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Valorization of lubricant-based wastewater for bacterial neutral lipids production: Growth-linked biosynthesis

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ABSTRACT

Lipids produced by microorganisms are currently of great interest as raw material for either biofuels or oleochemicals production. Significant biosynthesis of neutral lipids, such as triacylglycerol (TAG) and wax esters (WE) are thought to be limited to a few strains. Hydrocarbonoclastic bacteria (HCB), key players in bioremediation of hydrocarbon contaminated ecosystems, are among this group of strains. Hydrocarbon rich wastewaters have been overlooked concerning their potential as raw material for microbial lipids production. In this study, lubricant-based wastewater was fed, as sole carbon source, to two HCB representative wild strains: *Alcanivorax borkumensis* SK2, and *Rhodococcus opacus* PD630. Neutral lipid production was observed with both strains cultivated under uncontrolled conditions of pH and dissolved oxygen. *A. borkumensis* SK2 was further investigated in a pH- and OD-controlled fermenter. Different phases were assessed separately in terms of lipids production and alkanes removal. The maximum TAG production rate occurred during stationary phase (4 mg-TAG/L h). The maximum production rate of WE-like compounds was 15 mg/L h, and was observed during exponential growth phase. Hydrocarbons removal was 97% of the gas chromatography (GC) resolved straight-chain alkanes. The maximum removal rate was observed during exponential growth phase (6 mg-alkanes/L h). This investigation proposes a novel approach for the management of lubricant waste oil, aiming at its conversion into valuable lipids. The feasibility of the concept is demonstrated under low salt (0.3%) and saline (3.3%) conditions, and presents clues for its technological development, since growth associated oil production opens the possibility for establishing continuous fermentation processes.

1. Introduction

Lipids produced by microorganisms are currently of great interest as raw material for biofuels production (Röttig et al., 2010) as well as food applications, cosmetics and oleochemicals (Santala et al., 2011).

Biosynthesis of reserve polymers, such as polyhydroxyalkanoates (PHA), is a characteristic wide spread among bacteria (Koutinas et al., 2014). On the other hand, significant biosynthesis and accumulation of neutral lipids, such as triacylglycerol (TAG) and wax esters (WE) are thought to be limited to a few strains (Wältermann and Steinbüchel, 2006). Hydrocarbonoclastic bacteria (HCB), key players in bioremediation of hydrocarbon contaminated ecosystems, are among this group of strains. HCB are able to produce and accumulate neutral lipids, which production can be maximized up to 76% of cell dry weight, when submitted to growth-limiting conditions (Kosa and Ragauskas, 2011; Manilla-Pérez et al., 2010b). Growth-limiting conditions may encompass stress conditions such as nitrogen scarcity and dissolved oxygen tension (Alvarez et al., 2002; Bredemeier et al., 2003).

Neutral lipids have been reported to be biologically produced from separately fed hydrocarbons (HC) (Kalscheuer et al., 2007; Manilla-Pérez et al., 2010a). However, waste streams rich in HC have been overlooked concerning their potential as C-source for biological production of bacterial lipids (Naether et al., 2013). One example of such potential is the lubricant waste. The intensive usage of crude oil derivatives as lubricants was estimated to be around 30 million tonnes per year, about 1% of the World's total

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List of abbreviations

C-source	carbon source
COD	chemical oxygen demand
FA	fatty acid
FAME	fatty acid methyl esters
GC-FID	gas chromatography and Flame ionization detector
HC	hydrocarbons
HCB	hydrocarbonoclastic bacteria
HPLC	high-performance liquid chromatography
LW	lubricant waste-oil
NIR	near-infrared region
OD_x	optical density at X nm wavelength
SPE	solid phase extraction
TAG	triacylglycerol
TLC	thin layer chromatography
TPH	total petroleum hydrocarbons
WE	wax ester
WS/DGAT	wax ester synthase/acyl coenzyme A (acyl-CoA):diacylglycerol acyltransferase

mineral oil consumption, according United Nations - Industrial Commodity Statistics Database (2010), and is known to originate equivalent volumes of recalcitrant waste rich in HC. Treatment options for spent lubricant oil include incineration and landfill dumping, which disregards the energy, among other resources, that can still be recovered from these waste streams (Koutinas et al., 2014). Lubricant waste (LW) consists of a complex matrix containing a mixture of straight-chain alkanes ranging from C10 to C34. The apolar fraction of LW contains compounds that are unresolvable in regular HC analysis by gas chromatography (GC-FID).

In this research, model organisms were selected as representative of different branches of the hydrocarbonoclastic cluster: *Alcanivorax borkumensis* SK2 and *Rhodococcus opacus* PD630. *A. borkumensis* SK2 is a ubiquitous marine gram-negative bacterium (Manilla-Pérez et al., 2010a; Schneiker et al., 2006; Yakimov et al., 1998), which goes from nearly undetectable to dominant in open sea or coastal waters after HC-based contamination (Atlas, 1981). *R. opacus* PD630 is an actinomycete gram-positive soil bacterium capable of degrading a wide range of carbon sources (Alvarez et al., 1996; Gouda et al., 2008; Kalscheuer et al., 2000).

Combining valuable lipidic compounds production with industrial wastewater treatment can contribute to make waste management more economic and environmentally sustainable. This study aims at assessing the potential of HC-rich wastewaters as cheap raw-material for biological production of valuable neutral lipids.

2. Materials and methods

2.1. Inoculum

Rhodococcus opacus PD630 (DSM number 44 193, Braunschweig, Germany) was pre-cultured at 28 °C in Mineral Salts (MS) Medium according to Schlegel et al. (1961) and *Alcanivorax borkumensis* SK2 (DSM number 11 573) was cultivated in saline medium (ONR7a) according to DMSZ, Braunschweig, Germany.

The biomass was harvested at the end of the exponential phase and washed to remove the remaining carbon source: pyruvate. The culture was centrifuged (20 000 × *g*, 10 min and 10 °C) in a centrifuge Avanti J25 (Beckman Coulter), supernatant was discarded and the pellet was resuspended in NaCl 0.9% (w/w). This procedure was repeated three times. Fresh culture medium was used to resuspend the pellet after the last centrifugation. The inoculum volume was calculated according to the targeted initial concentration for each assay (initial $OD_{600} = 0.1$).

2.2. Carbon source (oily wastewater)

The concentrated emulsified lubricant-based wastewater (LW), used as substrate for lipid production, was collected from a heavy machinery maintenance service unit from Alstom Corporation in Maia, Portugal (2011). This concentrate corresponds to the floating phase of the wastewater generated in the machinery washing process, separated in a gravimetric oil/water separation unit.

2.3. Experimental design

Lubricant-based wastewater (LW) was fed to *A. borkumensis* SK2 and to *R. opacus* PD630 as sole carbon source in non-controlled 50 mL batch experiments. *A. borkumensis* SK2 was also cultivated in controlled conditions: 2 L batch experiments operated at constant pH and dissolved oxygen concentration.

Growth and lipid production were monitored in two different cultivation stages: in the first, referred to as growth-stage, bacteria were grown in adequate medium supplemented with nitrogen source in excess; and in the second stage, the so-called production-stage, the cultures grown in the first stage were fed with the same carbon source (LW) and limited nitrogen source concentration (ammonium chloride).

The transition between stages, in non-controlled shake flask experiments - E1 (*A. borkumensis* SK2) and E2 (*R. opacus* PD630) - was done manually by the transference of bacteria to the new media with three different levels of nitrogen scarcity (see sub-section 2.3.1. Uncontrolled cultures). In controlled batch experiments, where *A. borkumensis* SK2 was further studied (R1 and R2), the transition between the two stages was prompted by growth-related nitrogen depletion (see sub-section 2.3.2. Controlled cultures).

2.3.1. Uncontrolled cultures

A. borkumensis SK2 and *R. opacus* PD630 were cultivated in 250 mL shake flasks with 50 mL of ONR7a medium and MS medium, respectively. The incubation temperature was 28 °C and stirring speed 140 rpm. Each culture was composed of two distinct and consecutive stages. First, bacteria were grown in adequate medium supplemented with LW (3.9 g/L COD) as sole carbon source and excess ammonium chloride (0.3 g/L) as nitrogen source. The grown biomass was washed following the described 3-step centrifugation process (see section 2.1. Inoculum) and inoculated in the corresponding medium for the second stage. The medium for the second stage was supplemented with LW (3.9 g/L COD) and 3 different levels of nitrogen concentration: 29, 15, and 7 mg-N/L, which corresponds to fractions of the recommended for cultivation of both strains. Incubation in nitrogen limiting conditions was performed for 48 and 90 h.

2.3.2. Controlled cultures

A. borkumensis SK2 was further cultivated in controlled batch operation mode. A double-wall water-jacketed CSTR reactor with 2 L working volume was used. The culture was monitored and controlled by using an ez-Control device from Applikon Biotechnology, Inc. (Delft, The Netherlands). The data was compiled with BioXpert v2 software package from Applikon Biotechnology®. Dissolved oxygen (dO_2) and pH were monitored with AppliSens probes, and controlled by automated addition of NaOH (4 M) and