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

ARTICLE INFO

Keywords:
Environmental science
Polycyclic aromatic hydrocarbons
Heavy metals
Bacteria
Degradation
Oil refinery wastewater
Conjugative plasmids

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 $2.38 \times 10^6, 1.89 \times 10^6, 2.20 \times 10^6$ CFU/mL and $8.76 \times 10^5$ 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 rRNA 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 ortT 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...
2.4. Extraction of wastewater and GC-MS analysis

About effects of treated/partially treated oil refinery 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 crude oils 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 HNO3 (10 mL 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 up to 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) 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 braker-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).
2.7. 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, and 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 (up to 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:

\[
N_t = N_0 \times \left(\frac{1}{2}\right)^{t_{1/2}}
\]

Where:
- \(N_0\) = initial concentration
- \(N_t\) = remaining concentration
- \(T\) = Time of incubation (96 h)

\[
t_{1/2} = \frac{T}{\ln(2)}
\]

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

\[
\text{Mean life time (τ)} = \frac{\text{half life}}{\ln(2)}
\]

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:

\[
\text{Degradation rate} = \frac{1}{\text{Mean life time}}
\]

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 (25μM each), 3.75 mmol/L MgCl₂ (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 mg l⁻¹, 0.02–0.052 mg l⁻¹, 1.488–1.624 mg l⁻¹, 0.012–0.156 mg l⁻¹ and 0.504–0.66 mg l⁻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 excess 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 acid, etc).
acid etc), esters and phthalate etc (Table 1). Saien and Shahrezaei (2012) reported aliphatic and aromatic petroleum hydrocarbons at different concentrations viz. methyl-tetradecyl ether, phenol, 2,3,5,6-tetramethylphenol, naphthalene, xylene, tetradecane, 4-chloro-3-methylphenol and 3-tert-butylphenol in the pre-treated 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, as compared to control. The total aerobic heterotrophs decreased from 2.38 × 10^9 to 1.03 × 10^8 CFU/mL at 100 µg/mL of the phenanthrene, whereas CPU/mL of fungi and asymbiotic nitrogen fixers were 0.29 × 10^2 and 3.22 × 10^2 respectively on Rose Bengal and Jensen's agar plates containing 100 µg/mL 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 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).

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

Enrichment technique is a method of choice for isolating bacteria

Table 3: Minimum inhibitory concentration (MIC) of bacterial isolates from wastewater against heavy metals.

| S. No. | Isolates | Heavy Metals |
|--------|----------|--------------|
|        |          | Ni<sup>2+</sup> | Cu<sup>2+</sup> | Cr<sup>6+</sup> | Cd<sup>2+</sup> | Pb<sup>2+</sup> | Cr<sup>3+</sup> |
| 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    | 600           | 400           | 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         | 1800          |
| 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          |

0.0 indicates no growth inhibition.
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 \(\mu\)g/mL for Pb and Cr\(^{6+}\) 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 \(\mu\)g/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 \(\mu\)g/disc of the PAHs mixture. Out of 25 isolates, 9 were sensitive to higher concentrations of PAHs, exhibited inhibition even at 5\(\mu\)g. 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 \(\mu\)g 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 100\(\mu\)g 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 (\(\tau\)) and degradation rate/degradation constant (\(\lambda\)) was found to 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 \(\mu\)g 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...
fragmentation pattern and m/z values obtained by mass spectral analysis, several degradation products were tentatively identified as 3-(3,4-dihydroxy naphtalen-1-y)-2, 3-dioxopropanoic acid, benzoocumarin, 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 benzoocumarin 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 ludwigi* KWB3. Several authors have also reported the formation of benzoocumarin 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 ludwigi* KWB3 for subsequent degradation of other metabolites through aerobic respiration. Uma 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. Uma 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 ludwigi* (Fig. 3). Evolutionary analyses were carried out using MEGA6 software. The accession number of the partial 16S rRNA 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 ludwigi* 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.

**Funding statement**

Khalida Khatoon was supported by a Non-NET Fellowship from the University Grant Commission, New Delhi. Abdul Malik was supported by Deutsche Forschungsgemeinschaft (DFG, Germany) to work at Beuth University of Applied Sciences, Berlin, Germany under exchange
programme.

Competing interest statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.hellyon.2019.e02742.

Acknowledgements

Jawaharlal Nehru University, New Delhi, and Sophisticated Analytical Instrument Facility, Indian Institute of Technology, Mumbai, India are acknowledged for GC-MS analysis. Authors are thankful to Prof. Jawed Iqbal, Department of Chemistry, Aligarh Muslim University for mass spectral analysis.

References

Adam, I.K.U., Miltnner, A., Kästner, M., 2015. Degradation of 13C-labelled pyrene in soil compost mixtures and fertilized soil. Appl. Microbiol. Biotechnol. 99, 9813–9824.
Ahmad, K., Ejaz, A., Azam, M., Khan, Z.I., Ashraf, M., Al-Qurainy, F., Faridoua, S., Badal, S., Bayat, A.B., Valeem, E.E., 2011. Lead, cadmium and chromium contents of canola irrigated with sewage water. Pak. J. Bot. 43 (2), 1403–1410.
Ahmad, M., Yang, Q., Zhang, Y., Ling, J., Sajjad, W., Qi, S., et al., 2019. The distinct response of phenanthrene enriched bacterial consortia to different PAHs and their degradation potential: a mangrove sediment microcosm study. J. Hazard Mater. 380, 120863.
Alexandriou, M., 2006. Studies on the Mechanisms of Bacteria Elimination in Constructed Wetlands. PhD Thesis. Technical University, Berlin, Germany.
Anariz, M.L., Grohmann, E., Malik, A., 2008. Conjugative plasmids in multi-resistant bacterial isolates from Indian soil. Appl. Environ. Microbiol. 104, 1774–1781.
APHA. 2005. Standard Methods for the Examination of Water and Wastewater. American Public Health Association, American Water Works Association, Water Pollution Control Federation, and Water Environment Federation, twenty-first ed. APHA, Washington DC.
Bahl, M.I., Burmole, M., Meins, A., Hansen, L.H., Sorensen, S.J., 2009. All IncP-1 plasmid subgroups, including the novel epsilon subgroup, are prevalent in the influent of a Danish wastewater treatment plant. Plasmid 62 (2), 134–139.
Babri, M., Mahdavi, A., Mirzaei, A., Mansouri, A., Haghighat, F., 2018. Integrated oxidation process and biological treatment for highly concentrated petrochemical effluents: a review. Chem. Eng. Process. Process Intensification 125, 183–196.
Boczkal, G., Malos, P., Przyjazny, A., 2016. Application of dynamic headspace and gas chromatography coupled to mass spectrometry (DHS-GC-MS) for the determination of oxygenated volatile organic compounds in refinery effluents. Anal. Methods 8 (17), 3570–3577.
Brozat, M., Nacke, H., Blasi, R., Hübner, J., Daniel, R., Grohmann, E., 2014. Wastewater irrigation increases abundance of potentially harmful Gammaproteobacteria in soils from Mezquital Valley, Mexico. Appl. Environ. Microbiol. 80 (17), 5283-5291.
Darmawan, R., Nakata, H., Ohta, H., Niidome, T., Takikawa, K., Morimura, S., 2015. Isolation and evaluation of PAH degrading bacteria. J. Bioren. Biodegr. 6 (3), 1.
Dealtry, S., Nour, E.H., Holmgard, P., Ding, G.C., Schweitl, V., Dunon, V., Heuer, H., Hansen, L.H., Sorensen, S.J., Springael, D., Smalla, K., 2016. Exploring the complex response to linuron of bacterial communities from biopurification systems by means of cultivation-independent methods. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol. 92 (2).
Dhaker, A.K., Jain, P.K., 2011. Sewage pollutants and their bioremediation by using Bacillus subtilis metal resistant strain PN/Y via meta-cleavage of 2-hydroxy-1-naphthoic acid by Ochrobactrum sp. strain PWT-D. FEBS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 313 (2), 103–110.
Gotz, A., Pakulak, R., Smit, E., Tietze, E., Prager, R., Tschepe, H., van Elsas, J.D., Smalla, K., 1996. Detection and characterization of broad-host-range plasmids in environmental samples. Appl. Environ. Microbiol. 62, 2621–2626.
Gupta, P.K., 2004. Methods in Environmental Analysis: Water, Soil and Air. Upadhye Purnib, Agrobios, India.
Haleym, N., Shahsavari, E., Mansur, A.A., Koshlaf, E., Morrison, P.D., Osborn, A.M., 2016. Comparison of rapid solvent extraction systems for the GC-MS/MS characterization of polycyclic aromatic hydrocarbons in aged contaminated soil. Methods 3, 364–370.
Hara, R.V., Marin-Morales, M.A., 2017. In vitro and in vivo investigation of the genotoxic potential of waters from river under the influence of a petroleum refinery (São Paulo State—Brazil). Chemosphere 174, 521–530.