



P-ISSN: 2349-8528

E-ISSN: 2321-4902

[www.chemijournal.com](http://www.chemijournal.com)

IJCS 2021; 9(1): 1105-1110

© 2021 IJCS

Received: 04-09-2020

Accepted: 15-10-2020

**Arjita Punetha**

Department of Environmental  
Science, G.B. Pant University of  
Agriculture and Technology,  
Pantnagar, US Nagar,  
Uttarakhand, India

**Anamika Kumari**

Department of Environmental  
Science, G. B. Pant University of  
Agriculture and Technology,  
Pantnagar, US Nagar,  
Uttarakhand, India

**JPN Rai**

Department of Environmental  
Science, G. B. Pant University of  
Agriculture and Technology,  
Pantnagar, US Nagar,  
Uttarakhand, India

**Corresponding Author:****Arjita Punetha**

Department of Environmental  
Science, G.B. Pant University of  
Agriculture and Technology,  
Pantnagar, US Nagar,  
Uttarakhand, India

## Comparative effect of bioformulation and immobilization on bacterial cell viability under different storage conditions

**Arjita Punetha, Anamika Kumari and JPN Rai**

DOI: <https://doi.org/10.22271/chemi.2021.v9.i1p.11370>

### Abstract

This paper presents isolation and characterization of BaP degrading bacterial isolates and different bacterial entrapment methods (bioformulation and immobilization) to enhance their shelf life. Bacteria were isolated from coal tar contaminated sites and their BaP degrading efficiency was checked. Most potent bacterium i.e. 4d was biochemically characterized and immobilized using alginate beads and bioformulation was prepared by using talc and charcoal and kept at 25° C and 4°C for two months. Bacterium was subjected to serial dilution followed by spread plate method at subsequent intervals of 7<sup>th</sup>, 14<sup>th</sup>, 21<sup>st</sup>, 30<sup>th</sup>, 45<sup>th</sup> and 60<sup>th</sup> day to observe the CFU count. The bacterium immobilized in alginate beads was found to be most viable when kept in 4°C though the bacterium was also fairly viable in all other storage conditions. Further studies are required to assess the activity of the bacterium at BaP polluted site.

**Keywords:** Bioformulation, immobilization, Benzo a pyrene, cell viability

### Introduction

Rapid industrial growth and expansion has brought about the amassing of harmful and perilous substances to the environment, prompting adverse health impacts on people. Polycyclic Aromatic Hydrocarbons (PAHs) are an example of such toxic substances. PAHs are pervasively found since they are produced as results of processes involving partial combustion of organic materials like waste incineration, wood and vegetation burning, petroleum refining, volcanic eruptions and forest fires (Plaza *et al.* 2009) [1]. Benzo[a]pyrene (BaP) is a well known PAH with five rings of benzene fused together and is particularly perceived for being carcinogen, teratogen and mutagen (de Llasera *et al.* 2017, Delsarte *et al.* 2018) [3, 4]. BaP is hydrophobic in nature, having low volatility and it is quite importunate in the environment mainly because it has low water solubility. BaP has the propensity to get adsorbed on the soil, which diminishes its bioavailability and accessibility, particularly in profound soil where oxygen is scant. Numerous physical and chemical technologies for the treatment of BaP contaminated soil have been devised. Physical remediation strategies include inversion, incineration, thermal desorption and stripping of soil. Use of landfills is also a technique of removal of BaP from soil (Acharya and Ives, 1994, Harmon *et al.* 2001, Renoldi *et al.* 2003) [1-6, 14]. Chemical remediation of BaP contaminated soils is mainly involves processes such as leaching, chemical oxidation and photo-catalysis (Ferrarese *et al.* 2008, Tryba *et al.* 2014, Pardo *et al.* 2016) [5, 15, 10]. Physical and chemical methods of treatment require the use of high temperature or chemicals. These technologies are not fruitful as they significantly cause phase transfer instead of mineralization of the pollutant, thereby leading to secondary pollution and are quite expensive. In order to address these challenges, significant interest has been laid on microbial degradation. Being inexpensive, eco-friendly and efficient, microbial degradation has end up being a promising method to clean BaP contaminated soils (Qin *et al.* 2017, Mandal *et al.* 2018) [12, 9]. Since microorganisms are universally found and produce several enzymes that can degrade the pollutant, converting them to non-toxic products, this strategy is sustainable. To date, several microorganisms with the potential to degrade BaP have been identified. Microbes possess catabolic and catalytic activities often desired for degradation of the contaminant and secrete numerous enzymes like laccase, oxygenases, peroxidase and

dehydrogenases that act to metabolize BaP into smaller non toxic compounds. However, the course of microbial degradation is highly dependent on environmental factors that affect microbial growth like temperature, pH and nutrient availability. Bio-augmentation (addition of lab cultured or pre-grown microbes to contaminated soil) and bio-stimulation (supplying nutrients to accelerate microbial growth) are two commonly used mechanisms embraced to accelerate the growth of microbes and increase the effectiveness of microbial degradation.. However, the endurance of microorganisms introduced to contaminated sites through bio-augmentation is a challenge since such microbial strains are regularly exposed to prolonged storage periods and malicious environmental variables, which unfavorably influence their feasibility and eventually affect their proficiency. Bioformulations confer the microorganisms with the advantage of viability. Using carriers to deliver microbes to soil for the purpose of remediation has therefore been proposed (Wang *et al.*, 2019; Zhang *et al.* 2019, Kalsi *et al.* 2020) [12, 16, 7]. The carriers that assist in increasing bioavailability along with providing nutrients to microbes are better suited for degradation. Therefore, selecting carrier materials based on these criteria helps in enhancing remediation of the hazardous compound. The method of microbial cell entrapment within alginate beads has turn out to be one of the most extensively used immobilization method (Zommere and Nikolajeva 2017) [17]. Talc and charcoal have also been used for several years as bio-formulation carriers. This study aims to evaluate a suitable carrier for entrapment of BaP degrading bacteria and to determine appropriate conditions for its storage.

## Material and Methods

### Isolation of Bacteria

Soil samples were collected from sites contaminated with coal tar and wood burning for isolation of bacteria. Further, the techniques of enrichment and serial dilution were used for isolating bacteria from the soil samples. 10 g of soil sample was dissolved in 100ml minimal medium suspended with 100 ppm BaP and kept at 30°C for 1 week. After a week, 1 ml of enriched sample was taken and added to 9 ml Distilled water. This method of serial dilution was followed for up to 8 dilutions. 100 µl of sample from each dilution was subsequently taken and spread into petri plates containing nutrient agar and plates were kept on incubation at 28°C for 24hr. Bacterial colony having different morphology were selected and purified. Purified bacterial isolates were preserved in slants and glycerol stock for further use.

### Screening of bacterial strains for BaP Utilization

To test the BaP utilization property, bacterial cultures purified from nutrient agar plates were inoculated in minimal media supplemented with 100 ppm of BaP and kept at 28°C for 24hr. 16 bacterial isolates that could utilize 100 ppm concentration of BaP were then checked for their BaP tolerance capacity. All 16 bacterial cultures were inoculated in minimal medium supplemented with different concentrations of BaP. Bacterial isolates that were able to grow in minimal medium supplemented with 300 ppm of BaP were finally selected for further study.

### Morphological and Biochemical Characterization

Bacterial isolate 4d was streaked on nutrient agar and after 24hr of incubation its morphological characters such as margin, cell shape, size, elevation, surface and Gram's

staining were recorded. Moreover, catalase, gelatin liquefaction, casein hydrolysis, oxidase, carbohydrate utilization pattern and IMViC test were performed for its biochemical characterization.

1. Catalase: Actively growing culture was transferred to glass slide with a sterile loop. A drop of 3% H<sub>2</sub>O<sub>2</sub> was added to it and observed to check if bubbles of oxygen evolve.
2. Casein hydrolysis: Skim milk and nutrient agar was prepared in separate flasks and autoclaved. Contents of both the flasks were mixed just before pouring to plates. After pouring, plates were kept for solidification following which plates were inoculated with short thick streaks in centre and kept to incubate for 48-72 hrs. The plates were observed to check if clear zones appear, which indicates positive reaction.
3. Gelatin hydrolysis: Gelatin tubes were prepared by addition of 10% gelatin into nutrient broth (NB) and autoclaved. Actively growing bacterial culture was inoculated in the tubes at 30° C for 1-2 days. Afterwards, the tubes were put in ice and checked for liquefaction or solidification. Liquefaction of tubes is considered positive and solidification negative.
4. Oxidase test: A drop of actively grown culture in NB was put in oxidase disc to observe the change of color of disc from white to purple which indicated positive results.
5. Carbohydrate utilization: Carbohydrate utilization potential for 35 different carbohydrates was determined through HiCarbo kit (Hi-Media)
6. IMViC test: IMViC test was also performed using test kits from Hi-Media.

### Bio-formulation Preparation

Bacterial culture was inoculated in 300 mL Nutrient Broth (pH 7) and was kept for incubation at 28°C and 100 rpm for 24 hrs. Prepared active culture was then centrifuged with 10,000 rpm for 10 min. The bacterial pellets obtained were mixed homogenously with 10 g sterilized talc in aseptic conditions and then kept for drying at room temperature. Similar procedure was used to prepare charcoal bio-formulation. When the mixtures dried completely, both formulations were packed separately in sterilized air tight packets and sealed properly to avoid contamination. The prepared bio-formulations were stored at room temperature (25°C) and in a refrigerator at 4°C.

### Immobilization using Alginate Beads

Immobilization was carried out using a method adapted from the one described by Bashan (1986) [2]. In brief, 300 ml of 3% Sodium alginate and 100 ml of 0.2 M CaCl<sub>2</sub> were prepared and autoclaved separately. Bacterial cultures were grown in nutrient broth for 12 hours and centrifuged, from which pellets were obtained. The pellets were mixed with 50 ml 3% sodium alginate and stirred completely. The mixture was then filled into a sterile syringe and dispensed drop-wise into chilled solution of 0.2 M CaCl<sub>2</sub> where formation of beads took place. Then, beads were kept at room temperature for 60 min to allow their solidification. The beads were incubated with 2ml CaCl<sub>2</sub> in sterile glass tubes with caps and maintained at 4°C and 25° C for 60 days.

### Microbiological analysis

Samples of beads were ground to finely using a sterile pestle and mortar. Serial dilution was then performed to determine bacterial CFU on agar plates using spread plate method.

Bacteria were recovered from talc and charcoal based bio-formulation directly by serial dilution on agar plates using spread plate method following which plates were incubated at 30 ° C for 24-48hr.

## Results and Discussion

### Morphological Analysis

A total of 60 bacterial cultures were isolated from enriched soil, out of which 35 were selected for purification based on the differences in morphology of their colonies, which were then checked for growth in different concentration of BaP. Among all isolated bacterial cultures, bacterium 4d showed prominent growth in minimal medium that was supplemented

with 300ppm BaP, hence was chosen for further study. Morphological characterization and gram staining revealed that the bacterium 4d is gram negative and appears as small, circular shaped having smooth surface (Fig 1).

**Table 1:** Morphological characteristics of bacterium 4d

S. No	Feature	Type
1	Margin	Entire
2	Cell shape	Circular
3	Size	Small
4	Elevation	Convex
5	Surface	Smooth
6	Gram's stain	Negative

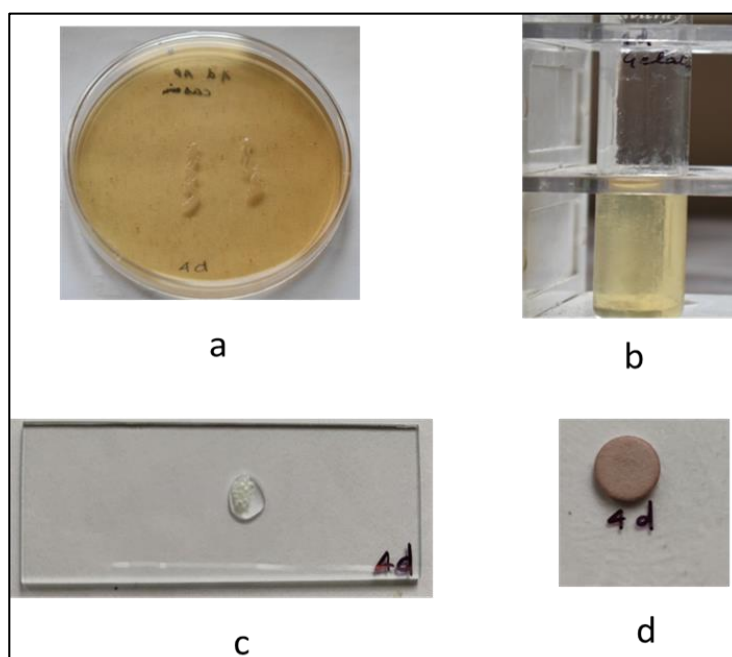


**Fig 1:** Morphology of bacterium 4d

### Biochemical analysis

Catalase is a commonly found enzyme among aerobic microbes that breaks hydrogen peroxide into water and oxygen (Kaushal *et al.* 2018) [8]. Positive result in the study i.e bubble formation (Fig 2) was observed that suggests that the bacterium 4d is able to produce catalase. Whereas, oxidase test determines the activity of enzyme cytochrome c oxidase involved in bacterial electron transport chain. Bacterium used in current study is negative for oxidase production. Moreover,

Casein hydrolysis demonstrates that the microorganism produces a proteolytic exo-enzyme named proteinase (Caseinase) that is able to degrade the casein and gelatin test indicates the ability of microbe to produce gelatinase, an enzyme that liquefies gelatin, of which both are also found negative (Table 2) for the bacterium 4d. Moreover, results of IMViC test (Table 2) demonstrated that bacterium 4d is positive for Indole and Methyl red and negative for Voges Proskauer and citrate utilization (Fig 3).



**Fig 2:** Biochemical characterization of bacteria: a) casein hydrolysis, b) gelatin liquefaction, c) catalase, d) oxidase

**Table 2:** Biochemical characteristics of bacterium 4d

S. No	Test	Result
1	Catalase	+
2	Oxidase	-
3	Casein hydrolysis	-
4	Gelatin hydrolysis	-
5	Indole	+
6	Methyl red	+
7	Voges Proskauer's	-
8	Citrate	-

### Carbohydrate Utilization Test

Carbohydrate utilization test determines whether or not a bacterium can utilize a certain carbohydrate. Results

demonstrate that the bacterium 4d has ability to utilize Xylose, Maltose, Fructose, Dextrose, Galactose, Raffinose, Trehalose, Sucrose, L-Arabinose, mannose, Insulin, Sodium gluconate, glycerol, salicin, cellobiose, ONPG, Esculin, D Arabinose and Glucose (Fig 3; Table 3) however, the bacterium cannot utilize lactose, melibiose, dulcitol, inositol, sorbitol, mannitol, adonitol, arabitol, erythritol, A Methyl D-glucose, Rhamnose, Melezitose,  $\alpha$  Methyl D-mannoside, Xylitol, malonate and sorbase (Table 3). Since the bacterium 4d is found to utilize a number of carbohydrates, it can easily grow in minimal nutrient supplementation. Additionally, these carbohydrates can also be provided to the growth medium of the bacterium to enhance its growth.

**Table 3:** Carbohydrate utilization pattern of bacterium

S. No	Carbohydrate	Result
1	Lactose	-
2	Xylose	+
3	Maltose	+
4	Fructose	+
5	Dextrose	+
6	Galactose	+
7	Raffinose	+
8	Trehalose	+
9	Melibiose	-
10	Sucrose	+
11	L-Arabinose	+
12	Mannose	+
13	Insulin	+
14	Sodium gluconate	+
15	Glycerol	+
16	Salicin	+
17	Dulcitol	-
18	Inositol	-
19	Sorbitol	-
20	Mannitol	-
21	Adonitol	-
22	Arabitol	-
23	Erythritol	-
24	A Methyl D- glucose	-
25	Rhamnose	-
26	Cellobiose	+
27	Melezitose	-
28	$\alpha$ Methyl D-mannoside	-
29	Xylitol	-
30	ONPG	+
31	Esculin	+
32	D Arabinose	+
33	Citrate	-
34	Malonate	-
35	Sorbate	-
36	Glucose	+
37	Control	-

**Fig 3:** a) Carbohydrate utilization pattern of bacterium, b) IMViC test



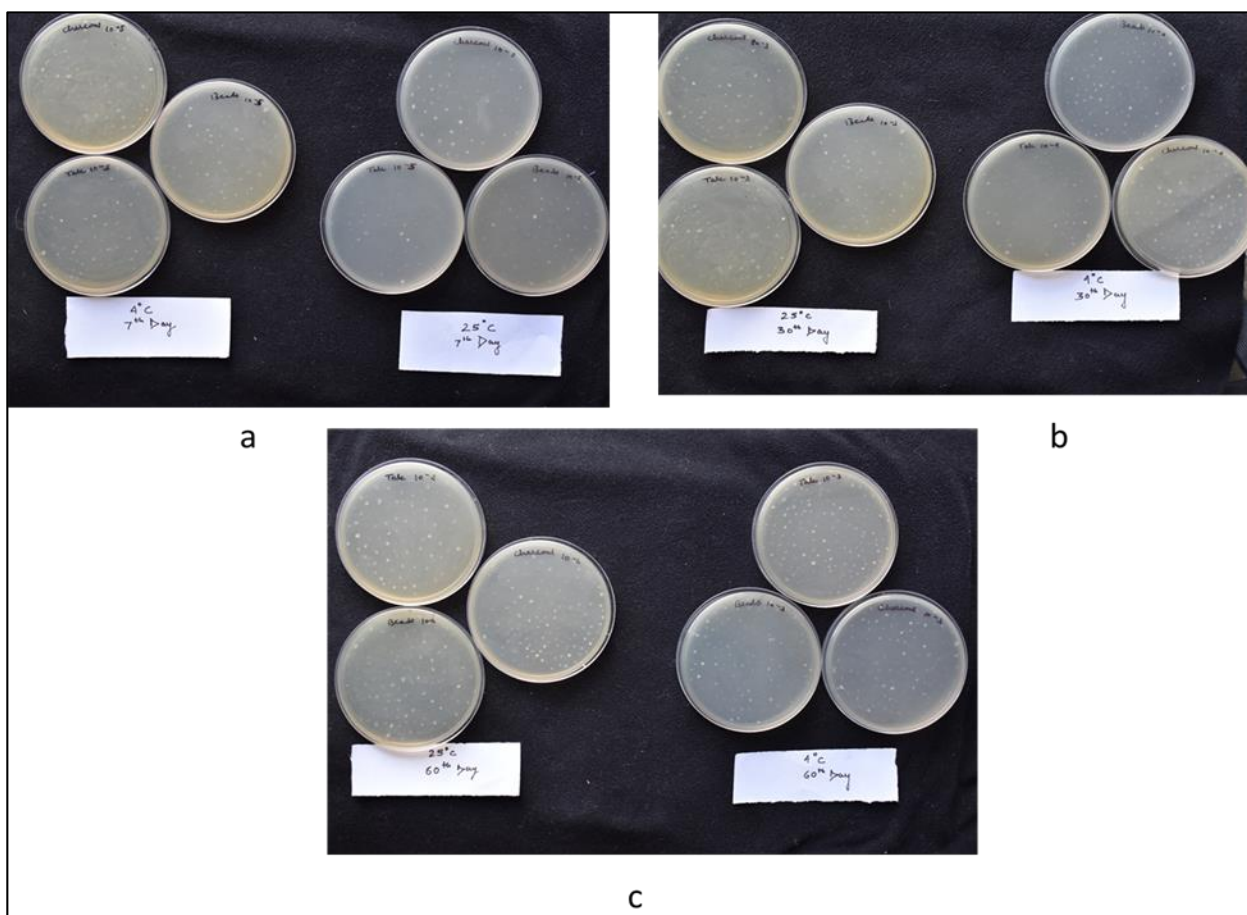
### Bioformulation

Bioformulations are biologically active products that contain beneficial microbial strains in some easy to use and economical carrier material. Previous studies have documented immobilization of microorganisms in several carrier materials like vermiculite, plant residue, activated carbon, etc through several processes like adsorption, entrapment or chemical bonding. Zomme and Nokalajeva (2017) also studied the viability of microbes by immobilization in alginate beads and reported that bacteria can thrive in entrapped conditions for 70 days. Power *et al.* (2011) [13] also recorded 100% recovery of active *Pseudomonas fluorescens* F113 after being stored in alginate beads at ambient temperature for 250 days and confirmed that

alginate beads are an efficient storage system. In order to evaluate the most suitable prolonged storage material and temperature, present study uses three carriers *viz* talc, charcoal and alginate beads to entrap bacterium 4d having potential to degrade BaP. The study was conducted in two different temperatures, 4°C and 25°C. Bacterial cells entrapped in alginate beads and kept at 4°C were found to be most viable, for which the CFU count on 60<sup>th</sup> day was detected to be highest amongst all the treatments (Table 4), followed by charcoal bioformulation at 4°C and then talc based formulation at the same temperature. Talc based formulation at 25°C was found to be the least effective condition of storage of the bacterium.

**Table 4:** CFU count after subsequent days in different conditions

SN	Bio formulation type	CFU mL <sup>-1</sup> at subsequent time interval (days)					
		7 <sup>th</sup> day	14 <sup>th</sup> day	21 <sup>st</sup> day	30 <sup>th</sup> day	45 <sup>th</sup> day	60 <sup>th</sup> day
1	Talc based bioformulation (4°C)	7.5x10 <sup>6</sup>	1.71x10 <sup>6</sup>	1.26x10 <sup>6</sup>	8.6 x10 <sup>5</sup>	1.83 x10 <sup>5</sup>	1.55 x10 <sup>5</sup>
2	Talc based bioformulation (25°C)	5.6 x10 <sup>6</sup>	9.2 x10 <sup>5</sup>	6.3 x10 <sup>5</sup>	7.9x10 <sup>4</sup>	1.13 x10 <sup>4</sup>	9.4 x10 <sup>3</sup>
3	Charcoal based bioformulation (4°C)	7.8 x10 <sup>6</sup>	1.73 x10 <sup>6</sup>	1.32 x10 <sup>6</sup>	9.4 x10 <sup>5</sup>	1.86 x10 <sup>5</sup>	1.63 x10 <sup>5</sup>
4	Charcoal based bioformulation (25°C)	5.7 x10 <sup>6</sup>	1.06 x10 <sup>6</sup>	7.4 x10 <sup>5</sup>	8.3x10 <sup>4</sup>	1.31 x10 <sup>4</sup>	9.9 x10 <sup>3</sup>
5	Alginate beads with 0.2M CaCl <sub>2</sub> (4°C)	8.7x10 <sup>6</sup>	1.74 x10 <sup>6</sup>	1.41 x10 <sup>6</sup>	9.8 x10 <sup>5</sup>	1.90 x10 <sup>5</sup>	1.65 x10 <sup>5</sup>
6	Alginate beads with 0.2M CaCl <sub>2</sub> (25°C)	7.4 x10 <sup>6</sup>	1.06 x10 <sup>6</sup>	8.6 x10 <sup>5</sup>	9.6 x10 <sup>4</sup>	1.47 x10 <sup>4</sup>	1.01 x10 <sup>4</sup>



**Fig 4:** CFU count after a) 7 days, b) 30 days, c) 60 days

### Conclusion

The present study demonstrates that bacteria can be used in immobilized forms or in the form of bioformulation for the degradation at contaminated sites. Conveying bacteria in such associated forms rather than free form confers them the advantage of increased shelf life. Suitable carriers can also provide additional advantage of increasing the bio-availability of pollutants along with providing nutrients to the bacteria. In

the current study of 60 days, alginate beads, talc and charcoal all proved to be effective carriers of BaP degrading 4d bacterium, where, alginate beads were found to be the best supporting carrier. Since alginate is naturally found polymer, it is biodegradable and thus eco-friendly. Talc and charcoal are also cheap, easily available and environment friendly substances, thereby mitigating the issues of cost effectiveness and secondary pollution. Using microbes with such

associations can be useful to degrade various pollutants. Extensive studies are required further to evaluate the competence of such associations to degrade contaminants in the field conditions.

## References

1. Acharya P, Ives P. Incineration at Bayou bounfouca remediation project. *Waste Management* 1994;14:13-26.
2. Bashan Y. Alginate beads as synthetic inoculant carriers for slow release of bacteria that affect plant growth. *Applied and Environmental Microbiology* 1986;51:1089-1098.
3. De Llasera MPG, Santiago ML, Flores EJJ, Toris DNB, Herrera MRC. Mini-bioreactors with immobilized microalgae for the removal of benzo(a)anthracene and benzo(a)pyrene from water. *Ecological Engineering* 2017;121:89-98.
4. Delsarte I, Rafin C, Mrad F, Veignie E. Lipid metabolism and benzo[a]pyrene degradation by *Fusarium solani*: an unexplored potential. *Environmental Science and Pollution Research* 2018;25:12177-12182.
5. Ferrarese E, Andreottola G, Oprea IA. Remediation of PAH-contaminated sediments by chemical oxidation. *Journal of Hazardous Materials* 2008;152:128-139.
6. Harmon TC, Burks GA, Aycaguer AC, Jackson K. Thermally enhanced vapor extraction for removing PAHs from lampblack-contaminated soil. *Journal of Environmental Engineering -ASCE* 2001;127:986-993.
7. Kalsi A, Mary Celin S, Bhanot P, Sahai S, Sharma JG. Microbial remediation approaches for explosive contaminated soil: critical assessment of available technologies, Recent innovations and Future prospects. *Environmental Technology and Innovation* 2020,100721.
8. Kaushal J, Mehandia S, Singh G, Raina A, Arya SK. Catalase enzyme: Application in bioremediation and food industry. *Biocatalysis and Agricultural Biotechnology* 2018,16192-199.
9. Mandal SK, Ojha N, Das N. Optimization of process parameters for the yeast mediated degradation of benzo[a]pyrene in presence of ZnO nanoparticles and produced biosurfactant using 3-level Box-Behnken design. *Ecological Engineering* 2018;120:497-503.
10. Pardo F, Peluffo M, Santos A, Romero A. Optimization of the application of the Fenton chemistry for the remediation of a contaminated soil with polycyclic aromatic hydrocarbons. *Journal of Chemical Technology and Biotechnology* 2016;91:1763-1772.
11. Plaza C, Xing BS, Fernandez JM, Senesi N, Polo A. Binding of polycyclic aromatic hydrocarbons by humic acids formed during composting. *Environmental Pollution* 2009;157:257-263.
12. Qin W, Zhu Y, Fan F, Wang Y, Liu X, Ding A *et al.* Biodegradation of benzo(a)pyrene by *Microbacterium* sp. strain under denitrification: degradation pathway and effects of limiting electron acceptors or carbon source. *Biochemical Engineering Journal* 2017;121:131-138.
13. Power B, Liu X, Germaine KJ, Ryan D, Brazil D, Dowling DN. Alginate beads as a storage, delivery and containment system for genetically modified PCB degrader and PCB biosensor derivatives of *Pseudomonas fluorescens* F113. *Journal of Applied Microbiology* 2011;110:1351-1358.
14. Renoldi F, Lietti L, Saponaro S, Bonomo L, Forzatti P. Thermal desorption of a PAH contaminated soil: a case study. *Ecosystems & Sustainable Development* 2003;4:1123-1132.
15. Tryba B, Homa P, Wrobel RJ, Morawski AW. Photocatalytic decomposition of benzo[a]pyrene on the surface of acrylic, latex and mineral paints. Influence of paint composition. *Journal of Photochemistry and Photobiology* 2014;286:10-15.
16. Zhang C, Pan X, Wu X, Dong F, Liu X, Xu J *et al.* Removal of dimethachlon from soils using immobilized cells and enzymes of a novel potential degrader *Providencia stuartii* JD. *Journal of Hazardous Materials* 2019;138:12606.
17. Zommere Z, Nikolajeva V. Immobilization of bacterial association in alginate beads for bioremediation of oil-contaminated lands. *Environmental and Experimental Biology* 2017;15:105-111.