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EFFICIENCY OF POLYCYCLIC AROMATIC HYDROCARBONS (PAHs) DEGRADING CONSORTIUM IN RESISTING HEAVY METALS DURING PAHs DEGRADATION

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Abstract
Polycyclic aromatic hydrocarbons (PAHs) comprised of many dangerous organic pollutants which affect human cell. The choice of phenanthrene and pyrene as model substrates was based on their classification among the most hazardous PAHs group by the US EPA where they belonged to low and high molecular weights PAHs respectively. Biodegradation of these PAHs is the best strategy that completely removes such pollutants in an environmentally friendly manner. However, the bacteria involved are challenged degradation difficulties as a result of PAHs inhibitory effects to the organisms. This research is aimed at formulating phenanthrene and pyrene degrading consortium that effectively perform best even in complex mixture with hazardous heavy metals. Different bacteria consortia were formulated using the compatibility testing and mathematical permutation approach and the best consortium selected. This selected consortium was then subjected to the degradation of both phenanthrene and pyrene separately in a combined mixture with the selected heavy metals from the inductively coupled plasma optical emission spectrophotometer (ICP-OES) analysis. Consortium composition of *C. sakazakii* MM045 (2%, v/v) and *Enterobacter* sp. MM087 (2%, v/v) were found to be much effective during phenanthrene (500 mg/L) and pyrene (250 mg/L) degradation. This consortium also resisted more than 6 mg/L each of Nickel (Ni), Cadmium (Cd), Vanadium (V) and Lead (Pb) in such complex degradation which was found to be more than the concentration in the natural habitat the consortium exists prior to isolation. Such performance makes the selected consortium to be an extremely efficient tool for the PAHs degradation application as many biodegradation agents were reported to be less effective when significant concentration of Ni, Cd, V and Pb are present.
Key words: PAHs, Biodegradation, Hazardous metals, Resistance

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Introduction

The toxicity nature of phenanthrene and pyrene necessitate the design of more proactive bio-removal strategies (Simarro *et al*., 2011; Wong *et al*., 2005). These PAHs normally exist in a combined mixture with heavy metals being co-contaminants (Tremolada *et al*. 2008; Katsoyiannis and Samara 2004). The complex existence of such PAHs with heavy metals as environmental pollutants has resulted in poor environmental management of petroleum based products (Prasad and Katiyar, 2010). This constituted over 40% of the global most dangerous wastes which negatively impacts on plants, animals and microbial ecosystems (Thavamani *et al*., 2011; Micó *et al*., 2006). The liphophilic nature of PAHs makes them to penetrate into the animal's cells which eventually causes covalent DNA adducts and alter the cell chromosomal arrangements (Joutey *et al*., 2013). Such alteration can cause severe complications such as mutation and cancer to the affected cells (Calvo *et al*., 2009).

The reported toxicity effects caused by hazardous heavy metals involving Cd, Ni, Pb, V, Cr and As were previously reported to inhibit the bacterial PAHs degradation response thereby limiting the organisms environmental applications (Ibarrolaza *et al*., 2009; Pereira *et al*., 2007). These metals inhibit the growth of the degrading bacteria by congesting the permeability nature of the cell membrane and prevent the organisms to recognise PAHs as potential substrates (Maliszewska and Smreczak, 2003). This forces the bacteria to replace basic functional groups of the enzymes involved in PAHs degradation with metal ions which eventually destroys the entire cells (Guzzo and DuBow 1994). Therefore, effective PAHs biodegradation is seriously affected by many hazardous metals (Cao *et al*., 2008).

Hazardous heavy metals toxicity during PAHs biodegradation provides the need to use mixed bacterial combinations called consortium (Ghazali *et al*., 2004; Bao *et al*., 2012; Plaza *et al*., 2008). The consortium when applied generates broad range of enzymes that effectively remove PAHs pollutants such as phenanthrene and pyrene due to synergistic cooperation (Ghazali *et al*., 2004; Sathishkumar *et al*., 2008; Rahman *et al*., 2002). Considering the importance of applying mixed bacterial consortium during PAHs biodegradation in a complex system, the previously identified effective degrading isolates *C. sakazakii* MM045 and *Enterobacter* sp. MM087 were used in this study (Umar *et al.,* 2017; in press; Darma *et al*., 2016). These bacteria were used for the formulation of more proactive PAHs degrading consortium whose main objective was to assess the resistance of the consortium to hazardous heavy metals during PAHs biodegradation.

Materials and Methods

Chemicals and Media

Standard Reference Material (SRM-2587) and Certified Reference Materials (CRMs) used were purchased from US National Institute of Standards and Technology (NIST, 2017) and Sigma-Aldrich, USA. All other chemicals used were of analytical grade and purchased from standard manufactures. Standards for the Inductively Coupled Plasma Optical Emission Spectrophotometry (ICP-OES) were prepared using 2% (v/v) HNO_3. All other reagents were prepared using Purelab de-ionised water (13.2 mΩ cm resistivity). Mineral salts medium (MSM) was also prepared using de-ionised water with the chemical compositions of Na_2HPO_4 (9.0 g/L), KH_2PO_4 (1.5 g/L), $MgSO_4 \cdot 7H_2O$ (0.2 g/L), $ZnSO_4 \cdot 7H_2O$ (0.2 g/L), $CoSO_4 \cdot 7H_2O$ (10.0 μg), $MnSO_4 \cdot H_2O$ (3.0 g/L), Ferric citrate (5.0 g/L), NH_4Cl (2.0 g/L), Titriplex III (0.01

g/L). Furthermore, phosphate buffer saline (PBS) was prepared using NaCl (8 g/L), KCl (0.2 g/L), Na_2HPO_4 (1.44 g/L), KH_2PO_4 (0.24 g/L).

Inoculums preparation

The previously identified *C. sakazakii* MM045 and *Enterobacter* sp. MM087 were used for the preparation of bacteria inoculums whose optimum degradation responses were determined (Umar *et al.,* 2017; in press; Darma *et al*., 2016). Bacteria resting cells of each individual bacterium strain was prepared separately as the pure isolates were initially grown at 35 ^{0}C for 24 hours under 160 rpm (Sogani *et al*., 2012). These cultures were centrifuged at 4000 rpm and the cells were washed and diluted in PBS until 10^6 cells/mL was achieved for each strain based on haemocytometer standard measurements (Doyle and Bryan, 1998).

Formulation of PAHs degradation consortium

Bacteria inoculums mixture involving *C. sakazakii* MM045 and *Enterobacter* sp. MM087 were used to formulate effective PAHs degrading consortium based on the protocols adopted from Sarkar *et al*., (2011). Initially, both isolates were simultaneously cultured on a single LB plate for 24 hours at 35 ^{0}C where tremendous colonies appearance indicates the isolates as compatible to one another (Raja *et al.,* 2006). Experimental replications for the consortia were then computed using combined permutation based on the following mathematical formula mentioned in Rosen, (2007).

$$E = \frac{n!}{(n-r)!r!}$$

Where E is the experimental replications, n is the consortium volume composition (5%, v/v), r is the quantity of different bacteria strains involved (2 strains).

Ten experimental replications were suggested from the permutation formula comprising bacteria mixture of *C. sakazakii* MM045 and *Enterobacter* sp. MM087. Phenanthrene (500 mg/L) and pyrene (250 mg/L) were then separately supplemented in different MSM as DCPIP was used to indicate PAHs degradation (Hilyard *et al*., 2008). All degradation cultures were incubated at 35 ^{0}C, 160 rpm for 24 hours where selection of degradation consortium was done based on the consortium's volume and degradation response.

Heavy metals selection

The selection of heavy metals for the bacteria tolerance was carried out using the soil analysis based on the ICP-OES analysis. These soil samples were the ones from which strain MM045 and MM087 were initially isolated. The samples were initially dried at 60 ^{0}C for 16 hours followed by crushing with mortar and pestle before sieving (63 µm) as powdered soil (Zulkifli *et al*., 2010a). About 0.5 g of the powdered soil was soaked in 10 mL aqua regia solution ($HClO_4$: HNO_3 at 3 : 1) for 16 hours (Zulkifli *et al*., 2010b). The soaked mixture was then digested for 2 hours through heating at 130 ^{0}C thereby filtered with 4.7 cm microfiber filter and filled with HNO_3 (2%, v/v) up to 50 mL final volume. The same digestion process was also carried out on the purchased standard reference materials soil (SRM-2587) for the validation of the ICP analysis.

The ICP-OES analysis was performed using PerkinElmer® Optima™ 7300 (PerkinElmer, Inc. Shelton, USA) which was equipped with the software WinLab32™ (Version 5.3). Multi-element CRM (TraceCERT, Sigma-Aldrich, Switzerland) was used as external standards for

the analysis and 2% (v/v) HNO_3 was the calibration blank. The ICP analysis targeted 10 different heavy metals (Co, Cd, Cr, Cu, As, Ni, Pb, Mn, V, Zn) that were previously reported to be commonly obtained from the used engine oil contaminated sites (Mielke *et al.*, 2000). The digested soils and SRM were separately introduced on the ICP instrument spray chamber using flow rate and concentration of 0.5 L/min were simultaneously displayed in mg/L before being converted to mg/kg by the following formula:

$$X_F = \frac{X_I \times V}{U \times S} \times D$$

Where X_F means metal concentration (mg/kg), X_I is the metal concentration from the ICP analysis (mg/L), V is the dilution volume of the digested soil (50 mL) before ICP analysis, U is the unit quantity of each analyzed soil (1.0), S is the powdered soil before digestion (0.5 g), D is the sample dilution during ICP analysis (1.0). Recovery of each individual metal analyzed was further calculated using the ICP results of the SRM soil (SRM-2587) based on the following formula:

$$Y = \frac{(S - B)}{S_A} \times 100$$

Y is the percentage recovered metal, S is the ICP result of SRM soil sample in mg/kg, B is the analyzed ICP control result (0 mg/kg), S_A is the standard metal concentration of SRM-2587 (mg/kg) based on NIST validated result. Hazardous metals selection was done based on toxicity preference from all the quantified metals (Goyer *et al.*, 2004).

Effects of the selected metals on PAHs biodegradation

The individual selected heavy metals were separately supplemented into the PAHs biodegradation culture using the best chosen consortium as inoculums (Varjani and Upasani, 2013). Initially, MSM medium were separately supplemented with phenanthrene (500 mg/L) and pyrene (250 mg/L) followed by adding varying concentrations of the CRMs (2 mg/L to 12 mg/L). For each PAH, different treatments were then inoculated with 4% (v/v) bacteria consortium and then incubated at 35 ^{0}C under 160 rpm for 24 hours. The PAH degradation was quantified using the residual concentration obtained from the 24 hours based on Spectrophotometry assessments (Hanson *et al*., 1993).

Data analysis

Data presentations were done in tabular and graphical forms while analysis were statistically carried out and presented in mean ± standard deviation for each triplicates treatments.

Results

PAHs degradation consortia formulations

A total of 10 different consortia were formulated where each consortium constituted mixed resting cells of strain MM045 and MM087 (10^6 cells/mL) at varying mixed bacteria volumes (Table 1). The combined degradation strength of each consortium was found to be effective even at 4% (v/v) resting cells containing equal bacteria volume composition which resulted in complete PAHs degradation (100%). This provides better opportunity in testing the degradation capability of such consortium as 5% (v/v) was previously reported as the best

optimum inoculums quantity for each bacterium. Hence, 4%, (v/v) bacteria consortium was selected for this study.

The selected consortium was used to effectively degrade phenanthrene (250 mg/L) and pyrene (250 mg/L) in a complex culture containing heavy metals. These metals were chosen based on the ICP-OES analysis conducted on different soil samples which were found to be heavily contaminated with different concentrations of Zn, Mn, Cu, Pb, Cr, Ni, V, Co, As, Cd whose toxicity may reduce degradation outcomes (Table 2). Based on the metals toxicity, Pb, Ni, V and Cd were selected as the most hazardous metals.

Table 1: Formulation of effective PAHs degrading consortia and their degradation responses

Consortium ratio for MM45 to MM87 (%, v/v)	**% Phenanthrene degraded ± s.d**	**% Pyrene degraded ± s.d**
1 : 4	101.00 ± 1.40	100.70 ± 0.71
2 : 3	100.20 ± 0.72	100.00 ± 9.2
3 : 2	100.50 ± 0.50	100.50 ± 1.38
4 : 1	100.20 ±0.31	100.20 ± 1.17
1 : 3	98.30 ± 0.90	94.30 ± 1.50
***2 : 2**	**100.10 ± 0.81**	**100.00 ± 0.92**
3 : 1	98.90 ± 0.70	94.90 ± 2.12
1 : 2	49.90 ± 0.90	63.90 ± 1.67
2 : 1	47.90 ±0.50	52.70 ± 3.34
1 : 1	0.00	10.40 ± 1.43

* Chosen degradation consortium

Table 2: The ICP-OES analysis of soil samples involving MM045, MM087 & SRM-2587 soils

Metal	MM045 Soil		MM087 Soil		SRM-2587 Soil		Certified NIST	Recovery
	mg/L	mg/kg	mg/L	mg/kg	mg/L	mg/kg	std (mg/kg)	(%)
As	0.05 ± 0.07	5.10 ± 6.56	0.03 ± 0.03	3.10 ± 3.20	0.11 ± 0.16	11.20 ± 15.84	13.70	81.50
***Cd**	**0.04 ± 0.02**	**3.50 ± 1.53**	**0.01 ± 0.01**	**1.20 ±0.62**	**0.02 ± 0.04**	**1.90 ± 3.76**	**1.92**	**97.20**
Co	0.12 ± 0.02	12.30 ± 1.93	0.03 ± 0.01	2.80 ± 0.90	0.13 ± 0.03	12.90 ± 2.90	14.00	92.10
Cr	0.76 ± 0.33	75.80 ± 32.93	0.23 ± 0.10	22.50 ± 10.33	0.74 ± 0.89	74.40 ± 89.14	92.00	80.80
Cu	5.52 ± 3.29	551.50 ± 328.7	0.56 ± 0.64	56.40 ± 64.25	1.54 ± 0.55	153.70 ± 55.17	160.00	96.10
Mn	7.24 ± 3.82	723.70 ± 382.07	3.33 ± 0.08	333.20 ± 8.02	5.55 ± 0.60	554.90 ± 60.13	651.00	85.20
***Ni**	**0.47 ± 0.15**	**46.50 ± 14.93**	**0.16 ± 0.05**	**16.30 ± 4.89**	**0.34 ± 0.06**	**33.70 ± 5.89**	**36.00**	**93.60**
***Pb**	**3.59 ± 1.98**	**358.50 ± 197.99**	**0.59 ± 0.06**	**59.50 ± 5.70**	**32.65 ± 9.77**	**3265.30 ± 976.59**	**3242.00**	**100.70**
***V**	**0.19 ± 0.06**	**19.20 ± 6.12**	**0.16 ± 0.06**	**15.80 ± 6.14**	**0.67 ± 0.27**	**66.60 ± 27.41**	**78.00**	**85.40**
Zn	9.51 ± 0.66	950.50 ± 66.01	3.49 ± 1.78	349.00 ± 178	3.34 ± 1.99	333.70 ± 199.06	335.80	99.40

* Selected heavy metals based on toxicity and concentration

Nickel (Ni) toxic effect during PAHs degradation

The initial toxicity effect of Ni was observed when 8 mg/L concentration of the certified reference material was applied to the PAHs biodegradation culture (Figure 1). Despite such initial effect, 50% phenanthrene and pyrene degradation was achieved at 8.24 mg/L and 9.11 mg/L of Ni concentrations respectively. This shows remarkably excellent consortium tolerance to Ni which was found to be less than 1 mg/L in the original sample where the consortium bacteria were isolated previously. The slope equations for such degradations were presented in Equation 1 and 2.

$$Y_{PHN} = -8.6393X_{Ni} + 121.27 \quad (1)$$
$$Y_{PYR} = -7.741X_{Ni} + 120.52 \quad (2)$$

Where Y_{PHN} and Y_{PYR} were the phenanthrene and pyrene degraded (%), X_{Ni} was the Ni concentration (mg/L) tolerated by the consortium.

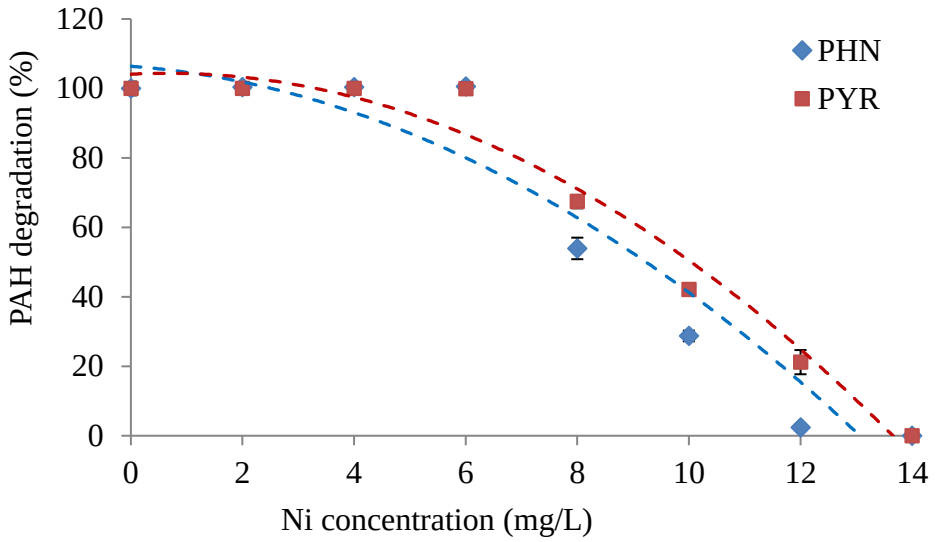

Figure 1: Degradation pattern of phenanthrene and pyrene using effective consortium as affected by different Ni concentrations (mg/L).

Cadmium (Cd) toxic effect during PAHs degradation

The Cd toxicity on phenanthrene degradation was initiated at 6 mg/L concentration of the CRM concentration while pyrene degradation response of the consortium tolerated more than such Cd quantity (Figure 2). This indicates different toxicity pattern as that of Ni while the 50% degradation response of phenanthrene and pyrene were attained at 8.10 mg/L and 8.29 mg/L of Cd concentrations respectively. Considering the Cd level contained within the soil where both MM045 and MM087 bacteria were obtained, it recorded less than 0.05 mg/L which was far below the tolerable concentration by the formulated bacteria consortium. Moreover, the PAHs degradation slopes were represented at Equations 3 and 4:

$$Y_{PHN} = -8.554X_{Cd} + 119.24 \quad (3)$$
$$Y_{PYR} = -8.8558X_{Cd} + 123.41 \quad (4)$$

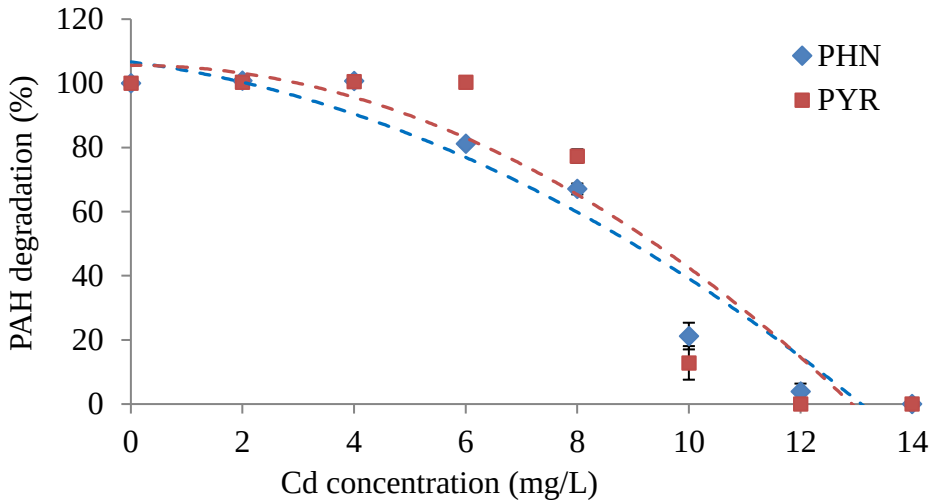

Figure 2: Degradation response for phenanthrene and pyrene using efficient consortium as affected by different Cd concentrations in mg/L.

Lead (Pb) toxic effect during PAHs degradation

The Pb toxicity was also initially observed at 8 mg/L CRM concentration as the consortium effectively tolerated 6 mg/L of Pb with 100% PAHs degradation response (Figure 3). The degradation slope for both phenanthrene and pyrene indicated 8.74 mg/L and 8.65 mg/L of Pb can generate good PAHs degradation which tremendously exceeded the previously quantified Pb in the original soil samples. Such slope equations were presented as Equation 5 and 6:

$$Y_{PHN} = -8.5242X_{Pb} + 124.53 \quad (5)$$

$$Y_{PYR} = -8.6498X_{Pb} + 124.81 \quad (6)$$

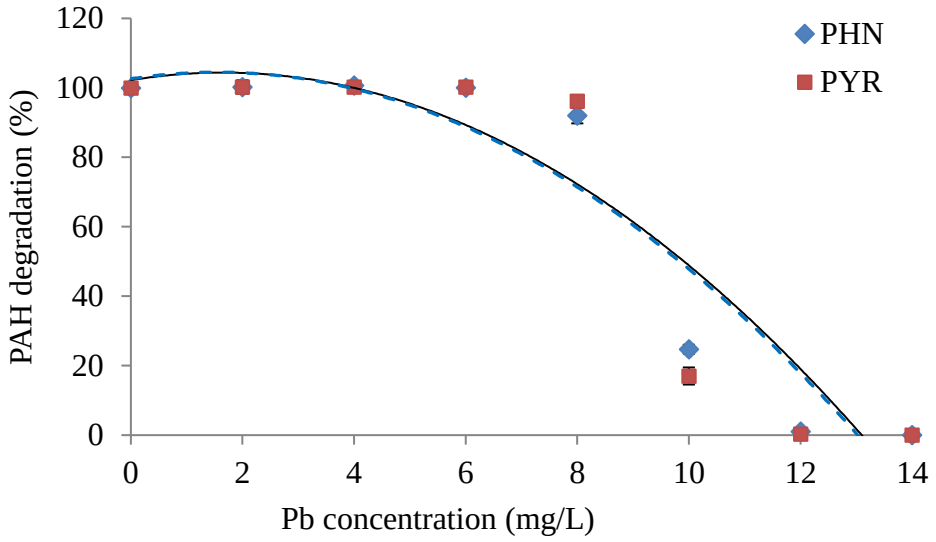

Figure 3: Degradation response for the phenanthrene and pyrene using efficient consortium as affected by different Pb concentrations (mg/L).

Vanadium (V) toxic effect during PAHs degradation

The vanadium toxic effect also indicates the bacteria in the formulated consortium could tolerate more than 6 mg/L CRM concentration while the previously analysed quantity was less than 0.2 mg/L of V (Figure 4). This shows good tolerable response during PAHs biodegradation as 50% phenanthrene and pyrene degradations were still achieved at 8.06 mg/L and 7.67 mg/L of V concentrations respectively. These were confirmed from the following mathematical slopes in Equations 7 and 8:

$$Y_{PHN} = -9.045X_V + 122.87 \quad (7)$$

$$Y_{PYR} = -9.0601X_V + 119.53 \quad (8)$$

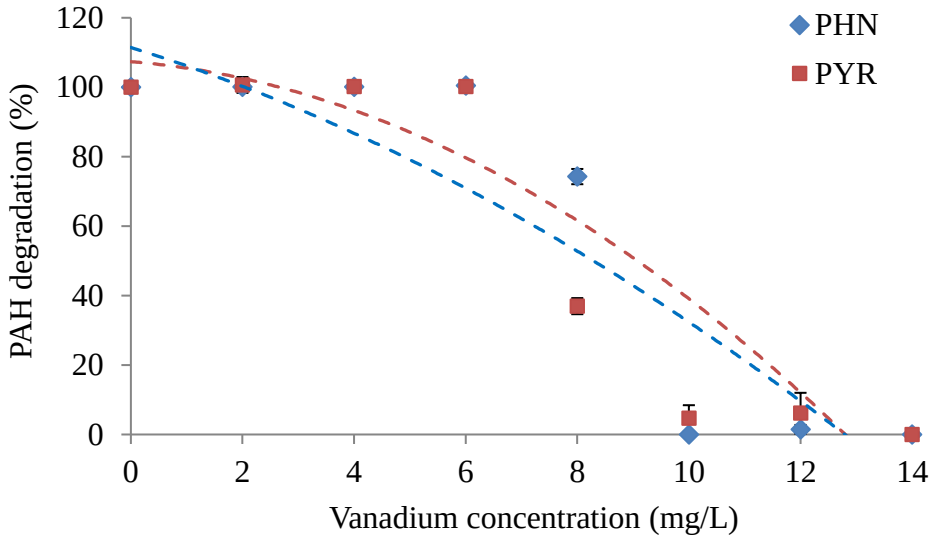

Figure 4: Degradation response for the phenanthrene and pyrene using efficient consortium as affected by different vanadium concentrations (mg/L).

Discussions

Heavy metals existence as PAHs co-contaminants in polluted environment portray dangerous effects to living cells which has more toxic effects than PAHs alone as each has critical toxic complications (Maliszewska and Smreczak 2003). Such metals reduce the PAHs biodegradation catabolism even when effective degrading isolates are employed (Hiroki, 1994). Therefore, formulation of effective degrading consortium provides better opportunity for synergistic cooperation among efficient bacteria to overcome the limitation (Sarkar *et al*., 2011; Raja *et al*., 2006). In this view, efficient PAHs degrading *C. sakazakii* MM045 and *Enterobacter* sp. MM087 were used to formulate better degrading consortium that will tolerate hazardous metals effect during PAHs biodegradation. Based on the catabolic synergy among both bacteria, very efficient PAHs degrading consortium was attained as different bacteria compositions degraded 100% PAHs quantities even at low composition of 4% (v/v) bacteria.

The previously reported PAHs degradation confirmed that *C. sakazakii* MM045 and *Enterobacter* sp. MM087 generate relevant degradation intermediates during their response (Umar *et al*., 2017; in press). Such effective degradation response could be the reason for excellent catabolic response of the formulated consortium. Based on the experimental results,

all the consortia within 5%, v/v (10^6 cells/mL) compositions were able to achieve complete (100%) PAHs degradation in 24 hours. The responses ascertained the results from the previous studies where single bacterium strain could effectively perform such degradation using 5.5%, v/v inoculums. Hence, bacteria consortium involving 4%, v/v of 10^6 cells/mL was selected for further PAHs biodegradation in a complex culture of PAH-metal mixture. This 4%, v/v of 10^6 cells/mL provides opportunity for effective phenanthrene and pyrene degradation even in an environment containing lower microbial population of *Enterobacter* sp. and *C. sakazakii* respectively (Khandai *et al.*, 2004).

The bacteria consortium selected was shown to have efficiently degraded significant phenanthrene and pyrene even in the presence of hazardous metals (Figures 1 to 4). These PAHs degrading efficiency was rarely reported in the previous literature especially metals involving Pb, Ni, V, and Cd due to their severe metabolic effects to microorganisms (Irha *et al.*, 2003; Abbas & Edwards, 1989). Additionally, the determination of several metals from the soil samples confirmed the previous reports on their frequent existence within PAHs contaminated sites (Irha *et al.*, 2003; Vig, 2003; Mielke *et al.*, 2000).

All the individual metals tested (Pb, Ni, V, and Cd) were found to be tolerated by the consortium even at higher concentrations (>4 mg/L) as 100% phenanthrene and pyrene degradations were successfully attained. Furthermore, the consortium recorded effective degradation response (>50%) in the presence of 8 mg/L of all the tested metal and the response decreases with metal increase as a result of bacteria DNA alteration. This alteration prevents replication of DNA by producing single stranded DNAs in excess which was not experienced by the consortium at 6 mg/L of Pb, Ni and V during both phenanthrene and pyrene degradations. This happened because of the consortium effectiveness in resisting higher concentrations of such hazardous metals due to efficient DNA repair strategy (Guzzo and DuBow, 1994). Such strategy caused the bacteria to sufficiently express LexA and RecA proteins which transcribed genes responsible for encoding the DNA repair enzymes (Ingraham *et al*., 1987).

Another strategy that increases the chances of mixed bacteria consortium to efficiently degrade PAHs in the presence of heavy metals is the ability to generate broad degrading enzymes (Joutey *et al.*, 2013). This helps in overcoming growth limitations faced from such metals and enhances the bioavailability of *C. sakazakii* and *Enterobacter* sp. respectively.

Additionally, the consortium also degraded 100% phenanthrene and pyrene in the presence of 4 mg/L and 6 mg/L of Cd concentrations respectively (Fig 3). The pyrene degradation recorded similar response as those of other tested metals while the bacteria tolerated lower Cd concentration during phenanthrene degradation. The bacteria consortium tolerated very high Cd concentration when compared to the Cd concentration of <0.04 mg/L recorded from the natural soil samples from which the organisms were isolated. All the PAHs degradation response by the bacteria in the presence of hazardous metals were observed to support the previous reports as the degradation decreases with increasing metal quantity (Duxbury and Bicknell, 1983). All the higher concentrations of Pb, Ni, V and Cd tolerated by the formulated consortium were extremely higher than the environmental contaminations where both bacteria were isolated previously. This portrays the biodegradation desirability of such consortium which can be applied to environment with much heavy metals toxicity (Ingraham *et al*., 1987).

The use of mixed bacteria consortium in this study caused considerable PAHs degradation increase based on synergistic bacteria cooperation which reduces hazardous metals effect on

the bacteria response. However, the bacteria responses were mainly controlled by the complex nature of the polluted environment which involves high concentrations of mixed pollutants involving PAHs and heavy metals that influences bacteria growth conditions (Duxbury and Bicknell, 1983). This strongly required continuous research in order to completely eliminated PAHs biodegradation limitations.

Conclusion

The study confirmed the effective degradation response of the constituted bacteria consortium in the presence of hazardous heavy metals. It was further demonstrated that both bacteria involved in the consortium could synergistically cooperate to overcome degradation limitation resulting from toxic metals effects. The natural environments where both bacteria were isolated contained several hazardous metals as additional contaminants as a result of anthropogenic activities. Hence, the formulated consortium could effectively perform better PAHs degradation within 24 hours even in the presence of toxic co-contaminants and therefore further research might help to improve such response.

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Conflict of Interest

This research study has no any conflict of interest whatsoever.

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