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Production of added value bacterial lipids through valorisation of hydrocarbon-contaminated cork waste

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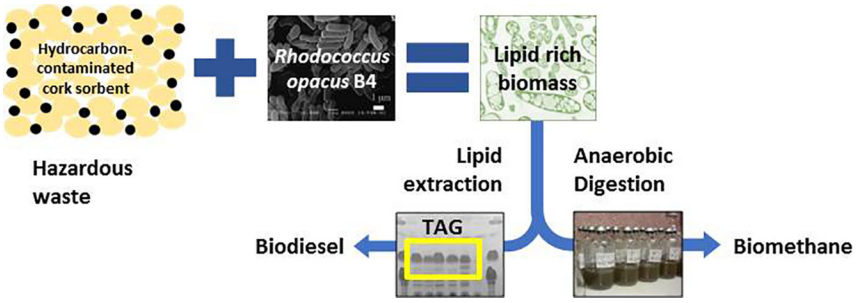
HIGHLIGHTS

- Hydrocarbon-contaminated cork sorbents are conventionally submitted to costly hazardous wastes treatments.
- *Rhodococcus opacus* B4 was able to efficiently treat hexadecane-contaminated cork wastes with concomitant lipids production.
- Triacylglycerol (TAG) was the main neutral lipid produced and palmitic acid was the predominant fatty acid present.
- Produced lipid-rich biomass can be used as feedstocks for biofuels production.

GRAPHICAL ABSTRACT



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ABSTRACT

This work demonstrates that cork used as oil-spill sorbents, contaminated with liquid hydrocarbons, herein demonstrated with hexadecane, can be biologically treated by *Rhodococcus opacus* B4 with concomitant lipids production. *R. opacus* B4 consumed up to 96% of hexadecane (C16) impregnated in natural and regranulated cork sorbents after 48 h incubation, producing 0.59 ± 0.06 g of triacylglycerol (TAG) g^{-1} of C16 consumed with a TAG content of 0.60 ± 0.06 g g^{-1} of cellular dry weight (CDW) and 0.54 ± 0.05 g TAG g^{-1} of C16 consumed with a TAG content of 0.77 ± 0.04 g g^{-1} (CDW), respectively. TAG was mainly composed by fatty acids of 16 and 18 carbon chains demonstrating the feasibility of using it as raw material for biodiesel production. In addition, the obtained lipid-rich biomass (whole cells) can be used for biomethane production, at a yield of 0.4 L CH_4 g^{-1} (CDW).

The obtained results support a novel approach for management of oil-spill contaminated cork sorbents through its valorisation by producing bacterial lipids, which can be used as feedstocks for biofuels production.

1. Introduction

Increasing demand for liquid petroleum has intensified industrial activities related to petroleum exploration increasing the risk of oil spillages in land or in marine systems, accidentally or deliberately (Lucas and MacGregor, 2006; Atlas, 1995). Crude oil is mainly composed by a mixture of hydrocarbons, with >20,000 chemical components (Marshall and Rodgers, 2003). Release of these compounds into the environment is a serious pollution threat, resulting in a high negative impact on the biotic and abiotic components of the ecosystems (Peterson et al., 2003; Teal and Howarth, 1984). To mitigate this problem, several technologies have been developed to treat hydrocarbon contaminated environments, employing biological, physical, chemical and thermal processes (Hu et al., 2013; Dewling, 1980). Among those, physical containment and recover, using a variety of equipment such as booms, barriers, skimmers, as well as natural and synthetic absorbent materials,

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are usually in the primary line of defence against oil spills. They present some advantages, since can be applied to all types of oils, no maintenance is required and are simple to use (Dave, 2011).

Cork is a good natural absorbent due to their hydrophobic nature (Silva et al., 2005) and is being used as biosorbent material in the treatment of oil spills and leaks (Silva, 2007), oil-in-water emulsions (Souza et al., 2016) and also vegetable oil refinery wastewaters (Pintor et al., 2015). After use, oil contaminated cork residues are treated by conventional physical-chemical methods, having an associated cost.

Rhodococcus are aerobic gram-positive bacteria widely distributed through different environments such as soils, sediments and water (Alvarez et al., 2004; Peng et al., 2008) and particularly in hydrocarbon-contaminated ecosystems (Van Hamme et al., 2003). Because this genus possess many catabolic genes conferring the capacity to transform a wide variety of pollutants including hydrocarbons, (Larkin et al., 2010), several *Rhodococcus* strains are being study for bioremediation of hydrocarbon-contaminated sites and industrial wastewaters (Auffret et al., 2014). *Rhodococcus* species are also interesting because they can produce storage lipids compounds, especially TAG, during cultivation on several carbon sources, including single hydrocarbons (Alvarez, 2003) and waste hydrocarbon mixtures (Da Silva et al., 2016). Bacterial lipids are valuable compounds with potential to replace fossil resources in many industrial processes, in particularly in biofuels production.

In this work, biological treatment of hydrocarbon-contaminated cork sorbents for its valorization by bacterial lipids production was assessed using *R. opacus* B4. The suitability of the produced lipids as feedstock for biofuels production, namely biodiesel and biomethane, was further evaluated.

2. Material and methods

2.1. Cork sorbents

Natural cork and regranulated cork were tested. Natural cork can absorb five times its weight in oil while regranulated cork, due to a thermal treatment at 450 °C can absorb ten times its weight in oil (Silva, 2007).These products are commercially available under the name CORKSORB (https://www.corksorb.com/en). Both materials were provided by Cortiçeira Amorim, S.A., Portugal.

2.2. Bacterial strain, media and cultivation conditions

2.2.1. Strain and media

R. opacus B4 (NBRC 108011) was purchased from the National Institute of Technology and Evaluation, Biological Resource Center, Japan (NBRC). This strain was selected due to the ability to produce lipid storage compounds, namely TAG when cultivated on hexadecane ($C_{16}H_{34}$) (Castro et al., 2016).

Mineral salts (MS) medium was used for cell maintenance and growth (Schlegel et al., 1961). Hexadecane was chosen as a model contaminant since it is usually used as the main representative compound of aliphatic hydrocarbons, one of the dominant groups found in crude oil and derivatives (Pacini-Petitjeana et al., 2015). This compound was used in the assays as carbon and energy source at 1 g L^{-1}.

2.2.2. Preparation of seed culture

Cells from a single colony of *R. opacus* B4 grown on MS medium agar plates (1.5% agar) at 30 °C during 4 days were inoculated in 50 mL of 802 medium (rich medium) in a 250 mL flask. The seed culture was incubated on a rotary shaker (150 rpm) at 30 °C until the middle of the exponential growth phase was reached (48 h). Growth was determined by measuring optical density at 600 nm wavelength with a spectrophotometer (U-1500 Hitachi, Tokyo, Japan).

2.2.3. Growth and lipid accumulation experiments

The experiments were carried out under sterile conditions, in duplicate, using 250 mL conical flasks containing 50 mL of defined medium. The cultures were incubated on a rotary shaker (150 rpm) at 30 °C.

Cells of seed culture were harvested, washed with sterile sodium chloride solution (0.9%, *w*/*v*), and re-suspended in fresh MS medium. Then, cells were used to inoculate flasks to an optical density at 600 nm (OD_{600nm}) of 0.1 and cultivated in MS medium supplemented with hexadecane (1 g L^{-1}) as sole carbon source. Nitrogen was supplied at a molar carbon to nitrogen ratio of 4 (C/*N* = 4). These conditions will promote growth in order to have high amounts of biomass. Cells were grown until the middle of the exponential growth phase which corresponded to 150 h of cultivation. Cells from the grown inoculum were thereafter collected by centrifugation (4 °C; 10 min, 10,000 g), washed twice with sterile sodium chloride solution (0.9%, *w*/w) and transferred to new flasks previously prepared with fresh MS medium and cork contaminated with hexadecane. Considering the maximum absorption capacity of both cork sorbents, 0.01 g of natural cork or 0.005 g of regranulated cork granules were weight together with 50 mg of hexadecane (final concentration of 1 g L^{-1} in the culture medium) to the flasks. After 24 h of contact between cork and hexadecane, MS medium containing nitrogen at a molar C/N ratio of 300 (storage lipid accumulation conditions) was added.

In parallel, several control experiments were performed: 1) *R. opacus* B4 growing on MS medium supplemented with hexadecane 0.1% (*w*/*v*) and without cork – B4·C16; 2) MS medium supplemented with hexadecane 0.1% (*w*/*v*) and natural cork granules (NC) – NC·C16; 3) MS medium supplemented with hexadecane 0.1% (*w*/*v*) and regranulated cork granules (RC) – RC·C16; 4) MS medium supplemented with natural cork granules but without hexadecane – B4·NC and 5) MS medium supplemented with regranulated cork granules (RC) but without hexadecane – B4.RC. After 48 h of cultivation, cells were harvested, washed and kept at −80 °C until further lyophilisation.

2.3. Analytical methods

2.3.1. Chemical oxygen demand (COD)

Chemical oxygen demand (COD) was determined using the cuvette-test Lck414 (Hach-Lange, Germany). These measurements were made in triplicate, using the manufacturer's procedures.

2.4. Hexadecane extraction and quantification

Hexadecane extraction and quantification was performed according to Castro et al. (2016). Briefly, hexadecane in the culture medium was sequentially extracted from each replicate flask (total content of the flask analyzed) in a separation funnel using hexane as solvent. Hexadecane concentration was determined in a gas chromatograph coupled to a flame ionization detector (GC-FID) (GC Varian® star 3400CX, USA). The column used was the model VF-1 ms (Agilent, USA) 30 m length long × 0.025 mm internal diameter, made from fused silica coated with dimethylpolysiloxane as stationary phase. Helium was used as carrier gas at a flow rate of 1 mL min^{-1}. The temperature of the detector and the injector were set at 300 and 285 °C, respectively. The column's temperature was maintained at 60 °C for 1 min and then raised up to 290 °C at a rate of 8 °C min^{-1}.

2.5. Extraction and analysis of neutral lipids

2.5.1. Total lipid extraction

Total lipid were extracted from 10 mg of lyophilized cells (or cork granules) using chloroform: methanol (2:1; *v*/v) as extraction solvent, according to Folch method (Folch et al., 1957). The mixture was incubated at room temperature with shaking for two hours. Afterwards, the lipid extracts were separated from the cells by filtration through glass wool and evaporated to dryness.