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Research note

Anaerobic Bioconversion of Heavy Hydrocarbons Using Native Consortia

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Abstract
This research work is concerned with the bioconversion of a heavy hydrocarbon cut from a petroleum refinery using native anaerobic consortia. The heavy cut is taken from one of the end-cuts of the vacuum distillation column of Tehran refinery which is normally fed into Isomax unit (a catalytic cracking process) called Isofeed. The consortia for this study were prepared from the petroleum-contaminated sludge and soils from Abadan, Shiraz, Isfahan and Tehran petroleum refineries of Iran. Nine microbial samples were collected (A, B, C,…and I) and examined. Microorganisms were cultivated in an anaerobic medium to which Isofeed from Tehran refinery was added under anaerobic conditions. After twice sub-culturing the samples, the oil was separated from the liquid and examined to identify its bioconversion extent. For this purpose, a column chromatograph method was used to separate saturates, aromatics and resins content of the oil. The results demonstrate that consortia A and C were able to increase the saturated and aromatics fractions of the oil and reduce the resins content of the oil. The saturated fractions of the bio-treated oil were also analyzed by a GC-MS analytical instrument. The GC-MS results revealed that the amount of normal chain hydrocarbons are reduced and the branched and cyclic hydrocarbons are increased.

Keywords: *Anaerobic, Bioconversion, Petroleum, Hydrocarbons, Native bacterial consortia*

1. Introduction
Anaerobic bacteria are usually considered to be any organism that does not require oxygen for growth and may even die in its presence. Microorganisms that degrade hydrocarbons anaerobically can be divided into two groups, at least in relation to molecular oxygen [1]. The first group includes facultative anaerobes, which are normally associated with anaerobic condition but can grow under aerobic condition as well [2], such as nitrate, iron, and manganese reducing micro-organisms. The second group comprises obligate anaerobes that can not grow in the presence of oxygen, such as sulfate-reducers [3, 4]. It has been proposed that differences in oxygen tolerance among anaerobes may be related to the effectiveness of defense mechanisms possessed by bacteria against toxic products of oxygen reduction [5].

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Metabolic reduction of oxygen results in the production of highly toxic and reactive oxygen species, such as superoxide anion (O_2^{-}), hydrogen peroxide (H_2O_2) and the hydroxyl radical $(^{\bullet}OH)$ [6]. Most aerobes have developed appropriate protective mechanisms to overcome the toxic effect of hydrogen peroxide and the superoxide anion [7].

It is well known that petroleum hydrocarbons can be degraded by microorganism under aerobic and anaerobic condition [3]. The driving force for petroleum biodegradation is the ability of microorganisms to utilize hydrocarbons to satisfy their cell growth and energy needs [8]. As alkanes are the major components of petroleum, numerous studies have been conducted on their biodegradability by different microorganisms. However, most of the earlier studies have focused on aerobic degradation, in which alkanes are initially oxidized to alcohols by monooxygenases [9]. In contrast, much less is understood about the mechanism of alkane degradation under anaerobic condition [9]. Some reports indicate that alkanes can be enzymatically dehydrogenated to alkenes [10], and the reaction may serve as an initial reaction for anaerobic alkane degradation [11]. The possibility of anaerobic oxidation of n-alkanes of various chain lengths has been repeatedly investigated since the 1940s [12]. It is suspected that n-alkanes serve as growth substrates for anaerobes such as sulfate-reducing bacteria and denitrifying bacteria, and are only slowly attacked in the absence of oxygen [9, 13, 14]. Several strains of alkane oxidizing anaerobes were isolated [15, 16] that are specialized either on long chain (C_{12}-C_{20}) or on medium chain (C_6-C_{16}) alkanes and use either sulfate or nitrate as an electron acceptor. No anaerobic degradation of short chain alkanes ($<C_6$) has been observed so far [15, 16]. Species of sulfate reducing and denitrifying bacteria that utilize alkanes are listed in Table 1.

Many aromatic petroleum hydrocarbons are substituted by one or more alkyl chains of varying length, thus exhibiting much structural diversity. Anaerobic biodegradation of aromatics depends on the type and position of substitutes. For methyl substitutes, the anaerobic biodegradability of meta substitution is better than that of ortho substitution. The existence of –COOH and –OH on an aromatic ring will promote its anaerobic biodegradability [18]. Species of sulfate reducing and denitrifying bacteria that utilize aromatics are listed in Table 2.

In this research work, the extent of anaerobic conversion of a heavy hydrocarbon cut in terms of the variation of its different constituents including saturates (aliphatics and cyclics), aromatics and resins is looked into. Mixed cultures of anaerobes have been isolated from the oil polluted sites of Iran and used for this purpose.

Table 1. Reported bacteria isolates with the capacity of anaerobic oxidation of alkanes [9, 12,15-17]

Genus and Species or Strain Designation	Hydrocarbon Used for Isolation	Range of n-alkane Utilized	References
Sulfate-Reducing Bacteria:			
Hxd3	n-Hexadecane	C_{12}-C_{20}	15
HD3	n-Decane	C_6-C_{14}	20
Pnd3	n-Pentadecane	C_{14}-C_{17}	15
AK01	n-Hexadecane	C_{13}-C_{18}	18
Dnitrifying Bacteria:			
Azoarcus sp. (HxN1)	n-Hexane	C_6-C_8	21
OcN1	n-Octane	C_8-C_{12}	21
HdN1	n-Hexadecane	C_{14}-C_{20}	21

Table 2. Reported bacteria isolates with the capacity of anaerobic oxidation of aromatics

Genus and Species or Strain Designation	Aromatic Used for Isolation	Aromatics Utilized	References
Sulfate-Reducing Bacteria:			
Desulfobacula toluolica	Toluene	Toluene	19
oXyS1	o-Xylene	Toluene o-Xylene, o-Ethyltoluene	23
EbS7	Ethylebenzene	Ethylebenzene	21
NaphS2	Naphtalene	Naphtalene	20
Dnitrifying Bacteria:			
PbN1	n-Propylbenzene	Ethylebenzene, n-Propylbenzene	22
CyN2	p-Cymene	Alkenoic monoterpens, p-Cymene	23

2. Materials and methods

2.1. Collection of samples

The anaerobic consortia for this study are collected by sampling from the petroleum-contaminated sludge and soils from Abadan, Shiraz and Tehran Oil Refineries of Iran, making 9 samples in total. These samples are taken from 30 cm below the ground surface. The sampling locations are indicated in Table 3.

2.2. The substrate

The substrate in this research work is a heavy cut from the vacuum distillation column of Tehran refinery, which is normally fed into an Isomax unit (catalytic cracking process), and is called Isofeed.

2.2. The preparations of consortia

The preparations of the anaerobic cultures from the soil and sludge samples were made inside a glove box chamber providing anaerobic conditions (see Section 2.4 for preparation of the glove box). For this purpose, 2.5 grams of each sample were added to 250 ml flasks containing 10 ml buffer phosphate solution of 0.5 mM for 2-3 hours. After this time, the contents were centrifuged for 5 min in order to separate the soil. The liquid phases were then used as the inoculation cultures. The consortia prepared for this study are available in the Environment Group of Materials and Energy Research Center, Karaj, MeshkinDasht. They can also be prepared by the procedure stated in this research work from the soil samples of the same locations.

Table 3. Soil and sludge sampling locations for the present research work

Consortia	Type of Sample	Location
A	Soil	Vicinity of tank NO. 2035, Shiraz Oil Refinery
B	Soil	Vicinity of Storage of oily sludge, Tehran Oil Refinery
C	Soil	Vicinity of tank NO. 802B, Abadan Oil Refinery
D	Sludge	Vicinity of tank NO. 2044, Shiraz Oil Refinery
E	Sludge	Storage of oily sludge, Tehran Oil Refinery
F	Soil	Vicinity of tank NO. 802A, Abadan Oil Refinery
G	Soil	Vicinity of the Storage of oily sludge, Arak Oil Refinery
H	Soil	Vicinity of the Storage of oily sludge, Isfahan Oil Refinery
I	Sludge	Storage of oily sludge, Abadan Oil Refinery

2.3. The culture medium

The culture media used in this work was prepared according to DSMZ-Media358. This media is one of the most popular media used for cultivation of anaerobic micro-organisms. The compositions of the media per liter are: $(NH_4)_2SO_4$, 1.3 g; $FeCl_3.6H_2O$, 0.02 g; KH_2PO_4, 0.28 g; $MgSO_4.7H_2O$, 0.25 g; $CaCl_2.2H_2O$, 0.07 g; Sulfur, 1.00 g; Yeast extract, 0.5 g; $Na_2B_4O_7.10H_2O$, 4.5 mg; Na_2S, 0.025 mg; $MnCl_2.4H_2O$, 1.8 mg; $CuCl_2.2H_2O$, 0.05 mg; $VoSO_4.2H_2O$, 0.03 mg; $CoSO_4$, 0.01 mg; $ZnSO_4.7H_2O$, 0.22 mg; $Na_2MoO_4.2H_2O$, 0.03 mg. The pH of the media was adjusted to 2.5 at room temperature. Oxygen was removed from the media by boiling the contents for 2 minutes and subsequently cooled in the glove box under atmosphere of 80% N_2 and 20% CO_2. Finally, the residual molecular oxygen of the media was bounded chemically by the addition of a reducer such as sodium sulfide. Also, 1 mg/l of resazurine was used for indication of oxygen depletion. This indicator solution is colorless at an $E_h < -100$ mv. The medium was prepared in capped bottles and sterilized at 121 ºC for 15 minutes.

2.4. The cultivation of consortia

The anaerobic cultures were inoculated inside an anaerobic Glove-Box. The Glove-Box was first evacuated and then flushed with a mixture of N_2 and CO_2 (80/20, v/v). The Glove-Box atmosphere was sterilized using a UV lamp (265 nm) fixed in the box. Each consortium was inoculated into two identical previously autoclaved capped bottles containing the media and the substrate in the same conditions. Therefore, each bottle contained 40 ml of the media, 10 ml of inoculum and 8 ml of Isofeed, which was filter sterilized. The bottles were sealed and incubated at 30 ºC for 21 days in a dark room on a rotary shaker with 100 rpm. Also, one sterile control bottle was set up as Blank. The control bottle was prepared in the same way except that the media did not contain inocula. After incubation for 21 days, the liquid phases were used for sub-culturing in new media supplemented with the same feed.

2.5. The microbial growth

After twice sub-culturing, the microbial growth of the samples were investigated visually and also using microscopic examinations indicating the shape of species in the aqueous phases. Table 4 shows the observed characteristics of the samples.

Table 4. Visual characteristics of the aqueous phase

Sample	Turbidity of Aqueous Phase	Microbial Accumulation at Oil-Water Interface	Emulsification of Oil
A	+	+	++
B	-	=	-
C	++	+	+
D	=	=	=
E	-	=	-
F	=	=	=
G	-	=	-
H	=	=	=
I	=	=	=

+ : High, ++ : Very High, - : Low, = : Very low

2.6 The analysis of the hydrocarbon bioconversion

After the second subculturing, the oil phase was separated from the liquid using 15 ml dichloromethane as the solvent. The dichloromethane was removed from the oil by evaporation in a water bath at 40 ºC. The oil phases were then used for analysis of their constituents using the gravimetric method and Gas chromatography-Mass spectroscopy (GC-MS) method. The gravimetric method is used as an indication of different fractions of the oil sample; *i.e.*, saturates (aliphatic and cyclic hydrocarbons), aromatics and resins, while GC-MS analysis indicates the chemical analysis of the sample from a molecular point of view.

2.6.1 The gravimetric method

The column chromatography method was used for the fractionation of different constituents of the oil (saturates, aromatics and resins) also called a gravimetric method. A glass column 50 cm in length having a 1 cm inner diameter was used and packed with silica gel for about 30 cm of its length. The silica gel with 100-200 mesh was activated by heating at 120ºC for about 16 h. A vibrator was used to pack the silica gel inside the column. Initially, 10 ml of normal hexane as the elution solvent was added to the top of the column to wet the adsorbent. About 0.5 g of the oil was diluted with 2 ml normal hexane added to the top of the column while the solvent level reached the top of the silica gel bed. Again, when the solution level reached the top of the bed, a 50 ml mixture of normal hexane and 60 ml of normal hexane and dichloromethane (60/40, v/v) was inserted into the column. It should be noted that 60 ml of normal hexane - dichloromethane was added after the level of normal hexane reached the top of the silica gel bed. The elution rate was controlled at 1-2 ml per min by the end valve of the column. The initial 40 ml of n-hexane which was eluted from the column was labeled as the saturate cut. The remaining output of the column was labelled as the aromatics cut. The resins remained in the column, since the solvents can not elute them. The solvents of the separated cuts were evaporated in a water bath at 40°C and weighed. Table 5 shows the weight percent of different cuts of each bio-treated sample A and sample C , as well as the control.

2.6.2 The gas chromatography-mass spectroscopy Analysis

The saturated fractions of Isofeed as well as the bio-treated oil samples were analyzed using a Gas chromatography-Mass spectrometry (GC-MS) system. The GC (Agilent Technologies 6890N) is equipped with an HP-5 capillary column (30 m × 0.25 mm × 1 mm film thickness) and MS (Agilent technologies 5973N) used as a detector. The oven was operated in an initial temperature of 100ºC and was held for 3 min at this temperature. Then the temperature was increased to 300°C at 15ºC/min and held for 20 min. The helium gas flow rate was 25 cm/sec. Figures 1, 2 and 3 show the chromatograms of the saturate cut of Isofeed, sample A and sample C respectively.

Table 5. The weight percent of saturates, aromatics and resins fractions of the oil samples in Iso-Feed, sample (A) and sample (C)

Cuts	Isofeed	Sample (A)		Sample (C)	
	Weight (%)	Weight (%)	Percent of Variations	Weight (%)	Percent of Variations
Saturates	65.4	69.2	+5.8	78.6	+20.2
Aromatics	12.2	25.6	+110	17.8	+46
Resins	22.2	5.2	-76.6	3.6	-83.8

3. Results and discussions

As listed in Table 5, the saturated fractions of both samples A and C are increased by about 6% and 20% respectively. This is also the same for the aromatics but with a higher rise in their percent of variations. In contrast, the resins are decreased by about 77% and 84% for samples A and C respectively. These results indicate the good ability of the selected anaerobic consortia in the conversion of the heavy fractions of the oil (resins) into the lighter ones. In other words, it seems that the resins are converted to the saturated and aromatic hydrocarbons.

As previously mentioned, Fig. 1 shows the GC-MS analysis of the saturated fraction of Isofeed as control. Each peak is related to a specific saturated compound. The main peaks are related to normal saturated hydrocarbons. This figure shows that Isofeed mainly contains n-C_{10} to n-C_{12} and n-C_{20} to n-C_{26} compounds but, n-C_{22} to n-C_{25} are dominant. These compounds exit the gas chromatography column at retention times of 13.73 to 15.41 min.

Figs. 2 and 3 illustrate the GC-MS chromatograms of the saturated fraction of samples A and C. However, due to the presence of an enormous number of compounds, the chromatograms do not have enough resolution to show the details, therefore, tracking the changes of the bio-treated samples is not easy. In this way detailed analytical reports of GC-MS analysis of the samples (not shown) reveals that some intermediate peaks emerged, while others disappeared compared to that of the control. However, for sample A, some changes are observed in the peaks at a time span of 12-13 min and also two new peaks appeared at 17.2 min, and many branched saturates were found in the bio-treated oil samples.

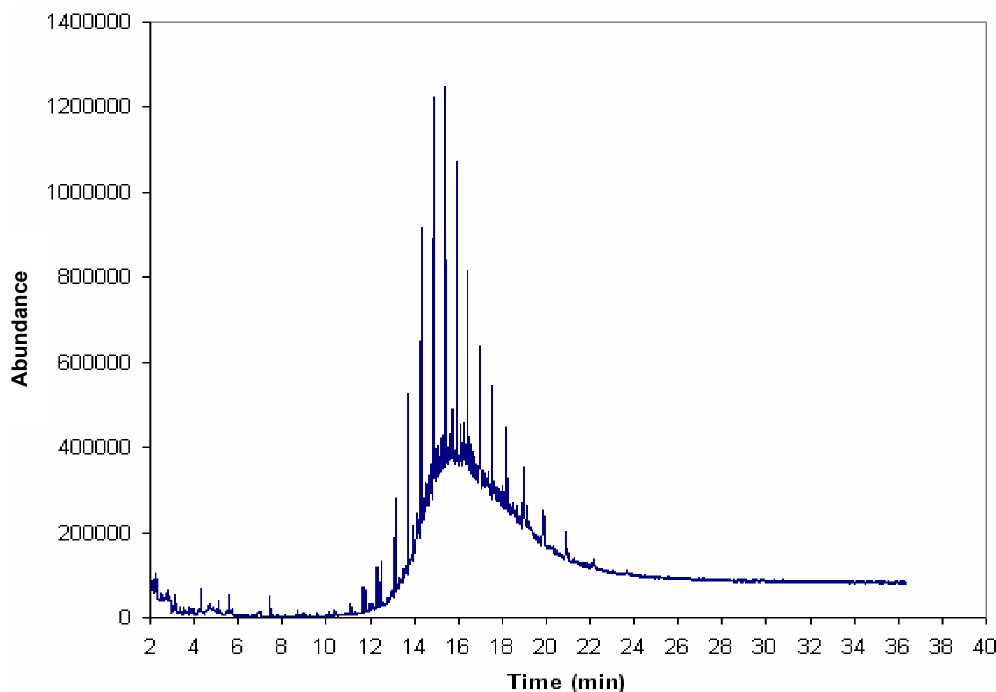

Figure 1. GC-MS analysis of the saturated hydrocarbons fraction of Isofeed.

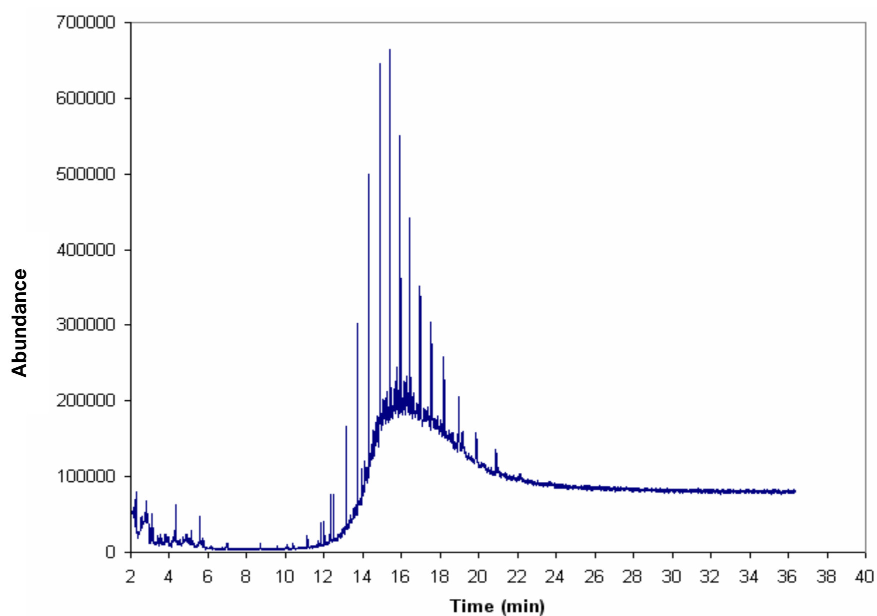

Figure 2. GC-MS analysis of the saturated hydrocarbons fraction of biotreated sample A

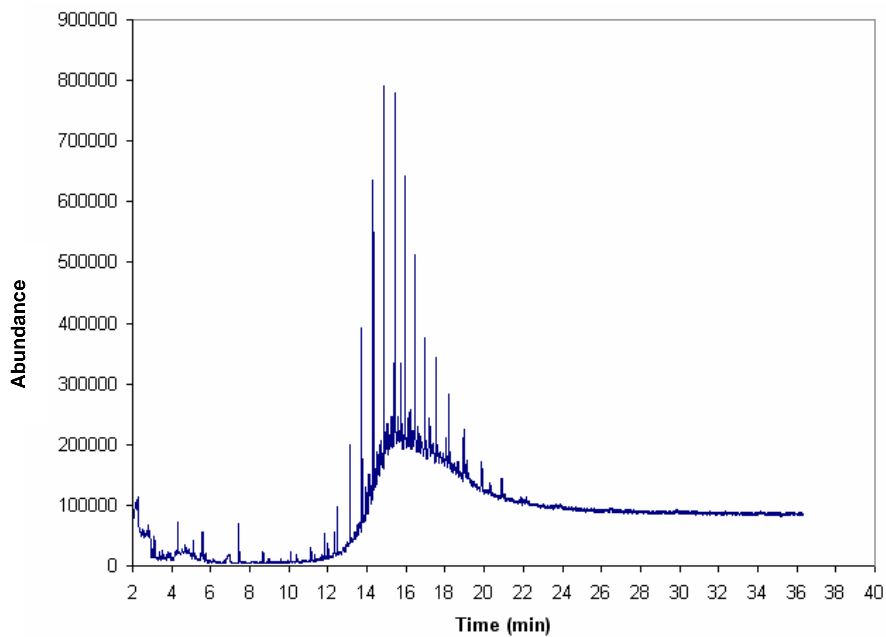

Figure 3. GC-MS analysis of the saturated hydrocarbons fraction of biotreated sample C.

Table 6 shows the weight percent of n-alkanes taken from the GC-MS analysis report. In both samples, the major reduction of compounds is related to n-C_{10} to n-C_{12}. Of which, approximately, 80% to 85% decrease in sample A and 53% to 42% decrease in sample C. The rate of reduction is less for longer chain hydrocarbons. As shown in Table 6, the percent of variations of n-C_{20} to n-C_{24} are reduced from -78.1% to -58.8% in sample A and -49.1% to -25.7% in sample C, respectively. In contrast, the weight percent variations of n-C_{25} and n-C_{26} are increased to 3.6% and 58.7%, respectively. This could be attributed to breakage of high molecular resins into long chain hydrocarbons or biopolymerization of smaller chain hydrocarbons [18].

4. Conclusions

The gravimetric analysis of the results demonstrates that consortia A and C are able to increase the saturated and aromatics hydrocarbons of Isofeed (a heavy oil faction) as well as decrease the resins' content. The saturated components are increased by 6% and 20% for samples A and C, respectively. Also, the aromatic fractions are increased by about 110% and 46% in sample A and C respectively. In contrast, the reduction of resin content for sample A and C are 77% and 84% respectively. This reveals that anaerobic treatment (as a pretreatment stage) of heavy oil fractions could improve the oil quality in terms of reducing the heavier part of the oil, i.e., resins. In other words, the resins are converted to saturated and aromatic compounds. The GC-MS results of the saturated fraction of the oil show that the normal hydrocarbons are mainly decreased and, in contrast, the cyclic and branched alkanes appear. This could be partly due to consumption of lighter hydrocarbons resulting in an apparent increase of the other ones and also partly due to the breakage of resins into long chain alkanes. Further investigation is needed to understand the phenomena behind this process.

Table 6. Weight percent of normal saturated hydrocarbons of Isofeed, sample A and sample C

Retention Time(min)	Component	Isofeed	Sample A		Sample C	
			Weight (%)	Percent of variations	Weight (%)	Percent of variations
3.11	10n–C	0.84	0.13	-84.5	0.39	-53.6
4.32	11n–C	1.41	0.20	-80	0.66	-53.2
5.57	12n–C	0.88	0.13	-85.2	0.51	-42
6.74	13n–C	-	-		-	
7.78	14n–C	-	-		-	
8.72	15n–C	-	-		-	
9.57	16n–C	-	-		-	
10.37	17n–C	-	-		-	
11.12	18n–C	-	-		-	
11.82	19n–C	-	0.11		0.27	
12.49	20n–C	1.14	0.25	-78.1	0.58	-49.1
13.12	21n–C	1.96	0.43	-78.1	1.11	-43.4
13.73	22n–C	3.92	1.21	-69.1	2.34	-40.3
14.32	23n–C	6.84	2.35	-65.6	4.45	-34.9
14.88	24n–C	9.70	3.95	-59.2	7.21	-25.7
15.41	25n–C	9.18	3.78	-58.8	9.51	+3.6
16.25	26n–C	1.79	-		2.86	+58.7

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