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# Effect of carbon sources on lipid accumulation in *Rhodococcus* cells

Maria Ana L.R.M. Cortes, Carla C.C.R. de Carvalho*

*Institute of Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Department of Bioengineering, Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1049-001 Lisboa, Portugal*

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ABSTRACT

New regenerative and ecologically friendly processes to produce energy are required to replace petroleum-based derived combustibles. The objective of this work was to assess the usefulness of *Rhodococcus erythropolis* DCL14 and *Rhodococcus opacus* PWD4 cells to produce lipids for biodiesel blends. These cells accumulated triacylglycerols (TAGs), the most relevant lipids for biodiesel production, in addition to other lipids, including apolar lipids and phospholipids, which contained fatty acids that could be esterified. The proportion between phospholipids and storage lipids was dependent on carbon source and culture age. The estimated cetane number of the fatty acid blends produced ranged between 60.5 and 61.7 for *R. erythropolis* and 50.5 and 69.8 for *R. opacus*.



## 1. Introduction

The quest for renewable fuels from suitable, sustainable, and ecologically friendly processes to replace petroleum-derived combustibles has included as a promising candidate biodiesel. Presently, biodiesel is largely produced from vegetable oils which are chemically in the form of triacylglycerols (TAGs). However, the use of food crops as the starting material has raised several ethical and economical issues. Algae, yeast, and bacteria are known to produce TAGs with fatty acid composition similar to vegetable oils and are considered oleaginous if the lipid content surpasses 20% [1–4]. Microorganisms produce these lipids under stressful conditions such as an excess of carbon and a limiting quantity of nitrogen sources [5,6]. The lipid content of oleaginous microorganisms, grown under conditions allowing high lipid accumulation, makes their use very promising for biodiesel production [4,7].

Microalgae have a high lipid accumulation capacity: e.g., *Schizochytrium* sp. cells accumulate 50–77% of the dry weight as lipids [3]; *Chlorella protethecoides* presented a lipid content of 89.3% when subjected to Cu(II) stress [8]; *Chlorella emersonii* accumulated 63% of the dry weight as lipids under nitrogen limitation [9]. Although it has been claimed that algal biodiesel will be economically viable in the next few years, problems related to microalgae harvesting, oil extraction, and the supply of carbon dioxide and light still limits the use of this feedstock [3,10].

Yeast and filamentous fungi are also known to accumulate lipids which might be used to produce biodiesel: the oleaginous yeast *Cryptococcus curvatus* contained ca. 38% of lipids with reasonable cetane number when grown on organic waste from the brewery industry [11]; a Japanese research team isolated a *Cryptococcus* sp. strain, from a collection of 500 yeasts, with a lipid content of 62% [12]; the filamentous fungus *Mortierella isabellina* ATCC42613 presented a lipid content around 40% when grown on glucose and xylose, with the content being dependent on carbon source concentration [13]; lipid content in *Lipomyces starkeyi* reached ca. 70% of dry matter in synthetic medium and some accumulation could also be attained from pre-treated sewage sludge [14]. However, growth of oleaginous yeasts and filamentous fungi are usually slow and represent a minor proportion of the total known populations [15,16].

In bacteria, the accumulation of TAGs has been described in actinomycetes, including the genera *Mycobacterium*, *Streptomyces*, *Acinetobacter*, *Nocardia*, and *Rhodococcus* [1]. The principal function of TAG in bacteria is, apparently, to act as storage compounds but could also serve to regulate the cellular membrane fluidity by preventing unusual fatty acids to be incorporated in the membrane phospholipids or to act as a sink for reducing equivalents [1]. Recent findings have suggested that lipid body formation and accumulation of TAGs are important in the metabolism of pathogenic bacteria such as *Mycobacterium tuberculosis* [17,18]. Strain *Rhodococcus opacus* PD630, grown on gluconate, is capable of accumulating up to

* Corresponding author. Tel.: +351 218419594; fax: +351 218419062.
*E-mail address:* ccarvalho@tecnico.ulisboa.pt (C.C.C.R. de Carvalho).

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76% of the cell dry weight as TAGs [19,20]. When grown on in a batch culture with 240 g/L glucose, strain PD630 yields ca. 38% of cell dry weight as TAGs [21]. However, bacterial cells can also produce other specialized lipids containing fatty acids that can be used for biodiesel production: phospholipids used in the cellular membrane; glycolipids used as exopolymers and biosurfactants; polydroxyalkanoates and wax esters used as storage compounds.

The aim of this study was to assess the ability of two strains of *Rhodococcus erythropolis* and *R. opacus* to produce fatty acids that could be used in biofuels.

## 2. Materials and methods

### 2.1. Bacterial strain, medium, and growth conditions.

*R. erythropolis* DCL14 was isolated by the Division of Industrial Microbiology of the Wageningen University, The Netherlands [22]; it is now deposited and maintained at the Institute for Biotechnology and Bioengineering, Lisbon, Portugal. Both *R. erythropolis* DCL14 and *R. opacus* PWD4 were grown at 28 °C and 200 rpm in mineral medium (MM) containing per liter of demineralised water: 0.01 g EDTA, 0.002 g $ZnSO_4{\cdot}7H_2O$, 0.001 g $CaCl_2{\cdot}2H_2O$, 0.005 g $FeSO_4{\cdot}7H_2O$, 0.0002 g $Na_2MoO_4{\cdot}2H_2O$, 0.0002 g $CuSO_4{\cdot}5H_2O$, 0.0004 g $CoCl{\cdot}6H_2O$, 0.001 g $MnCl_2{\cdot}4H_2O$, 0.1 g $MgCl_2{\cdot}6H_2O$, and 1.55 g $K_2HPO_4$ and 0.85 g $NaH_2PO_4{\cdot}H_2O$ for buffering (all chemicals were from Sigma–Aldrich). The nitrogen source, $(NH_4)_2SO_4$, was added at 2 g/L unless stated otherwise (e.g., 0.01 g $L^{-1}$ during nitrogen source limitation). Growth was promoted until an optical density (OD; 600 nm) of ca. 1–1.5 before promoting nitrogen or phosphorus limitation. Glucose, ethanol, sodium gluconate, *n*-hexane, *n*-heptane, *n*-pentadecane, *n*-hexadecane and toluene, and both toluene and hexadecane, were tested as carbon sources at 0.25% and 1%. Initial concentrations of 5% glucose and ethanol were also tested but higher concentrations of hydrocarbons were prevented due to extremely low solubility of organic solvents in water. Nevertheless, carbon source was still available during the stationary phase (glucose analyzed by HPLC, the remaining by GC; the system was not carbon limited). Cell growth was started by adding 2% of inoculum to 20 mL MM in 100 mL Erlenmeyer flasks. Two milliliter samples were collected along the culture growth (during the exponential and stationary phases) and at least three independent assays were performed per growth condition. Cell dry weight was determined after 24 h at 65 °C using a Mettler Toledo AG104 balance and lipid composition was evaluated by gas chromatography.

### 2.2. Lipid production

Lipid accumulation was promoted by reducing the nitrogen source to 0.01 g $L^{-1}$ with an excess of carbon source on the MM.

#### 2.2.1. Visualization of lipids using Nile Red

Intracellular lipids in *R. erythropolis* were stained using Nile Red (Molecular Probes, Life Technologies, USA) as follows: a stock solution of 1.3 mg/mL of Nile Red in acetone was prepared, added to phosphate buffer suspended cells ($1–2 \times 10^6$ cells/mL) to achieve a 1:100 dilution, and incubated for 5 min. The samples were observed by fluorescence microscopy using an Olympus CX40 microscope, with an Olympus U-RFL-T burner and an U-MWB mirror cube unit (excitation filter: BP450-480; barrier filter:BA515). Images were captured by an Evolution™ MP5.1 CCD color camera using the software Image-Pro Plus, both from Media Cybernetics, Inc. (USA).

#### 2.2.2. Lipid extraction and fractioning

Lipids were extracted by a modified Bligh and Dyer method [23] according to Findlay et al. [24] using chloroform:methanol:milli-Q water (at a ratio of 1:2:0.8). The chloroform fraction, containing all lipids, was dried under a nitrogen stream using a 6-Port mini-vap (Supelco) and placed in fresh chloroform. The lipids were fractioned in classes with different polarity on a heat-activated silicic acid column (Merck, Darmstadt) by sequential elution with chloroform, acetone, and methanol [25,26]. The chloroform and acetone fractions contained neutral lipids and glycolipids, respectively, whilst the methanol fraction contained mainly polar membrane lipids. Fatty acid methyl esters (FAMEs) were obtained from the apolar fractions by derivatization with $H_2SO_4$-methanol and by mild alkaline transmethylation with methanolic NaOH from the polar lipid fraction. The fatty acid C19:0, which is not produced by the cells, was added to each lipid fraction prior to the transesterification to be used as internal standard for lipid quantification. Since the exactly amount of C19:0 added was known, the final FAME concentrations could be corrected for losses of FAME occurring during the transesterification reaction and during the following extraction with *n*-hexane, and thus, the correct weight of each fatty acid could be calculated. The percentage of each lipid fraction was determined after calculating the weight of all fatty acids in the three fractions.

#### 2.2.3. Lipid analyses

FAMEs were analyzed by gas chromatography on an Agilent 6890N gas chromatograph with a FID and a 7683 B series injector equipped with a 30 m HP-5 capillary column from J&W Scientific as described previously [27]. Peak identification was achieved using standards of bacterial fatty acid methyl esters and of polyunsaturated fatty acids (both from Supelco), and a methyl *cis*-11-octadecenoate standard solution (Sigma–Aldrich). Peak identification was confirmed by injecting both standards and selected samples on a gas chromatograph–mass spectrometer (5975B inlet MSD from Agilent) equipped with a DB-1 column from J&W Scientific.

### 2.3. Cetane number

The cetane number (CN) was calculated according to Eq. (1) [28]:

$$CN = 61.1 + 0.088x_2 + 0.133x_3 + 0.152x_4 - 0.101x_5 - 0.039x_6 - 0.243x_7 - 0.395x_8 \quad (1)$$

where $x_n$ are the percentage compositions of FAME.

### 2.4. Error analysis

The average error associated with the GC quantification of each FAME was ±1.8% and of each *n*-alkane was 2.7%, quoted for a confidence interval of 99.5%. The errors were calculated based on five independently prepared standard solutions.

## 3. Results and discussion

### 3.1. Effect of fermentation conditions on the production of specialised lipids

A wide array of carbon sources has been identified as being used by bacteria for the synthesis of TAGs, including sugars, organic acids, crude glycerol, alcohols, aliphatic-, phenyl- and branched alkanes, oils, and coal [1,6]. TAG accumulation has been observed predominantly during the stationary growth phase in *Rhodococcus* and when the carbon source is present in the medium in excess when compared to the nitrogen source [19]. Both *R. erythropolis* and *R. opacus* cells produced storage lipids depending on: cell age; type and concentration of carbon, nitrogen, and phosphorous sources used for growth; and culture conditions (Fig. 1 and Table 1).