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Synergistic degradation of phenanthrene by constructed *Pseudomonas* spp. consortium compared with pure strains

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are highly persistent compounds as well as they have a toxic impact to all living organisms. Removal of these xenobiotic compounds by mixed bacteria is achieving a maximum degradation rate over the pure strains. So, this study substantiates the application of constructed bacterial consortium for degradation of 400 mg/L phenanthrene within 30 days of incubation at 30 °C. The consortium consisted of two bacterial species *Pseudomonas pseudoalcaligenes* (B1) and *Pseudomonas aeruginosa* (B2). It exhibited a significantly higher phenanthrene degradation efficiency (97.5%) compared to pure strains B1 and B2 (52.5 and 47.6%), respectively. The phenanthrene degradation rate by the individual strains was enhanced in the presence of salicylic and phthalic acids as intermediates pathway. On the other side, the degradation rate was reduced in the presence of catechol suggesting a feedback inhibition of catabolic enzymes. The ring-cleavage dioxygenases were induced in all treatments. However, the induction of ortho cleavage dioxygenase (2.44 U/mg protein) was higher than meta cleavage dioxygenase (0.42 U/mg protein). Six main metabolites were detected by GC–MS analysis. Among them, four metabolites were detected in the extract of phenanthrene growing consortium suggesting three main pathways, phthalate, salicylic and benzocoumarin by the constructed consortium. Therefore, this study provides a measuring of phenanthrene degradation efficacy by bacterial consortium and paved the way to increase the potential of using the consortium for successful removal of PAHs from the polluted environment.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are toxic, mutagenic, and carcinogenic environmental pollutants (Bacosa and Inoue, 2015). PAHs pollution release in the environment naturally or due to anthropogenic activity. Natural sources of PAHs are forest fires and volcanic eruptions (Cerniglia, 1993). However, the anthropogenic sources include petroleum products processing, combustion of fossil fuels, industrial waste incineration and coal gasification and liquefaction (Heitkamp and Cerniglia, 1988). Phenanthrene (three fused aromatic ring) is a model compound for low molecular weight

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(LMW-PAHs). It is categorized as priority pollutant by the United States Environmental Protection Agency (USEPA) (Abdel-Shafy and Mansour, 2016). The water solubility of phenanthrene is very low (1.24 mg/L) which strongly reduces its bioavailability (Okere and Semple, 2012).

There are many approaches for removal of PAHs from the environment which may be physically, chemically or biologically (Mawad et al., 2016; Oleszczuk, 2007). Bioremediation is considered one of the most effective approaches to get rid of PAHs from polluted sites because its completely breakdown without formation of toxic byproducts and cost effectiveness as well (Kanaly and Harayama, 2010). Many bacterial species capable of decomposing a variety of PAHs have been isolated from various polluted sites (Haritash and Kaushik, 2009). The majority of the previous literatures have focused on the pure bacterial culture of PAH degraders The biodegradability of a pure strains has been found limited (Lafortune et al., 2009). However, constructing of consortium by mixing and adapting variable PAH-degrading bacteria is paid more attention due to achieving maximum biodegradation efficiency and complete removal of PAHs (Dastgheib et al., 2012). The existence of potentially diverse degradation pathways, effective synergism, and organized metabolic activities are the key advantages of microbial consortia (Mawad et al., 2020a; Mnif et al., 2017).

Three mechanisms, involved in improving the efficiency of PAH degradation by mixed bacterial cultures (consortium), have been reported. (i) Mixed bacterial cultures may be able to overcome the inhibitory effects of recalcitrant PAHs on the growth of certain PAH-degrading bacterial strains, resulting in higher degradation rates (Bouchez et al., 1995), (ii) Interdependence between various bacterial genotypes in mixed cultures may lead to different PAH degradation pathways, resulting in faster degradation than pure strains (Wang et al., 2006) and (iii) Because some strains were capable of breakdown metabolites that produced when the parent PAHs were degraded by other strains, the mixed bacterial cultures could perform a complete PAH degradation pathway (Dastgheib et al., 2012).

Some enzymes which catalyze this process have been characterized (Hesham et al., 2014b; Okere and Semple, 2012; Sivaram et al., 2019). Phenanthrene degradation is initiated by a ring-hydroxylating dioxygenase to yield cis-3,4-dihydroxy-3,4 dihydrophenanthrene, which is subsequently catabolized to 1 hydroxy-2-naphthoic acid (1-H2NA). The later generated is metabolized to either phthalic acid, salicylic acid or 1-naphthol (Al Farraj et al., 2020; Deveryshetty and Phale, 2009). The lower pathway of phenanthrene metabolism is catalyzed by ring-cleaving dioxygenases that catalyze the addition of two atoms of molecular oxygen into substrates. According to the mechanism of ring-cleavage, they are grouped as (i) extradiol dioxygenases (catechol 2,3 dioxygenases), that split the aromatic ring proximal to hydroxylated carbon atoms, producing a semialdehyde (Siegbahn and Haeffner, 2004); and (ii) intradiol dioxygenases (catechol 1,2 dioxygenases), that split the aromatic ring between the two hydroxylated carbon atoms, producing muconic acid (Borowski and Siegbahn, 2006).

This study has been focused on the degradation potential of phenanthrene by bacterial consortium consisted of two *Pseudomonas* species and comparing the growth, biodegradability, and the induction of catabolic enzymes of individual bacterial strains and constructed consortium. Also, the impact of intermediate compounds on phenanthrene degradation efficiency and the expected metabolic products from biodegradation process have been investigated.

2. Materials and methods

2.1. Bacterial strains

Bacterial strains B1 and B2 were originally isolated from oily sludge. They were genetically identified as *Pseudomonas pseudoalcaligenes* (KC342252) and *Pseudomonas aeruginosa* strain ASU-B6 (KU900217) based on 16S rDNA gene sequencing (Hesham et al., 2014a; Mawad et al., 2020a), respectively. The bacterial strains were separately preserved in 20%, v/v sterile glycerol solution at –20 °C for further use.

Each bacterial strain was grown in LB liquid medium at 150 rpm and 30 °C for 36 h. The cells were harvested by centrifugation and then were washed with sterile water for three times. Finally, the absorbance (OD600) of cell suspensions was adjusted to 0.5 by diluting suspension with sterile water.

2.2. Preparation of bacterial consortium and phenanthrene degradation

The mineral basal salt (MBS) medium consists of 0.5 g KH_2PO_4, 1 g NH_4Cl, 0.33 g $MgCl_2 \cdot 6H_2O$, 0.05 g $CaCl_2 \cdot 2H_2O$, 10 g NaCl and 1 mL trace element solution per liter of distilled water (Mnif et al., 2017). The pH was adjusted to 7 with 10 M NaOH solution. The medium was sterilized by autoclaving at 121 °C for 20 min. Phenanthrene was added as a sole carbon and energy source at 400 mg/L from a concentrated stock solution (10 g/L) prepared in ethyl acetate. The bacterial inoculum was added after evaporation of solvent (Mawad et al., 2016).

Phenanthrene degradation experiments were conducted in 500 mL Erlenmeyer flasks containing 100 mL of MBS medium. The experimental treatments were set up as follows: (1) phenanthrene-MBS without inoculum (phen-C) which serves as negative control, (2) phenanthrene-MBS inoculated with ASU-0116 (phen-B1); (3) phenanthrene-MBS inoculated with ASU-06 (phen-B2) and (4) phenanthrene-MBS inoculated with ASU-016 and ASU-06 (1:1; v:v) (phen-consortium). All experiments were performed in triplicate with an inoculum size of (OD_{600}, 0.5). All treatments were incubated at 150 rpm, 30 °C for 30 days.

The bacterial growth was evaluate by measuring the absorbance at 600 nm using UV–Vis Spectrophotometer, according to Barathi and Vasudevan (2001).

2.3. Impact of phenanthrene intermediate byproducts

The effects of pathway intermediates on degradation were investigated by adding 0.1% (w/v) either catechol, salicylic acid or phthalic acid in MBS medium supplemented with 400 mg/L of phenanthrene. The inoculum size and incubation conditions were the same as described above in Section 2.2 (Patel et al., 2019).

2.4. Analysis of synergistic phenanthrene biodegradation

An aliquot of each treatment (20 mL) was withdrawn at 10 days intervals. It was extracted using an equal volume of ethyl acetate to quantify the residual concentration of phenanthrene. The extract was exposed to anhydrous sodium sulfate (Na_2SO_4) to remove water content. The extracts were divided to two equal portions: one for High Performance Liquid Chromatography (HPLC) (LC-20AD, Shimadzu, Japan) and another for GC–MS analysis

For determination of phenanthrene degradation percentage: the solvent was evaporated and then replaced by 80% acetonitrile using Speed Vacuum Concentrator (Thermo Electron Corporation, Massachusetts, United States). The residual phenanthrene was determined using (HPLC) equipped with photodiode array detector (PDA) and Nucleosil 100-5 C18 PAH column (250 mm length, 4.6 mm inner diameter, 5.0 μm particle size). The analysis was performed at 254 nm using 80% acetonitrile as a mobile phase with 1 mL/min isocratic flow rate under ambient temperature (Hesham et al., 2014b).

For detection of phenanthrene tentative metabolites: The extract was then dissolved in hexane and introduced for GC–MS analysis. GC–MS analysis was performed using an HP 6890 gas chromatograph with an HP 5973 mass spectrometer system. The column was a TR-5MS (5% phenyl polysilphenylene siloxane) (30 m × 0.25 mM diameter, 0.25 uM film thickness). Helium was the carrier gas, at 1 mL/min constant flow. The column temperature was held at 70 °C for 5 min, increased at a rate of 4 °C/min to 290 °C, and held for 10 min. To remove any remaining compounds, the analysis was finished with a ramp of 20 °C /min to 320 °C held for 20 min. The mass spectrometer was operated in electron impact (EI) mode at 70 electrons volts (EV) in the full scan mode from 85 to 450 m/z over 6.5–85 min. Injector and detector temperatures were 270 °C and 280 °C, respectively (Hesham et al., 2014b).

2.5. Determination of phenanthrene catabolic enzymes

2.5.1. Preparation of crude enzyme extracts

An aliquot (5 ml) of phenanthrene-growing bacterial cells was harvested by centrifugation (5000 rpm for 10 min). The obtained pellet was washed twice with phosphate buffer (50 mM, pH 7.5). The cells were homogenized in 1 mL ice-cold phosphate buffer using an ultrasonic processor (1-s pulse with 1-s interval, cycle duration 20 s, power output 8 W) at 4 °C in three cycles of ten pulses. The homogenized cells were centrifuged at 10000rpm for 15 min and the supernatant served as cell free extract for determining enzymes activity (Mawad et al., 2016). The total protein was estimated as the method described by Lowry et al. (1951) using bovine serum albumin as a standard protein.

2.5.2. Determination the specific activities of ring-cleaving dioxygenases

Catechol 2,3-dioxygenase was determined as follows: a volume of 100 μL of cell free extract was added to 2.9 mL of potassium phosphate buffer (50 mM, pH 7.2) consisting of catechol (1 mM) which serves as substrate to start the reaction at 55 °C. The reaction product was 2-hydroxymuconic semialdehyde that monitored at 375 nm. An enzyme activity's unit was defined as the amount of enzyme catalyzing the formation of 1-mmol 2-hydroxymuconic semialdehyde per minute at 55 °C (Silva et al., 2012; Zaki, 2006).

For determination of catechol 1,2-dioxygenase activity, the reaction conditions were the same as the catechol 2,3-dioxygenase assay except the formation of cis, cis-muconic acid, which was measured at 260 nm (Silva et al., 2012).

2.6. Cell-surface hydrophobicity

Affinity of cells towards phenanthrene was monitored using the method of Rosenberg and Ron (1999). The bacterial cells were centrifuged, washed twice, and resuspended in phosphate buffer (50 mM, pH 7.5. The assay mixture (6-mL) consisted of 3.0 mL cell suspension ($OD_{600} = 0.3$) and 3.0 mL test phenanthrene (200 mg/L). The mixture was incubated for 5 min, vortexed for 1 min then incubated for additional 15 min at room temperature. The OD of the aqueous phase was measured at 600 nm. The cell-surface hydrophobicity (CSH) was expressed as a percentage of cells transferred to hydrocarbon phase by measuring the OD of the aqueous phase before and after vortexing. It was estimated using the following formula:

$$\%CSH = 100 - [OD_{600} \text{ after mixing}/OD_{600} \text{ before mixing}] * 100 \quad (1)$$

2.7. Statistical analysis

All experiments were performed in triplicate. The mean value of the degradation percentage was considered, and the standard deviation was represented in the error bar to show variation within same experiments. The obtained results were analyzed with one-way ANOVA and significance amongst the treatments were considered with $p \leq 0.05$ using GraphPad prism software. Tukey's Post hoc test was applied to compare the means.

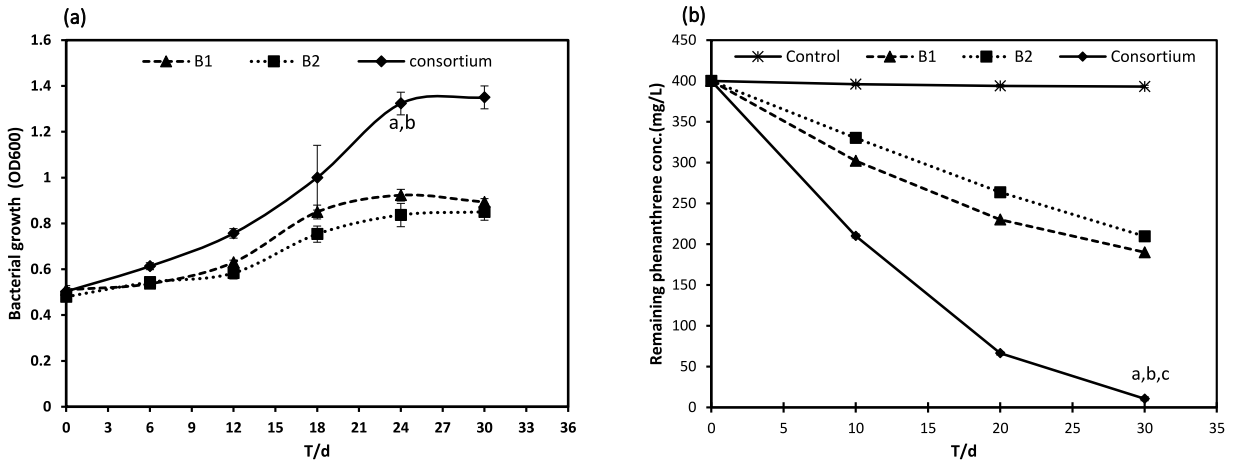

Fig. 1. The growth at OD600 (a) and residual phenanthrene degradation (mg/L) (b) of individual B1, B2 and constructed consortium growing on MBS medium supplemented with 400 mg/L phenanthrene at 30 °C and 120 rpm for 30 d. Error bars represent the standard deviation (SD±) of three replications.[a,b,c] significant variation of consortium ($P \leq 0.05$) compared to B1, B2 and control (during degradation experiment only), respectively.

3. Results

3.1. Synergistic degradation of phenanthrene by constructed bacterial consortium

The bacterial growth has been studied by measuring optical density (OD600) each 6 days interval for 30 days. The growth of individual strains B1, B2 and consortium markedly increased after 12 days of incubation with 400 mg/L of phenanthrene. There was no noticeable increase in the growth of the three treatments after 24 days of incubation. There is no significant difference in the growth of individual stains (B1 and B2) however, the consortium exhibited a significant ($p \leq 0.05$) growth higher than the individual strains Fig. 1a.

The efficiency of constructed bacterial consortium for phenanthrene degradation was revealed by comparing the degradation potential of different treatments. The results in Fig. 1b illustrated that the bacterial consortium significantly ($p \leq 0.05$) enhanced the rate of phenanthrene degradation. The remaining phenanthrene concentration significantly ($p \leq 0.05$) decreased from 190.2 mg/L (B1) and 209.6 mg/L (B2) to 10.5 mg/L (consortium) after 30 days of incubation when the initial concentration of phenanthrene was 400 mg/L. It was noticed that the individual B1 showed superior capability of phenanthrene degradation comparing to B2.

3.2. Impact of metabolic byproducts on phenanthrene degradation rate

The degradation rate of phenanthrene in the presence of intermediate compounds like catechol, phthalate and salicylic acid was investigated in the three treatments Fig. 2. Phenanthrene without any added intermediate compounds served as a control. The presence of salicylic acid had no impact i.e., phenanthrene degradation rate showed non significantly ($p \leq 0.05$) affected in the whole treatments within 10 days compared to control sample. However, the degradation rate increased to 66.2% and 63.8% in the individual strains of B1 and B2 within 30 days, respectively. Interestingly, the presence of phthalic acid significantly ($p \leq 0.05$) enhanced phenanthrene degradation rate from 45.2 to 63.2% within the first 10 days in consortium sample, however, there was no remarkable effect was observed in the individual B1 or B2. While the phenanthrene degradation rate was shifted ($p \leq 0.05$)from 53.2 to 80.3% in the individual B2 within 30 days compared to the control.

In contrast, addition of catechol showed a negative impact on phenanthrene degradation rate. It reduced the degradation rate to 40% and 75.4% ($p \leq 0.05$) within 30 days of incubation with the individual strain B1 and mixed culture (consortium), respectively however, no effect was determined with the individual strain B2.

3.3. Cell surface hydrophobicity (CSH)

The results confirmed that many interactions could be happened during microbial remediation of hydrophobic pollutants to make them more available to their cells. As shown in Fig. 3 the individual bacterial cells of B1 and B2 as well as consortium exhibited affinity towards phenanthrene. The ability of individual cells of B1 to adhere phenanthrene was significantly ($p \leq 0.05$) higher than B2 along the incubation period. However, the consortium showed a significant ($p \leq 0.05$) increased in CSH% (60%) compared to individual cells of B1 (30%) and B2 (40%) within 30 days of incubation.

Moreover, the results of the CSH agreed with the bacterial growth. By comparing the results in Figs. 1 a and 3, it could be depicted that the bacterial cells showed an noticeable affinity towards phenanthrene in the early stationary phase of growth (18 days for individual strains B1 and B2 and 24 days for consortium).

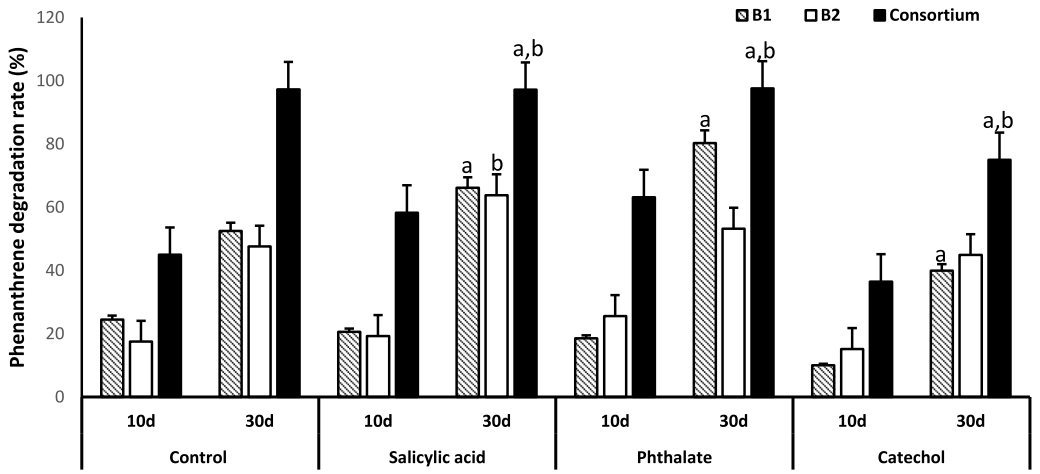

Fig. 2. Effect of pathway intermediates on phenanthrene degradation rate (%). Error bars represent the standard deviation (SD±) of three replications. [a,b] significant difference of individual B1, B2 and consortium ($P \leq 0.05$) compared to control.

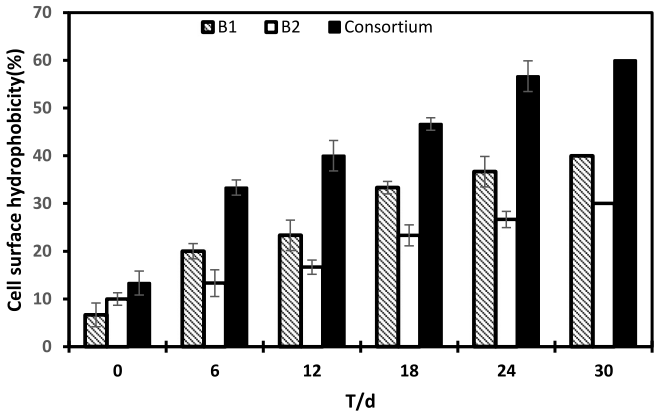

Fig. 3. Percentage of cell surface hydrophobicity of individual B1, B2 and consortium growing in MBS supplemented with phenenthrene as a sole carbon source for 30 d at 30 °C. Error bars represent the standard deviation (SD±) of three replications.

3.4. Phenanthrene degradative enzymes in cell free extract

Bacterial degradation of PAHs like phenanthrene is mostly catalyzed by dioxygenases. Therefore, two dioxygenase enzymes; catechol-1,2-dioxygenase (C12O) and catechol-2,3-dioxygenase (C23O) were monitored within 30 days of incubation (6 days intervals) in a batch culture. The results in Fig. 4a & b showed the specific activities of catechol-1,2-dioxygenase and catechol-2,3-dioxygenase were demonstrated in. C12O could be induced up to the maximum level two times during the growth of the bacterial consortium. The first time within 6 days and the second one within 24 days of incubation. However, the induction of C12O at the last time (2.44 nm min^{-1} mg $protein^{-1}$) was significantly more than the first one (1.6 U/ mg $protein^{-1}$). Moreover, C12O exhibited maximum induction of 0.86 and 0.15 U/ mg $protein^{-1}$ for the individual strains B1 and B2 with 24 and 6 days, respectively as shown in Fig. 4a. On the other hand, C23O exhibited a clear induction in all treatments however, the maximum induction was noticed in bacterial consortium (0.42 U/ mg $protein^{-1}$) (Fig. 4b). The results of C12O and C23O illustrated that, the induction of C12O was significantly ($p \leq 0.05$) higher than C23O.

3.5. Determination of phenanthrene metabolic products by GC–MS

Based on GC–MS analysis, the molecular ion (M+) at m/z, retention time (Rt (min)) and the tentative identification of metabolites were illustrated in Fig. 5 and Table 1. Six main metabolites were detected as byproducts of phenanthrene degradation by the individual strains B1, B2 and bacterial consortium after 30 days of incubation at 30 °C. The results showed that the peak of phenanthrene was detected in the control sample, individual strain B1 and B2 at 44.59 min (1) with a molecular ion (M^+) at m/z 178. The peak of 3-hydroxyphenanthrene was detected in the individual strains B1,

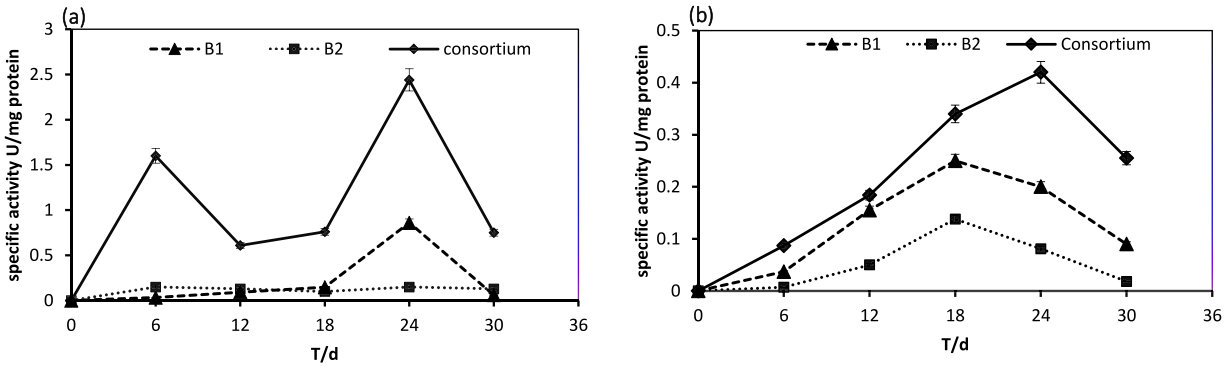

Fig. 4. Specific activities of some Catechol 1,2 dioxygenase (a) and Catechol 2,3 dioxygenase (b) (U/mg protein) of individual B1, B2 and consortium growing in MBS supplemented with phenenthrene as a sole carbon source for 30 d at 30 °C. Error bars represent the standard deviation (SD±) of three replications.

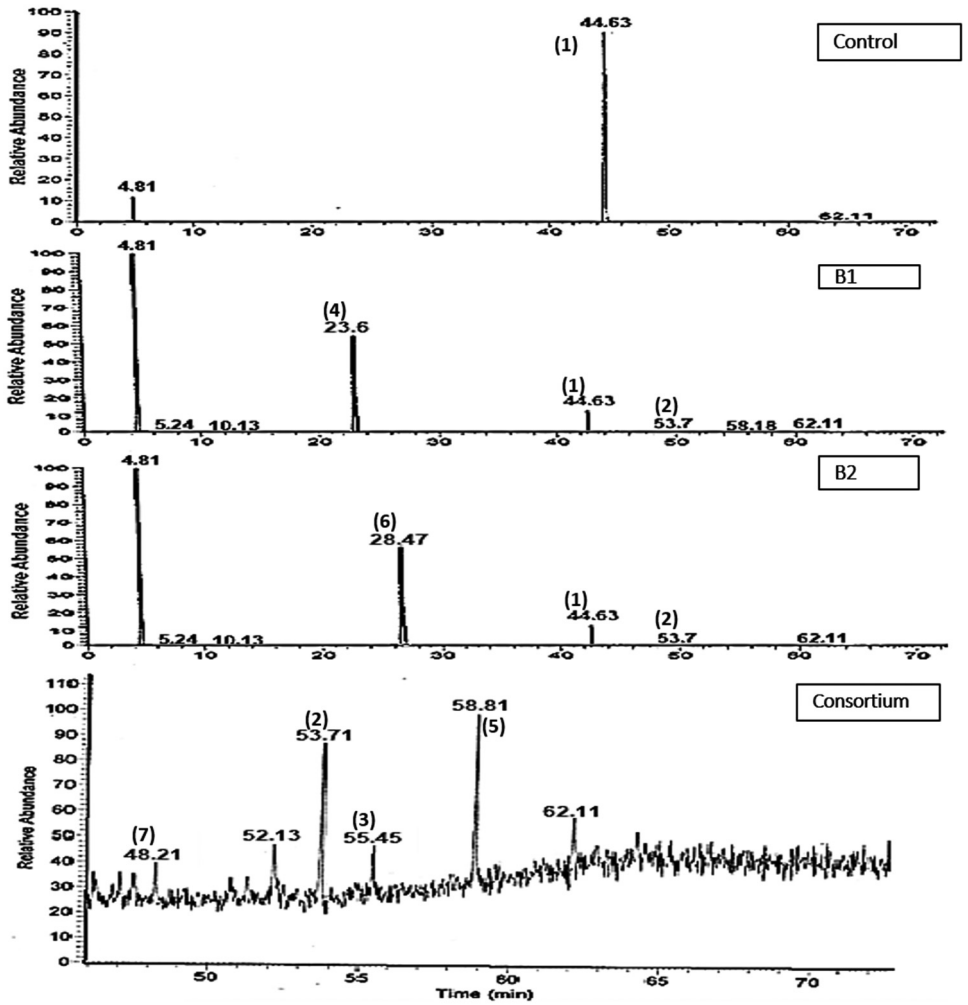

Fig. 5. GC–MS chromatograms of metabolic extracted from control, individual B1, B2 and constructed consortium after 30 d of incubation. (1): phenanthrene; (2): 1-Hydroxy-phenenthrene (3): 3,4 dihydroxyphenenthrene (4): 1-Hydroxy-2-naphtoic acid; (5): phthalic acid (6): salicylic acid (7): Benzocoumarin.

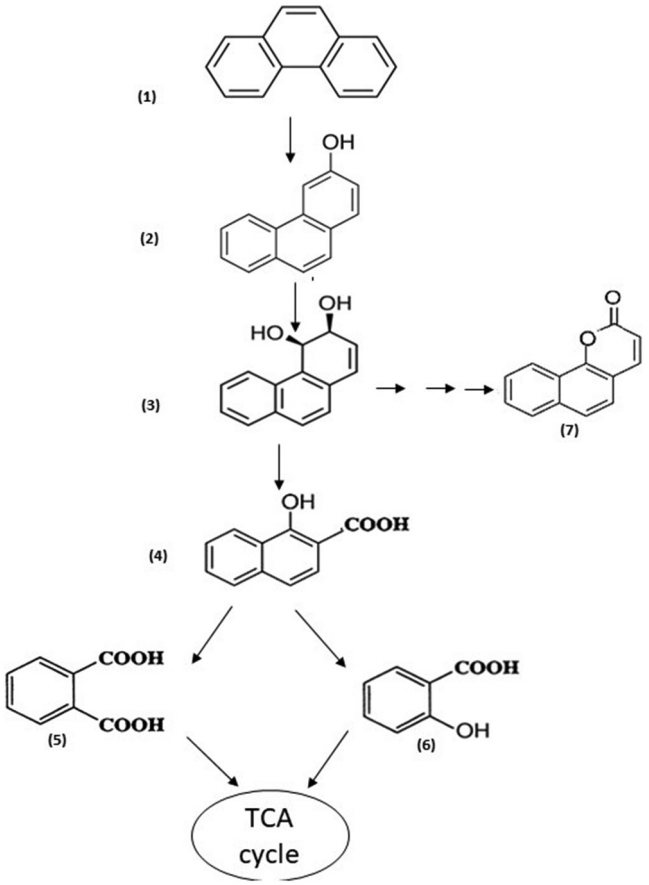

Fig. 6. Proposed Tentative phenanthrene metabolic pathway of consortium based on the detected intermediates from GC–MS analysis. (1): phenanthrene; (2): 1-Hydroxy-phenenthrene (3): 3,4 dihydroxyphenenthrene (4): 1-Hydroxy-2-naphtoic acid; (5): phthalic acid (6): salicylic acid (7): Benzocoumarin.

Table 1
MS analysis of phenanthrene metabolites detected during degradation by the individual B1, B2 and constructed consortium.

No.	Metabolites	RT (min)	m/z (%)
1	Phenanthrene	44.59	178(M^+,100),152(6),76(5)
2	1-hydroxyl phenanthrene	53.17	197(M^+, 5),193(100),178(14),127(12),95(26),69(16),60(23)
3	Dihydroxyl phenanthrene	55.17	210(M^+, 22), 194 (10), 178(100), 161(58),133(30), 55(15)
4	1-hydroxy-2-naphthoic acid	23.6	202(M, 35), 170(100), 142(42),114(32),92(10)
5	Phthalate	58.97	279(M^+,12),219(3),167(42),149(100), 57(14)
6	Salicylate	28.43	168(M,20), 153(12), 137(100), 99(26), 97(84), 57 (40)
7	Benzocoumarin	48.21	197(M^+,10), 193(100),178(14),127(12),95(26),69(16),60(23)

B2 and bacterial consortium at 53.7 (2) with a molecular ion (M^+) at m/z 197. However, it showed very low abundance with the individual strains compared to the abundance with consortium (50%). The peak of 1-hydroxy-2-naphthoic acid appeared in the individual strain B1 at retention time 23.6 (4) with a molecular ion (M^+) at m/z 202. Also, the peak of salicylate was detected only in the individual strain B2 at 28.35 (6) min with a molecular ion (M^+) at m/z 168.

On the other hand, the peak of phthalate was detected at 58.9 (5) with a molecular ion (M^+) at m/z 279 in the individual B1 and in the consortium as well. The peaks of 3,4 dihydroxyphenanthrene and benzocoumarin were detected only in the consortium at 55.17 (3) and 48.2 (7) with molecular ion (M^+) at m/z 210 and 197, respectively.

3.6. Proposed tentative metabolic pathway

The phenanthrene degradative pathway by the three treatments has been proposed based upon the intermediate byproducts that were detected by GC–MS analysis (Fig. 6). It was noticed that, the degradation of phenanthrene initiated by ring hydroxylating that lead to formation of 3-Hydroxyphenanthrene (2) followed by formation of 3,4 dihydroxyphenanthrene. The last metabolic byproduct may go through two different pathways. The first one is the formation

of 1-hydroxy-2-naphthoic acid (1-H2NA) that will differentiated to either salicylic acid or phthalic acid formation. The second one is the formation of benzocoumarin pathway.

4. Discussion

Phenanthrene is listed as a prior hazardous compound according to USEPA (Abdel-Shafy and Mansour, 2016). Therefore, the removal of such toxic compound from the environment is emergent (Jie-Ting et al., 2015). The bacterial strains that have been isolated from contaminated sites exhibited a good capability of utilization of pollution as carbon and energy source (Murínová and Dercová, 2014). Mixed cultures bacteria showed improved the potentiality of degradation comparing to single (pure) strains due to a synergistic mechanisms, and variability of enzymes that catalyze complete degradation (Kumari et al., 2018; Mikesková et al., 2012; Vandermeer and Daugulis, 2007).

This study investigated the degradation of phenanthrene, a three-ring aromatic hydrocarbon by constructed consortium of two *Pseudomonas* species at high concentration 400 mg/L for 30 days and the results were compared to the individual strain as well as the previous studies. The results showed that the degradation percentage by the consortium (97.5%) was about two times higher than the individual strains of either *P.pseudoalcaligenes* (B1) (52.7%) or *P. aeruginosa* (B2) (47.6%) and. The bacterial strain B1 completely removed phenanthrene within 15 days of incubation when initial concentration is 100 mg/L as mentioned in the previous study (Hesham et al., 2014a). *Pseudomonas* sp. strain BZ-3 could remove 17.7% of phenanthrene at initial concentration 200 mg/L while it could remove 75% at an initial concentration of 50 mg/L at 20 g/L NaCl within 7days (Lin et al., 2014). In a recent study, Singh and Haritash (2021) reported that *Kocuria flava* and *Rhodococcus pyridinivorans* could remove 55.13 and 62.03% of phenanthrene at 10 mg/L initial concentration during 15 days of incubation. Therefore, it was suggested that the application of high initial phenanthrene concentration on pure bacterial strain may lead to hinder degradation rate due to the imposed toxic effect. Moreover, more attention was paid to investigate the degradation of phenanthrene by bacterial mixed culture and adapted consortia. *K. flava* and *R. pyridinivorans* have an evolutionary relationship and have the same PAHs-catabolic genes (Sakshi Singh and Haritash, 2020). However, there was no synergistic or antagonistic effect between them in PAHs degradation process. The capacity of microbial consortium (*Sphingobacterium* sp., *Bacillus cereus* and *Achromobacter insolitus*) enriched from a petrochemical refinery field to remove phenanthrene at 100 mg/L was reported by Janbandhu and Fulekar (2011). The results reported by Kim et al. (2009) came in accordance with ours. They reported that, the rate of phenanthrene degradation was very low when *Acinetobacter baumannii* Ab, *Klebsiella oxytoca* Ko and *Stenotrophomonas maltophilia* Sm, were individually applied. However, the degradation rate was greatly improved in the presence of the combined cultures.

There was a correlation between the bacterial growth and the rate of hydrocarbon degradation (Yuan et al., 2018). In this study, the growth and degradation rate of bacterial consortium was significantly ($p \leq 0.05$) higher than the individual strains. this could be attributed to the accumulation of metabolites in the medium of pure strains that may retard their growth and subsequently delay the degradation potential. While in the case of consortium, the accumulated metabolites may serve as a substrate for the growth of another. This assumption has been reported by Dastgheib et al. (2012) and confirmed by our findings.

During the investigation of the impact of intermediate compounds on phenanthrene degradation rate. The individual strains B1and B2 consumed salicylic acid and phthalic acid as an individual energy source to attack phenanthrene rings within 10 days then they used them individually as a carbon source to increase the induction of phenanthrene catabolism as well as their growth enzymes. This explains why the addition of salicylic acid and phthalic acid (separately) in the media improved the phenanthrene degradation rate of consortium after 10 days and individual strain of B1 and B2 after 30 days. In contrast, negatively effects of catechol on the phenanthrene degradation rate of the individual strain B1 and the consortium may be attributed to its potential toxic impact on growth or enzymes of strain B1. So, it delayed the degradation rate of B1 and consortium (Dastgheib et al., 2012; Seo et al., 2009). At the same time, catechol did not affect the degradation rate of the individual B2 possibly due to it accumulated as a byproduct.

On the other hand, the main limiting factor in the PAHs degradation is the low water solubility and the lack of bioavailability to the cells leading to their persistence in the environment. Microorganisms could tackle the PAHs uptake problem by direct contact to these compounds and increasing microbial cell surface hydrophobicity (Prabhu and Phale, 2003). It was noticed that the affinity of individual B1, B2 and the consortium towards phenanthrene was growth dependent. However, the CSH% of the consortium was higher than the individual B1 or B2. This could be interpreted the increasing of active sites on the surface of mixed cultures compared to the single one. Increasing of CSH may enhance the transfer of substrate (phenanthrene) from the medium to the bacterial cell surface particularly in a well-stirred system and subsequently increase the bioavailability of hydrophobic substances to the cells. This interaction is considered a limiting factor in PAHs degradation (Mnif et al., 2017).

The key enzymes catalyze attacking aromatic ring of PAHs by aerobic bacteria are dioxygenases which are substrate specific (Cerniglia, 1993; Haritash and Kaushik, 2009; Sawulski et al., 2014). The upper pathway of phenanthrene produced cis-3,4-dihydroxy-3,4-dihydrophenanthrene Fig. 6 (3) that is subsequently metabolized to 1-hydroxy-2-naphthoic acid (1-H2NA) Fig. 6 (4) (Deveryshetty and Phale, 2009). This was confirmed by the appearance of 1-H2NA peak in the GC–MS chromatogram of the individual B1 while it was absent in the consortium and individual B2 after 30 days which meant that 1-H2NA is catabolized to further compounds.

Ortho- and meta ring cleavage dioxygenases is produced by bacteria to metabolize 1-H2NA and handle lower phenanthrene pathway. Phthalic acid is a yield of ortho cleavage and salicylic acid is a yield of meta-cleavage (Gao et al.,

2013). So, the detection of catechol-1,2-dioxygenase activities at λmax at 260 nm in phenanthrene extract of grown B1, B2 and consortium suggested that the degradation pathway as: 1-H2NA metabolized to phthalic acid Fig. 6 (5) which is converted to muconic acid that will be involved in tricitric acid cycle (TCA) of bacterial metabolism (Mallick et al., 2007).

On the other side, detection of catechol-2,3-dioxygenase activities at λmax at 375 nm in phenanthrene extract of grown B1, B2 and the consortium suggested that the degradation pathway as: 1-H2NA metabolized to salicylate Fig. 6 (6) which is converted to catechol then 2'-hydroxymuconicsemialdehyde that will be involved in tricitric acid cycle (TCA) of bacterial metabolism. However, the specific activity of C23O in B2 (0.25 U/mg protein) was double increased than B1(0.12 U/mg protein). Induction of C12O in the consortium was exceed its induction in individual strains because substrate was more available in the mixed cultures. The *C12O* catabolic gene expression may be enhanced in the presence of consortium over the *C23O* catabolic gene. This suggested that the constructed consortium enhanced the catabolic enzymes activity which enhanced phenanthrene degradation (Gao et al., 2013).

This may explain why the phenanthrene degradation rate increased in the presence of salicylic acid and not decreased in the presence of catechol as intermediate metabolites in the extract of individual B2. It may be attributed to feedback inhibition of enzymes catalyzed in metabolism of phenanthrene (Seo et al., 2009). It could be assumed that individual B2 preferred meta-cleavage pathway despite catechol was not detected in GC–MS analysis. Similar results were reported by Deveryshetty and Phale (2009) who detected the specific activity of both *C12O* and *C23O* in phenanthrene degrading *Pseudomonas* sp. equivalent to 1.4 U/mg protein. Interestingly, increasing the induction of C12O in the individual B1 and the consortium than C23O indicating that ortho cleavage pathway is preferable for degradation of phenanthrene than meta cleavage (Ghosh et al., 2017). These was confirmed by the positive impact of phthalic acid on the phenanthrene degradation rate.

In a previous study; *Pseudomonas fluorescence* AH-40 metabolized the 1-hydroxynaphthoic acid through two separate pathways, the phthalic acid pathway or the naphthalene pathway (Mawad et al., 2020b). However, benzocoumarin was detected as one of the phenanthrene and anthracene metabolites of *Cryptococcus* sp. MR22 (Al Farraj et al., 2020), *Mycobacterium* sp. PYR-1 (Moody et al., 2001), and *Rhodococcus* species (Dean-Ross et al., 2002), and showed that benzocoumarin is a common metabolite for the first reaction of three-ring PAHs transformation in most bacteria. So, additional experiments is being conducted to confirm the detected metabolites and catabolic enzymes.

The data collected from enzymes analysis illustrated that, the constructed consortium contained two bacterial species belonging to the genera *Pseudomonas* are able to assimilate phenanthrene via ortho (phthalic) and meta (salicylic acid) pathway. However, the phthalic acid pathway is preferable for the degradation process. Identification of benzocoumarin Fig. 6 (7) only with constructed consortium strongly suggested that the initial dioxygenase used for phenanthrene degradation by constructed consortium has a broad substrate specificity (Pinyakong et al., 2000). In previous study, it was reported that the pure *Pseudomonas pseudolacaligense* degraded PAHs via ortho and meta cleavage pathway (Hesham et al., 2014a). Patel et al. (2012) discussed the capability of pure *Pseudoxanthomonas* sp. strain DMVP2 to assimilate 300 mg/L of phenanthrene via phthalic acid however, *Pseudomonas* sp. strain PP2 removed it by salicylic acid pathway (Prabhu and Phale, 2003). In addition to, C23O activity was detected in reported that *Kocuria flava* and *Rhodococcus pyridinivorans* during the degradation of three-ring PAHs indicating a vital role of this enzyme in catabolizing these compounds Singh and Haritash (2021).

5. Conclusion

The constructed consortium of *Pseudomonas* spp was assessed as an effective method for removal of PAHs. The degradation rate of phenanthrene was enhanced by constructed consortium compared to the pure single strains. The presence of the consortium increased the active sites and surface area which increase the contact of phenanthrene to the bacterial cells and consequently overcome the problem of low water solubility. Phenanthrene degradation rate was enhanced in the presence of phthalate and salicylic acid while it was retarded in the presence of catechol as intermediate metabolite. The ring cleavage dioxygenases are highly induced in phenanthrene growing consortium. Four main metabolites were detected suggesting the complete removal of phenanthrene by constructed consortium. So, this study provides precious information to set up a cost-effective method for bioremediation of PAH contaminated sites.

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CRediT authorship contribution statement

Asmaa Mawad: Conceptualization, Supervision, Writing – original draft. **Hiba Albasri:** Methodology, Data analysis. **Abdel-Gawad Shalkami:** Writing – review & editing. **Saad Alamri:** Project administration, Funding acquisition. **Mohamed Hashem:** Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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