isolates were also able to tolerate PAHs at the concentration of 1000 μg/mL.

3.5. Tolerance of bacteria to polycyclic aromatic hydrocarbons

The radius of growth inhibition zone (mm) of bacteria in the presence of different concentrations of a mixture of Nap, Ant, Phe and Pyr are shown in Table 3. Bacterial isolates KWB3, KWB7, KWB12, KWB15, KWB18, KWB22 showed the highest tolerance (no inhibition) when exposed to the concentrations up to 50 μg/disc of the PAHs mixture. Out of 25 isolates, 9 were sensitive to higher concentrations of PAHs, exhibited inhibition even at 5μg. However, solvent control (acetone:-water 1:1) as PAHs carrier did not cause any adverse effect on the growth of bacterial isolates (Table 3). Zafra et al. (2014) evaluated the tolerance of PAHs to various group of microorganisms at extreme concentrations and selected highly tolerant microbial consortium (fungal and bacterial isolates) for the removal of PAHs and found that 87.76 % Phenanthrene, 48.18 % Pyrene, and 56.55 % Benzo(a)pyrene was removed after 14 days of incubation.

3.6. Analysis of phenanthrene degradation

Out of 25 isolates, 6 exhibiting significantly highest MIC towards metal ions and PAHs were selected for their ability to degrade PAHs in minimal medium supplemented with 100 μg mL^{-1} of Phenanthrene and showed different degree of degradation ranging from 34.89-66.07% after 96 h of incubation (Fig. 1). Out of 6, one of the bacterial isolate KWB3 showed 66.07 % removal after 96 h of incubation at 37 °C in the presence of 100μg phenanthrene/L under shaking condition (200 rpm) and at neutral pH. Utilization of phenanthrene by KWB3 was confirmed by its removal from phenanthrene amended minimal media (O.D. at 251 nm) with a corresponding increase in bacterial biomass. The half-life ($t_{1/2}$) of the phenanthrene degradation was found to be 61.58 h while mean lifetime (τ) and degradation rate/degradation constant (λ) was found to

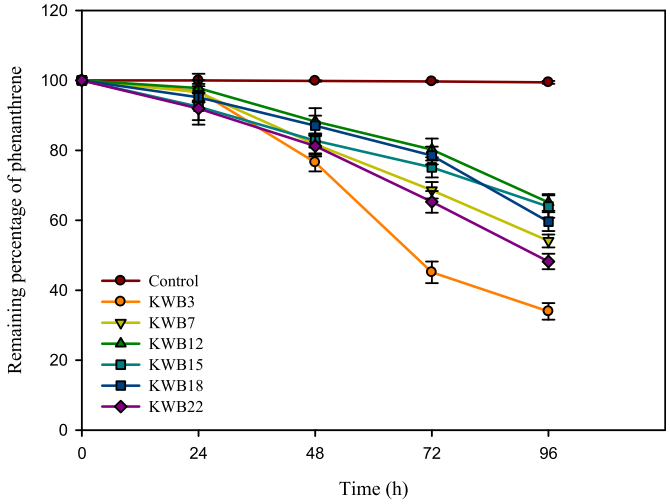

Fig. 2. Degradation and residual percentage of phenanthrene by selected wastewater bacterial isolates (KWB3, KWB7, KWB12, KWB15, KWB18, KWB22).

be 88.84 h and 0.011 per hour respectively. Besides Phenanthrene, KWB3 also utilized Naphthalene, Anthracene and Pyrene individually as sole carbon and energy source.

Gas chromatogram of the control sample (minimal broth supplemented with 100 μg mL^{-1} of phenanthrene and without bacteria) showed a peak with retention time of 24.250 (m/z 178) reflects no removal of phenanthrene (Fig. 2a). GC-MS analysis shows that approximately 44.53% phenanthrene was removed by KWB3 isolate in the absence of glucose; however, removal of phenanthrene was enhanced upto 50.78% in the presence of glucose. Addition of a primary carbon source (e.g., glucose) provide energy needed for the degradation of target compounds as well as building material for cell synthesis, causing an increase in the growth of microbial population (Yuan et al., 2000). The ethyl acetate extract of minimal medium containing phenanthrene degraded products using KWB3 revealed the presence of 9 different peaks with retention time of 11.824, 13.860, 14.063, 14.984, 19.210, 19.460, 23.577, 23.950 and 37.647 (Fig. 2b) while the extract in which glucose was provided as a co-substrate displayed 25 peaks with retention time of 10.471, 11.821, 12.567, 13.444, 13.851, 14.044, 14.979, 19.202, 23.539, 23.947, 24.901, 25.145, 25.430, 26.067, 26.473, 27.466, 27.533, 27.600, 28.252, 28.310, 31.139, 31.423, 35.223, 35.546 and 37.627 (Fig. 2c). A peak of undegraded phenanthrene was also observed in the samples at retention time of 37.647 and 23.947 min in the absence and presence of glucose (Fig. 2b-c). From the

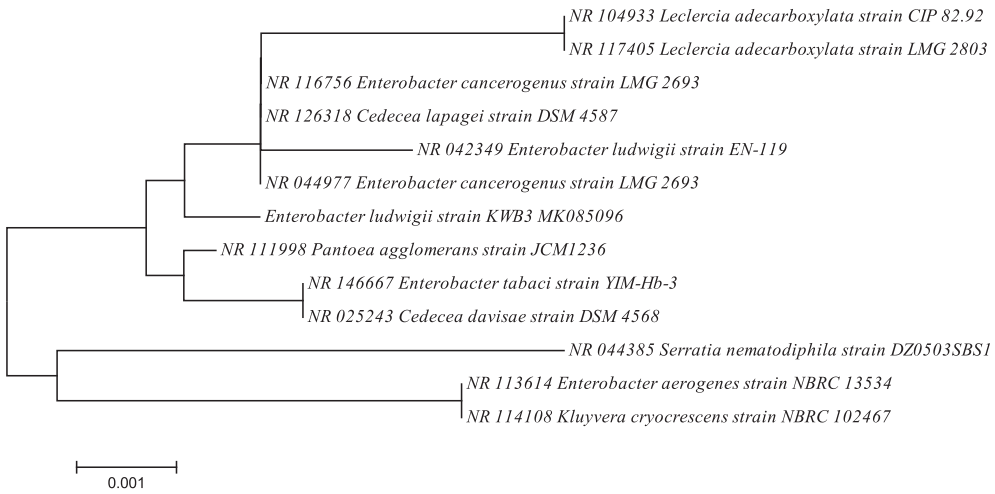

Fig. 3. Phylogenetic analysis of 16S rRNA gene of KWB3 strain most similar to *Enterobacter ludwigii* (Accession NO. MK085096) and other related spp. using Mega 6.0 software neighbor-joining method. The scale bar indicates 0.001 nucleotide substitutions per nucleotide position.

fragmentation pattern and m/z values obtained by mass spectral analysis, several degradation products were tentatively identified as 3-(3,4-dihydroxynaphthalen-1yl)-2, 3-dioxopropanoic acid, benzocoumarin, 4-hydroxyphenylacetic acid, Phthalic acid, 2-formyl benzoic acid, Pyruvic acid, Fumaric acid and Oxalic acid. Present results indicated that the KWB3 isolate has the ability to degrade phenanthrene to phthalic acid through benzocoumarin and converted into catechol as well as several organic acids including pyruvate, fumarate, lactate, acetate and oxalate in the presence of glucose (suppl. table 3; Suppl. Fig.1), which confirms the phenanthrene degradation by *Enterobacter ludwigii* KWB3. Several authors have also reported the formation of benzocoumarin as a ring-fission product of oxidative metabolism of PAHs (Mallick et al., 2007; Seo et al., 2009; Ghosal et al., 2010). PAHs are oxidised to aromatic dihydroxy compounds (catechols) and finally transformed via ortho- or meta-cleavage pathway (Johnsen and Karlson, 2005) to pyruvate that provides energy or can be used to form amino acids (Seo et al., 2009). Many researchers have reported that an alternative soluble carbon source is beneficial for PAHs degradation (Teng et al., 2010; Adam et al., 2015). In the present study phthalic acid was the intermediate metabolite of KWB3 which is further degraded by central energy yielding pathway based on ring cleavage and pyruvic acid is produced; being simple metabolite provides energy to the *Enterobacter ludwigii* KWB3 for subsequent degradation of other metabolites through aerobic respiration. Umar et al. (2017) reported formation of phthalic acid on the degradation of pyrene and phenanthrene by *Cronobacter sakazakii* MM045 which is further catabolised through ring cleavage and dioxygenation into pyruvic acid and ultimately reduced to lactic acid, acetic acid and oxalic acid. Umar et al. (2018) found carboxylic acid metabolites such as pyruvic acid, acetic acid and formic acid from the degradation of pyrene and phenanthrene by *Enterobacter sp*. MM087.

3.7. Phylogenetic analysis of bacterial isolate

16S rRNA gene of the KWB3 isolate was PCR amplified and the amplicon was sequenced. BLAST analysis of the 16S rRNA gene sequence showed maximum similarity to *Enterobacter ludwigii* (Fig. 3). Evolutionary analyses were carried out using MEGA6 software. The accession number of the partial 16S rDNA sequence (MK085096) obtained in this study is submitted and available at NCBI (http://www.ncbi.nlm.nih.gov/BLAST).

3.8. Inc-specific PCR

Total DNA isolated from multi-metal resistant and PAHs tolerant isolates were PCR amplified with *Inc*-specific primers i.e. IncP, IncN, IncW, IncQ and pMV158 rolling circle type. Test samples (DNA from bacteria) gave PCR products with *oriT* and *trfA2* primers of the IncP group (Suppl. Table 4). Bahl et al. (2009) studied the presence and diversity of IncP plasmids in the wastewater treatment plant by PCR amplification of *trfA2* and confirmed that the wastewater constitutes a reservoir of conjugative IncP plasmids. Therefore, these isolates carrying conjugative IncP plasmids have gene mobilizing capabilities, which can result in the spread of multi-metal resistance and PAHs tolerant genes to the native bacterial population in soil by wastewater irrigation. The abundance of IncP plasmids may contribute to the accumulation and spread of resistance genes in the environment. The resistance of bacterial isolates may be attributed to these plasmids carrying the genes coding for enzymes that enable bacteria to resist heavy metals or to degrade xenobiotics.

Our findings suggest that wastewater in the vicinity of Mathura oil refinery is polluted with several heavy metals, organic compounds including PAHs as determined by AAS and GC-MS. The indigenous microbes present in wastewater have been found to be resistant to multiple metal ions and PAHs. The *Enterobacter ludwigii* KWB3 isolated from the oil refinery wastewater was found to remove phenanthrene efficiently under laboratory condition, which showed remarkable tolerance to high concentrations of PAHs mixture (up to 5000 mg L^{-1}). GC-MS analysis of the crude extracts of the metabolites of the isolated strain confirms the phenanthrene degrading capability and the bacterial strain may be useful in developing a technology for decontamination of PAHs-polluted environment. The presence of IncP plasmids in these bacterial isolates also suggests that metals and PAHs contamination applies selective pressure for proliferation of these plasmids, as IncP are involved in catabolic pathways of pollutants.

Declarations

Author contribution statement

Khalida Khatoon: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Abdul Malik: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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