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

Ana Rita Castro [a], Pedro T.S. Silva [a, 1], Paulo J.G. Castro [a, 2], Eliana Alves [b], M. Rosário M. Domingues [b], Maria Alcina Pereira [a, *]

[a] CEB - Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-57 Braga, Portugal
[b] Mass Spectrometry Centre, Department of Chemistry & QOPNA, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

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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.

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,

* Corresponding author. Universidade do Minho, Departamento de Engenharia Biológica, Campus de Gualtar, 4710-057 Braga, Portugal.
E-mail address: alcina@deb.uminho.pt (M.A. Pereira).
[1] Present address: Department of Chemical Engineering, Loughborough University, Epinal way, Loughborough, Le11 3EP, UK.
[2] Present address: Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Av. da República, 2780-157 Oeiras, Portugal.

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List of abbreviations

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