Eco. Env. & Cons. 29 (January Suppl. Issue) : 2023; pp. (S109-S116)
Copyright@ EM International
ISSN 0971–765X

DOI No.: http://doi.org/10.53550/EEC.2023.v29i01s.017

Bioremediation of Crude Oil using Hydrocarbon degrading *Pseudomonas luteola* by Immobilization methods

V. Gayathri[1] and K. Krishnaprema[2]*

[1,2]Department of Microbiology, Ethiraj College For Women, Chennai, Tamil Nadu, India

(Received 7 May, 2022; Accepted 20 July, 2022)

ABSTRACT

Bioremediation is a technique which uses living organisms, like bacteria, to convert toxic contaminants into harmless compounds in the environment. As crude oil spills cause a major environmental hazard, methods to clear oil spills using biological systems is the need of the hour. Four different bacteria were isolated from hydrocarbon contaminated soil. Among the four bacteria isolated, *Pseudomonas luteola* was immobilized in four different carriers such as banana peel, banana stem, sediment and gravel due to its hydrocarbon degrading efficiency. The immobilized bacteria were incubated with crude oil along with growth medium and the degradation was studied after 5 days. Degradation was checked with gas chromatography–mass spectrometry, and the efficiency of each biocarriers were noted. It was found that *Pseudomonas luteola* immobilized with banana peel and banana stem were more efficient in degrading the crude oil than the *Pseudomonas luteola* immobilized with sediment and gravel.

Key words : *Biodegradation, Crude oil, Pseudomonas luteola*

Introduction

Crude oil spillage can cause environmental hazards as it takes many years to degrade completely. Though chemical and physical processes clear up oil spill, the marine bacteria play an important role in oil degradation (Della Torre *et al*., 2012). There is an urgent need to find eco-friendly methods to clear oil spills in oceans and land after the recent oil spill (2017), in Ennore, Chennai, Tamil Nadu, India, presented a challenge to the current oil bioremediation techniques. The process of Bioremediation is an effective, economical, and environment friendly and bioremediation can be skilfully performed by controlling the physical factors like moisture, temperature, pH, Oxygen and nutrient levels (D. de Souza Pereira Silva *et al*., 2015). The effective way of bioremediation is immobilization of the bacteria on affordable carriers. The absorption and stability of biocarriers are two major criteria for the selection of a suitable supporting material (Hsu *et al*., 2004). Bacterial immobilization could occur via adsorption, covalent binding, entrapment, and encapsulation. Excellent degradation is enhanced by the high immobilization efficiency of bacterial cells onto the carrier material and the high affinity between the hydrophobic immobilization material and the substrates (Bayat *et al*., 2015; Cassidy *et al*., 1996).

Four different carriers were chosen for bacterial immobilization (banana peel, banana stem, sediment and gravel) due to their availability and economical aspect. Many bacteria were identified as Hydrocarbon degraders including *Pseudomonas* sp) *Rhodococcussp* (Hassanshahian *et al*., 2012;

Hassanshahian *et al*., 2010) and *Alcanivorax* sp (Yakimov *et al*., 2007; Hassanshahian *et al*., 2010). The predominant bacterium from oil-contaminated environment was chosen and was immobilized to four different carriers and was checked for the degradation of crude oil. The use of local microbial strains is preferred as these strains are well adapted, reconciled, and suitable to be propagated in bioremediation processes.

Materials and Method

Oil-contaminated soil samples (including petrol and diesel) were collected from different automobile workshops in Vandavasi, Tiruvannamalai district, Tamil Nadu, aseptically. The soil was transferred into sterile polythene bags and labelled and stored at 4 °C. Sea water was collected from the East Coast of Chennai and was autoclaved. Crude oil was collected from Indian Oil Corporation, Manali, and Chennai and was autoclaved. The diesel used in this experiment was purchased from a local fuel station and stored in dark at ambient temperature throughout the study. Before use, the diesel was sterilized by using the membrane filtration technique.

Isolation of Hydrocarbon degrading bacteria was performed using one gram of diesel-contaminated soil which was mixed in 9 ml of Bushnell Hass (BH) broth. Diesel (0.1ml) was layered on BH agar plates, and 0.1 ml of the soil dilution was plated in BH agar plates and was incubated at room temperature for 3 days. Bacteria having the ability to use oil as carbon and energy source and consequently showing colony formation were taken as HC-degrading bacteria. The isolated bacteria were identified by biochemical methods and VITEK-MS (MALDI-TOF)

Ten grams of each bio carrier material, banana peel and banana stem, was cut into small pieces (nearly 1 cm in dimension), washed in sterile distilled water and dried at 70°C in hot air oven. The sediment and gravel were sterilized in hot air oven. The prepared bio carrier materials were immersed in culture flasks containing 50 ml of BH broth and one ml bacterial culture (10^5 CFU/ml). The culture flasks were incubated at 30 °C for 3 days in a shaker at a speed of 120 rpm. The bacterial strain *Pseudomonas luteola* was grown in batch cultures in 100 ml Erlenmeyer flasks containing 50 ml of BH broth supplemented with 0.5 ml of crude oil. Each flask was added with 0.5 ml of crude oil for 5 days at 37! with rotation of 120 rpm. An uninoculated control flask was incubated in parallel to monitor abiotic losses of the crude oil substrate. All experiments including uninoculated controls were performed in duplicate.

After incubation the remaining oil was extracted by the solvent extraction procedure using a separating funnel by adding equal volume of *N*-hexane (Environmental Protection Agency., 2010). The filtrate which had hydrocarbon residues was analysed using GCMS. To determine the extent of degradation, various HC components were quantified by interpreting the areas of individual peaks and expressed as a percentage of degradation relative to the amount of the corresponding peak remaining in the appropriate abiotic control samples. The degradation of crude oil as a whole was expressed as the percentage of crude oil degraded inrelation to the amount of the remaining fractions in the appropriate abiotic control samples.

Results

Identification of Bacterial Strains

The isolated colonies in the BH agar were found to be *Pseudomonas luteola, Pseudomonas oryzihabitans, Pseudomonas stutzeri* and *Serratiamarcescens* using classical tests. The results of the morphological and biochemical tests carried out to characterize four microorganisms are given in Table 1. Furthermore, the isolated bacteria were confirmed using VITEK-MS (MALDI-TOF).

Four different bacteria were isolated and identified from oil-contaminated soil samples. They were *Pseudomonas luteola*, *Pseudomonas oryzihabitans*, *Pseudomonas stutzeri* and *Serratiamarcescens*. The predominant bacteria *Pseudomonas luteola* was chosen for the degradation of crude oil.

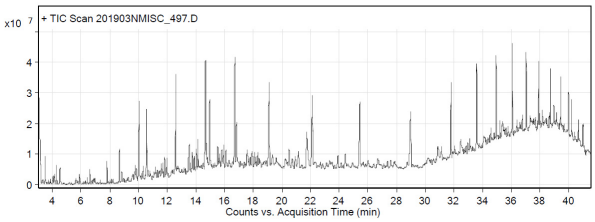
Around 251 compounds were present in the crude oil, and 210 compounds were detected from GC-MS. HC compounds such as hexadecane, tetradecane, pentadecane, heptadecane, nonadecane, tetracosane, and naphthalene 1,3 were detected in sample 1 (Graph 1).

Around 251 compounds were present in sample 2, and 55 compounds were detected from GC-MS. The HC compounds such as hexadecane and nonadecane were detected in sample 2 (Graph 2).

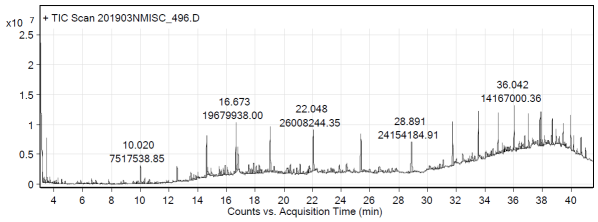
Around 231 compounds were present in sample 3, and 104 compounds were detected from GC-MS. The HC compounds such as hexadecane and

Table 1. Morphological and biochemical identification of the four strains

Items	Isolates			
	C1	C2	C3	C4
Colony Color	White	White	Yellow	Yellow
Colony Surface	Moist	Dry	Moist	Glistening
Gram's Staining	Gram-negative	Gram-negative	Gram-negative	Gram-negative
Motility	+	+	+	+
Catalase Test	-	-	-	+
Urease Test	-	+	+	+
Gelatin Test	+	+	+	+
Glucose Fermentation	+	+	+	+
Lactose Fermentation	-	-	-	-
Citrate Test	+	+	+	+
Gelatin Liquefaction	+	+	+	+
Starch Hydrolysis	+	+	-	-



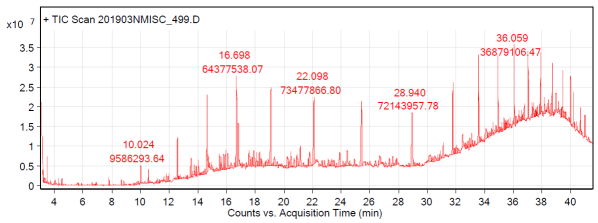
Graph 1. GC–MS profile for sample 1 (crude oil)



Graph 2. GC-MS profile for crude oil sample 2 (crude oil + *Pseudomonas luteola)*

heptadecane were detected in sample 3 (Graph 3).

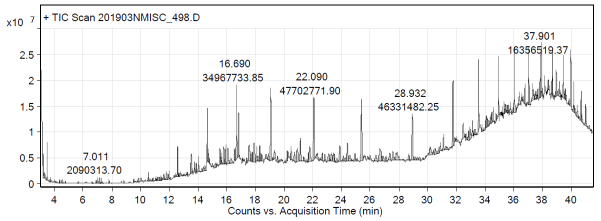
Around 231 compounds were present in the crude oil, and 104 compounds were detected from GC-MS. The HC compounds such as hexadecane, tetradecane, heptadecane, and nonadecane were detected in sample 4 (Graph 4).



Graph 3. GC–MS profile for sample 3 (crude oil + *Pseudomonas luteola*-immobilized bananapeel)

Table 2. Enumeration of *Pseudomonas luteola* for immobilization

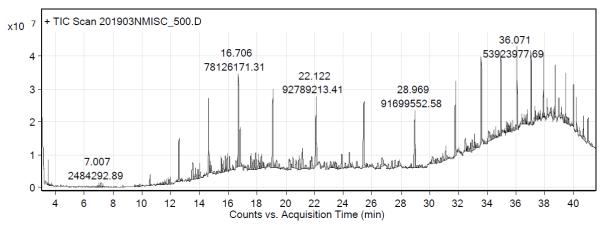
S. No.	Dilutions of Broth			
	10^4	10^5	10^6	Control
1	68	TNTC	TLTC	No growth
2	50	TNTC	TLTC	No growth



Graph 4. GC–MS profile for sample 5 (crude oil + *Pseudomonas luteola*-immobilized banana stem)

Around 337 compounds were present in the crude oil, and 67 compounds were detected from GC-MS. The HC compounds such as hexadecane, pentadecane, heptadecane, nonadecane, and tetracosane were detected in sample 5 (Graph 5).

Around 224 compounds were present in the crude oil, and 215 compounds were detected from



Graph 5. GC–MS profile for sample 5 (crude oil + *Pseudomonas luteola*-immobilized sediment)

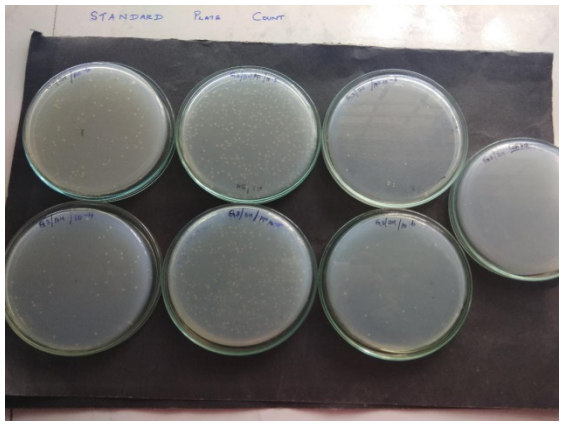


Fig. 1. Standard plate count

Fig. 2. Gelatin Hydrolysis Test

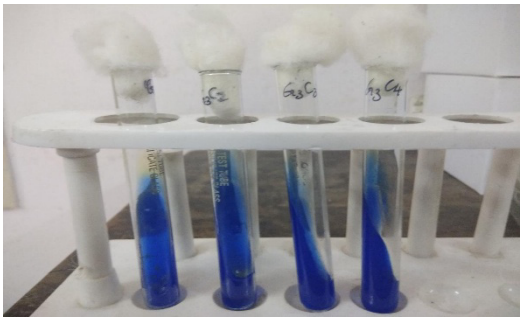


Fig. 3. Starch Test

Fig. 4. Urease Test

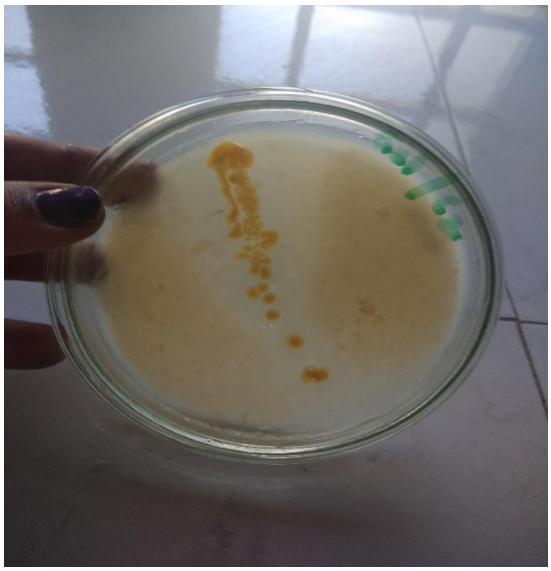


Fig. 5. Citrate Test

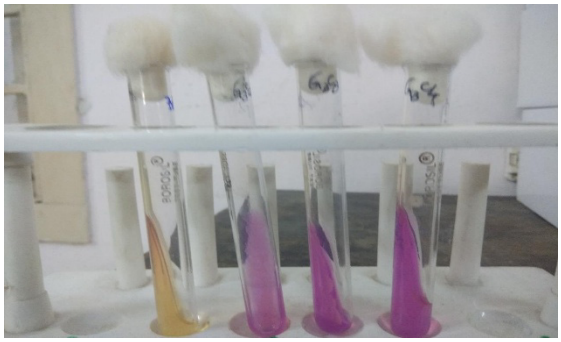


Fig. 6. Sugar Fermentation Test

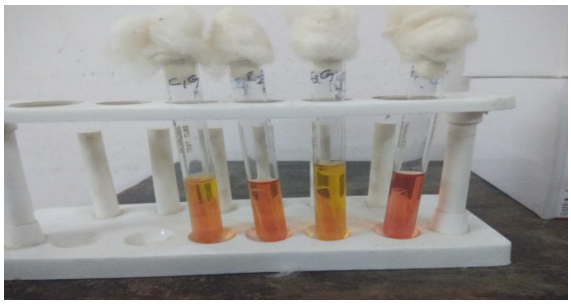
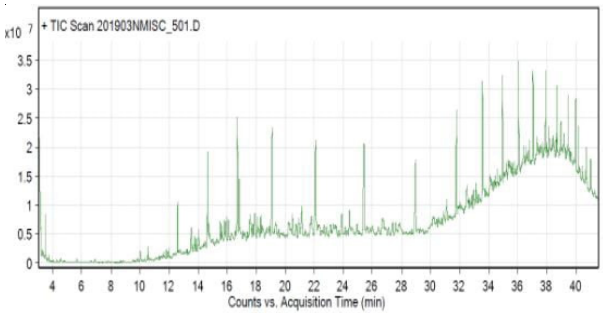


Fig. 7. Immobilized Carriers

GC-MS. The HC compounds such as hexadecane and tetradecane were detected in sample 6 (Graph 6).

Discussion

In this study, the four identified isolates were identified as C1 (*Pseudomonas oryzahabitans*), C2



Graph 6. GC–MS profile for sample 5 (crude oil + *Pseudomonas luteola* immobilized gravel)

(*Pseudomonas stutzeri*), C3 (*Pseudomonas luteola*), and C4 *Serratiamarcescens*). Among these, the predominant isolate was *Pseudomonas luteola*, which was immobilized on four different types of carriers for the remediation of oil-contaminated waters.

Several studies have shown that P*seudomonas* species are superior in crude oil degradation, which could be due to their metabolic diversity, abundance in microbial communities, and their resistance to chemical agents. *Pseudomonas luteola* is a Gram-negative, motile aerobic bacteria. An ideal carrier should be nontoxic, non-polluting, biodegradable, having high cell mass loading capacity, biologically and chemically stable, having a long shelf life, low cost, diffusible by bacteria, easy to handle from media, and easy to regenerate (Chettri *et al.*, 2016; Bayat *et al.*, 2015).

In this study, two samples (banana peel and banana stem) from agricultural waste and two samples (sediment and stones) from the environment were selected as carriers. The GC fingerprints showed that the aliphatic and aromatics were degraded while using *Pseudomonas luteola* immobilized

Table 3. Compounds detected in the crude oil blank/sample 1 from the chromatogram peak list

S. No	Name of the compound	RT	Area	Area %	Area sum %
1	Hexadecane	6.62	7990606.38	7.05	0.25
2	Hexadecane	12.605	81306007.51	71.71	2.5
3	Tetradecane	8.672	23355303.91	20.6	0.72
4	Pentadecane	10.577	56021526.26	49.41	1.72
5	Heptadecane	14.674	101666648.99	89.67	3.13
6	Nonadecane	19.113	107916841.16	95.18	3.32
7	Tetracosane	28.969	102417975.8	90.33	3.15
8	Naphthalene 1,3	9.216	1943207.32	1.71	0.06

Table 4. Compounds detected in sample 2 (crude oil + *Pseudomonas luteola*) from the chromatogram peak list

S. No	Name of the compound	RT	Area	Area %	Area sum %
1	Hexadecane	6.617	1436917.42	5.12	0.2
2	Tetradecane	-	-	-	-
3	Pentadecane	-	-	-	-
4	Heptadecane	-	-	-	-
5	Nonadecane	19.054	24236832.92	86.35	3.42
6	Tetracosane	-	-	-	-
7	Naphthalene 1,3	-	-	-	-

Table 5. Compounds detected in sample 3 (crude oil + *Pseudomonas luteola*-immobilized banana peel) from the chromatogram peak list

S. No	Name of the compound	RT	Area	Area %	Area sum %
1	Hexadecane	12.576	12970878.04	23.23	0.88
2	Tetradecane	-	-	-	-
3	Pentadecane	-	-	-	-
4	Heptadecane	14.637	22188465.51	39.74	1.5
5	Nonadecane	-	-	-	-
6	Tetracosane	-	-	-	-
7	Naphthalene 1,3	-	-	-	-

with banana peel and banana stem. The sharp peaks represent the aliphatics, whereas the short peaks denote the aromatics. The degradation showed nearly less peaks of aliphatic, nonaliphatic, and aromatic peaks in flasks inoculated with strain *Pseudomonas luteola*, which show the ability of this organism in degrading crude oil. The adherence of oil to the bacterial consortium-coated bio carriers is a simple way to boost the substrate uptake speed (Chettri *et al.,* 2016). In the above study, banana peel and banana stem show the adsorption ability of the carriers, and the current study proves that they are good adsorbents of bacteria and crude oil and have degraded crude oil effectively.

In this study, GC–MS showed that the removal of crude oil from contaminated water was more by the application of HC-degrading bacteria immobilized on the carrier in free and immobilized cells. The charge mass ratio for the qualitative ion peaks and the quality control of chromatographic conditions were similar as in the study of Kim *et al.* (2013).

The studies of (Atanaskoviæ *et al*., 2016), show

Table 6. Compounds detected in sample 4 (crude oil + *Pseudomonas luteola*-immobilized banana stem) from the chromatogram peak list

S. No	Name of the compound	RT	Area	Area %	Area sum %
1	Hexadecane	12.578	24710850.6	31.03	1.21
2	Tetradecane	8.666	-	-	-
3	Pentadecane	-	-	-	-
4	Heptadecane	14.648	48616477.73	61.05	2.38
5	Nonadecane	19.092	72635364.91	91.21	3.56
6	Tetracosane	-	-	-	-
7	Naphthalene 1,3	-	-	-	-

Table 7. Compounds detected in sample 5 (crude oil + *Pseudomonas luteola*-immobilized sediment) from the chromatogram peak list

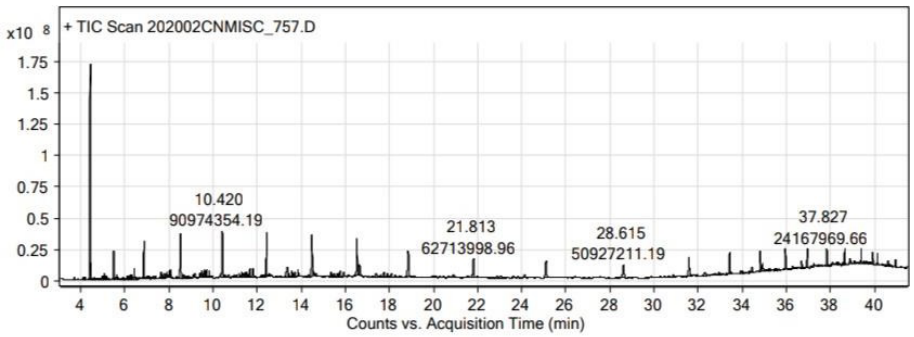
S. No	Name of the Compound	RT	Area	Area %	Area sum %
1	Hexadecane	12.579	31456432.24	30.57	1.35
2	Tetradecane	-	-	-	-
3	Pentadecane	10.554	7398626.53	7.19	0.32
4	Heptadecane	14.651	59822025.02	58.13	2.56
5	Nonadecane	19.108	95761606.37	93.06	4.1
6	Tetracosane	28.97	91699552.58	89.11	3.93
7	Naphthalene 1,3	-	-	-	-

Table 8. Compounds detected in sample 6 (crude oil + *Pseudomonas luteola*-immobilized gravel) from the chromatogram peak list

S. No	Name of the compound	RT	Area	Area %	Area sum %
1	Hexadecane	12.58	18879911.54	24.98	1
2	Tetradecane	14.645	33745693.85	44.65	1.79

Table 9. Compounds detected in crude oil (control)

Compound Name	RT	Molecular Formula	Molecular Weight	Peak Area %
Nonane 4,5	4.1	C11H24	156.31g/mol	57.1
Benzene 1,4	4.17	C10H14	134.22 g/mol	57.1
Nonane, 5	4.98	C12H26	170.33g/mol	57.1
Benzene 1,2,3,5-	4.75	C10H14	134.22g/mol	119.1
Phosphorochloric Acid, dipentyl	4.41	C18H24O4	304.4g/mol	117.1
[4]Cyclohexane,	4.89	C11H22	154.29 g/mol	55.1
Nonane, 5 propyl	4.98	C12 H26	170.33g/mol	57.1
Benzene 1,3	5.12	C11H16	148.24g/mol	119.1



Graph 7. GC–MS profile for sample 6 (crude oil as control)

that *Pseudomonas luteola* can be used in bioremediation of crude oil-contaminated environments very similar to our studies.

Wiesel *et al.* (1993) reported that an immobilized bacterial culture exhibited good growth, and demonstrated equivalent degradation potential of polycyclic aromatic HCs compared with freely suspended cells. The isolated strain degraded crude oil, so it could find a great use in bioremediation technologies.

Conclusion

In this study, four oil-degrading bacterial strains (C1, C2, C3 and C4) were isolated from a HC-contaminated soil sample, Vandavasi, Chennai. They were attributed to *Pseudomonas luteola*, *Pseudomonas stutzeri*, *Pseudomonas oryzihabitans* and *Serratiamarcescens*, respectively. The predominant strain was chosen, which showed the degradation efficiency of crude oil in the free state, as well as immobilizing carriers such as banana peel and banana stem.

Immobilized bacteria performed better than free bacteria in degrading crude oil. After 5 days of biodegradation, the degradation efficiencies of crude oil of the immobilized cells (*Pseudomonas luteola* with banana peel and banana stem) were more when compared to immobilized ones. The degradation process of crude oil by immobilized bacteria was more efficient compared with that of the free bacteria. Biodegradation of the bacterial consortium in bio carriers was the main moving force for crude oil removal, and the bio carrier provided an arena for bacterial cell protection and better biodegradation. Further research should be carried out to optimize the efficiency of the degradation of this strain.

Acknowledgement

I sincerely thank management of Ethiraj College and Department of Microbiology, Ethiraj College for Women for providing financial support and Laboratory facilities to conduct this research work.

References

Atanaskovic, I.M, Jovièiæ Petrovic, J. P., Bioèanin, M.B., Karlièic, V. M., Raièevic, V.B. and Laleviæ, B.T. 2016. Stimulacija degradacije dizela i produkcija biosurfaktanata pomoæu aminoglikozida i nove bakterije *Pseudomonas luteola* pro23 koja razgrađuje naftu. *Hem. Ind.* 70 (2) : 143–150, 2016, doi: 10.2298/HEMIND141127020A.

Bayat, Z., Hassanshahian, M. and Cappello, S. 2015. Immobilization of Microbes for Bioremediation of Crude Oil Polluted Environments: A Mini Review. *Open Microbiol. J.* (9) : 48–54, 2015, doi: 10.2174/1874285801509010048.

Cassidy, M.B., Lee, H. and Trevors, J.T. 1996. Environmental applications of immobilized microbial cells: A review. *J. Ind. Microbiol.* 16 (2) : 79–101, doi: 10.1007/BF01570068.

Chettri, B., Mukherjee, A., Langpoklakpam, J.S., Chattopadhyay, D. and Singh, A.K. 2016. Kinetics of nutrient enhanced crude oil degradation by *Pseudomonas aeruginosa* AKS1 and *Bacillus* sp. AKS2 isolated from Guwahati refinery, India. *Environ. Pollut.* 216 : 548–558, doi: 10.1016/j.envpol.2016.06.008.

Das, N. and Chandran, P. 2011. Microbial Degradation of Petroleum Hydrocarbon Contaminants: An Overview. *Biotechnol. Res. Int.* 1–13, doi: 10.4061/2011/941810.

De Souza Pereira and Silva, D. 2015. Bio-removal of diesel oil through a microbial consortium isolated from a polluted environment. *Int. Biodeterior. Biodegrad.* 97:85–89, doi: 10.1016/j.ibiod.2014.09.021.

Della Torre, C., Tornambè, A., Cappello, S., Mariottini, M., Perra, G., Giuliani, S., Amato, E., Falugi, C., Crisari, A., Yakimov, M.M. and Magaletti, E. 2012. Modulation of CYP1A and genotoxic effects in European seabass (*Dicentrarchus labrax*) exposed to weathered oil: a mesocosm study. *Mar Environ Res.* 76 : 48-55. doi: 10.1016/j.marenvres.2011.08.006. Epub 2011 Sep 8. PMID: 21963215.

Environmental Protection Agency, 2010. Method 1664B: n-Hexane Extractable Material and Silica Gel Treated n-Hexane Extractable Material by Extraction and Gravimetry. *United States Environ. Prot. Agency*, no. February, 2010, [Online]. Available: https://www.epa.gov/sites/production/files/2015-08/documents/method_1664b_2010.pdf.

Hassanshahian, M., Emtiazi, G. and Cappello, S. 2012. Isolation and characterization of crude-oil-degrading bacteria from the Persian Gulf and the Caspian Sea. *Mar. Pollut. Bull.* 64 (1) : 7–12, doi: 10.1016/j.marpolbul.2011.11.006.

Hassanshahian, M., Emtiazi, G., Kermanshahi, R.K. and Cappello, S. 2010. Comparison of oil degrading microbial communities in sediments from the Persian Gulf and Caspian Sea. *Soil Sediment Contam.* 19 (3) : 277–291, doi: 10.1080/15320381003695215.

Hsu, C.H., Chu, Y.F., Argin-Soysal, S., Hahm, T.S. and Lo, Y.M. 2004. Effects of surface characteristics and xanthan polymers on the immobilization of *Xanthomonas campestris* to fibrous matrices. *J. Food Sci*. 69(9): 441–448, doi: 10.1111/j.1365-2621.2004.tb09928.x.

Kim, D.W., Suhaimi, M.A., Kim, B.M., Cho, M.H. and Chen, F.F. 2013. Rough cut machining for impellers with 3-axis and 5-axis NC machines. *Lect. Notes Mech. Eng.* 7: 609–616, doi: 10.1007/978-3-319-00557-7_50.

Wiesel, S. M. Wübker and Rehm, H.J. 1993. Degradation of polycyclic aromatic hydrocarbons by an immobilized mixed bacterial culture. *Appl. Microbiol. Biotechnol.* 39 (1) : 110–116, doi: 10.1007/BF00166858.

Yakimov, M.M., Timmis, K. N. and Golyshin, P.N. 2007. Obligate oil-degrading marine bacteria. *Current Opinion in Biotechnology.* 18(3) : 257–266. https://doi.org/10.1016/j.copbio.2007.04.006