Original Research Article

Viability of hydrocarbon-degrading bacterial consortium immobilized on different carriers

Abstract

Aim: Viability of hydrocarbon-degrading bacterial consortium immobilized on different carriers was studied. **Methodology:** Hydrocarbon-degrading bacteria were isolated from crude oil contaminated sites in Gio and K-Dera, Rivers State, Nigeria using enrichment method. Proximate analyses were carried out on the best carrier materials. Immobilization was by direct adsorption of the isolates onto the carrier materials and viability was determined by plate count method. The carrier materials tested included soya bran, sugarcane bagasse, corn cob, brown saw dust, white saw dust, cassava peel and red mud (bentonite). **Results:** The bacterial isolates demonstrated varied degradation capacity. The best carrier material was saw dust (103.6% survival) and corn cob (103.6% survival) followed by soya bran (94.4% survival rate) and cassava peel (94.4% survival rate). The saw dust had moisture content, 5.92%; ash content, 7.49%; crude protein, 2.2%; volatile matter, 74.28; and fixed carbon, 12.34%; whereas, the percentage chemical composition observed for soya bran were 10.11, 4.08, 5.22, 42.61, 18.37 and 8.89 for moisture content, ash content, crude fibre, crude protein, crude fat and carbohydrate, respectively. There was significant difference (p=0.05) between viability rate observed with the different carrier materials. **Conclusion:** This study showed that the agro-wastes used in this study can effectively retain the viability of hydrocarbon-utilizing bacterial. The result is significant as it shows the possibility of using these carrier materials for biodegradation of hydrocarbon contaminated media.

Keywords: Carrier materials; hydrocarbon-utilizing bacteria; immobilization; viability; saw dust and soya bran.

1.0 INTRODUCTION

Immobilization refers to the process of limiting the mobility of microbial cells or their enzymes in order to preserve their viability and and/or catalytic function [1-4]. Not all materials qualify as carrier or support materials for immobilization. For a material to be suitable for immobilization, the material must be insoluble, cost-effective, readily available, non-toxic to the environment and the material being immobilized, stable and fit for regeneration. Immobilization of microorganisms on suitable carriers is widely acknowledged to be beneficial for the maintenance of long-term viability.

Different immobilization techniques exist and these techniques are employed based on certain pre-determined criteria [5]. Amongst the many criteria to consider in choosing a suitable carrier for use in immobilization is the property of the carrier material. The aim of immobilization is another important factor to consider; for instance, carriers for bioaugmentation should be readily biodegradable. A few of the characteristics of a carrier material that influences the immobilization technique to be employed is its porosity and surface area. These features are especially important in adsorption technique. Kariminiaae-Hamedaani *et al*. [6], Martin *et al*. [7] and Bayat *et*

al. [8] noted that carriers used for binding or adsorption on the surface must possess high porosity so that the immobilized material and the carrier will have large enough contact area.

Carrier materials to be used for immobilization may be classified as either organic and inorganic or natural and synthetic. Natural organic carriers possess several functional groups which aid biocatalysts' stabilization. Examples of natural organic carriers frequently employed in immobilization process include bagasse [9], rice [8], corn cob [10], saw dust [11], straw [12], charcoal [13], plant fibres [14], alginate [15], diatomite [16] etc. The features of these materials that have encouraged their use are their hydrophilic nature, biodegradability, cost effectiveness and biocompatibility. However, natural organic carrier materials may not be suitable for certain applications.

Several studies have reported enhanced petroleum hydrocarbon degradation by immobilized hydrocarbon-degrading bacteria compared to free-living cells [17,18]. This observation has increase the interest in immobilization of microbial cells for bioremediation purposes. This study investigated the viability of hydrocarbon-degrading bacterial consortium immobilized on different carriers. This is important in determining the suitability of these carrier materials for use in bioremediation.

2. MATERIALS AND METHODS

2.1 Isolation of Hydrocarbon-Degrading Bacteria

Soil samples were collected from K-Dere and Gio local government areas in Rivers State, Nigeria and processed by drying in an oven at 40 ^{o}C for 3 h, sieving with a 2 mm mesh sieve to remove unwanted debris and then refrigerating at 4 ^{o}C prior to use. Hydrocarbon-degrading bacteria were isolated by enrichment method as described by [19] and [20] with slight modification. In brief, 10 g of the processed soil was dissolved in 250 mL Erlenmeyer's flask, containing 100 mL of sterilized normal saline. The flask was votexed at maximum speed for 2 min and the suspension allowed settling for 5 min. A volume of 5 mL of the supernatant was used to inoculate another separate flask containing 100 mL Bushnell Haas Broth (BHB) (Hi Media, India) (containing in g/L: 0.2 $MgSO_4.7H_20$; 0.02 $CaCl_2.2H_2O$; 1 KH_2PO_4; 1 K_2HPO_4; 1 NH_4NO_3; 0.05 $FeCl_3$; nystatin- 0.1g; and pH 7.0) supplemented with crude oil (1%, v/v) as the sole carbon and energy source. Tween 80 (0.05% v/v) was added to the broth to enhance hydrocarbon degradation. The procedure was repeated for each of the soil samples and the set-up performed in duplicate. The flasks were incubated in a rotary shaker incubator at 150 rpm for 7 days. At the end of 7 days, successive sub-culturing were done by transferring 5 mL of BHB culture into fresh BHB medium supplemented with crude oil (1%, v/v). The sub-culturing ensured isolation of only oil-tolerant and oil-degrading bacteria [21].

After three sub-culturing stages, 1 mL of the broth was pipetted aseptically and serially diluted to make 10^{-1} to 10^{-6} dilutions. The 10^{-3}, 10^{-5} and 10^{-6} dilutions were plated out on freshly prepared Bushnell Haas agar plates supplemented with crude oil (1% v/v). The inoculated plates were incubated for 4 days at 30 ^{o}C. Discrete colonies on the plates were picked and purified by repeated streaking on Bushnell Haas Agar supplemented with crude oil (1%, v/v). The pure isolates were further purified on nutrient agar and stored in Bushnell Haas Agar slants supplemented with 1% (v/v) crude oil.

2.2 Screening of Carriers

Seven (7) agro-wastes materials (corn cob, sugarcane bagasse, white saw dust, soya bran, brown saw dust, cassava peel, and white mud (bentonite)) were screened as potential carriers of the bacterial consortium inoculant. This test was carried out to determine the biocompatibility of the carriers with bacterial consortium inoculant.

2.2.1 Decontamination of carriers

Prior to inoculation with the bacterial consortium, carrier materials were decontaminated following slight modification of the method described by [22]. In brief, carriers were first oven-sterilized at 140 ^{o}C for 1 h. After 1 h, the materials were transferred into an autoclave and sterilized further at 121 ^{o}C (15 psi) for 30 min. A volume of 0.2 mL sterile crude oil (corresponding to 2% (v/w) of the carrier) was added to the carrier and the mixture votexed to ensure proper mixing. To confirm the absence of viable cells in the carriers, 1 g of each of decontaminated carriers was dissolved in 10 mL of normal saline and 0.1 mL spread-plated on fresh nutrient agar plates. The plates were incubated at 30 ^{o}C for 48 h and thereafter observed for growth. Absence of growth confirmed successful decontamination.

2.2.2 Immobilization (by adsorption) of bacterial consortium on the carriers

Individual bacterium, used for the consortium, was grown in a Bushnell Haas Broth medium for 48 h in a shaker incubator set at 150 rpm [23]. From each broth, 1 mL inoculum with turbidity corresponding to 1.0 McFarland standard was used to make the bacterial consortium inoculant. The consortium was homogenised by shaking with hand. Thereafter, 10 mL of the broth was dispensed into each flask containing 50 g of the carrier material. The inoculated carriers were mixed properly and incubated in a shaker incubator for 21 days at 30 ^{o}C. The first sample was taken immediately after inoculation for bacterial count; subsequent counts were carried out at days 7, 14 and 21.

2.3 Enumeration of Total Culturable Heterotrophic Bacteria Hydrocarbon-Utilizing Bacteria

Total culturable heterotrophic bacterial (TCHB) and Hydrocarbon-utilizing bacterial (HUB) counts were taken at days 0, 7, 14 and 21 using Bushnell Haas agar (amended with 1% v/v Bonny light crude oil and 0.01% w/v nystatin) [24] and nutrient agar (Merck, Germany) plates, respectively. The plates were prepared according to manufacturer's instruction. A volume of 100 µL each of 10^{-3}, 10^{-5}, and 10^{-6} dilutions of the samples was spread onto corresponding agar plates. The inoculated plates were incubated at 30 ^{o}C for 24 h for TCHB and 96 h for (HUB). After incubation, the plates with discrete colonies ranging between 30 and 300 were selected [25]. Total viable cell (TVC) was calculated in cfu/g using the formula in Eq. 1.

$$\text{TVC (cfu/g)} = \frac{\text{No of colonies} \times \text{dilution factor}}{\text{Volume of inoculum}} \qquad \text{Eq. 1}$$

2.4 Proximate Analysis of Agro-Waste Materials

Chemical characteristics of the agro-waste carrier materials used in the study were determined using standard methods. Crude protein, crude fat, crude fibre, total carbohydrate, moisture content and ash content were determined using the methods described by [26] and [27].

3. RESULTS

3.1 Hydrocarbon-utilizing bacteria

Eight (8) hydrocarbon-utilizing bacteria were used in the study. The bacterial isolates all grew on Bushnell Haas agar amended with 1% crude oil. The isolates also showed high turbidity when subjected to biodegradability test using turbidometric technique.

3.2 Chemical composition of the carrier materials

Table 1 shows the chemical composition of the different carrier materials used in the study. Soya bran had the highest crude protein (42.61±2.1%). Sugarcane bagasse had the highest carbohydrate content.

Parameters	Saw Dust	Soya Bran	Cassava peel	Corn cob	Bagasse
Moisture content (%)	5.92±0.09	10.11±0.11	8.03±0.89	5.11±0.05	6.34±0.08
Ash content (%)	7.49±0.08	4.08±0.05	9.5±0.7	7.2±0.6	4.3±0.5
Volatile matter (%)	74.28±2.02	ND	ND	ND	ND
Fix carbon (%)	12.34±1.1	ND	ND	ND	ND
Crude fibre	ND	5.22±0.07	ND	ND	ND
Crude protein (%)	2.2±0.09	42.61±2.1	4.8±0.3	4.3±0.3	3.8±0.1
Crude fat	ND	18.37±1.01	0.9±0.02	0.7±0.04	0.6±0.04
Carbohydrate	ND	8.89±0.09	69.6±1.2	68.0±2.3	70.3±1.9

Table 1. Chemical composition of carrier materials used in this study

ND: not determined

3.3 Viability of the bacterial consortium on different carrier materials

Figure 1 shows the growth of the bacterial consortium (Log cfu/g) on different carrier materials over a period of 21 days. The growth of the consortium with almost all the carrier materials peaked at day 7. Figure 2 shows the survival rate of the bacterial consortium on different carrier materials. From the figures, best carrier materials were saw dust (103.6% survival) and corn cob (103.6% survival) followed by soya bran (94.4% survival rate) and cassava peel (94.4% survival rate).

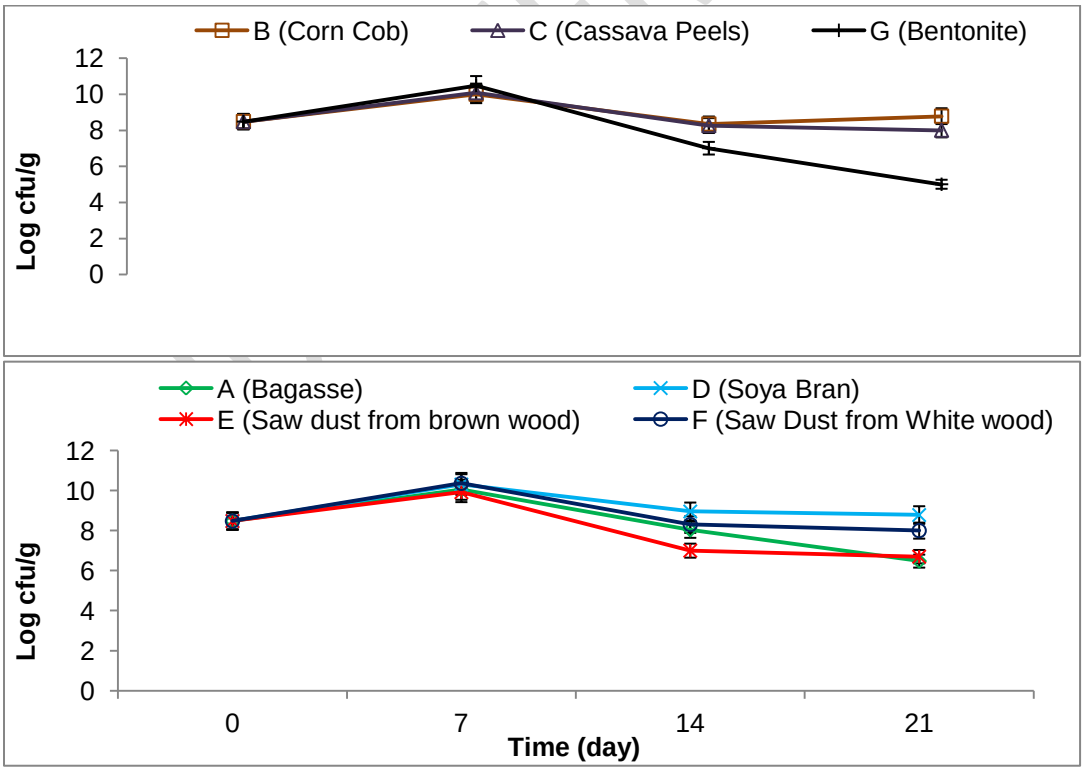

Figure 1: Growth of the hydrocarbon-degrading bacterial consortium immobilized on different carriers

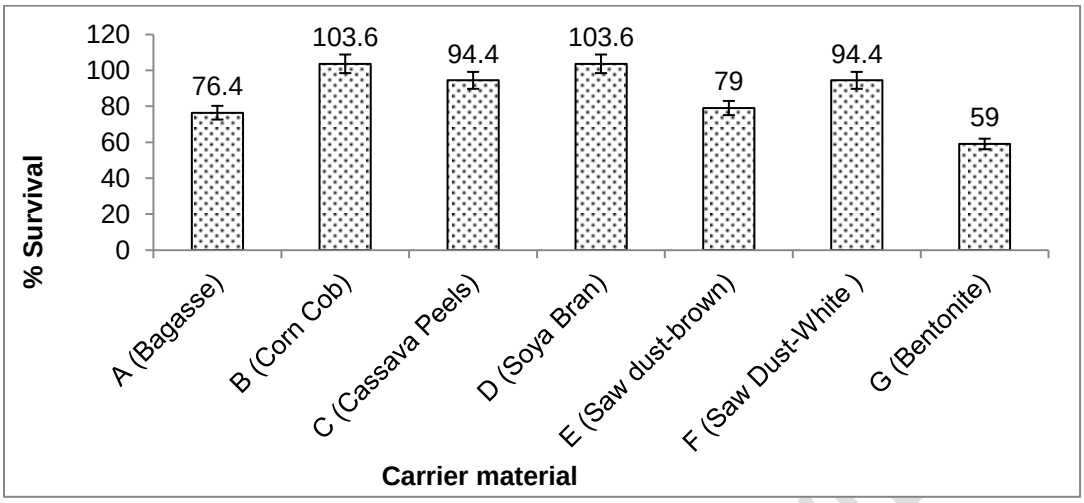

Figure 2: Percentage survival of the bacterial consortium immobilized on the different carrier materials

4. DISCUSSION

This study investigated the viability of hydrocarbon-utilizing bacterial consortium immobilized on different carrier materials. Immobilization enhances the capacity of bacterial isolates to survive and degrade organic contaminants. According to Martin [28] immobilization provides high biomass, provides cell reuse and reduces the costly processes of cell recovery and cell recycle, eliminates cell washout problems at high dilution rates, high flow rates allowing high volumetric productivities, provides suitable micro environmental conditions, improves genetic stability, protects cells against shear damage and improves resistance to toxic chemicals, pH, temperature, solvents and heavy metals.

Immobilization of bacterial cultures has been generally reported to increase their viability than when left in free-living form. Obuekwe and Al-Muttawa [29] immobilized *Arthrobacter* sp., a Gram-negative bacillus isolated from Kuwait oil lakes, using sawdust, Styrofoam or wheat bran, as carriers, under low nutrient conditions and reported the production of stable exopolysaccharide. When they investigated the ability of the immobilized cells to survive and degrade hydrocarbons for 6 weeks at 45 ^{o}C against free suspensions of the same bacterium they discovered that the immobilized cells performed better than the freely suspended cells. Similarly, Quek *et al.* [30] reported the immobilization and performance of *Rhodococcus* sp. F92 on polyurethane foam (PUF) in the bioremediation of petroleum hydrocarbons. They discovered that the immobilized cells were able to degrade a variety of petroleum products such as Arabian light crude, Al-Shaheen crude, diesel and oil slops more than the freely suspended cells.

This study employed the following carrier materials for the immobilization process: Bagasse, cassava peels, corn cob, saw dust, soya bran and benthonite. These carrier materials have been used by different researchers at different times to immobilize bacteria with outstanding result. Most of the reports focussed on single organisms but in this study bacterial consortium were immobilized using these different carrier materials. Bagasse has been used as a carrier for the immobilization of bacterial isolates. Mohammadi and Nasernejad [9] studied the enzymatic degradation of anthracene by the white rot fungus *Phanerochaete chrysosporium* immobilized on sugarcane bagasse. The result was compared with the unimmobilized cell with the immobilized

resulting in higher degradation than the unimmobilized cell. Liu *et al*. [31] in another study investigated the efficiency of sugarcane bagasse as support material for the immobilization of *Bacillus pumilus* HZ-2 and thereafter applied the immobilized cells in the bioremediation of mesotrione contaminated soils. The result showed a better degradation of the contaminant with immobilized cells than the freely suspended cells.

Rivelli *et al*. [32] studied persistence and degrading activity of free and immobilised allochthonous bacteria during bioremediation of hydrocarbon-contaminated soils and reported better degradation with immobilized cells than freely suspended cells. Paliwal *et al*. [33] successfully immobilized bacterial consortium on corn cob with slightly enhanced biodegradation of chlorophenol when immobilized cells were used compared to the freely suspended cells.

The saw dust used as a carrier material in this study showed one of the highest survival rates. Obuekwe and Al-Muttawa [29] immobilized bacterial consortium on saw dust and reported enhanced petroleum hydrocarbon degradation by the immobilized cells compared to the freely suspended cells. Similarly, Hazaimeh *et al*. [34] enhanced crude oil hydrocarbon degradation by self-immobilized bacterial consortium culture on sawdust and oil palm empty fruit bunch. From the discussion, it is obvious that immobilization enhances bacterial survival rate compared to when the bacterial isolates are freely suspended in media. This is important in bioremediation of hydrocarbon contaminated soil as the longer the bacterial cultures survive in the media the higher their chances of degrading the contaminant.

5. CONCLUSION

This study has demonstrated the capacity of the agro-wastes used to effectively retain the viability of hydrocarbon-utilizing bacterial consortium. The study showed that saw dust, corn cob, soya bran and cassava peels can effectively be used as carrier agents in immobilizing hydrocarbon-utilizing bacteria. The result is significant as it shows the possibility of using these carrier materials for biodegradation of hydrocarbon contaminated media. These wastes are easily available and besides their use may help mitigate the cost involved in managing them thereby reducing pollution. Further study on the effectiveness of these immobilized cells in bioremediation of different hydrocarbon contaminants should be carried out.

Conflict of interest

The authors declare that they have no conflicts of interest to this work.

References

1. Kourkoutas Y, Bekatoroua A, Banat, IM, Marchant R, Koutinasa AA. Immobilization technologies and support materials suitable inalcohol beverages production: a review. Food Microbiol. 2004; 21:377–397.
2. Wojcieszyńska D, Hupert-Kocurek K, Guzik U. Influence of the entrapment of catechol 2,3-dioxygenase in κ-carrageenan on its properties. Pol J Environ Stud. 2013;22(12):19–25. http://www.pjoes.com/pdf/22.4/Pol.J.Environ.Stud.Vol.22.No.4.1219-1225.pdf.
3. Guzik U, Hupert-Kocurek K,Marchlewicz A,Wojcieszyńska D. Enhancement of biodegradation potential of catechol 1, 2-dioxygenase through its immobilization in calcium alginate gel. Electron J Biotechnol. 2014;17:83–8. http://dx.doi.org/10.1016/j.ejbt.2014.02.001.

4. Guzik U, Hupert-Kocurek K,Wojcieszyńska D. Immobilization as a strategy for improving enzyme properties—Application to oxidoreductases. Molecules. 2014;19:8995–9018. http://dx.doi.org/10.3390/molecules19078995.
5. Dzionek A, Wojcieszy D, Guzik U. Natural carriers in bioremediation: A review. Electron J Biotechnol. 2016;23:28–36.
6. Kariminiaae-Hamedaani HR, Kanda K, Kato F. Wastewater treatment with bacteria immobilized onto a ceramic carrier in an aerated system. J Biosci Bioeng, 2003;95:128–32. http://dx.doi.org/10.1016/S1389-1723(03)80117-2.
7. Martins SCS,Martins CM, Fiuza LMC. Immobilization of microbial cells: A promising tool for treatment of toxic pollutants in industrial wastewater. Afr J Biotechnol, 2013;12:4412–8. http://dx.doi.org/10.5897/AJB12.2677.
8. Bayat Z, Hassanshahian M, Cappello S. Immobilization of microbes for bioremediation of crude oil polluted environments: A mini review. Open Microbiol J. 2015;9:48–54. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4676050/pdf/TOMICROJ-9-48.pdf.
9. Mohammadi A, Nasernejad B. Enzymatic degradation of anthracene by the white rot fungus *Phanerochaete chrysosporium* immobilized on sugarcane bagasse. J Hazard Mater. 2009;161:534–7. http://dx.doi.org/10.1016/j.jhazmat.2008.03.132.
10. Zhang A, Wang G, Gong G. Immobilization of white rot fungi to carbohydrate-rich corn cob as a basis for tertiary treatment of secondarily treated pulp and paper mill wastewater. Ind Crop Prod. 2017;109: 538-541.
11. Abarian M, Hassanshahian M, Esbah A. Degradation of phenol at high concentrations using immobilization of *Pseudomonas putida* P53 into sawdust entrapped in sodium-alginate beads. Water Sci Technol. 2019;79(7):1387-1396. doi: 10.2166/wst.2019.134.
12. Huang DL, Zeng GM, Jiang XY, Feng CL, Yu HY, Huang GH. Bioremediation of Pb-contaminated soil by incubating with *Phanerochaete chrysosporium* and straw. J Hazard Mater. 2006;134:268–76. http://dx.doi.org/10.1016/j.jhazmat.2005.11.021.
13. Ullah H, Shah AA, Hasan F, Hameed A. Biodegradation of trinitrotoluene by immobilized *Bacillus* sp. YRE1. Pak J Bot. 2010;42:3357–67.
14. Pattanasupong A, Nagase H, Sugimoto E, Hori Y, Hirata K, Tani K. Degradation of carbendazimand 2,4-dichlorophenoxyacetic acid by immobilized consortium on loofa sponge. J Biosci Bioeng. 2004;98:28–33.
15. Sinha A, Pant KK, Khare SK. Studies on mercury bioremediation by alginate immobilized mercury tolerant Bacillus cereus cells. Int Biodeter Biodegr. 2012;71:1–8. http://dx.doi.org/10.1016/j.ibiod.2011.12.014.
16. Wang JY, De Belie N, Verstraete W. Diatomaceous earth as a protective vehicle for bacteria applied for self-healing concrete. J Ind Microbiol Biotechnol. 2012;39:567–77. http://dx.doi.org/10.1007/s10295-011-1037-1.
17. Moreno-Medina DA, Sánchez-Salinas E, Ortiz-Hernández ML. Removal of methyl parathion and coumaphos pesticides by a bacterial consortium immobilized in *Luffa cylindrica*. Rev Int Contam Ambiental 2014;30:51–63. http://scielo.unam.mx/pdf/rica/v30n1/v30n1a5.pdf.
18. Carabajal M, Perullini M, Jobbágy M, Ullrich R, Hofrichter M, Levin L. Removal of phenol by immobilization of *Trametes versicolor* in silica-alginate fungus biocomposites and loofa sponge. Clean Soil Air Water 2015;44:180–8. http://dx.doi.org/10.1002/clen.201400366.
19. Mittal A, Singh P. Isolation of Hydrocarbon degrading bacteria from soils contaminated with crude oil spills. Indian J Expt Biol. 2015;47:760-765.
20. Mnif S, Sayadi S, Chamkha M. Biodegradative potential and characteriza-tion of a novel aromatic-degrading bacterium isolated from a geothermal oil field under saline and thermophilic conditions. Int Biodeterior Biodegrad. 2014;86:258

21. Wedulo A, Atuhaire DK, Ochwo A, Muwanika V, Rwendeire AJJ, Nakavuma JL. Characterization and evaluation of the efficiency of petroleum degrading bacteria isolated from soils around the oil exploration areas in western Uganda. Afr J Biotechnol. 2014;48:4458-4470.
22. Cubitto MA, Gentili AR. Bioremediation of crude oil — Contaminated soil by immobilized bacteria on an agro-industrial waste — Sunflower seed husks. Biorem J. 2015;19:277–86. http://dx.doi.org/10.1080/10889868.2014.995376.
23. Hsu CH, Chu YF, Argin-Soysal S, Hahm, TS, Lo YM. Effects of surface characteristics and xanthan polymers on the immobilization of *Xanthomonas campestris* to fibrous matrices. J Food Sci. 2006; 69(9): 441-448. doi:10.1111/j.1365-2621.2004.tb09928.x.
24. Agu IV, Ibiene, AA, Okpokwasili GC. Effect of micronutrients and macronutrients on the biodegradation of phenol in biological treatment of refinery effluent. Microbiol Res J Int. 2017;18(3):1-12.
25. APHA. Standard Methods for the examination of water and wastewater. American Public Health Association, Washington, DC; 2005.
26. Ezebuiro V, Ogugbue CJ, Oruwari B, Ire FS. Bioethanol production by an ethanol-tolerant *Bacillus cereus* strain GBPS9 using sugarcane bagasse and cassava peels as feedstocks. J Biotechnol Biomater. 2015; 5:213. doi:10.4172/2155-952X.1000213.
27. Ire FS, Ezebuiro V, Ogugbue CJ. Bioresour Bioprocess. 2016; 3:26. https://doi.org/10.1186/s40643-016-0104-x.
28. Martin AM, ed. Bioconversion of waste materials to industrial products. New York: Springer 1998.
29. Obuekwe CO, Al-Muttawa EM. Self-immobilized bacterial cultures with potential for application as ready-to-use seeds for petroleum bioremediation. Biotechnol Lett. 2001;23:1025–32. http://dx.doi.org/10.1023/A:1010544320118.
30. Quek E, Ting YP, Tan HM. *Rhodococcus* sp. F92 immobilized on polyurethane foam shows ability to degrade various petroleum products. Bioresource Technology. 2006; 97(1): 32-8.
31. Liu J, Chen S, Ding J, Xiao Y, Han H, Zhong G. Sugarcane bagasse as support for immobilization of *Bacillus pumilus* HZ-2 and its use in bioremediation of mesotrione contaminated soils. Appl Microbiol Biotechnol. 2015 99:10839–51.
32. Rivelli V, Franzetti A, Gandolfi I, Cordoni S, Bestetti G. Persistence and degrading activity of free and immobilised allochthonous bacteria during bioremediation of hydrocarbon-contaminated soils. Biodegradation. 2013;24:1–11. http://dx.doi.org/10.1007/s10532-012-9553-x.
33. Paliwal R, Uniyal S, Rai JPN. Evaluating the potential of immobilized bacterial consortium for black liquor biodegradation. Environ Sci Pollut Res. 2015;22:6842–53. http://dx.doi.org/10.1007/s11356-014-3872-x.
34. Hazaimeh M, Mutalib SA, Abdullah PS, Kee WK, Surif S. Enhanced crude oil hydrocarbon degradation by self-immobilized bacterial consortium culture on sawdust and oil palm empty fruit bunch. Ann Microbiol. 2014;64:1769–77. http://dx.doi.org/10.1007/s13213-014-0821-3.