International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2016): 79.57 | Impact Factor (2017): 7.296

Degradation of used Engine Oil Alkanes by its Indigenous Bacteria, Production of PHA and Secretion of an Elastomer-Like Biopolymer

Manel Ghribi[1,2,3], Fatma Meddeb-Mouelhi[1,2,3], Marc Beauregard[1,2,3]

[1]CRML, Centre de Recherche sur les Matériaux Lignocellulosiques, C.P. 500, Trois-Rivières (Québec), G9A 5H7 Canada,

[2]Université du Québec à Trois-Rivières

[3]PROTEO, Université Laval

Abstract: ***Microbes have been shown to adapt to extreme environments and to develop the ability to use various substrates. Here in collaboration with a local oil recycling company (Québec, Canada), we assessed the biodiversity of bacteria and studied their ability to metabolize used engine oil (UEO). The bacterial species identified (Paenibacillus, Klebsiella, Micrococcus, Microbacterium, Bacillus, Pseudomonas, Rhodococcus and Stenotrophomonas) were similar to those found elsewhere in the world, including countries with much warmer climate than Canada (like China, India, and Egypt to name a few). This suggests that UEO as main carbon source -and not climate- is determinant for the biodiversity on UEO collection sites. Many bacterial strains identified here resisted to exposure to UEO at higher concentration (20% v/v) than reported earlier. As observed in earlier reports for many bacteria under various growth conditions, bacteria screened from this UEO source were also able to produce an intracellular biopolymer that was identified as a polyhydroxyalkanoate (PHA). We also identified an interesting consortium (composed of Stenotrophomonas and Rhodococcus) that metabolized UEO alkanes, and appeared suitable for two applications: bioremediation and biopolymer production. Under specific conditions, this same bacterial consortium secreted an elastomer-like biopolymer. The biopolymer formed at the surface of broth cultures where it was easily recovered. Based on FTIR analyses, the biopolymer was tentatively identified as ethylene propylene diene monomer (an EPDM rubber). To our knowledge, the conversion of UEO into an extracellular elastomer by bacteria has not been previously reported. Both species were found to be necessary for production of this biopolymer, suggesting a synergy among their respective metabolic machinery.***

Keywords: used engine oil, bacteria, biopolymer, PHA

1. Introduction

The intensive use of petroleum hydrocarbon products, such as diesel and engine oil, can lead to unintentional soil pollution via leakage or dispersion, and represents a major environmental problem. In most countries, oil spills at auto-mechanic workshops have been left un-remediated for the last hundred years, and the hazards associated with the continuous accumulation of petroleum products in this environment remains a serious concern.

The Environmental Protection Agency (EPA) defines used oil as any oil that has been refined from crude petrochemical oil, or any synthetic oil, that has been already used. Here, we defined used engine oil (UEO) as any petroleum-based or synthetic oil that has been used and degraded by heat and mechanical stress in combustion engines [1]. The result of such use has several consequences, including contamination by physical or chemical impurities.

During engine operation, lubricants are modified by both chemical and physical actions, as a consequence of the high temperature and heavy mechanical shear. After usage, engine oil contains a wide variety of new substances. In fact, engine oil is chemically transformed by oxidation, nitration, cracking of polymers and decomposition of metallic compounds. In addition to physically degraded and shortened hydrocarbon chains, UEO contains different contaminants such as organo-metallic compounds whose synthesis is made possible via atmospheric dust, metals, metal oxides and combustion products [2][3]. Waste engine oil also contains other contaminants such as alkyl benzenes, naphthalene, methylnaphthalenes and polycyclic aromatic hydrocarbons (PAHs). The presence of these compounds makes UEO one of the most polluting, toxic, and dangerous waste products existing on Earth [4][5]. Furthermore, due to its ability to form thin films over large surfaces of water, oil spills have harmful consequences for the life forms associated with aquatic environments [6].

The importance of reducing oil spills of any size is now widely recognized, and many countries have imposed regulations and procedures for collecting used oils [7]. In addition, extensive research efforts aimed at minimizing the environmental consequence of oil spills have been developed using chemical, physical and natural processes. Such pollution control methods include: bioremediation (use of microorganisms to degrade pollutants); phytoremediation (use of plants to clean pollutants by bioaccumulation into the plant's tissues), chemical modifications, dispersion (using surfactants), and for large spills, containment at surface (with floating barriers). Among these approaches, decontamination using bacteria has been a successful approach to remediate many pollutants.

Bacteria have shown their value for the treatment of industrial wastewater [8], production of industrial enzyme [9], bioconversion of substrate to value-added products [10],

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and other applications such as the treatment and biodegradation of tire rubber and related compounds [11].
Previous works on hydrocarbon-degrading microorganism showed that different microbes such as fungi and bacteria (*Brevibacterium* and *Bacillus* to name a few) were common in most biological-based treatments. The indigenous microbes found in used oil environments (garages or recycling plants) also represent a high potential for remediation in terms of their adaptation to locally available carbon sources such as cyclic and linear hydrocarbons found in used oil. In fact, most of the previously described micro-organisms were isolated from contaminated motorcycle workshop or petrochemical refinery soils [12][13][14]. Various fungi such as *Aspergillus niger, Penicilliumdocumbens, Cochliobolus lutanus* and *Fusariumsolani* biodegrade crude oils [13]. *A. niger*was shown to be very active for degradation of different kinds of oil compounds and was the most efficient metabolizer of hydrocarbons in the study of Nasrawi 2012[15].The hydrocarbon biodegradation ability of many other microorganisms has been reported over the years [16][17]. Importantly, the use of microorganisms has an advantage over chemical approaches in terms of not introducing additional chemicals into the environment.

Currently, UEO are seldom used as substrates or starting materials for synthesis of value-added products. For instance, they can be used as low-cost additive for tar and mixed with asphalt for road construction or repair [7]. Another interesting fate for UEO involves its conversion to diesel[18]. However, this operation is associated to time consuming pre-treatment (chemical filtrations and blending process) and the yield of produced diesel is still low. Unfortunately, in Canada, most UEO ends up being burned in heat generating equipment [19].

The use of microbes to convert UEO into valuable products could represent a more effective use of this common waste product. Several bacteria have been shown to metabolize nutrients in industrial and municipal wastewaters for production of industrially relevant enzymes [20][11]. Other laboratories have reported the production of bioplastics such as PHA or PLA by microbes using hydrocarbons as substrate [21][22].

Obviously, such microbes should thrive in UEO collection sites where hydrocarbons are in essentially an unlimited supply.

Here, we have isolated different species of bacteria sampled from a number of surfaces such as containers, machines, floors, soils samples on the premises of an important East Canada UEO collecting site.These bacteria were characterized and identified based on morphology, biochemical and genetic analysis. The biodegradation potential of these strains was explored, both individually and in mixed cultures. Finally, we studied their ability of converting used oil carbon into biopolymers of value. As far as we know, this is the first microbiology study done on UEO in Canada with focus on bioremediation and biopolymer production.

2. Materials and Methods

2.1 Used engine oil sampling

Different samples of UEO were collected at a local recycler (Phoenix Environment, QC, Canada). Sites for samples collection included the main UEO reservoir, a contaminated plastic waste container, and from the surfaces of several machines and equipment located at the production site. The samples were collected in plastic Ziploc bags and/or in Falcon tubes under aseptic conditions. All samples were identified according to location, and stored at 4°C.

2.2. Isolation and growth of bacteria from UEO samples

Isolation of bacteria contained within each UEO sample was performed as followed: 10g UEO was diluted in 100 mL of saline water (0.85 % NaCl), mixed by vortexing, and incubated for 1 h at room temperature (RT), then filtered through a sterile filter paper to remove solids. A 500µL aliquot of filtrate from each UEO sample was spread on LB media and minimum media (Mm) agar plates (0.1% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.1% KCl, 0.05% yeast extract and 1.5 % Bacto agar) supplemented with 10% UEO (final concentration, UEO was collected on site). Since the water extracted from bulk UEO had a pH ranging from 6 to 7, the pH of media was adjusted to 7.0 which we assumed should allow for growth of most bacteria present. Plates were incubated at 37°C for a minimum of 48 h, or until visible colonies were present. Following incubation, colonies having macroscopically different morphologies were picked from each plate and re-streaked onto LB agar media to obtain pure cultures. For long term storage, isolates were grown overnight in 10 mL LB medium (pH 7.0) with agitation (230 rpm). A 1 ml aliquot was mixed with glycerol (30% v/v final concentration) and stored at -80°C.

2.3. Morphological identification and biochemical characterization

For each isolate the colony characteristics (i.e. cell morphology) and Gram stain reactions were determined. Catalase and oxidase activities were also determined as previously described [23]**(Table S2).**

2.4. PCR amplification of 16S rRNA gene and sequencing

In order to identify the isolated bacteria, genomic DNA was purified from each isolate by performing 2 cycles of heat shock (15 min at -80 °C and 10 min at 100 °C). In some cases where genomic DNA was not obtained, a TransDirect Animal Tissue PCR KIT (Biocivic#AD201) was used as an alternative. Amplification of 16S rRNA gene was performed using the following universal primers: 1522R (5′-AAGGAGGTGATCCANCCGCA-3′) and 27F (5′-AGAGTTTGATCMTGGCTCAG-3′). The PCR products were separated and visualized on a 0.8% agarose low melting gel and the bands corresponding to 1.5 kb 16S rRNA gene were purified using QiagenMinElute PCR purification kit and then quantified by measuring OD at 260 nm using a Biotek plate-reader (Take 3 DNA quantification application). For DNA sequencing, quantification of purified PCRs was verified again on 0.8% agarose gel. Sequencing of the 16S

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rRNA genes with the primers described above (27F and 1522R) was performed using ABI Prism 3100 automatic sequencers at the Biomolecular analysis platform (University Laval, QC).

For a formulated bacterial consortium, the abundance of individual constituents was performed by first cloning the community 16S rRNA genes. For this, bacterial culture was prepared and total genomic DNA was extracted with Favor Prep Soil DNA kit (#FASOI 000-Mini Kit from Biocivic). Then PCR amplification was carried out using the G7 primer mix and PCR fragments were ligated into pEasy-Blunt zero vector and then transformed into Trans1-T1 competent cells (Biocivic#CB501-01). Transformed cells were plated onto LB-agar plate containing 100 µg/ml ampicillin and then incubated at 37°C for overnight. Transformant plasmid was isolated from ten colonies and sequenced for 16S rRNA gene analysis.

The 16S rDNA sequences analysis of were performed using Clone Manager professional 7.0 (Sci Ed Centra) and verified by Blast2Go software. The resulting sequences were compared to the Genbank non-redundant nucleotide database using NCBI BLAST (National Center for Biotechnology Information (NCBI) website) [24][23].

2.5. Tolerance of bacteria to UEO high concentration

Isolates were tested for their ability to grow in presence of UEO. Each isolate was spread on Mm media agar (described in section 2.2) supplemented with up to 20% (v/v) of UEO and incubated for 3 days at 37°C. Bacteria growing within the indicated incubation time were identified as being highly tolerant to UEO.

2.6. Chemical analysis of the UEO

Duplicate samples of UEO were analyzed to determine the chemical composition (e.g. available alkane), compound structures, and their percentages.

2.6.1. TGA

Thermo Gravimetric Analysis (TGA) experiments were undertaken three times at different temperatures and aging intervals with a Machine Universal v4.5A (TA Instruments) with ~2 mg of oil under nitrogen atmosphere at a flow rate of 100 mL min^{-1}. Four different sample-heating rates (5, 10, 20 and 50 °C min^{-1}) were chosen in order to evaluate the thermal stability of UEO and kinetics of ageing. All samples were heated from ambient temperature to 600 °C. The ageing experiments were performed in presence of air.

2.6.2. FTIR

Fourier-transform infrared spectroscopy (FTIR) was used to determine the chemical composition of the UEO. The spectra were recorded using transmission mode with cell path lengths ranging from 0.1126 mm to0.1173 mm and normalized to a constant path-lengthprior to data analysis.

2.6.3. NMR Spectroscopy

NMR experiments were carried for analysis of UEO. Deuterated chloroform (CDCI3) was added to 5 ml of oil sample which was then positioned in a JEOL Eclipse +400 NMR instrument. NMR data and spectra were processed using the Delta NMR processing and control software version 3.1.

2.6.4. GC-MS/FID

Hexane extracts (1.0 L) of residual waste engine oil were analyzed with a Hewlett Packard 5890 Series II gas chromatograph equipped with a 30-m long HP-5 column (internal diameter, 0.25 mm; film thickness, 0.25 mL) and a flame ionization detector (FID). Nitrogen was used as carrier gas. The injector and detector temperatures were maintained at 250 and 350°C respectively. The column temperature was programmed to ramp from 60 to 500°C for 27 min.

2.7. Monitoring degradation of alkanes by FTIR

A bacteria consortium was formulated by mixing equal proportions (OD at 600 nm) of pure bacterial cultures that were isolated from UEO samples. A preliminary evaluation of the capacity of the formulated consortium to degrade petroleum hydrocarbons was conducted using Mm liquid medium supplemented with 1% (v/v) of UEO. Unsterilized UEO was added to the medium and then emulsified for 15 min in order to achieve a homogenized solution. The medium (200 ml) was inoculated and placed under four different conditions:

1) Mm media supplemented with 1% of UEO pH 7, incubated at 37°C without agitation
2) Mm media supplemented with 1% of UEO pH 7, incubated at 37°C with agitation at 180 rpm
3) Mm media supplemented with 1% of UEO pH7, incubated at room temperature (RT) without agitation and
4) Mm media supplemented with 1% of UEO pH7, incubated at RT with agitation at 180 rpm.

Negative controls (non-inoculated) were run under the same experimental conditions. Cultures were monitored weekly for up to 7 weeks, and examined for the loss of UEO-associated dark color. FTIR was used to ascertain the decrease and/or the disappearance of chemical compounds in the UEO by the bacteria consortium. For this, a sample of each culture (500 µl) was centrifuged for 15 min (5000 g). One drop from each supernatant was used for FTIR spectroscopy.

2.9. Production and analysis of extracellular bacterial biopolymer

The ability of the formulated bacterial consortium to produce biopolymers was investigated. For this, bacteria were incubated at room temperature in Mm media supplemented with 1% UEO at pH 7 without agitation for several days. Different methods were then used for analysis of biopolymers.

2.9.1 FTIR biopolymer analysis

FTIR spectroscopy was used to monitor the degradation of UEO under the various culturing conditions and to analyze the extracellular biopolymer produced by the bacterial consortium. For the analysis of the biopolymer, a section (0.5 x 1cm) was cut from the pellicle and washed 5 times with 2 mL hexane, then 3 times with 2 mL acetone and final with water. The polymer was left to dry for 4 hours under vacuum

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and FTIR spectra were recorded with FTIR Thermofisher instrument (model Nicolet iS10). The sample was exposed to irradiation in reflectance mode in the range of 500 to 4000 cm^{-1}.

2.9.2 SEM-EDS analysis of extracellular biopolymer

Scanning electron microscopy (SEM) and energy dispersive spectrometry (EDS) were performed on biopolymers after drying overnight at 50°C. The 0.5 X 1cm biopolymer sample was then applied onto a double face adhesive tape and then placed in the SEM microscope. SEM-EDS imaging was performed with a Hitachi SU1510 microscope, EDS attached to the detector oxford x-Max 20 mm^2 and AZtec software was used for element analysis.

2.10 Detection of Polyhydroxyalkanoate (PHA) bacterial production

Bacteria isolated from UEO were tested for their ability to produce PHA using two different staining methods: Sudan Black B (SBB) and Nile Blue A (NBA) as described by [9].

3. Results

3.1 Analysis of UEO

Table 1: UEO composition

Compounds and proportions	*Composition*
Non-volatile, 78%	Linear Alkanes: C_{10} to C_{40} Dibutyl-phthalate Bis-(2-ethylhexyl)-phthalate
Volatiles, 17%	Toluene Undecane Ethylbenzene 1,3-Dimethylbenzene 1-ethyl-3-methylbenzene 1,2,3-trimethylbenzene 1-ethyl-4-methylbenzene Mesitylene 2-butoxy-ethanol Trimethyl-bicyclo2.2.1hept-2-yl acrylate
Heavy compounds (high molecular weight), 4%	Not analyzed
Minerals, 1%	Not analyzed

UEO samples were provided by the Phoenix Environment staff and analyzed using analytical chemistry techniques (GS-MS/FID, TGA and NMR). TGA results pointed to four groups of molecules in UEO: Volatiles (25° C to 159° C), non-volatiles (heavy) (159° C to 383 ° C), molecules (383° C to 500° C) and minerals (500° C to 800° C). Based on these analyses we estimated that 17% of UEO consisted of volatile compounds, 78% non-volatile compounds, 4% heavy compounds and 1% minerals.

The NMR spectra were characterized by intense peaks that were located in the region of 0 at 2 ppm which usually correspond to carbon-hydrogen bonds found in alkanes. In combination with the GC-MS/FID, results confirmed that most molecules found in UEO are alkanes (Additional file: Figure S1, S2 and S3). In the non-volatile fraction, alkanes were predominantly linear of lengths ranging from C10 to C40. Among these, we found two predominant molecules; dibutyl phthalate and bis-(2-ethylhexyl) phthalate (Table1). The volatile fraction was predominately composed of phenolic compounds like toluene, and ethylbenzene (Table1). As expected, the heavy compounds fraction contains very little volatile products. Given the nature of the sample, it can be assumed that they are alkanes with chain lengths between C40 and C50. (Additional file: Figure S1, S2 and S3). One might expect that chemical composition of UEO may vary depending on the original crude oil depending the processes used during refining, the efficiency and type of engine it was used in, the gasoline combustion products, the additives added to the fuel and the length of time that the oil remains in the engine [5]. The analysis presented in Table 1 is in general agreement with previous analyses of UEO despite differences in origins and use [25].

3.2 Morphological and biochemical characterization of bacteria isolated from UEO

Fifty-five bacterial strains were isolated from the UEO samples collected from various surfaces across the oil collection site. All bacterial strains were characterized using standard methods including morphology, Gram-stain, detection of oxidase, catalase activities and ability to grow at different pH (Additional file: Table S1). Bacterial cell morphology showed that thirty-six strains had a bacilli rod shaped form, that six out fifty-five were coccobacilli while the remaining strains were cocci. Twenty-four strains displayed oxidase and catalase activities, twenty-two showed only catalase and the rest displayed oxidase activity. Approximately half of the bacterial strains isolated were Gram-positive (Additional file: Table S1). Most isolates (31/55) had the ability to grow over a wide pH range (pH 6 to 12), while eleven strains could only grow at pH 7. These 11 bacterial strains belong to the genera *Micrococcus* and *Klebsiella*.

3.3 Bacteria identification by analysis of 16S RNA gene sequences

The amplified 16S rRNA sequences were analyzed using BLASTN available on NCBI portal. Bioinformatic analysis of the 16S rRNA gene sequences (from bacteria collected from UEO) revealed DNA sequence similarity with nine bacterial species (*Paenibacillus*, *Raoultella*, *Klebsiella*, *Micrococcus, Microbacterium*, *Bacillus, Rhodococcus*, *Pseudomonas* and *Stenotrophomonas*) as shown in figure 1. Among these, *Bacillus* and *Pseudomonas* species accounted for 66% of all strains isolated from the site. This result is consistent with previous studies showing that *Pseudomonasspp.* can degrade linear alkanes having medium length chains ranging from $C_{20-}C_{34}$ [26][27], while *Bacillus* species were found to degrade linear alkanes ranging from C_9 to C_{30}[28][29]. The presence of *Arthobacterspo*n diesel contaminated sites was reported earlier [30][31] but was not found here. In regards to *Microbacterium,* this genus was previously detected in soil containing fluoranthene and was recently isolated from crude oil in a Chinese oilfield [32]. Other bacteria species such as *Micrococcus* were also found on used engine oil contaminated sites in India [33][34]. In general, the range of genera isolated here, are similar to those from UEO from South Africa, Saudi Arabia, India, Egypt

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and China (see above-cited studies) despite the profound differences in climatic conditions comparing to Canada. This observation suggests that bacterial species populating UEO are determined by availability and specificity of UEO substrates, rather than climatic conditions such as average temperatures. One possible limitation of this work is that both the isolation media and approach utilized may have affected the final biodiversity of strains found in the sampled UEO. Many other strains, including anaerobic and uncultivable bacterial strains, might be detected under different culture conditions.

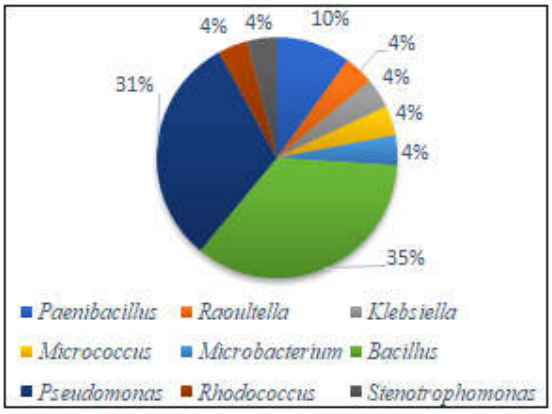


Figure 1: Percentage of bacterial strains with 16S DNA sequences similarity to known genera. All bacteria grown after sampling at the UEO recycling site had a high degree of sequence homology (97% to 99%) to known bacteria.

3.4 Bacterial resistance to high concentration of UEO

Certain isolates were better adapted to UEO and survived following exposure to a high concentration. Six out of 55 strains continued to grow despite the presence of 20% (v/v) UEO dispersed in growth medium. A majority of the strains found on site (26 bacteria) were able to grow on agar plate supplemented by up to 20% of UEO (pH 7, 7 days growth). As previously mentioned, some specific species of bacteria can degrade alkanes, benzene, toluene or naphthalene as carbon and/or energy sources [15][35]. But here, we tested the isolated bacteria for their resistance to all petroleum contaminants contained in UEO.

Isolates able to grow in the higher concentration of UEO were mainly *Bacillus, Stenotrophomonas, Rhodococcus, Pseudomonas* and*Microbacterium*species while the other genera (*Klebsiella, Micrococcus, Raoultella* and majority of *Paenibacillus*) did not tolerate 20% UEO in the medium (Additional file: Figure S7 and S8). Earlier studies have reported on bacteria resistant to the individual components of UEO, whereas others also used UEO but at lower concentrations [36]. For example, some have reported the growth of bacterial strains in single compounds (such as pyrene or fluorene) from UEO or diesel [34][37][38]. To our knowledge, the ability of a large portion of our isolates to grow on medium containing 20% UEO is higher than reported in previous similar studies [37].

3.5 PHA detection in bacteria screened from used engine oil

UEO could be a potential source of feedstock for bioplastic (PHA) production. Twenty-three strains studied here produced PHA as determined by staining with Sudan black B and Nile blue A (Additional file: Figure S5 and S6). They were identified as strains of *Klebsiella,Bacillus, Paenibacillus, Stenotrophomonas* and *Rhodococcus*. The other bacterial strains did not grow under our test conditions (PD agar, pH 7.0 and 37°C) or might produce undetectable amounts of PHA under our conditions. Among all strains, those within the genera *Bacillus* and *Paenibacillus* showed the most intense degree of staining and may hold the best potential for production of PHA [39]. Generally, other types of feedstock such as lipids, fats, palm oil, and sludge [10][21][40]are used for bacterial bioplastic production. As far as we are aware, this is the first time that bacteria isolated from used engine oil have been shown to hold potential for production of PHA.

3.6 Degradation of alkanes of used engine oil

In order to investigate the potential of these bacteria for bioremediation, we studied their ability to degrade the compounds found in UEO using liquid medium. The best results were achieved with a consortium of *Stenotrophomona s*and *Rhodococcus*. Following 47 days of incubation in 1% UEO, the dark colored phase disappeared when these bacteria were present in the media (Figure 2). The degradation of UEO was further investigated by FTIR. Spectra were recorded for two negative controls and for two samples obtained after bio-treatment with bacteria consortium incubated at 2 different temperatures (RT and 37°C). The FTIR spectra of initial media (i.e. before bacterial growth) showed absorption bands (2954-2923 cm^{-1}, 2858-2153 cm^{-1}) that are typical for alkanes (–CH and $–CH_2$ asymmetric stretching). Such bands are absent from the control sample (no UEO added) and in samples where the bacteria were allowed to grow at RT (Additional file: Figure S9). The apparent degradation of alkanes by the consortium was not as efficient when growth was performed at 37°C.
A: Negative control without bacteria
B: Bio-treatment of 1% of UEO with bacteria consortium



(a) **(b)**

Figure 2: Visual biodegradation of UEO by bacterial consortium compared to the control without bacteria (RT).

It has been documented in many studies that bacteria having potential for the degradation of petroleum hydrocarbons can be found in areas contaminated with oil [30]. One of the two main genera identified in our study (*Rhodococcus*) was discussed in previous work of bioremediation, and was found to degrade hydrocarbons such as benzenes [36][38]. Studies of degradation of used oil by *Stenotrophomonas* isolates[41][42] showed that species within this genus are capable of significantly decreasing the content of

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polyaromatic hydrocarbons when used for the bioremediation of crude oil. The two microorganisms we used in our consortium (identified as *Stenotrophomonas*and *Rhodococcus*) have been reported to have enzymatic systems which empower them to degrade and utilize petroleum hydrocarbon as source of both carbon and energy[13][42]. Our study fully supports earlier studies showing their superior ability for oil degradation [40] [42]. However, under our conditions, we found that both bacteria were required in the same culture for bioremediation to occur (Figure2, FigureS9). An analysis of these bacteria transcriptome is under way that should aid in understanding the contribution of the enzymatic machinery of each bacterium to the observed phenomenon.

3.7 Extracellular biopolymer production and characterisation

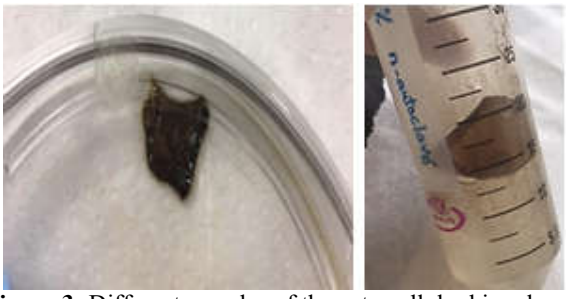


Figure 3: Different samples of the extracellular biopolymer produced by the bacteria consortium grown in 1% UEO.

The bacteria consortium isolated from UEO (*Stenotrophomonas* and *Rhodococcus*) promoted the formation of an extracellular polymer that floated on the surface of the medium, which was not PHA (as shown in the Figure 3 above). Production of this material required specific conditions: the consortium was incubated in Mm media supplemented with 1% UEO at RT, without agitation over an incubation period ranging from 55 to 60 days. The extracellular biopolymer material was recovered and dried (Figure 4). FTIR analysis (Addition file: Figure S10) revealed the presence of a large peak at 3300 cm^{-1}, typical for OH stretching. This may be a consequence of alcohol groups in the polymer, or from contamination of the biopolymer with water. Further drying of the material revealed the presence of several peaks were in the 2800-3000 cm^{-1} region, which is a typical signature for alkanes. Finally, the biopolymer had vibration modes corresponding to either alkenes, aromatics and/or carbonyl groups in the 1400-1700 cm^{-1} range. When comparing the biopolymer FTIR spectra with those of different polymers available in our database, we found that the two top matches were poly (ethylene:propylene: ethylidene:norborene) and an unidentified thermoplastic (HR Nicolet sampling library). The former is a well-known polymer that belongs to the EPDM rubbers or elastomers. EPDM rubbers have been extensively characterised and used for the last 20 years for controlling mechanical and thermal properties of many plastic or rubber materials synthesized using petroleum-based substrates [43][44][45].

Control experiments were also performed with individual strains and with both strains in plastic tubes and in glass Erlenmeyer. UEO was not discolored when the individual strains were inoculated into the medium. Formation of the extracellular polymer required the presence of the consortium and occurred in glass tubes as well as in plastic tubes, indicating that its formation was not a result of plastic degradation or conversion.

Surface morphology characteristics and internal structure of the extracellular biopolymer were investigated using scanning electron microscopy (SEM) in the environmental mode. SEM suggests the existence of two different biopolymer surfaces: a rough side (A) and a smooth side (B) (Figure 4). We suggest that the latter side is the surface in contact with the medium. SEM imaging also confirmed that the extracellular polymer was not a bacterial biofilm.

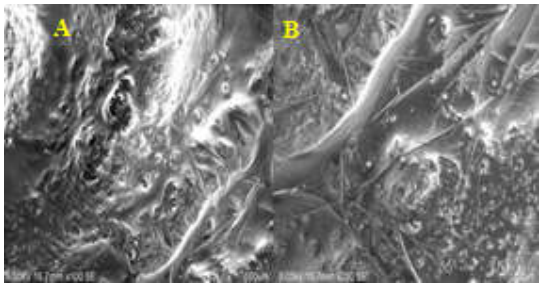


Figure 4: SEM images showing two faces of the extracellular biopolymer produced by bacteria consortium: rough side of the polymer (A) and smooth side (B) of the biopolymer produced from UEO both detected by SEM.

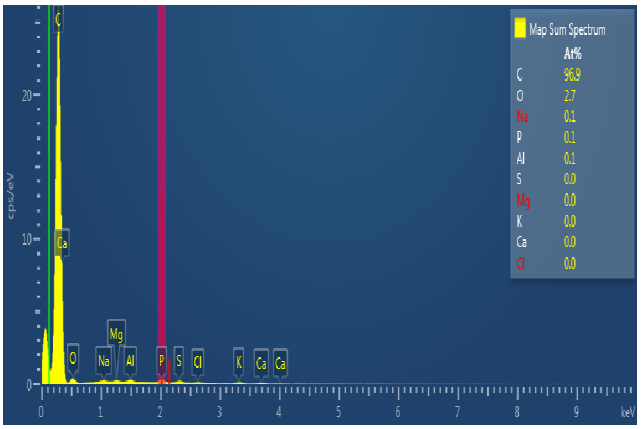


Figure 5: EDS spectra showing the main composition of the produced polymershowing map sum spectrum (Carbon C: 96.9%)

SEM-EDS results show that the peak associated with carbon was clearly dominant, indicating that the biopolymer is carbon rich. A small amount of oxygen as well as negligible proportions of other elements (Na, P and Al) were also detected (Figure 5), although some of this may result from contamination from the culture medium.

Different studies have shown that strains of *Stenotrphomonas* and *Rhodococcus* species can produce PHA[29][40][46]. In contrast, our observations (visual, SEM and FTIR) suggest that this extracellular floating polymer (probably an elastomer) was produced by the bacterial consortium using UEO as substrate. Indeed, this EPDM-like biopolymer does not resemble PHA. Furthermore, we report

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a synergy among these strains that appears to be necessary to produce this new extracellular biopolymer.

Since this biopolymer is extracellular, it has a definitive advantage over PHA regarding production costs: PHA extraction from bacterial remains a limiting step where chemical or mechanical methods for cell lysis are required prior to PHA isolation[39][47]. Depending on intended use, additional steps such as further purification may be required. This increases the cost of production of PHA. In contrast, the EPDM-likebiopolymer that we identified in this study is extracellular and easy to recover from the broth. It can be generated using a low-cost substrate (UEO) and the cultures do not require agitation or heating. The potential industrial importance of this discovery is not limited to this biopolymer. This study could open the way to tailored biopolymer of desired composition or properties by bacteria having appropriate enzymatic machinery. Once the key enzymes have been identified, it would be possible to evolve them by directed evolution for instance, and ultimately design the structure of the final biopolymer. UEO would certainly be a substrate of choice, considering the several advantages associated with the conversion of this waste into valuable products.

4. Conclusion

This study is the first description of bacterial strains found in a UEO recycling plant in Quebec, Canada. The identified isolates found here belong to genera also found in previous international biodiversity studies all over the world. Many of the bacterial strains isolated were able to grow in a high concentration of used engine oil, up to 20%. Therefore, these bacteria could be useful for the abatement of ecosystems that may be contaminated with hydrocarbons. Accordingly, our FTIR results showed complete decontamination of used engine oil when a formulated consortium screened from this oil is exposed to this waste. Moreover, we report the first observation of the formation/production of an extracellular biopolymer that resembles the elastomer EPDM, an elastomer that has been successfully used in several applications for decades. We also highlighted for the first time the fact that some of the isolated bacteria from this waste (UEO) can produce PHA.

Our study opens new horizons for approaches to the problem of petroleum waste biodegradation and bio-valorization. Elucidation of enzymatic pathways in bacteria found here and their directed evolution might open the way to tailor-made biopolymer production using inexpensive and dangerous UEO ingredients as substrate.

Authors Contributions

MG, FM and MB have planned sampling strategy and experimental work. MG and FM have sampled and manage strains reported here. Characterization was performed by MG Initial draft was prepared by MG and edited by FM and MB who performed final corrections leading to submitted manuscript. All authors read and approved the final manuscript.

Acknowledgements

Our heartfelt thanks to Dr. Martin Kalmokoff for his generous help in editing this manuscript. MB acknowledges financial support from NSERC (CRDPJ 44514), CRIBIQ and Buckman North America. MG was supported by a scholarship from Proteo.

Competing interests

The authors declare that they have no competing interests.

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