



Tuning culturing conditions towards the production of neutral lipids from lubricant-based wastewater in open mixed bacterial communities

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ABSTRACT

Production of bacterial lipid-based biofuels using inexpensive substrates, as wastes, is an emerging approach. In this work, a selective process using carbon feast-famine cycles was applied to obtain an indigenous microbial community of hydrocarbon-degrading and lipid-accumulating bacteria, using a real lubricant-based wastewater as carbon source. In the conditions applied, the enriched bacterial community, dominated by members of the genus *Rhodococcus*, *Pseudomonas* and *Acinetobacter*, was able to degrade almost all hydrocarbons present in the wastewater within 24 h' incubation and to accumulate, although in low levels, triacylglycerol (TAG) (<5% of cell dry weight (CDW)) and polyhydroxyalkanoates (PHA) (3.8% ± 1.1% of the CDW) as well as an unknown lipid (29% ± 6% of CDW), presumably a wax ester-like compound. The influence of culture conditions, namely carbon and nitrogen concentrations (and C/N ratio) and cultivation time, on the amount and profile of produced storage compounds was further assessed using a statistical approach based on a central composite circumscribed design and surface response methodology. The regression analysis of the experimental design revealed that only nitrogen concentration and C/N ratio are significant for neutral lipid biosynthesis ($p < 0.05$). Maximum neutral lipid content, i.e. 33% (CDW basis), was achieved for the lowest carbon and nitrogen concentrations evaluated (10 g COD L⁻¹ and 0.02 g N L⁻¹). PHA accounted for less than 5% of CDW. In these conditions, neutral lipid content was mainly composed by TAG, about 70% (w/w). TAG precursors, namely monoacylglycerols (MAG), diacylglycerols (DAG) and fatty acids (FA), accounted for 22% of total neutral lipids and WE for about 7%. Nevertheless, according to the applied response surface model, further improvement of neutral lipids content is still possible if even lower nitrogen concentrations are used. The fatty acids detected in TAG extracts ranged from myristic acid (C14:0) to linoleic acid (C18:2), being the most abundant palmitic acid (C16:0), stearic acid (C18:0) and oleic acid (C18:1).

This study shows the feasibility of combining treatment of hydrocarbon contaminated wastewater, herein demonstrated for lubricant-based wastewater, with the production of bacterial neutral lipids using open mixed bacterial communities. This approach can decrease the costs associated to both processes and contribute to a more sustainable waste management and production of lipid-based biofuels.

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1. Introduction

Increasing request for petroleum-derived products has led to a rapid development of several types of oil-based industries. As a consequence, considerable amounts of different hydrocarbon contaminated wastes are produced, the most significant being oily sludge and oily wastewaters. Oily wastewaters are produced from several industries, namely crude oil production and refinement,

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List of abbreviations

CCCD	Central composite circumscribed design	MAG	Monoacylglycerol
CDW	Cellular dry weight	MAH	Monocyclic aromatic hydrocarbons
C/N	Carbon to nitrogen ratio	OD	Optical density
COD	Chemical oxygen demand	PAH	Polycyclic aromatic hydrocarbons
DAG	Diacylglycerol	PHA	Polyhydroxyalkanoate, PHB Polyhydroxybutyrate, PHV Polyhydroxyvalerate
DGGE	Denaturing gradient gel electrophoresis	Q-TOF	Quadrupole time-of-flight
ESI-MS	Electrospray ionization-mass spectrometry	SPE	Solid phase extraction
FA	Fatty acid	TAG	Triacylglycerol
GC-FID	Gas chromatography coupled to flame ionization detection	TLC	thin-layer chromatography
GC-MS	Gas chromatography coupled to mass spectrometry	TN	Total nitrogen
		WE	Wax esters

lubricants and petrochemical manufacturing, automotive repair stations and industrial equipment maintenance unit and automobile repair shops. This type of wastewaters is mainly composed by complex mixtures of alkanes, aromatic hydrocarbons, phenols, asphaltenes, emulsified oils, solvents and lubricants (Bhattacharya et al., 2003), which present a major environmental concern. Currently applied treatment and disposal methods are neither effective nor efficient solutions. In particular, they are not cost-effective neither eco-friendly, and this type of waste corresponds to a loss of economic competitiveness for their source companies.

Production of bacterial storage lipids during biological treatment of hydrocarbon-based wastewaters has been recently proposed as a way to make management of these wastes more economic and environmentally sustainable (Da Silva et al., 2016). Hydrocarbonoclastic bacteria have the ability to degrade a wide range of hydrocarbon compounds: branched, unbranched and cyclic alkanes as well as aromatics, namely monocyclic aromatic hydrocarbons (MAH) and polycyclic aromatic hydrocarbons (PAH). These bacteria can be found in several distinct ecosystems, including marine waters, soils and sediments, especially in oil-polluted ones (Bragg et al., 1994; Harayama et al., 2004; Head et al., 2006). Some of the most representative genera are *Alcanivorax*, *Pseudomonas*, *Acinetobacter* and *Rhodococcus* (Koma et al., 2001; Larkin et al., 2005; Cappello et al., 2007; Palleroni et al., 2010). In addition to biodegradation capabilities, members of these groups can produce storage compounds, namely triacylglycerol (TAG), wax esters (WE) and polyhydroxyalkanoates (PHA) (Alvarez et al., 2002; Manilla-Pérez et al., 2011; Chen et al., 2014), which can be used as raw materials for the production of feed additives, cosmetics, lubricants, biofuels and bioplastics (Steinbüchel and Fuchtenbusch, 1998; Alvarez and Steinbüchel, 2010).

The process of bacterial neutral lipids production and accumulation is complex and the amount and type of carbon storage is highly dependent on several parameters, such as the species itself, carbon source type and cultivation conditions. Neutral lipids accumulation is promoted under unbalanced growth conditions, when an excess of carbon is present but low levels of nitrogen occur, resulting in a high ratio of carbon to nitrogen in the culture medium (Manilla-Pérez et al., 2011; Alvarez and Steinbüchel, 2010; Olukoshi and Packter, 1994). In general, cells in the initial and exponential growth phase do not produce significant amounts of lipids, whereas in the stationary phase, there is a drastic increase (Packter and Olukoshi, 1995; Wältermann et al., 2005). Wältermann and Steinbüchel 2005 described that under conditions of cell proliferation, *Rhodococcus opacus* PD630 produced low levels of TAG and *Acinetobacter calcoaceticus* ADP1 accumulated low TAG and WE. However, when transferred to nitrogen limiting conditions, both bacteria increased significantly TAG and WE content

after 24 h. Several works reported differences occurring between species in terms of quantity and type of carbon storage compounds production. Several *Rhodococcus* strains grown on glucose and gluconate accumulated TAG as a major storage compound and PHAs in lower amounts, whereas with hexadecane only TAG were produced (Alvarez et al. 1997, 2008; Alvarez, 2003). *Rhodococcus ruber* is a peculiar strain since it accumulates considerable PHA and TAG levels, but PHA production occurs already in exponential growth phase and TAG are only produced after, when cells enter the stationary growth phase (Alvarez et al., 2000). Members of the genera *Alcanivorax* and *Acinetobacter* present different neutral lipids profiles, when compared to *Rhodococcus*. In a general way, WE are the most dominant storage lipid in these bacteria, whereas TAG are produced in lower amounts, depending on the carbon source used. *Alcanivorax borkumensis* SK2 accumulated predominantly TAG when cultivated on pyruvate and similar levels of TAG and WE with hexadecane (Manilla-Pérez et al., 2011). More recently, *A. borkumensis* SK2 fed with lubricant-based wastewater, produced the highest TAG levels in stationary growth phase, whereas maximum production of WE-like compounds was achieved during exponential growth phase (Da Silva et al., 2016).

Due to the complexity of factors influencing storage compounds production, several works regarding the optimization of lipid storage compounds and the influence of cultivation conditions were reported using pure bacterial cultures (Gouda et al., 2008, Kurosawa et al., 2010, Kurosawa and Sinskey, 2014). From a practical point of view, however, studies with open mixed microbial cultures should be considered since mixed cultures facilitate the use of cheap complex substrates (wastes) and avoid the need for sterilization and sterile operation conditions (Reis et al., 2003).

In this work, a mixed bacterial community able to produce storage compounds was enriched with a real lubricant-based wastewater and the influence of several culture conditions, namely carbon and nitrogen concentrations (C/N ratio) and cultivation time, on the profile of storage compounds produced was further assessed using a statistical approach, based on a design of experiments.

2. Materials and methods

The experimental approach included two consecutive experiments, experiment 1 and experiment 2, as depicted in Fig. 1.

2.1. Experiment 1 – Selection of indigenous hydrocarbonoclastic bacteria able to accumulate neutral lipids

2.1.1. Inoculum and wastewater source

Sludge collected in February 2011 (5 L) from the aeration vessel

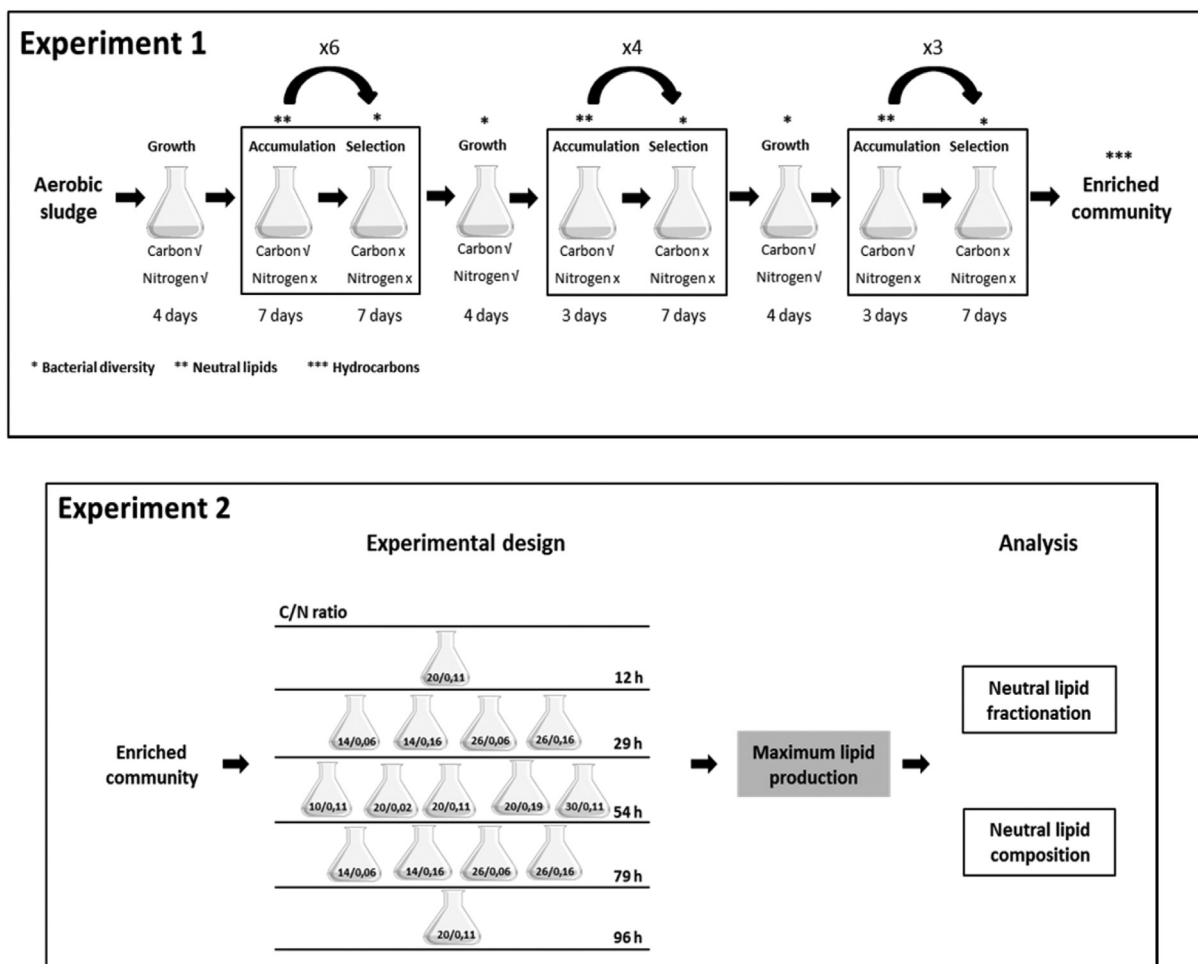


Fig. 1. Schematic diagram representing the experimental procedure of experiment 1 and experiment 2.

of a wastewater treatment plant located in a heavy machinery maintenance service unit (ALSTOM Portugal, S.A., Maia) was used as inoculum. Wastewater collected from the same service unit (10 L), containing lubricant and engine oil waste, was used as carbon source for the enrichment process. The wastewater treatment plant included a gravimetric oil/water separation unit and subsequent aeration of the separated water fraction in the collector vessel (to promote aerobic degradation of remaining hydrocarbons by indigenous microorganism) before being discharged to the municipal collector. The wastewater was kept at 0 °C to guarantee the original composition. It was characterized by a high Chemical Oxygen Demand (COD) ($307 \pm 14 \text{ g L}^{-1}$), and high amounts of total petroleum hydrocarbons (720 g kg^{-1} dry weight (DW)). Total ammonia nitrogen was present in very low amounts ($0.13 \pm 0.4 \text{ mg L}^{-1}$). The concentration of total and organic nitrogen was $172 \pm 18 \text{ mg L}^{-1}$ and $77 \pm 9 \text{ mg L}^{-1}$, respectively, whereas nitrates concentration was $94 \pm 9 \text{ mg L}^{-1}$. No nitrites were detected. The wastewater pH was slightly acid (5.5).

2.1.2. Selective enrichment process

The enrichment process was performed in batch mode using 250 mL flasks with 50 mL of mineral salt (MS) medium (Schlegel et al., 1961) at room temperature (22 °C) under agitation (150 rpm). The inoculum was first submitted to a growth stage (carbon rich, nitrogen rich) in MS medium supplemented with the hydrocarbon-rich wastewater (5 g COD L^{-1}) as sole carbon source and ammonium chloride (0.5 g L^{-1}) as nitrogen source, during 4

days, to obtain high biomass levels. Afterwards, selective pressure was applied in the form of alternating accumulation (carbon rich (5 g COD L^{-1}), no nitrogen) and selection (no carbon and no nitrogen) stages. Cells were collected by centrifugation (4 °C, 10 min, 10 000 g) and transferred to the next stage in fresh MS medium. The accumulation and selection stages lasted 7 days each. After 6 cycles of accumulation and selection (C1–C6), a growth phase of 4 days was promoted in order to obtain higher biomass levels. To increase the selective pressure, 7 cycles of accumulation and selection were performed, changing the accumulation phase from 7 to 3 days (C7–C13). Between cycles 9 and 10 and at the end of cycle 13 a growth stage was again performed to obtain higher biomass levels. Cyclosporine ($10 \mu\text{g mL}^{-1}$) was used to inhibit fungal growth. The total process lasted 13 cycles, corresponding to 4 months. Biomass samples were collected at the end of the accumulation stages of cycles 3, 5, 6, 10, 11 and 13 for analysis of carbon storage compounds production, at the end of selection and growth stages of cycles 3, 6, 10 and 13 for molecular characterization of bacterial community diversity and at the end of the enrichment process for hydrocarbon analysis.

2.2. Experiment 2- Effect of culturing conditions on neutral lipids accumulated by the mixed bacterial community

2.2.1. Inoculum and assay conditions

Production of carbon storage compounds by the bacterial community obtained in the selective enrichment process

(experiment 1), under different conditions of COD, N, and incubation times, was studied in 250 mL flasks containing 50 mL of MS medium supplemented with ammonium chloride (NH₄Cl) as nitrogen source at room temperature (22 °C) under agitation (150 rpm). The enriched microbial community was first grown in the presence of a COD concentration of 5 g L⁻¹ of wastewater and 0.5 g L⁻¹ of NH₄Cl to obtain high biomass levels. Grown cells were used to inoculate the flasks to an optical density (OD) at 600 nm of 1.0.

2.2.2. Factorial experimental design and statistical analysis

A factorial central composite circumscribed design (CCCD) based on surface response methodology was used to study the effect of cultivation conditions (factors) and its possible interactions, on neutral lipids accumulated by the enriched bacterial community. The three factors (independent variables) selected were COD concentration, N concentration and incubation time. For each independent variable five levels were chosen (: -a, -1, 0, +1, +a, where a = 2n/4; n = number of parameters and 0 corresponded to the central level), resulting in a total of 32 experiments (including duplicates and a quadruplicate central point). The experimental levels were selected by varying the parameters above and below the respective central level. The coded and uncoded levels of the independent variables are given in Table 1. The experiments were performed in duplicate in a random way. The software package Design-Expert® (Stat-Ease, Inc., Minneapolis) was used to perform the design matrix of the experiments subjected to regression analysis and their statistical evaluation.

The experimental results were analysed to assess the effects of each variable as well as their interaction effects by the response surface regression procedure using the following equation:

$$Y_i = \beta_0 + \sum \beta_i X_i + \sum \beta_i X_i^2 + \sum \beta_{ij} X_i X_j \quad (1)$$

Where Y_i is the response variable, β₀ is the constant, β_i is the linear effect, β_{ij} is the interactive effect and X_i is the coded factor level. The results were analysed by using ANOVA, i.e. analysis of variance, suitable for the experimental design. Statistical significance was established at the p < 0.05 level.

At the end of each experiment, all cell content of the flasks was collected, centrifuged, lyophilized and stored in a desiccator until further lipid and PHA analysis.

2.3. Bacterial community analysis

2.3.1. DNA extraction

Aliquots of well-homogenized biomass were immediately frozen at the time of sampling and stored at -20 °C. Total genomic DNA was extracted using a FastDNA SPIN Kit for Soil (Qbiogene, Carlsbad, CA, USA) according to the manufacturer's instructions.

Table 1

Independent variables and levels used in central composite circumscribed design (CCC) for the production of storage compounds by enriched bacterial community.

Variable	Units	Level				
		Coded values				
		-1.68	-1	0	+1	+1.68
		(-)				(+)
		Real values				
COD	g L ⁻¹	10.0	14.05	20.0	25.95	30.0
N	g L ⁻¹	0.056	0.083	0.129	0.16	0.250
Time	Hours	12	29	54	79	96

2.3.2. DGGE analysis

The 16S rRNA genes were amplified by PCR using a Taq DNA Polymerase kit (Life Technologies, Gaithersburg, MD, USA). All primers used were synthesized commercially by Invitrogen (Life Technologies). Primer set 968-f/1401-r (Muyzer et al., 1993) was used for amplification of the regions V6 to V8 of the 16S rRNA gene, for denaturing gradient gel electrophoresis (DGGE) purpose. Primer 968-f was modified by the addition of a 40-bp GC clamp at the 5' end of the sequence. The thermocycling program used for this amplification was: predenaturation at 95 °C for 2 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 40 s and elongation at 72 °C for 90 s, and postelongation at 72 °C for 5 min.

DGGE analysis of the amplicons was performed in a Dcode system (Bio-Rad, Hercules, CA, USA) with 8% (v/v) polyacrylamide gels and a denaturant gradient of 30–60% or 40–60%. A 100% denaturing solution was defined as 7 M urea and 40% formamide. Electrophoresis was performed for 16 h at 85 V in 0.5X TAE buffer at 60 °C. DGGE gels were stained with AgNO₃ according to the procedure previously described by (Sanguinetti and Simpson, 1994).

DGGE profiles were compared with the Neighbour Joining clustering method (Saitou and Nei, 1987) using the PyElph software version 1.4 (Pavel and Vasile, 2012). The distance matrix was calculated using the Dice coefficient (Dice, 1945).

2.3.3. Sequencing by Illumina

The composition of bacterial community was assessed from the variable V1-V3 regions of the 16S rRNA gene by high throughput sequencing, using the specific bacterial primer set 28F/388R. Amplification, library preparation, sequencing and taxonomic assignment of 16S rRNA reads were done by Research and Testing Laboratory-RTL, using an Illumina MiSeq platform and an Illumina two-step method described by (Caporaso et al., 2012). Chimeric sequences and sequences with less than 100 bp were discarded. Clustering was performed at a 4% divergence using the USEARCH algorithm (Edgar, 2010) and OTUs selection (97% identity threshold) was performed using the UPARSE algorithm (Edgar, 2013). The centroid sequence from each OTU cluster was run against the USERCH global alignment algorithm and taxonomic assignment done using a database of high-quality sequences derived from the NCBI nucleotide database. Detailed information on the bioinformatics analysis steps can be found at the RTL website (<http://rtlgenomics.com/amplicon-bioinformatics-pipeline>).

Bacterial community diversity was assessed by the Shannon diversity index (Shannon (1948)).

Illumina sequencing data was submitted to the European Nucleotide Archive, under the study accession number PRJEB25660, and can be viewed by following the URL: <http://www.ebi.ac.uk/ena/data/view/PRJEB25660>.

2.4. Analytical methods

Chemical oxygen demand (COD), total nitrogen (TN), ammonium (NH₄⁺), phosphate (PO₄³⁻), nitrates, nitrites and total ammonia nitrogen present in the wastewater were determined using the cuvette-tests Lck914, Lck338, Lck386, Lck305, Lck340, Lck341 and Lck303, respectively (Hach-Lange, Germany). These measurements were made in triplicate, according to the manufacturer's procedures.

2.5. Neutral lipids analysis

2.5.1. Neutral lipids extraction and analysis by TLC

Lipids were extracted according to Folch method (Folch et al., 1957), with some modifications. For this, 15 mg of lyophilized cells were extracted with 3 mL of a mixture of chloroform/

methanol (2:1, v/v) (Wältermann et al., 2000). The mixture was incubated at room temperature with shaking for two hours. Afterwards, the cell extracts were obtained after filtration through glass wool and evaporated to dryness.

Analysis of lipid profiles was performed by thin-layer chromatography (TLC). The extracts were dissolved in chloroform/methanol (2:1, v/v) and applied on 10×10 cm glass DC-Fertigplatten plates, precoated with 0.25 mm silica gel 60 with fluorescent indicator UV254 (60F254 silica gel plates - Merck, Darmstadt, Germany). The TLC was performed applying the following developing solvent system: hexane/diethyl ether/acetic acid (80:20:1, v/v/v) as mobile phase for separation of acylglycerols (Wältermann et al., 2000).

Neutral lipids were visualized on the-plates after brief exposure to iodine vapor. Olive oil, oleic acid, and oleyl oleate were used as standard substances for TAG, fatty acids and WE, respectively.

2.5.2. Fractionation of different lipid classes

Total lipid extracts were subjected to a separation process based on lipid class using a solid phase extraction (SPE) technique (Revellame et al., 2012). The extract was reconstituted in *n*-hexane (1 mL) and applied on a SPE silica column (SiOH) Chromabond 1000-mg, 6 mL, (Macherey-Nagel, Düren, Germany), previously conditioned with *n*-hexane. The sample was then fractionated into four main fractions: fraction A, enriched in hydrocarbons; fraction B, enriched in wax esters and sterol esters; fraction C enriched in triacylglycerols and fraction D, enriched in free fatty acids, monoacylglycerols and diacylglycerols. Fraction A was eluted by applying 3.7 mL of 94:6 (v/v) *n*-hexane/diethyl ether; fraction B was obtained by applying 4.5 mL of 94:6 (v/v) *n*-hexane/diethyl ether; fraction C was eluted by applying 10 mL of 94:6 (v/v) *n*-hexane/diethyl ether; fraction D was obtained applying 15 mL of 85:15:2 (v/v/v) *n*-hexane/diethyl ether/acetic acid. The fraction volumes were optimized based on TLC analysis. All fractions were collected, evaporated to dryness and weighted.

Fractions B, C and D, corresponding to total neutral lipid compounds, were gravimetrically quantified. Each fraction was scraped from the TLC plates transferred to a Pasteur pipet filled with cotton wool and eluted from the silica, using hexane as solvent (Santala et al., 2011).

2.5.3. Neutral lipids analysis by ESI-MS

Fractions B, C and D were analysed by mass spectrometry (MS) through electrospray ionization (ESI) obtained in a quadrupole time-of-flight (Q-TOF) mass spectrometer (Q-TOF2, Micromass, Manchester, UK) and fraction D was also analysed in an LXQ linear ion trap mass spectrometer (Thermo Scientific, San Jose, CA, USA). ESI-MS and MS/MS conditions in the Q-TOF mass spectrometer were the following: electrospray voltage was 3 kV in positive-ion mode with a cone voltage of 30 V; the source temperature was 80 °C and the desolvation temperature was 150 °C. Collision energy used for the MS/MS analysis varied between 30 and 40 eV. Data acquisition and treatment of results was carried out with the MassLynx™ software V4.0 (Waters, Manchester, UK). ESI-MS and MS/MS conditions used in the linear ion trap mass spectrometer were the following: electrospray voltage was 4.7 kV in negative-ion mode; capillary temperature was 275 °C, and the sheath gas (He) flow rate was 25 units. For MS/MS acquisition, the normalized collision energy (CE) varied between 33 and 40 (arbitrary units). Data acquisition and treatment of results were carried out with the Xcalibur® Data System 2.0 (Thermo Scientific, San Jose, CA, USA).

2.6. PHA extraction and quantification

Accumulation of PHA (polyhydroxybutyrate and

polyhydroxyvalerate) by the microbial community was determined according to the procedure described in Smolders et al. (1994). Freeze-dried biomass of each sample was weighed (8–20 mg) into digestion tubes. 1.5 mL of internal standard benzoic acid in dichloromethane (1 mg mL^{-1}) and 1.5 mL of 1-propanol:hydrochloric acid (75:25 v/v) were added. The tubes were placed in a digester block at 100 °C for 3.5 h.

Samples were analysed by gas chromatography (GC) with flame ionization detector (GD-FID). The GC equipment was a GC Varian 3800, Varian Inc. USA with a TR-WAX (Teknokroma, Spain) column, $30 \text{ m} \times 0.32 \text{ mm} \times 0.25 \text{ mm}$. The injector and detector temperatures were 220 °C and 250 °C, respectively. Column temperature was 50 °C for 2 min, with a $15 \text{ }^\circ\text{C min}^{-1}$ ramp to 225 °C, and a final hold at 225 °C for 5 min. Helium was used as carrier gas at 1 mL min^{-1} .

2.7. Total hydrocarbons extraction and analysis

Hydrocarbon content in the wastewater was analysed at SGS Portugal (Sociedade Geral de Superintendência, S.A.) according to DIN EN 14039/EN ISO 16703.

Extraction of the hydrocarbons present in the culture media was performed based on procedures disclosed in NJDEP EPH 10/08 (NJDEP, 2010) and began with the acidification of the media with hydrochloric acid to a pH below 2.0. Acidified media were transferred to 50 mL separatory funnels. Then, 7.5 mL of chloroform:methanol (3:1, v/v) was added to each flask and transferred to the corresponding separatory funnel (Koma et al., 2001). The separatory funnels were vigorously stirred for 2 min and then allowed to rest for 5 min to allow the organic and aqueous phase separation and the organic phases were carefully collected. This process was performed three times. This process was also performed in control flasks, corresponding to the beginning of the assays.

The extracts were cleaned on Florisil Sep-Pak columns 6 cc (Waters, USA) adapted to a vacuum manifold. The columns were equilibrated with 6 mL of methanol:chloroform (3:1, v/v) and the samples were passed through the columns to new flasks. Then, additional 6 mL of solvent were passed through the columns to remove any remaining hydrocarbons. The samples were allowed to evaporate in the fume hood, suspended in 1.5 mL of hexane and analysed by GC-FID.

Gas chromatography was performed in a GC Varian® star 3400CX equipped with a VF-1ms column, 30 m long and 0.25 mm internal diameter, and made from fused silica coated with dimethylpolysiloxane as stationary phase. The column temperature was held at 60 °C for 1 min and then programmed to raise up to 290 °C at $8 \text{ }^\circ\text{C min}^{-1}$. The injector temperature was set to 285 °C. Helium was used as carrier gas at a flow rate of 1 mL min^{-1} . The detector was operated at 300 °C.

3. Results and discussion

3.1. Selection of lipid-accumulating hydrocarbonoclastic communities

Changes in the bacterial community structure during the selective enrichment process were followed by DGGE. The obtained DGGE profiles showed a decrease in bacterial diversity (decrease in number of bands) throughout time until the establishment of a relatively stable profile at the end of cycle 13 (C13f) (Fig. S1a). In particular, two predominant bands (stronger intensity) were detected at this time point, which probably corresponds to the most dominant bacteria in the developed community.

During the selective process, a significant shift in bacterial diversity was observed between cycle 6 and 10 (C6f and C10f profiles)

(Fig. S1b) possibly related to the differences applied in terms of the duration of the accumulation phases. Until cycle 10 the accumulation stage lasted 7 days and in the further cycles the selective pressure was intensified, and it was decreased to 3 days. Therefore, the duration of the accumulation steps seems to significantly affect bacterial diversity of the enriched community.

The identification of bacterial species present in the microbial community at the beginning (Inoculum – I) and at the end of the enrichment process (cycle 13 – C13f) was performed by Illumina sequencing. From the results presented in Fig. 2 it can be seen that the inoculum had a high bacterial diversity (Shannon diversity index (H') = 1.59), with most abundant 16S rRNA gene sequences belonging to the phylum *Proteobacteria* (67% of relative dominance) and to orders *Rhodocyclales* (14%), *Rhizobiales* (13%), *Chromatiales* (12%) and *Burkholderiales* (7%). After the enrichment process, the bacterial community showed a considerable decrease in diversity (the H' index decreased from 1.59 to 0.89). The most dominant sequences corresponded to those with high similarity to members of the genus *Acinetobacter* (26% relative dominance), *Pseudomonas* (31%) and *Rhodococcus* (12%) which are well known for their ability to produce wax esters (WE), polyhydroxyalkanoates (PHA) and triacylglycerols (TAG), respectively (Wältermann et al., 2000; Ishige et al., 2002; Tobin and O'Connor, 2005). The remaining sequences showed high similarity with members of the genera *Acidovorax* (14%), *Zoogloea* (5%) and *Variovorax* (3%), which include species that have been described as capable of producing PHA (Fukui et al., 1976; Renner et al., 1996; Gasser et al., 2009).

The profile of neutral lipid storage compounds accumulated by the enriched bacterial community was evaluated through thin layer chromatography (TLC) (Fig. S2). In the initial phase of the enrichment (cycle 3), it was only possible to detect faint bands at the level of fatty acids, however, throughout the enrichment two distinct bands corresponding to TAG became visible (C5 and C6). These results are in agreement with the results of Illumina sequencing, where an increase in the dominance of sequences corresponding to *Rhodococcus* sp. and *Rhodococcus erythropolis*, known as TAG producers, was detected. A shift in TAG profile was observed in the next stages and only one single band was detected (C11 and C13), which can be related to the imposed decrease of the accumulation phase duration (from 7 to 3 days). TAG content produced by the bacterial community was however very low, below 5% of the cellular dry weight (Table S1). In the last 3 cycles of the enrichment (C11 and C13, Fig. S2) an intense band was detected between TAG and WE standards, which corresponded to 29% of the cellular dry weight. This unknown lipid may be a wax diester-like compound, as previously found by (Kalscheuer et al., 2003). According to this author, *Acinetobacter calcoaceticus* strain ADP1 is able to accumulate wax diesters under nitrogen limiting conditions, which resolve at similar TLC zone of the unknown lipid detected in this work. This result is supported by the phylogenetic identification of bacteria present at the end of the enrichment, where one of the most dominant bands corresponds to a member of the genus *Acinetobacter*.

In spite of the several PHA producers identified in the enriched bacterial community, PHA content was very low, around 4% of cellular dry weight (Table S1).

During the selective enrichment process, pH varied between 7.25 and 7.56 and dissolved oxygen concentration between 5 and 6 mg L⁻¹ (Table S1).

The capacity of the enriched bacterial community to degrade the hydrocarbons present in the lubricant-based wastewater was further assessed at the end of the enrichment process. This wastewater is mainly composed by medium chain hydrocarbons, between C9 and C12, and a complex mixture in the range between C17 and C30 characterized by the presence of two significant GC-

unresolved humps (Fig. S3 (A)). After 24 h' incubation, the bacterial community was able to fully degrade the GC resolved hydrocarbons and a considerable part of the GC-unresolved ones. These results clearly demonstrated the feasibility of the developed enriched community to treat hydrocarbon-based wastewaters, supporting the phylogenetic identification that indicated the presence of hydrocarbon degrading-bacteria such as *Pseudomonas*, *Acinetobacter* and *Rhodococcus* genus (Larkin et al., 2005; Palleroni et al., 2010; Kostka et al., 2011).

The applied multistage enrichment process, with successive cycles of feast and famine, allowed the development of a community able to use the hydrocarbons present in the wastewater and produce storage lipids. The use of complex wastewater has the advantage to mimic a real treatment scenario. Real wastewater may contain indigenous microorganisms already adapted to the wastewater contaminants and to an environment of high carbon concentration and low levels of nitrogen, which favours lipid accumulation (Riesenberg and Guthke, 1999). The obtained lipid content was quite low suggesting that the cultivation conditions applied in the enrichment process can be improved.

3.2. Influence of culturing conditions on storage compounds accumulation

The microbial community enriched in hydrocarbonoclastic and lipid accumulating bacteria was further cultivated in different concentrations of carbon and nitrogen (resulting in different C/N ratios) and different incubation times. The interactive effects of the tested variables (COD concentration (A), N concentration (B) and cultivation time (C)) in PHA and neutral lipid production (response variable) were assessed.

In all assays, PHA content was lower than 5% and, thus, further analysis concerning this storage compound was not performed.

3.2.1. Neutral lipids accumulation

Neutral lipids accumulation in the assays performed in the conditions determined by the experimental design is described in Table 2. The lowest neutral lipid content measured was between 1 and 5% of the CDW for 79 h of cultivation at a concentration of COD and nitrogen of 26 g L⁻¹ and 0.0557 g L⁻¹, respectively (C/N ratio = 116). The highest values of neutral lipids detected in the enriched bacterial community was around 33% of the CDW cultivated on 20 g COD L⁻¹, 0.0216 g N L⁻¹ (C/N ratio = 231) after 54 h of cultivation. This result is in the range of values reported in other works, including the ones obtained with pure cultures of oleaginous bacteria proposed for neutral lipid production (more than 20% of lipids in cellular dry weight) (Manilla-Pérez et al., 2011; Revellame et al., 2012; Alvarez et al., 1996; Hernández and Alvarez, 2010; Tamis et al., 2015).

The experimental data described in Table 2 were used in the statistic Design-Expert software to perform multiple regression analysis and to generate a model representing the relation between independent and response variables. The generated second-order polynomial equation was established as follows:

$$Y = 9.81 + 0.14 \times A - 2.97 \times B - 1.57 \times C + 2.98 \times A \times B + 0.033 \times A \times C + 1.72 \times B \times C - 1.68 \times A^2 + 2.15 \times B^2 + 2.50 \times C^2 \quad (2)$$

In this equation, Y represents the neutral lipid content obtained by the bacterial community (% CDW) and the terms A, B and C are the coded values of the independent variables for carbon content (g COD L⁻¹), nitrogen content (g L⁻¹) and cultivation time (hours),

Table 2

Experimental design to assess the effects of independent variables (COD concentration – A; nitrogen concentration – B and cultivation time - C) on neutral lipid production.

Experiment	Coded values			Actual values			C/N ^a	% Neutral lipids (g g ⁻¹ CDW)
	A: COD (g L ⁻¹)	B: N (g L ⁻¹)	C: t (h)	A: COD (g L ⁻¹)	B: N (g L ⁻¹)	C: t (h)		
15	0	0	-1.682	20	0.1058	12	47	23.9
22	0	0	-1.682	20	0.1058	12	47	24.1
2	-1	-1	-1	14	0.0557	29	63	15.4
24	-1	-1	-1	14	0.0557	29	63	15.5
6	1	-1	-1	26	0.0557	29	116	10.5
5	1	-1	-1	26	0.0557	29	116	10.8
7	-1	1	-1	14	0.1559	29	23	7.5
18	-1	1	-1	14	0.1559	29	23	10.2
29	1	1	-1	26	0.1559	29	42	6.9
27	1	1	-1	26	0.1559	29	42	14.7
20	-1.682	0	0	10	0.1058	54	24	5.9
9	-1.682	0	0	10	0.1058	54	24	8.3
31	1.682	0	0	30	0.1058	54	71	11.0
8	1.682	0	0	30	0.1058	54	71	9.3
32	0	-1.682	0	20	0.0216	54	231	33.5
21	0	-1.682	0	20	0.0216	54	231	30.0
10	0	1.682	0	20	0.1900	54	26	10.4
26	0	1.682	0	20	0.1900	54	26	3.9
12	0	0	0	20	0.1058	54	47	14.6
25	0	0	0	20	0.1058	54	47	7.0
16	0	0	0	20	0.1058	54	47	12.5
21	0	0	0	20	0.1058	54	47	7.6
28	-1	-1	1	14	0.0557	79	63	8.6
13	-1	-1	1	14	0.0557	79	63	14.7
11	1	-1	1	26	0.0557	79	116	1.2
17	1	-1	1	26	0.0557	79	116	4.6
23	-1	1	1	14	0.1559	79	23	8.8
14	-1	1	1	14	0.1559	79	23	4.6
3	1	1	1	26	0.1559	79	42	8.1
1	1	1	1	26	0.1559	79	42	15.1
30	0	0	1.682	20	0.1058	96	47	12.3
4	0	0	1.682	20	0.1058	96	47	21.4

CDW – Cell dry weight.

^a Molar carbon was estimated considering a ratio between COD and total organic carbon of about 4, based on the theoretical values calculated for saturated hydrocarbons with the general formula of C_nH_{2n} + 2.

respectively. In order to investigate the adequacy of the model, multiple regression analyses on the data were applied and the statistical significance of the model was evaluated by means of ANOVA. Table 3 displays the coefficients and p-values of the independent variables as well as of their interaction on neutral lipids accumulation. ANOVA of the quadratic model indicated that the model is significant (p-value < 0.05), suggesting that the model was statistically significant with a confidence interval of 95% and suitable for revealing the relation of carbon concentration, nitrogen concentration and cultivation time and predicting the response values in terms of neutral lipid production (Fig. S4). The regression analysis of the experimental design revealed that only nitrogen concentration and the interaction between carbon and nitrogen concentrations are significant for neutral lipid biosynthesis (p < 0.05).

Fig. 3 shows the effect of the interaction between carbon and nitrogen concentrations on neutral lipid accumulation at different cultivation times. Highest neutral lipid level, about 33%, was predicted for 29 h' cultivation time with the lowest carbon and nitrogen concentrations analysed, namely 10 g COD L⁻¹ and 0.02 g N L⁻¹ (C/N = 126). The lowest level of neutral lipids (less than 1%) was obtained by decreasing carbon concentration and increasing nitrogen concentration (C/N = 43). The amount of neutral lipids

Table 3

Degree of positive or negative effects of the experimental variables on neutral lipids production.

Factor	Neutral lipids	
	Coefficient	P-value
Model	—	<0.0429*
A – COD	0.14	0.9105
B – N	-2.97	0.0215*
C – t	-1.57	0.2050
AB	2.98	0.0408*
AC	0.33	0.8364
BC	1.72	0.2834
A ²	-1.68	0.3000
B ²	2.15	0.1872
C ²	2.50	0.1278

*p value < 0.05.

showed a less significant decreased to approximately 26% (for a C/N ratio of 126). The response surface model does not attain an optimum value in the tested conditions suggesting that further improvement on neutral lipids levels accumulated may be obtained when even lower N concentrations are used. Previous reports have demonstrated that neutral lipid accumulation is highly dependent

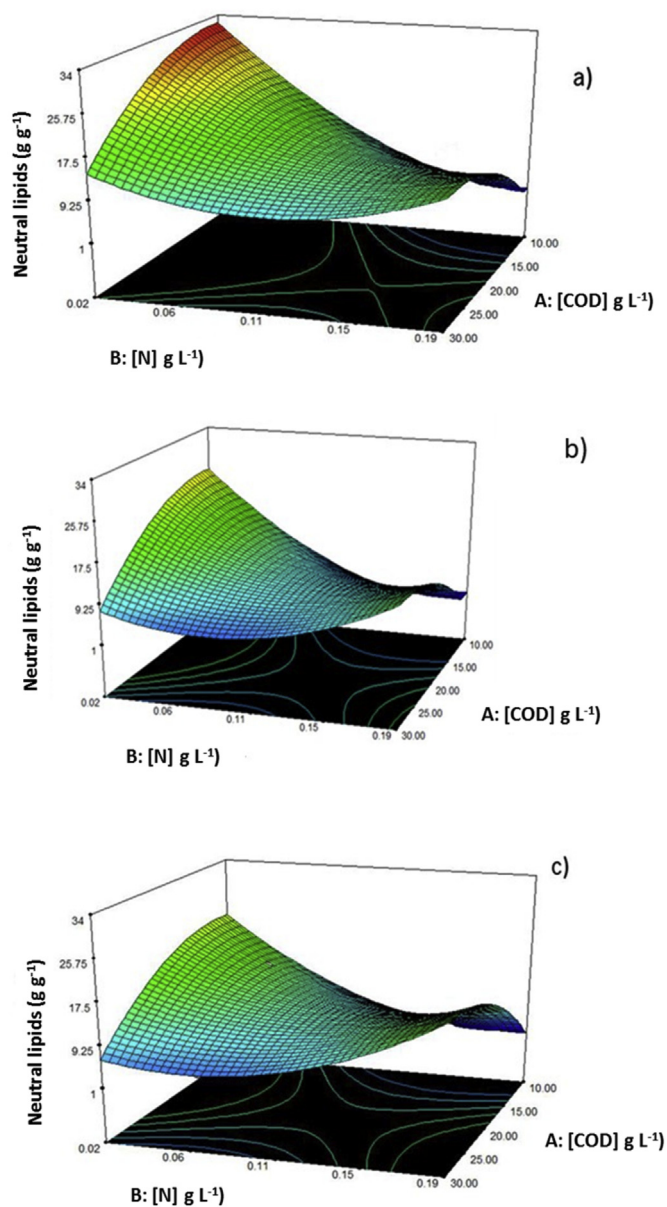


Fig. 3. Three-dimensional response surface plots showing the effect and interactions between the independent variables in neutral lipids accumulation. a) Interaction between carbon and nitrogen concentrations at 29 h of cultivation; b) Interaction between carbon and nitrogen concentrations at 54 h of cultivation; c) Interaction between carbon and nitrogen concentrations at 79 h of cultivation.

on several culturing conditions, C/N being one of the most relevant in several types of bacteria (Riesenberg and Guthke, 1999). *R. opacus* PD630 cultivated on orange waste and glucose accumulated more TAG (45–47% w/w) at C/N ratios (g/g) of 2400 and 16, respectively, than at lower ratios (C/N = 266 and C/N = 6) (Gouda et al., 2008; Kurosawa et al., 2010). *A. borkumensis* SK2 grown in pyruvate presented the same behaviour. At a C/N ratio of 50 accumulated more TAG than at C/N = 7 (Manilla-Pérez et al., 2011). Some authors reported a C/N ratio ranging between 50 and 500 as optimal for TAG accumulation in *Rhodococcus* species grown on hydrocarbons and sugars (Alvarez et al., 1996, 1997; Alvarez, 2003; Gouda et al., 2008; Kurosawa et al., 2010). However, these studies were done with simple substrates, making it impossible to directly compare these values with the values used in this work.

The classes of neutral lipids accumulated by the bacterial community cultivated in conditions promoting maximum neutral lipid content (about 33% of CDW) were further analysed by thin layer chromatography (TLC) (Fig. S5). TAG was the most abundant lipid, representing 70% (w/w) of total neutral lipid content. Fatty acids (FA), monoacylglycerols (MAG) and diacylglycerols (DAG) were also found, accounting for 22% of total neutral lipids, whereas WE were produced in lower amounts, corresponding to 7% of total neutral lipids. The low levels of WE production may be related to the low dominance of WE producing bacteria present in the obtained enrichment (namely *Acinetobacter* genus) as suggested by Illumina sequencing (Fig. 2).

The composition of lipids extract fractions B to D (Fig. S5), was analysed by ESI-MS and ESI-MS/MS. The mass spectrometry analysis of fraction B showed no molecular species corresponding to wax esters or sterol esters. Table 4 describes the several TAG structures identified in fraction C.

The most abundant TAG ions were found at m/z 850.9, 876.8 and 902.8 identified as $[M+NH_4]^+$ adducts of TAG(50:1), TAG(52:2) and TAG(54:3), respectively. The fatty acyl chains identified in TAG molecules ranged between myristic acid (C14:0) and linoleic acid (C18:2), being the most abundant species composed mainly by palmitic acid (C16:0), stearic acid (C18:0) and oleic acid (C18:1). The free fatty acids identified in fraction D are presented in Table 5.

A wide range of free fatty acids were produced by the bacterial community, with carbon chain length ranging between 14 and 25 carbon atoms. Interestingly, odd-chain fatty acids, namely C15:0 and C17:0, and unsaturated fatty acids between one and five double bonds were also detected.

The structures of MAG and DAG produced by the enriched community are listed in Table 6. MAG are mainly composed by palmitic acid (C16:0), stearic acid (C18:0) and linoleic acid (C18:2), whereas DAG are composed by a mixture of palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3).

This wide range of TAG, free fatty acids, MAG and DAG structures possibly reflects the complex mixture of hydrocarbon types present in the wastewater used as carbon and energy source. In fact, these results are in agreement with previous reports, where a wide range of fatty acids were produced by a mixed microbial population present in municipal activated sludge and also by pure bacterial cultures, namely *R. opacus* PD630 and *Gordonia* sp. DG, when grown on real and complex agro-industrial wastes and municipal wastewaters (Gouda et al., 2008; Revellame et al., 2012). The fatty acid profile of the bacterial neutral lipids obtained in this work consisted

Table 4

Molecular species of triacylglycerols identified as $[M+NH_4]^+$ ions detected by ESI-Q-TOF-mass spectrometry in fraction C of the cell extract obtained from the enriched community cultivated in conditions promoting maximum lipid content.

m/z $[M+NH_4]^+$	Triacylglycerol (C:N)	Fatty acyl chains
822.9	48:1	16:0/16:0/16:1 14:0/16:0/18:1
848.9	50:2	16:0/16:1/18:1 16:0/16:0/18:2 18:1/18:1/14:0 16:1/16:1/18:0 14:0/18:0/18:2
850.9	50:1	16:0/16:0/18:1 14:0/18:0/18:1 16:0/16:1/18:0
874.9	52:3	16:0/18:1/18:2 16:1/18:1/18:1
876.9	52:2	16:0/18:1/18:1
902.9	54:3	18:1/18:1/18:1

Table 5

Molecular species of free fatty acids identified as $[M-H]^-$ ions detected by ESI-ion trap -mass spectrometry in fraction C of the cell extract obtained from the enriched community cultivated in conditions promoting maximum lipid content.

m/z $[M-H]^-$	Fatty acyl chains
227.2	14:0
241.2	15:0
253.2	16:1
255.2	16:0
269.2	17:0
281.3	18:1
283.3	18:0
301.2	20:5
303.2	20:4
307.2	20:2
309.2	20:1
311.3	20:0
325.2	21:0
337.2	22:1
339.2	22:0
367.3	24:0
381.3	25:0

Table 6

Molecular species of monoacylglycerols and diacylglycerols identified as $[M+NH_4]^+$ ions detected by ESI-Q-TOF-mass spectrometry in fraction C of the cell extract obtained from the enriched community cultivated in conditions promoting maximum lipid content.

m/z $[M+NH_4]^+$	Diacylglycerols	
	Monoacylglycerols C:N	C:N Fatty acyl chains
348.3	16:0	
372.4	18:2	
376.4	18:0	
608.6		34:3 16:0/18:3
610.6		34:2 16:0/18:2
612.6		34:1 16:0/18:1
630.6		36:6 18:3/18:3
632.6		36:5 18:2/18:3
634.6		36:4 18:2/18:2

C:N means total number of carbons:total number of double bonds.

mainly of the five most common C16–C18 fatty acids present in the feedstocks traditionally used for biodiesel production, showing its suitable use for lipid-based biofuels production.

4. Conclusions

This study shows the feasibility of combining treatment of hydrocarbon contaminated wastewater, herein demonstrated for lubricant-based wastewater, with the production of bacterial neutral lipids, using open mixed bacterial communities. In the tested conditions, neutral lipids content in the biomass could be increased up to 33% (CDW basis), mainly composed by TAG (about 70% w/w), by changing the culturing conditions, namely carbon and nitrogen ratio. However, further improvement of this content is still possible, as predicted by the response surface model applied to study the effect of culture conditions on neutral lipids biosynthesis, if even lower concentrations, in particular of nitrogen, are used.

This study also showed that indigenous microbial community (present in the wastewater/wastewater treatment plant) can be selectively enriched on lipid-accumulating bacteria, being potential resources for obtaining high-value compounds. Additionally, the use of hydrocarbon-rich wastewaters, which are cheap raw materials, can significantly reduce the production and commercialization costs of the obtained lipids as well as to mitigate

environmental pollution by decreasing the amount of wastes that have to be treated. In this way, this study opens new perspectives to develop more economic and environmentally sustainable processes to manage this type of wastes.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.watres.2018.07.068>.

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