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Biological Conversion of Normal Chain Octadecane by Native Microbial Consortia

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Abstract

Long-chain alkanes are a major constituent of crude oils and their conversions into other compounds are of interest depending on the specific application. Here, five native microbial consortia obtained from petroleum polluted sites were examined for biological conversion of n-octadecane as a representative of long chain alkanes. The experiments were implemented in 250 mL flasks containing 0.5 g n-octadecane in 40 mL culture media kept on a shaker at 160 rpm and 30 ℃ for one week. A pure culture of Psedumonas putida was inoculated at the same condition for comparison. Amongst the consortia, ABN52 imposed more obvious changes on n-octadecane. The GC-MS analysis of daily samples showed the appearance of lighter branched compounds at the first and second days of incubation but disappeared in the following days. At the end of incubation time up to 20 (w/w%) of the initial substrate was turned into polyhydroxyalkanoates (PHAs). It also suggested higher activity of the consortia compared to the pure culture of Psedumonas putida.

Keywords: *Bioconversion, Aalkanes, Polyhydroxyalkanoate, PHA, Pseudomonas Putida*

1. Introduction

Biotechnology was introduced into the oil industry a few decades ago. So far, some progress has been made going through applications for microbial enhanced oil recovery (MEOR) along with improvements in biological soil and water treatment technologies, e.g. bioremediation processes. Some other areas are succeeding but not applicable yet. These include: bio-desulphurization, bio-denitrogenation, bio-demetallization and bio-upgrading of crude oils and intermediates [1]. Others that are not often mentioned thus far may include biocracking of heavy hydrocarbons, biopolymerization of small chain molecules and bio-dehydrogenation.

Crude oil and its fractions contain thousands of different hydrocarbons which could be categorized as alkanes, aromatics, resins and asphaltenes [2-3]. Due to the large variety of components in hydrocarbon oils and their complexity, biotechnology faces many difficulties in this area [1]. Practical implementation of biotechnology in the oil industry undoubtedly needs an understanding of what is happening to the individual

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compounds or hydrocarbon groups during a bioconversion process.

Alkanes are a major part of oil compounds, overwhelming several researches on their biological degradation and conversion [4-6]. Alkanes are linear or branched saturated hydrocarbons known as paraffins. They are good carbon sources for microorganisms and so many organisms utilize a proper mechanism for their consumption [7-9]. Many microorganisms are able to grow on linear alkane**s** by virtue of alkane hydroxylases. Alkane**s** are first converted to 1-alkanols which are then further metabolized to fatty acids by alcohol and aldehyde dehydrogenases [10-11]. Das and Chandran (2011) reported the efficiency of alkanes biodegradation in the range of 6% to 82% by soil fungi, 0.13% to 50% by soil bacteria and 0.003% to 100% by marine bacteria based on other research works [2].

A large number of studies report that low molecular weight alkanes are biologically most rapidly converted than the heavier ones due to their low hydrophobicity [12]. Therefore, many researches have been done on short and medium chain length *n*-alkanes that are easily degraded and mineralized. Here, little information is available on bioconversion of longer chain length *n*-alkanes (octadecane and higher) [13-14]. Also, most of the studies are carried out by pure cultures on different individual alkanes. However, while dealing with alkanes as a part of petroleum oils, mixed cultures may carry out more extensive biodegradation and bioconversion processes than pure cultures due to broad enzymatic capabilities [15-17].

Microbial cultures containing strains of various genera have been isolated in hydrocarbon-contaminated soil, water or sediment samples. This signifies that each strain or genus has a specific role in the degradation process [18]. Rambeloarisoa *et al*. (1984) demonstrated a consortium of 8 strains made up of 6 genera for effective degradation of crude oil. However, when 3 strains were removed from the consortium, the effectiveness of the mixed culture was remarkably reduced [19].

This research investigates the impacts of five native consortia on biological conversion of normal chain octadecane as a representative of long chain alkanes. The consortia were already prepared from petroleum polluted sites for the purpose of biological conversion of a heavy hydrocarbon cut. The main objective of the research is to understand the impact of the consortia on bioconversion of long chain alkanes of heavy hydrocarbon oils.

2. Materials and methods

2-1. Chemicals

Normal chain octadecane (99% of purity), n-hexane and dichloromethane (99% of purity) of Merck products were used for the experiments. Other chemicals used for preparation of culture media were also obtained from Merck.

2-2. Microorganisms

Five native microbial consortia were supplied by Energy and Environment Laboratory of Materials and Energy Research Center of Iran. The consortia were prepared by sampling from the oily contaminated soils and were already adapted with a heavy hydrocarbon cut from Tehran Refinery containing a complex

mixture of alkanes, aromatics and resins. The consortia were ARN13 from Arak Refinery, ABN52 from Abadan Refinery, SHN110 and SHN112 from Shiraz Refinery and ISN205 from Isfahan Refinery. A pure culture of *Pseudomonas putida* which was isolated from oil contaminated sites by Research Institute of Petroleum Industry (RIPI) of Iran was also used for comparison. This bacterium was chosen because of its high occurrence in oil polluted environments.

2-3. Culture media

The mineral salts medium (MSM) used for the growth of native consortia was the BH medium (Bushnell and Hass) with minor modifications [20]. It contained (g/L): 2.75 K_2HPO_4, 2.25 KH_2PO_4, 1 $(NH_4)_2SO_4$, 0.2 $MgSO_4.7H_2O$, 0.1 NaCl, 0.02 $FeCl_3.6H_2O$, 0.01 $CaCl_2.2H_2O$. The pH of the culture solution was adjusted at 6.8-7.

2-4. Bioconversion of n-octadecane by the native consortia

Duplicate batch cultures were grown in 250 mL Erlenmeyer flasks containing 40 mL MSM supplemented with 0.5 g n-octadecane (1.25 w/v%) as the only carbon source. One mL of each consortium (ARN13, ABN52, SHN110, SHN112, ISN205) and *Pseudomonas putida* were inoculated into the flasks. Incubation was performed at 30˚C on a rotating shaker at 160 rpm for one week. An un-inoculated flask also served as the blank. At the end of the designated time, the culture media were inspected by visual observations for the growth of microorganisms. Some of the samples were selected for further examinations, based on visual alterations of the aqueous and organic phases. The organic phases of the selected samples were then separated from the media by solvent extraction methods. Also, white granules were precipitated during the extraction procedure. The separated organic phases were weighed and analyzed by Gas Chromatography Mass Spectroscopy (GC-MS) in order to detect any biological conversion of n-octadecane. The deposited granules were analyzed by FTIR spectroscopy for structural analysis.

2-5. Daily assessment of n-octadecane bioconversion

Here, the biological conversion of n-octadecane was assessed over time in a daily experimental procedure. The experiment was applied on one of the consortia with the greater activity on n-C_{18} conversion. Based on the result of previous section 3.4, consortium ABN52 had the most significant bioconversion effect on n- C_{18} and therefore was selected for the daily assessment. The consortium was cultured on n- C_{18} at the same conditions as the previous experiment and incubated at 30ºC for daily inspection up to 7 days. Here, 21×250 mL flasks were prepared so that 14 flasks were with inoculations and 7 flasks without inoculations as blanks. Each day, 3 flasks were removed from the incubator (two inoculated flasks and one blank) for the experimental tests. The organic phases (residual oils) were separated from the aqueous phases by solvent extraction methods. The organic phases were then analyzed by GC-MS.

2-6. Extraction of the organic phases

The solvent extraction method was used for separation of the organic phases from aqueous phases. First, the pH was decreased to 3 by HCl. Then, 60 mL n-hexane was added into the solution in a decanter funnel in 3 steps, shaken well and allowed to settle each time. Three separate phases appeared; an aqueous phase, an organic phase and an intermediate thin layer phase containing cell debris. The organic phases were driven off and dichloromethane (DCM) was added into the decanter as the second extraction solvent. The hexane-extracts and DCM-extracts were weighed after evaporation. Also, white granules were deposited during the extraction procedure. The deposited granules were analyzed by FTIR spectroscopy in order to identify the functional groups.

2-7. Instrumental analysis and identification of products

The extracted organic phases were analyzed by a GC-MS instrument, Agilent Technologies model 6890 N equipped with a mass selective detector model 5973 Network, column HP-5 (30 m length and 0.25 mm width). The temperature was programmed from 100°C to 300°C at 10°C/min increment and the injected temperature was 280°C. FTIR instrument, Bruker model Vector 33 was used to identify the white granules produced during bioconversion of n-C_{18} by the native consortia.

3. Results and discussion

3-1. Comparing the activity of the native consortia

The visual observation of one week incubation of five microbial consortia in n-C_{18} revealed that ABN52 and ISN205 imposed more significant changes. The organic phases of the samples were yellow and the aqueous phases were yellowish milky. Fig. 1 shows the substrate reductions and the produced granules after bioconversion of n-C_{18} by the selected consortia and *Pseudomonas putida*. As can be seen, the average substrate reductions by ABN52 and ISN205 were 48.2% and 22.9 (w/w%) and the produced granules were 20.4% and 4.8 (w/w% of initial substrate), respectively. Whilst *Pseudomonas putida* produced the least deposited solids compared to the consortia (1.1%). This reveals the higher activity of the native consortia on bioconversion of n-C_{18} at the examined condition compared to *Pseudomonas putida*.

The GC-MS analysis of the extracted organic phases of the blank and the biotreated samples did not show notable discrepancy (the chromatograms are not shown here). This suggests that the intermediate products produced in the initial days may have been removed or converted to white deposits in the following days. The daily experiments were therefore set up to investigate it.

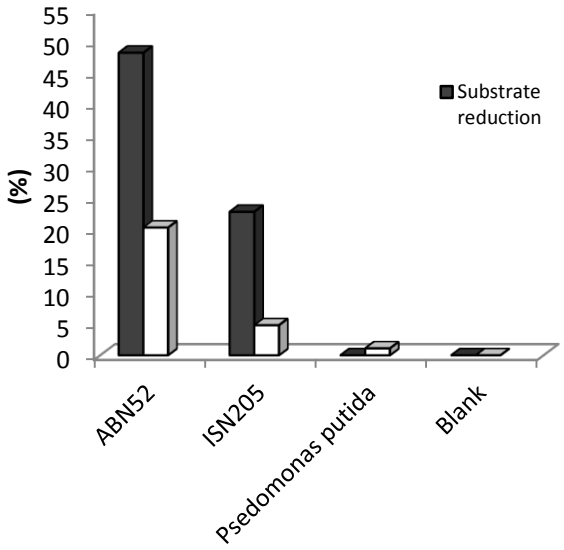

Figure 1. The substrate reduction and the amount of produced granules after bioconversion of n-octadecane by different microorganisms after seven days incubation at 30°C.

3-2. Daily assessment of n-octadecane bioconversion

The visual observations of ABN52 culture media of the daily experiment flasks showed that both organic and aqueous phases gradually turned into yellow from white. This indicated the progress of biological activities which could be due to bioconversion, biodegradation or both. Fig. 2a shows the GC-MS chromatogram of the blank after seven days incubation. The largest peak (No. 5) in the chromatogram relates to n-C_{18} and the other smaller peaks are identified as pentadecane,7-methyl (peak No. 1), tetradecane,5-methyl (peak No. 2), 2-piperidinone-1methyl (peak No. 3) and heptadecane,3-methyl (peak No. 4) which are the impurities of the substrate. Fig. 2 (b to e) represents the daily chromatograms of the organic phases after biotreatment of n-C_{18} by consortium ABN52.

Fig. 2b shows that the impurities disappeared after 1 day while two other peaks (No. 1 and 2) appeared at 11.73 min and 11.83 min retention times. The Wiley digital library of GC-MS instrument identified the peaks as hexadecane and nonadecane (~ 60% probability) for peaks 1 and 2, respectively. Since the identification probability of nonadecane is low and it cannot appear before n-octadecane, it is probably a lighter branched octadecane. This indicates the appearance of lighter compounds after 1 day incubation of n-C_{18}. However, these peaks disappeared in the second day chromatogram (Fig. 2c). Only a small peak at 12.38 min is observable which is not identified by GC-MS. The chromatograms of the following days were identical to that of the blank. The seventh day chromatogram is only shown here (Fig. 2d).

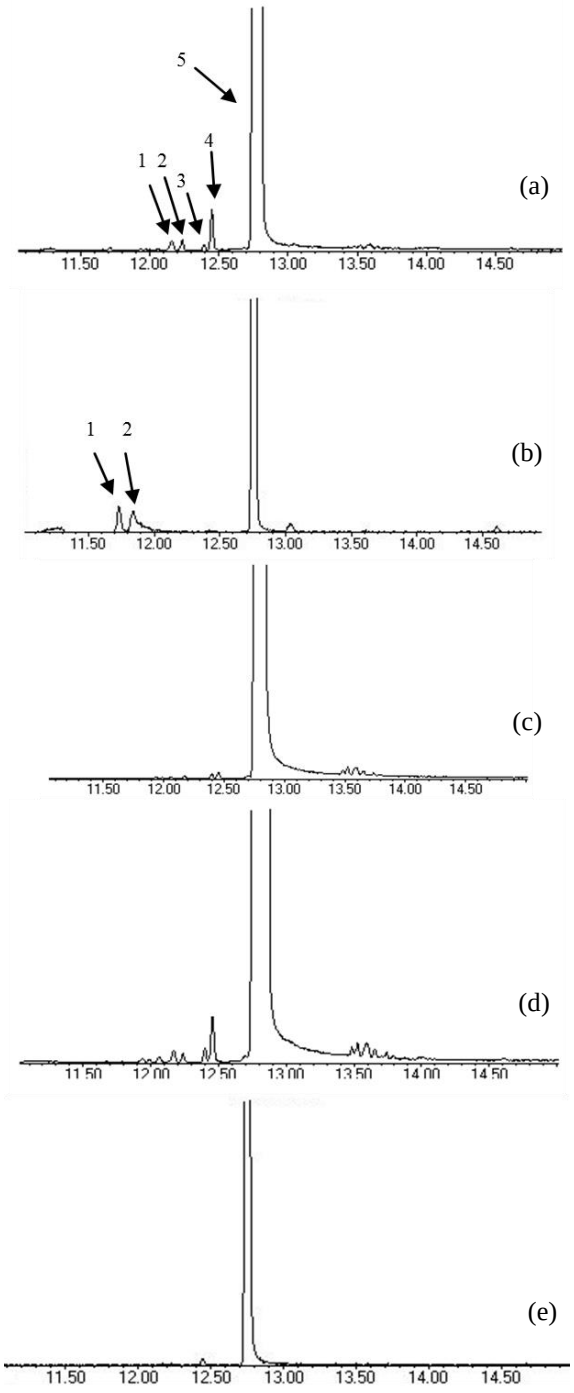

Figure 2. The GC-MS chromatograms of the extracted organic phases after biotreatment of n-octadecane by ABN52: the blank (a) peak 1: pentadecane, 7-methyl, peak 2: tetradecane, 5-methyl, peak 3: 2-piperidinone-1methyl, peak 4: heptadecane, 3-methyl, peak 5: n-C_{18}; first day incubation (b) peak 1&2: lighter branched octadecane; second day incubation (c); third day incubation (d); seventh day incubation (e).

3-3. FTIR analysis

The FTIR spectra of the produced granules from bioconversion of n-C_{18} by consortia ABN52 and ISN205 are shown in Fig. 3. The peaks were observed from 4000-400 cm^{-1} to identify the functional groups of the granules. The spectrum of ABN52 shows characteristic absorption bands for –C–O–C– stretching vibration at 1097 cm^{-1} and C–H stretching bands at 2854 cm^{-1}, 2927 cm^{-1} and 2966 cm^{-1}. The peak at 1634 cm^{-1} could be due to C=O amide compounds, salts of carboxylic acids or C=C band. The peak in the region of 3410 cm^{-1} corresponds to O-H stretching. The FTIR spectrum confirms the presence of alcohols, ether and ester bands.

The spectrum of ISN205 also indicates similar results with some differences in the exact peak locations. This is due to the extent of crystallinity of PHA and the polymer chain length [21-22]. The ISN205 spectrum shows characteristic absorption bands for–C–O–C– stretching vibration at 1049 cm^{-1} and 1156 cm^{-1}. The peak at 1746 cm^{-1} indicates C=O band and C–H stretching bands at 2852 cm^{-1}, 2926 cm^{-1} and 1376 cm^{-1}. The peaks of the FTIR spectra were similar to the peaks obtained by Hong *et al.* and Arcos-Hernandez *et al.* indicated the presence of PHAs [21-22]. As described by Lageveen *et al.* and Odian *et al.*, the produced alcohols from biodegradation of alkanes change to carboxylic acids which then form polyester monomers and finally convert to PHAs [23-24].

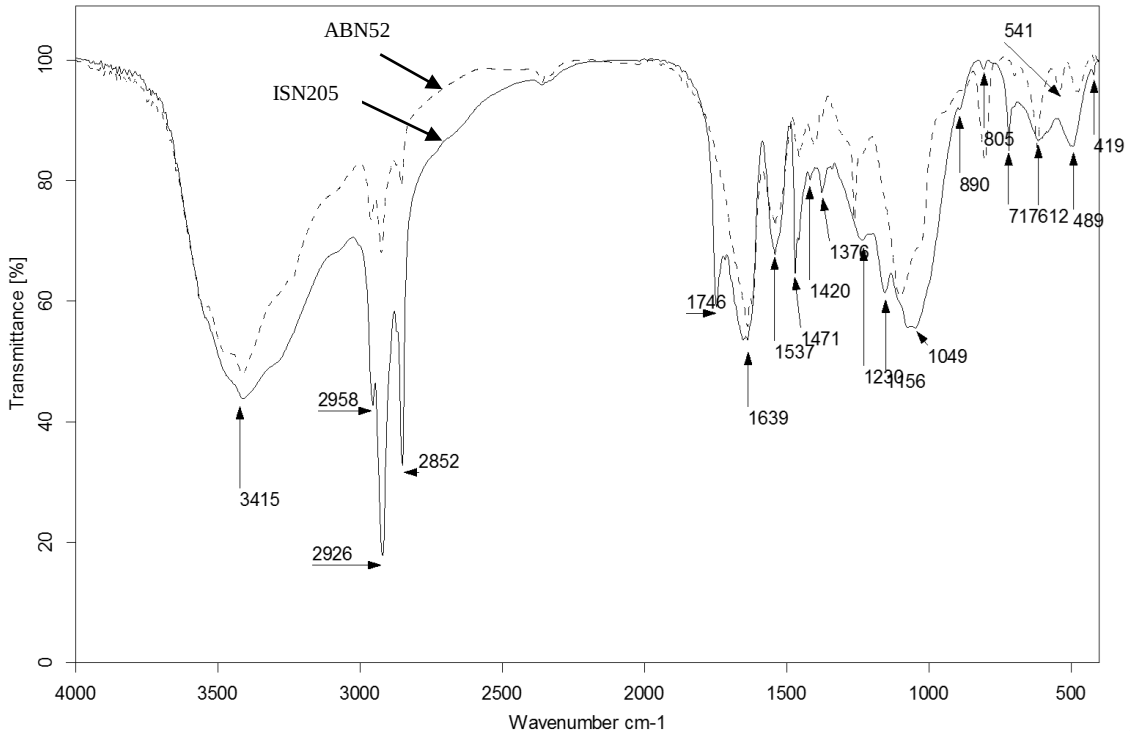

Figure 3. The FTIR spectra of the deposited granules produced from consortium ABN52 and ISN205 during bioconversion of n-octadecane.

4. Conclusions

Five native microbial consortia which were prepared from the oily contaminated soils were tested to evaluate bioconversion of n-C_{18} as the representative of long chain alkanes. Initial experiments showed that consortia ABN52 and ISN205 produced greater impact on n-C-18 than the others. The subsequent experiment revealed that most changes happened in the first two days of incubation. Compounds lighter than n-C_{18} which appeared in the first one or two days vanished in the subsequent days. The produced intermediates were converted to polyhydroxyalkanoates by polymerization and accumulated inside cells. According to literature [25-27], this happens at high F/M (Feed/Microorganism) ratio and lack of nutrients which were also the case here.

During the experiments, about 48% of the substrate (1.25 w/v%) was assimilated by ABN52 consortium and 20 (w/w%) of the initial substrate was converted to PHAs. This is equivalent to 2.42 g/L PHAs production by the consortium. The results were almost similar to that of other researchers. Santhanam *et al.* in 2010 obtained a maximum PHAs content of about 4.14 (g/L) by 2% inoculums of *A. eutrophus* on glucose after 48 h [28]. Bajpai *et al.* in 1998 showed that the octadecane uptake (o.2 w/v%) by *Acinetobacter calcoaceticus* S19 was 70% during growth time (4 h), of which 60% were incorporated into cell and 40% was oxidized [13].

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References

[1] Vazquez-Duhalt, R. and Quintero-Ramirez, R., "Studies in surface science and catalysis, Petroleum Biotechnology Developments and Perspectives", *Elsevier*, **151**, 29 (2004).

[2] Das, N. and Chandran, P., "Microbial Degradation of Petroleum Hydrocarbon Contaminants, an Overview", *Biotechnol. Res. Int*., **2011** (2011).

[3] Yanto, D. H. Y. and Tachibana, S., "Biodegradation of petroleum hydrocarbons by a newly isolated *Pestalotiopsis* sp. NG007", *Int. Biodet. Biodeg.*, **85**, 438 (2013).

[4] Labinger, J. A. and Bercaw, J. E., "Understanding and exploiting C-H bond activation", *Nature*, **417**, 507 (2002).

[5] Tapilatu, Y., Acquaviva, M., Guigue, C., Miralles, G., Bertrand, J. C. and Cuny, P., "Isolation of alkane-degrading bacteria from deep-sea Mediterranean sediments", *Lett. App. Microbiol.*, **50** (2), 234 (2010).

[6] Roj, F., "Minireview Degradation of alkanes by bacteria", *Environ. Microbiol.*, **11** (10), 2477 (2009).

[7] Das, K. and Mukherjee, A. K., "Crude petroleum-oil biodegradation efficiency of *Bacillus subtilis* and *Pseudomonas aeruginosa* strains isolated from a petroleum-oil contaminated soil from North-East India", *Biores. Technol*., **98**, 1339 (2007).

[8] Alzaga, R., Montuori, P., Ortiz, L., Bayona, J. M. and Albaige´s, J., "Fast solid-phase extraction-gas chromatography-mass spectrometry procedure for oil fingerprinting - Application to the Prestige oil spill", *J. Chromatography A*, **1025**, 133 (2004).

[9] Ta-Chen, L., Chang, J. Sh. and Young, C. C., "Exopolysaccharides produced by *Gordonia alkanivorans* enhance bacterial degradation activity for diesel", *Biotechnol. Lett.*, **30**, 1201 (2008).

[10] Van Beilen, J. B., Funhoff, E. G., Van Loon, A., Just, A., Kaysser, L., Bouza, M., Holtackers, R., Röthlisberger, M., Li, Z. and Witholt, B., "Cytochrome P450 Alkane Hydroxylases of the CYP153 Family Are Common in Alkane-Degrading Eubacteria Lacking Integral Membrane Alkane Hydroxylases", *Appl. Environ. Microbiol.*, **72**, 59 (2006).

[11] Hassanshahian, M., Ahmadinejad, M., Tebyanian, H. and Kariminik, A., "Isolation and characterization of alkane degrading bacteria from petroleum reservoir waste water in Iran (Kerman and Tehran provenances)", *Marine. Pollution. Bulletin.*, **73**, 300 (2013).

[12] Balba, M. T., Al-Awadhi, N. and Al-Daher, R., "Bioremediation of oil-contaminated soil: microbiological methods for feasibility assessment and field evaluation", *J. Microbiol. Methods.*, **32**, 155 (1998).

[13] Bajpai, U., Kuhad, R. C. and Khanna, S., "Mineralization of [C $_{14}$] octadecane by *Acinetobacter calcoaceticus S19*", *J. Microbiol.*, **44**, 681 (1998).

[14] Lal, B. and Khanna, S., "Mineralization of [C_{14}] octacosane by *Acinetobacter calcoaceticus* S30", *J. Microbiol.*, **42**, 1225 (1996).

[15] Ghazali, M. F., Zaliha, N. R., Abdul, R. N., Salleh, A. B. and Basri, M., "Biodegradation of hydrocarbons in soil by microbial consortium", *Int. Biodet. Biodegr.*, **54**, 61 (2004).

[16] Gerdes, B., Brinkmeyer, R., Dieckmann, G. and Helmke, E., "Influence of crude oil on changes of bacterial communities in Arctic sea-ice", *FEMS Microbiol. Ecology.*, **53**, 129 (2005).

[17] Ghollami, M., Roayaei, M., Ghavipanjeh, F. and Rasekh, B., "Bioconversion of Heavy Hydrocarbon Cuts Containing High Amounts of Resins by Microbial Consorsia", *Pet. Environ. Biotechnol.*, **4**, 139 (2013).

[18] Varjani, S. J. and Upasani, V. N., "Comparative Studies on Bacterial Consortia for Hydrocarbon Degradation", *Int. J. Innov. Res. Sci., Eng. Technol.*, **2** (10), 5377 (2013)

[19] Rambeloarisoa, E., Rontani, J. F., Giusti, G., Duvnjak, Z. and Bertrand, J. C., "Degradation of crude oil by a mixed population of bacteria isolated from sea-surface foams", *Mar. Biol.*, **83**, 69 (1984).

[20] Bushnell, L. D. and Hass, H. F., "The utilization of certain hydrocarbons by microorganisms", *J. Bacteriol.*, **41**, 653 (1941).

[21] Hong, K., Sun, Q., Tian, W. D., Chen, G. Q. and Huang, W., "A rapid method for detecting bacterial polyhydroxyalkanoates in intact cells by Fourier-transform infrared spectroscopy", *Appl. Microbiol. Biotechnol.*, **51**, 523 (1999).

[22] Arcos-Hernandez, M., Gurieff, N., Pratt, S., Magnusson, P., Werker, A. and Vargas Lant, P., "Rapid quantification of intracellulal PHA using infrared spectroscopy: An application in mixed cultures", *Biotechnol.*, **150**, 372 (2010).

[23] Lageveen, R. G., Huisman, G. W., Preusting, H., Ketelaar, P., Eggink, G. and Witholt, B., "Formation of Polyesters by *Pseudomonas oleovorans*: Effect of Substrates on Formation and Composition of Poly-I-3-Hydroxyalkanoates and Poly-1-3-Hydroxyalkanoates", *Appl. Environ. Microbiol.*, **54** (12), 2924 (1988).

[24] Odian, G., Principles-of-Polymerization, 4rd ed., Wiley J & Sons, New Jersey, USA, p. 40 (2004).

[25] Janarthanan, O. M., Yu, Y., Laycock, B., Werker, A. and Pratt, S., "Fractionation of microbial populations in a PHA accumulating mixed culture and associated PHA content and composition", *Int. J. Biol. Macromol*., **71**, 53 (2014).

[26] Laycock, B., Halley, P., Pratt, S., Werker, A. and Lant, P., "The chemomechanical properties of microbial polyhydroxyalkanoates", *Prog. Poly. Sci.*, **39**, 397 (2014).

[27] Hori, K., Abe, M. and Unno, H., "Production of triacylglycerol and poly (3-hydroxybutyrate-co-3-hydroxyvalerate) by the toluene-degrading bacterium *Rhodococcus aetherivorans* IAR1", *J. Biosci. Bioengin.*, **108**, 319 (2009).

[28] Santhanam, A. and Sasidharan, S., "Microbial production of polyhydroxy alkanotes (PHA) from *Alvaligenes* spp. and *Pseudomonas oleovorans* using different carbon sources", *African J. Biotechnol.*, **9**, 3144 (2010).