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ORIGINAL PAPER

Rhodococcus bacteria as a promising source of oils from olive mill wastes

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Abstract

The accumulation of triacylglycerols (TAG) is a common feature among actinobacteria belonging to *Rhodococcus* genus. Some rhodococcal species are able to produce significant amounts of those lipids from different single substrates, such as glucose, gluconate or hexadecane. In this study we analyzed the ability of different species to produce lipids from olive oil mill wastes (OMW), and the possibility to enhance lipid production by genetic engineering. OMW base medium prepared from alperujo, which exhibited high values of chemical oxygen demand (127,000 mg/l) and C/N ratio (508), supported good growth and TAG production by some *rhodococci. R. opacus, R. wratislaviensis* and *R. jostii* were more efficient at producing cell biomass (2.2–2.7 g/l) and lipids (77–83% of CDW, 1.8–2.2 g/l) from OMW than *R. fascians, R. erythropolis* and *R. equi* (1.1–1.6 g/l of cell biomass and 7.1–14.0% of CDW, 0.1–0.2 g/l of lipids). Overexpression of a gene coding for a fatty acid importer in *R. jostii* RHA1 promoted an increase of 2.2 fold of cellular biomass value with a concomitant increase in lipids production during cultivation of cells in OMW. This study demonstrates that the bioconversion of OMW to microbial lipids is feasible using more robust rhodococcal strains. The efficiency of this bioconversion can be significantly enhanced by engineering strategies.

Keywords Olive mill wastes · Bioconversion · Lipid production · Engineered *Rhodococcus*

Introduction

The production of olive oil constitutes a significant economic activity in Europe, mainly for Mediterranean lands, as well as in other countries, such as in Argentina. Argentina is the tenth world producer of olive oils, occupying the first place in the American continent. The oil extraction process generates a great amount of olive mill waste (OMW) during short periods of time, which causes serious pollution problems when it is disposed in the environment without treatment, due to its high chemical oxygen demand (COD) and low pH values (Niaounakis and Halvadakis 2006). The effluents generated during olive processing are one of the main polluting industrial wastes (Paraskeva and Diamadopoulos 2006). It has been estimated that 10 million m^3 of this liquid effluent are generated per year, with corresponds to an equivalent load of the wastewater generated from about 20 million people (McNamara et al. 2008; Barbera et al. 2013). The type of OMW and its composition mainly depend on the variety of olive fruits and the process used for olive oil extraction (Bellou et al. 2014; Sarris et al. 2013). Multiple methods are used in the olive oil extraction, resulting in different waste products. The three-phase system usually generates pure olive oil, olive mill wastewater (OMWW) and a solid cake-like by-product called *orujo*, whereas the two-phase system generates olive oil plus a semi-solid waste known as the two-phase olive-mill waste (TPOMW), and olive wet cake or *alpeorujo* (Darvishi 2012; Morillo et al. 2009). The two-phase system reduces substantially the enormous amount of liquid waste produced by the three-phase system (0.5–1.5 m^3 per ton of processed olives), however it generates large amount of semi-soil waste (800 kg per ton of processed olives) (Paraskeva and Diamadopoulos 2006; Darvishi 2012; Barbera et al. 2013). Semi-solid and liquid

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OMW have a dark-colored, acid smell and corrosive properties (Azbar 2004). In general, OMW contains approximately 15% of organic matter, including polysaccharides (9.6–19.3), lipids (3.76–18%), proteins (6.7–7.2%), minerals and high concentration of recalcitrant compounds such as polyphenols (0.5–25 g/l) with strong antimicrobial and phytotoxic properties, which hinders its biological treatment (Aguilera et al. 2008; Öner 2013; Azbar 2004; Roig et al. 2006). The high polluting power of the OMW on watercourses, soil and air determines the imperative need to apply effective pre-treatment to its final disposal; thus, this represent one of the main problems affecting the olive oil industry. Different treatments including physical, chemical, biological and combined technologies have been proposed for reducing the pollutant power of OMW (Darvishi 2012; Morillo et al. 2009; Chiavola et al. 2010). In this context, numerous methods have been applied in the treatment of OMWW and TPOMW, such as thermal process (evaporation or combustion), physico-chemical treatments (flocculation, decantation, adsorption, electro-coagulation, reverse osmosis, dissolution of organic matter, photochemical degradation, chemical oxidation processes and ion exchange), extraction of valuable compounds (pectins, antioxidants, residual oil, sugars) and biological treatments. The biological treatments (composting and aerobic or anaerobic microbiological fermentation) are considered most favorable because they are friendly to the environment and less expensive compared to other procedures (Mantzavinos and Kalogerakis 2005). In addition, the biological treatment of OMW opens the possibility for its conversion into products of industrial interest. Diverse approaches have been designed to convert OMW into value-added products using different microorganisms, such as organic acids, biopolymers, enzymes, ethanol, single cell proteins, or microbial lipids, among others (Darvishi 2012). Bellou et al. (2014) reported the ability of *Zygomycetes* strains to grow on OMW and produce lipids containing high amounts of polyunsaturated fatty acids (PUFA). In other study, a bacterial strain belonging to *Gordonia* genus was able to grow and accumulate up to 30% (w/w) of triacylglycerols (TAG) from OMW (Gouda et al. 2008). In this sense, the search for lipid accumulating-microorganisms has been promoted to a large extent by the potential applications of these compounds in the oleochemical and pharmaceutical industry, among others. Similar to vegetable oils, microbial oils (TAG, wax esters, fatty acids) can be used to the production of soaps, detergents, resins, lubricants, plastics, inks, surface coating, lacquers, cosmetics, and as ingredients for pharmaceutical products (Alvarez 2010). Microbial oils containing PUFAs could be beneficial for human health (SanGiovanni and Chew 2005; Youdim et al. 2000). The production of cacao butter-like oils has been reported for *Apiotrichum curvatum, Yarrowia lipolytica* and *Rhodococcus fascians* (Papanikolaou et al. 2002; Herrero et al. 2016), however its use as edible oils would be not well accepted socially. Eventually, lipids from bacterial origin may be used for feeding pets or commercially raised fish (Alvarez 2010). On the other hand, microbial oils are being considered as a source of precursors for the production of biodiesel and other biofuels due to the increasing demand of energy and the need to mitigate the use of vegetable oils destined for human consumption for this purpose. Some bacteria belonging to *Rhodococcus* genus are specialists in the production and accumulation of TAG from diverse carbon sources, including industrial wastes (Alvarez and Steinbüchel 2010). Previous studies revealed some genetic and physiological differences among rhodococcal species/strains for the degradation of industrial wastes and lipid production. *R. opacus* was the most robust rhodococcal species for bioconversion of whey to oils, in comparison to *R. jostii, R. erythropolis, R. fascians* and *R. equi* (Herrero and Alvarez 2016). *R. opacus* possesses the complete genetic endowment for degrading lactose, galactose and whey as well as for lipid production from these substrates, whereas in the other species the genomic region including genes for the putative lactose binding protein transporter system (LacEFGK), LacA, LacB, GalE and DeoR family regulator, is not present (Herrero and Alvarez 2016). On the other hand, *R. fascians* and *R. erythropolis* were the most efficient species for accumulating TAG from glycerol as sole carbon source. These microorganisms possess a set of genes (*glpFK1D1*) involved in glycerol degradation which is absent in other rhodococcal species, such as *R. opacus, R. jostii* and *R. equi* (Herrero et al. 2016). Glycerol assimilation and oil production were significantly improved in *R. opacus* PD630 after heterologous expression of *glpFK1D1* from *R. fascians* (Herrero et al. 2016). Glycerol utilization was alternatively enhanced in *R. opacus* PD630 using an adaptive evolutionary approach (Kurosawa et al. 2015). All these results demonstrated that *rhodococci* are an amenable biological system for biotechnological applications based on the reutilization of industrial wastes.

In the present study we investigated the ability of strains belonging to six rhodococcal species (*R. opacus, R. jostii, R. wratislaviensis, R. erythropolis, R. fascians* and *R. equi*) to use OMW as a low-cost substrate for growth and lipid production. In addition, we analyzed the effect of the overexpression of a gene involved in the fatty acid import (*ltp*1) in *R. jostii* RHA1 (Villalba and Alvarez 2014), on the cellular biomass and lipid production during cultivation in OMW base media. In a previous study, we determined that the overexpression and deregulation of *ltp*1 gene led to an increase up to sixfold and threefold in cellular biomass and lipid production in *R. jostii* RHA1, respectively, after cultivation with fatty acids (FA) as sole carbon sources (Villalba and Alvarez 2014). This study showed the potential of *rhodococci* to convert a highly polluting waste into value-added bioproducts.

Materials and methods

Bacterial strains, media and growth conditions

The strains used in this study are listed in Table 1. Cells were grown aerobically at 28 °C overnight in Luria Bertani (LB) medium on a rotary shaker (200 rpm). After growth, cells were harvested, washed, resuspended with sterile saline solution (NaCl 0.85% w/v), and finally transferred to the corresponding culture media.

In order to study the use of OMW as feedstock for growth and lipids synthesis by *Rhodococcus* strains, OMW base medium was prepared. For this, 250 g of semi-solid olive mill waste (alperujo or TPOMW) were boiled in 1000 ml of tap water for 1 h, the extract was filtered and autoclaved (1.0 atm 20 min). The pH was adjusted to 7.0 after sterilization. If necessary, antibiotic kanamycin was added to culture media at a final concentration of 50 μg ml^{-1}. Induction of acetamidase promoter (P*ace*) of pJAM2 and its derivatives was achieved by addition of 0.5% (w/v) acetamide to the cultures.

Two different culture methodologies were tested: submerged culture (SMC) and surface culture (SC). In the case of SMC, 50 ml of OMW base medium were inoculated with 2 ml of a cell suspension (OD_{600} 4.0), and grown aerobically at 28 °C on a rotary shaker (200 rpm). For SC, polyethersulphone filters (PES filters, 47 mm diameter and pore 0.22 μ) were inoculated with 150 μl of the cell suspension (OD_{600} 20.0) and placed onto Petri plates (50 mm diameter) which contained 4 ml of agar OMW base medium (named here SC1), or 4 ml of liquid OMW base medium (named here as SC2). Plates were incubated at 28 °C for 7 days in both cases. In order to determine the most appropriate concentration of OMW, different amounts (50, 100, 250 and 500 g) of this residue were boiled in 1000 ml of tap water for 1 h, the extracts were filtered, autoclaved and the pH adjusted to 7.0. Finally, these media were used for bacterial growth applying SC2 method. In addition, NH_4Cl (0.5 g/l) and different mineral salts (KH_2PO_4, 1.5 g; $Na_2HPO_4.12H_2O$, 9 g; $MgSO_4 \cdot 7H_2O$; 0.2 g; NH_4Cl, 1 g; ammonium ferric citrate, 1 ml; $CaCl_2 \cdot 2H_2O$, 20 mg; SL6 solution, 0.1 ml) (Schlegel et al. 1961) were added to OMW base medium (100 g/l) in order to assess their effect on growth and lipid synthesis.

Table 1 Bacterial strains used in this study

Bacterial strains	References
Rhodococcus opacus PD630	DSMZ 44193
Rhodococcus opacus MR22	DSMZ 3346
Rhodococcus wratislaviensis V	This study
Rhodococcus jostii RHA1	Seto et al. (1995)
Rhodococcus erythropolis	DSMZ 43060
Rhodococcus fascians F7	BNM 542
Rhodococcus equi	ATCC 6939
Rhodococcus jostii RHA1 pJAM2	Villalba and Alvarez (2014)
Rhodococcus RHA1 pJAM2/*ltp*1	Villalba and Alvarez (2014)

Chemical characterization of the OMW base medium

OMW used for the preparation of culture media corresponds to alperujo or TPOMW, a semisolid waste composed by olive pulp, olive stones, and washwater. OMW samples were obtained from Indalo olive oil production plant (Northwest Argentina), which uses a two-phase centrifugation process for olive oil extraction. The samples were transported and stored under refrigeration conditions.

The OMW base medium that shown to be most appropriate for growth and lipid production (100 g/l), was chemically analyzed. COD and total organic nitrogen were analyzed by standard methods (APHA 2005). Total sugar concentration was assayed by phenol–sulfuric acid method (DuBois et al. 1956). For glucose quantification, a commercial kit was used (Wiener lab group, Argentina). The C/N ratio was calculated by COD/total nitrogen (Cheirsilp and Louhasakul 2013). Qualitative and quantitative analyses of FA from OMW base medium were performed as described below.

Cell biomass and lipid analyses

After 7 days of incubation the PES filters used for cell biomass production were removed from the culture medium and dried to constant weight. The cell dry biomass (cellular dry weight, CDW) was determined gravimetrically subtracting the weight of the filters.

Total lipids were extracted from cell dry biomass according to Folch et al. (1957). Qualitative and semiquantitative analyses of lipids were carried out by thin layer chromatography (TLC). For this, 5 mg of lyophilized cells were extracted by means of vigorous shaking with 200 μl chloroform/methanol (2:1, v/v) for 90 min at 4 °C. Twenty-five microliters of chloroformic phase was then subjected to TLC on silica gel 60 F254 plates (Merck) using hexane/diethyl ether/acetic acid (80:20:1, by vol.) as mobile phase (Wältermann et al. 2000). Tripalmitin (Merck) was used as TAG reference substance. The lipids were revealed with iodine vapors.

The lipid content was determined by the quantification of total fatty acids. For qualitative and quantitative determination of fatty acids, 5 mg of dry cells were subjected to methanolysis in presence of 15% (v/v) sulfuric acid (Brandl et al. 1988). The resulting acyl-methylesters were analyzed by gas chromatography (GC) using an HP 5890 A gas chromatograph equipped with an InnoWAX

capillary column (30 m × 0.53 mm × 1 µm) and a flame ionization detector (FID). The injection volume was 0.5 ml, and hydrogen the carrier gas (13 ml/min). A temperature program was used for efficient separation of the methyl esters (90 °C for 5 min, temperature increase of 6°C/min, 220 °C for 10 min). For quantitative analysis, tridecanoic acid was used as internal standard. Biomass and lipids determinations were performed in triplicate experiments and results expressed as average values with their corresponding standard deviation.

Electron microscopy analysis

Cells were washed, suspended in 0.1M potassium phosphate buffer (pH 7.5) and fixed with glutaraldehyde for 24 h. Then, cells were washed with a sucrose solution 0.32 M in phosphate buffer and embedded in low viscosity resin (Spurr 1969). Thin sections were contrasted with uranyl acetate and ruthenium red (Vogt et al. 1995). Images were obtained utilizing a Zeiss 109T electron microscope with a Gatan ES camera.

Phylogenetic characterization of strain V

Sequences of 16S rRNA gene, *catA, gyrB* and *alkB* were amplified using DNA extracted from strain V. Primers and PCR cycling conditions used for each gene amplification were shown in Table S1. PCR products were purified using Wizard SV Gel and PCR Clean-Up System (Promega) and then sequenced by the Genomic Unit of Biotechnology Institute in INTA Castelar, Argentina. The generated sequences were edited with BioEdit sequence alignment editor v 7.0.9 (Hall 1999). Most related strains based on 16S rDNA sequencing included *R. opacus* (strains PD630, R7 and M213), *R. jostii* RHA1, *R. wratislaviensis* IFP2016, *R. imtechensis* RKJ300. All of them were considered to perform a phylogenetic comparison. Sequences of functional genes used as phylogenetic markers were obtained from NCBI GenBank database. *catA, gyrB* and *alkB* sequences were translated into amino acid sequences and aligned using BioEdit sequence alignment editor v. 7.0.9 (Hall 1999). Neighbour-joining, with Kimura approximation for calculation of distance matrices, and Parsimony treeing methods for phylogenetic analysis were done using the package Phylip (Felsenstein 1985).

Accession numbers

Nucleotide sequences of 16S rRNA gene, *catA, gyrB* and *alkB* belonging to strain V were deposited in GenBank under accession numbers KT253459, KT248547, KT258448 and KT258449, respectively.

Cloning and overexpression of *ltp*1 gene

The lipid transporter gene *ltp*1 (RHA1_RS27545) was amplified from total genomic DNA of *R. jostii* RHA1 by PCR using the primers FRHA1 (5′GGATCCATGCCCGCG ACCGGTTCCT3′) and RRHA1 (5′TCTAGAGAACTCTCC GCCCCGACC3′). The thermocycling parameters were as follows: 5 min at 94 °C, 30 cycles of 1 min at 94 °C, 30 s at 65 °C, 2 min 20 s at 72 °C, and finally 5 min at 72 °C. Resulting PCR products were cloned in pGEM-T-easy vector (pGEM-T-easy/*RHA1_RS27545*) and verified by DNA sequencing. For overexpression, a *Bam*HI/*Xba*I fragment of pGEM-T-easy/*RHA1_RS27545* was subcloned into a *Bam*HI/*Xba*I site of the shuttle *E. coli–Mycobacterium–Rhodococcus* vector pJAM2, which contains an inducible acetamide promoter (P*ace*), the resulting derivative plasmid pJAM2/*RHA1_RS27545* was transferred into *R. jostii* RHA1 wild type cells by electroporation as previously reported (Hernández et al. 2013). Assays were performed using a Model 2510 electroporator (Eppendorf-Netheler-Hinz-GmbH), transformation was carried as described Kalscheuer et al. (1999). To obtain recombinant strains 400 µl of competent cells were mixed with DNA and preincubated at 40 °C for 5 min followed by 10 min of incubation on ice and then electroporated.

Results

Optimization of OMW base medium for cultivation of cells

Liquid OMW base medium containing 250 g/l of OMW was used for cultivation of *R. opacus* strain PD630, in order to analyze its ability to grow and produce TAG. Cells grown on OMW produced large flocs or cell aggregates in the medium (Fig. S1a), which remained attached to the flasks, preventing the collection of cellular biomass for analysis of lipid content. For this reason, we cultivated cells on the surface of polyethersulfone (PES) filters (0.2 µm), which were placed onto OMW agar plates (named here as surface culture SC1, Fig. S1b) or OMW liquid medium (SC2, Fig. S1c), respectively. In both cases, cellular biomass was easily removed from the filters without contamination with OMW lipids. Similar cellular biomass production (1.8 g/l) and lipid contents (75%, CDW) were obtained in SC1 and SC2. SC2 was a more simplified culture method than SC1 since the addition of agar was not necessary; thus, SC2 was used for further studies.

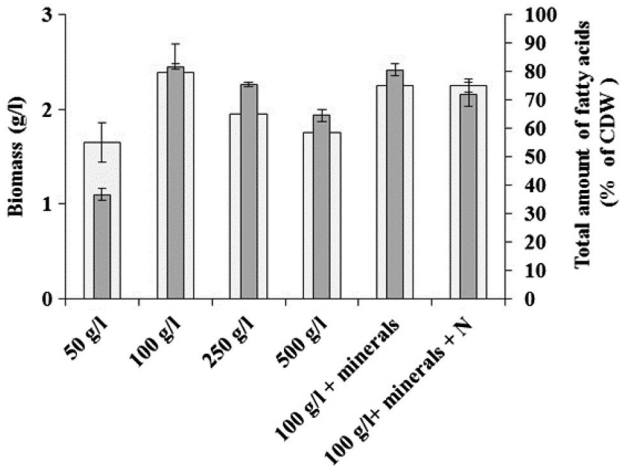

Fig. 1 Effect of different OMW concentrations or the addition of mineral salts and ammonium chloride on biomass and lipid production by *R. opacus* PD630 from OMW. The light gray bars represent the generated biomass expressed as CDW, the dark gray bars indicate the lipid content (total amount of FA as % of CDW). Data are expressed as means of triplicate experiments and their standard deviations

Chemical parameters	(a)
COD (mg/l)	127,200
Total organic nitrogen (mg/l)	256
C/N ratio	508.8
Total sugar (g/l)	18
Glucose (g/l)	4.2
Total lipids (g/l)	11
Total solids (g/l)	29.3
pH	2.2

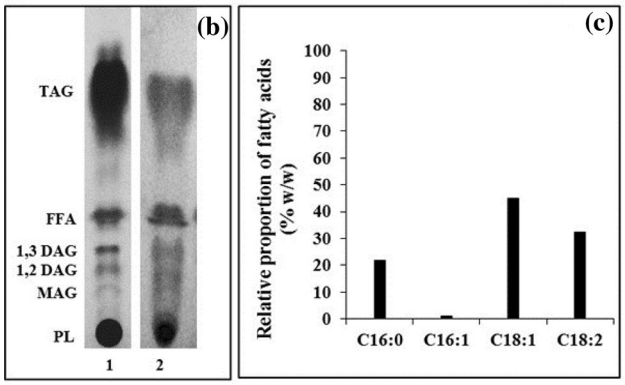

Fig. 2 Chemical characterization of OMW base medium used in this study (100 g residue/l water). **a** Physicochemical parameters. **b** TLC of neutral lipids extracted from OMW. Line 1, Mixture of reference lipids used as control (*TAG* triacylglycerol, *FFA* free fatty acid, *DAG* diacylglycerol, *MAG* monoacylglycerol, *PL* phospholipids); Line 2, OMW. **c** Relative proportion of FA contained in the OMW base medium

We analyzed the effect of different concentrations of OMW (50, 100, 250 and 500 g/l) and the presence/absence of mineral salts and a nitrogen source, on cellular biomass and lipid production (Fig. 1). The highest values of cellular biomass and lipids were obtained using a concentration of 100 g/l of OMW, whereas the addition of mineral salts and a nitrogen source (NH_4Cl) had no effect in this process (Fig. 1).

Chemical characterization of OMW base medium used in this study

We analyzed the chemical composition of the OMW base medium (100 g/l) used in this study. The OMW possessed high COD values indicating a significant content of organic matter, and a high C/N ratio (508.8), which resulted in a favorable condition for lipid accumulation by *Rhodococcus* (Alvarez et al. 2000) (Fig. 2a).

As is shown in Fig. 2b, OMW base medium possessed significant amounts of TAG and free fatty acids (FFA). The predominant FA occurring in this residual material were $C_{18:1}$ (45%), $C_{18:2}$ (32.2%), and $C_{16:0}$ (21.8%) species, in addition to $C_{16:1}$ (1%) (Fig. 2c). The OMW medium exhibited low pH (2.2); thus, it was necessary to adjust this parameter (to pH 7) for proper bacterial growth.

Biomass and lipid production by *Rhodococcus* strains during cultivation on OMW

OMW was utilized as base medium for cultivation of strains belonging to different species of *Rhodococcus* genus (Table 1) in order to analyze their ability to grow and produce TAG. The investigated strains exhibited different capability for producing cellular biomass and lipids from OMW base medium. *R. opacus* (PD630 and MR22), *R. jostii* RHA1 and the native strain V, exhibited good growth and lipid accumulation. The phylogenetic affiliation of strain V to *Rhodococcus wratislaviensis* was determined by comparison of its 16S rDNA (Fig. S2), AlkB, CatA and GyrB sequences with most related sequences found in databases (Fig. S3a, b, c). All these strains produced high amounts of cellular biomass (2.2–2.7 g/l) and lipids (77–84% of CDW, 1.8–2.2 g/l) after 168 h of incubation (Fig. 3a, b). In contrast, *R. erythropolis* DSMZ 43060, *R. fascians* F7 and *R. equi* ATCC6939 produced low amounts of cellular biomass (1.1–1.6 g/l) and lipids (7.1–14.0% of CDW, 0.1–0.2 g/l) during cultivation in OMW base medium (Fig. 3a, b).

R. opacus PD630, *R. jostii* RHA1 and *R. wratislaviensis* V produced large amounts of LD during cultivation of

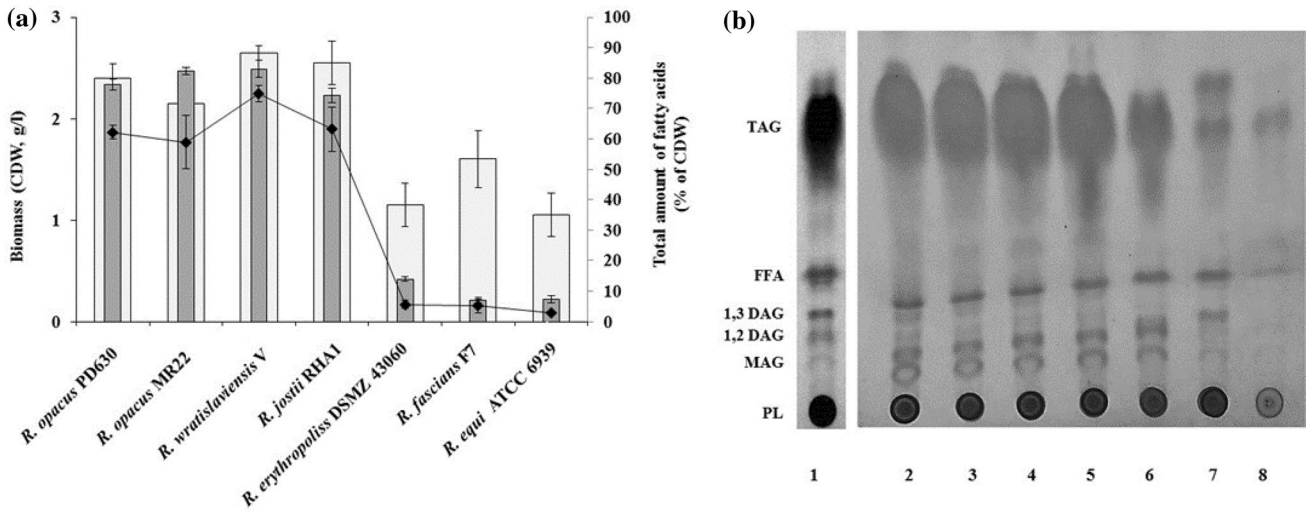

Fig. 3 Growth and lipid production by different *Rhodococcus* strains from OMW. **a** Production of cellular biomass and lipids. The light gray bars represent the generated biomass expressed as CDW, the dark gray bars indicate the lipid content as % of CDW, and continue line represent the lipid values in g/l. Data are expressed as means of triplicate experiments and their standard deviations. **b** TLC of neutral lipids extracted from OMW base medium-grown cells. Lanes: (1) Mixture of reference lipids used as control (*TAG* triacylglycerol, *FFA* free fatty acid, *DAG* diacylglycerol, *MAG* monoacylglycerol, *PL* phospholipids); (2) *R. opacus* PD630; (3) *R. opacus* MR22; (4) *R. wratislaviensis* V; (5) *R. jostii* RHA1; (6) *R. erythropolis* DSMZ 43060; (7) *R. fascians* F7; (8) *R. equi* ATCC 6939

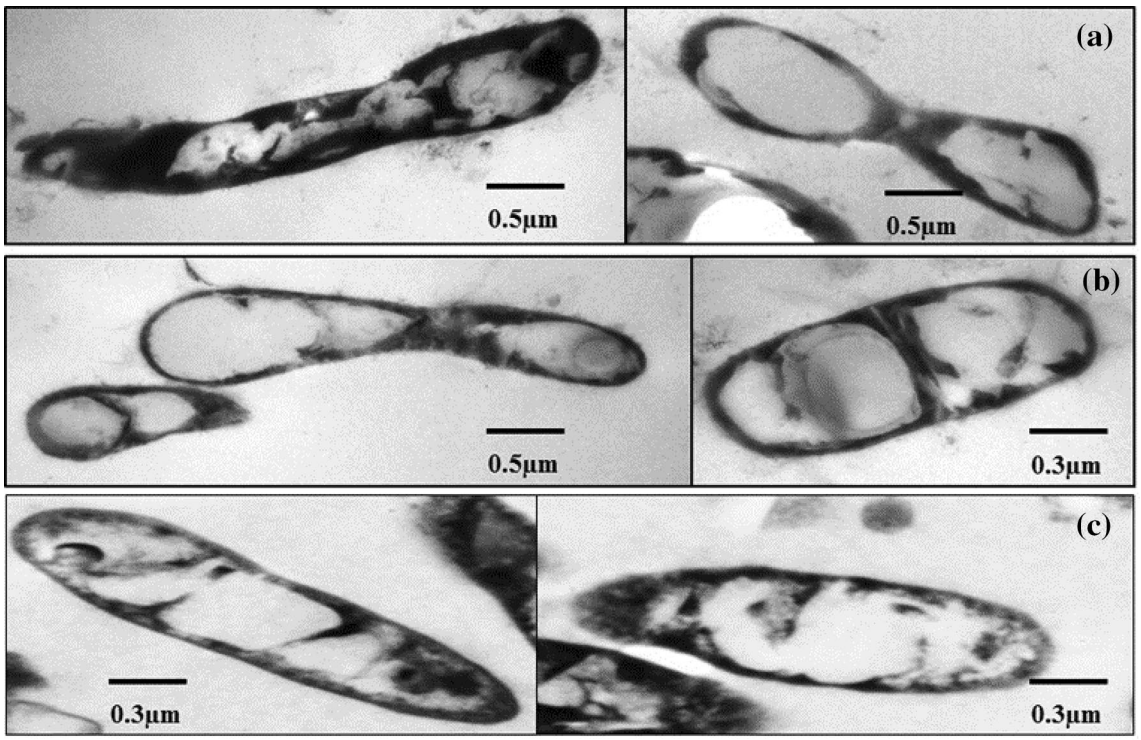

Fig. 4 Electron microscopy analysis revealed significant lipid inclusion when the cells grown in OMW. **a** *R. opacus* PD630, **b** *R. wratislaviensis* V and **c** *R. jostii* RHA1. The length of the bars are indicated on each micrograph

cells on OMW as is shown in Fig. 4, in agreement with lipid contents obtained by GC analyses. Lipids occurring in LD's consisted predominantly of TAG (Fig. 3b), which contained saturated and unsaturated straight long-chain FA as revealed by GC analyses (Table 2). Palmitic acid ($C_{16:0}$), oleic acid ($C_{18:1}$), which represented approximately 23 and

Table 2 FA composition of lipids accumulated by *R. opacus* (PD630 and MR22), *R. wratislaviensis* V and *R. jostii* RHA1 from OMW base media

Strains	Relative proportion of FA (% W/W)								Proportion SFA/UFA[a]
	C14:0	C15:0	C16:0	C16:1	C17:0	C17:1	C18:0	C18:1	
R. opacus PD630	1.3	4.3	23.6	3.9	11.1	16.4	7.4	32.4	0.9
R. opacus MR22	1.6	4.0	21.0	7.4	11.9	11.4	7.3	30.4	1.0
R. wratislaviensis V	1.5	5.7	24.8	5.8	10.6	17.7	6.6	27.3	1.0
R. jostii RHA1	1.1	4.8	23.7	5.0	15.0	15.3	6.7	28.4	1.0
R. jostii RHA1 pJAM2	1.3	4.7	24.6	3.3	14.2	12.4	11.6	27.7	1.3
R. jostii RHA1pJAM2 *ltp*1	1.3	4.7	32.7	4.9	13.4	12.3	11.1	19.3	1.7

[a]*SFA* saturated fatty acids, *UFA* unsaturated fatty acids

30% respectively of the total FA, were in all cases the predominant FA occurring in the accumulated lipids (Table 2). Other FA species, such as $C_{17:1}$, $C_{16:1}$, $C_{15:0}$ and $C_{14:0}$, also occurred in the produced TAG from OMW (Table 2).

Improvement of cellular biomass and lipid production in OMW base medium by engineered *R. jostii* RHA1 pJAM2/*ltp*1

In a previous study, we identified and characterized an ATP-binding cassette transporter (Ltp1) involved in the FA uptake in *R. jostii* RHA1 (Villalba and Alvarez 2014). Considering that OMW is a FA-rich industrial waste, we explored the possibility to improve cellular biomass and lipid production by overexpression of *ltp*1 gene in *R. jostii* RHA1 during cultivation on OMW. The expression of *ltp*1 gene in *R. jostii* RHA1 under the control of an acetamide-inducible promoter (RHA1 pJAM2/*ltp*1) improved cell growth (Fig. 5a), with an increase of 2.2 fold in cellular biomass production during OMW cultivation when compared to the control strain (RHA1/pJAM2), reaching values up to 8.66 g/l. In addition, genetically modified RHA1 strain accumulated higher amounts of lipids than control strain (2.28 g/l compared to 0.66 g/l). TLC analyses of the overexpressing strain revealed a significant increase in the free FA fraction, and a slight increase in DAG fraction, and almost no changes in TAG fraction (Fig. 5b).

Discussion

In this study, we analyzed the potential of different strains/species belonging to *Rhodococcus* genus for the bioconversion of OMW into cellular biomass and lipids. An OMW culture base medium was generated and optimized for the cultivation of cells. The final concentration of 100 g/l was most appropriate for rhodococcal cell growth, since higher OMW concentrations promoted an inhibition on

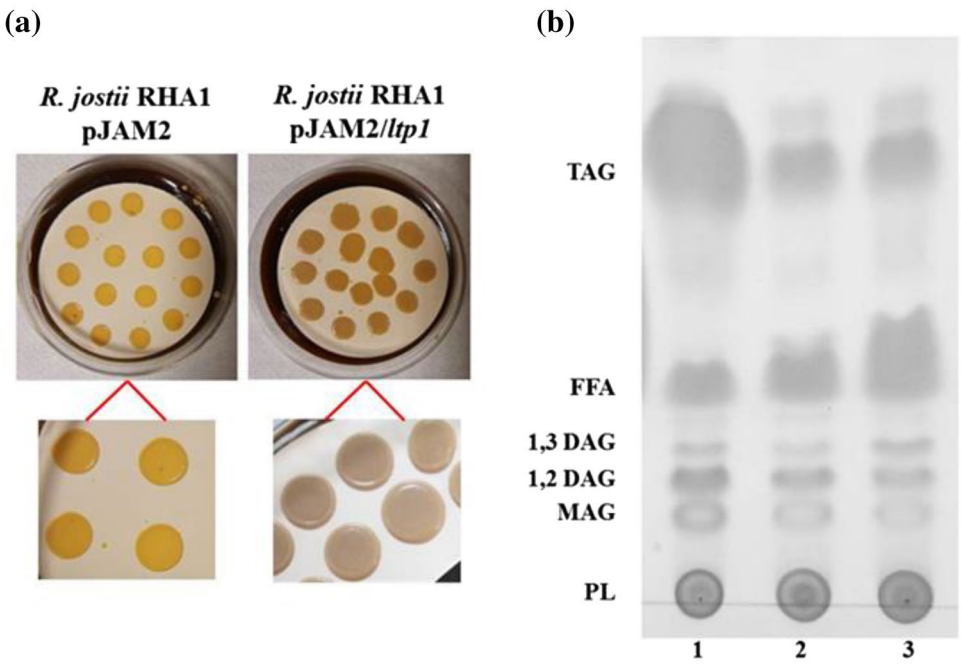

Fig. 5 Effect of *ltp*1-overexpression on cell growth and lipid production in *R. jostii* RHA1. **a** Cellular biomass production in the control strain *R. jostii* RHA1-pJAM2 and the recombinant strain *R. jostii* RHA1-pJAM2/*ltp*1 when grown on OMW. **b** TLC of neutral lipids extracted from OMW base medium-grown cells. Lanes: (1) Mixture of reference lipids used as control (*TAG* triacylglycerol, *FFA* free fatty acid, *DAG* diacylglycerol, *MAG* monoacylglycerol, *PL* phospholipids); (2) *R. jostii* RHA1-pJAM2; (3) *R. jostii* RHA1-pJAM2/*ltp*1

cell biomass and TAG production (Fig. 1), probably due to the presence of recalcitrant polyaromatics compounds with strong antimicrobial properties (Aguilera et al. 2008; Öner 2013). The OMW base medium resulted in a nutritional rich-substrate, which supported good growth and TAG production by rhodococcal cells without the addition of additional nutrients (mineral salts or nitrogen). Thus, OMW can be considered as a naturally lipogenic medium which serves as a renewable source of a variety of microbial lipid-derived compounds of industrial importance. However, the nutritional or toxic potential of the remaining solid residue obtained in this study after aqueous extraction is unknown. In this study, rhodococcal cells were attached to an inert support (PES filters) for cultivation on OMW, in order to avoid the formation of cell aggregates which hinders cell harvest for lipid extraction. This culture method allowed obtaining a rapid cell recovery with high biomass values (2.7 g/l) and TAG accumulation (84.4% PSC) in *R. opacus* PD630 without culture agitation. The use of immobilized cells of diverse *Rhodococcus* strains has been successfully applied for the degradation of pollutants or biotransformation processes (Mustacchi et al. 2005; Suttinun et al. 2010; Ivshina et al. 2012; Dinamarca et al. 2014), although it has not yet been proposed for the production of lipids in *rhodococci*. The immobilization of rhodococcal cells in an inert support dispenses the use of energy for agitation and harvesting of the biomass by centrifugation, which is an important advantage for its application in biotechnology, since both aspects affect significantly the production costs (Hughes and Benemam 1997; Subramaniam et al. 2010). Nevertheless, the boiling of the OMW/water mixture would contribute to an increase of the energy cost to the overall process. Thus, the economic feasibility to take this process to an industrial application should be thoroughly determined in the future.

The ability to grow and produce lipids from OMW was variable among the investigated rhodococcal species/strains. The different composition of FA observed in OMW and the lipids extracted from bacterial cells (Fig. 2; Table 2) indicates that the TAG come from the biosynthetic activity of bacteria and not simply from the transport to the interior of cells of the TAG contained in the OMW medium. *R. opacus* (strains PD630 and MR22), *R. wratilaviensis* V, and *R. jostii* RHA1 exhibited good growth (2.4–2.7 g/l of cell biomass) and lipid accumulation (78–84% of CDW) after 168 h of cultivation on OMW, whereas *R. erythropolis* DSMZ 43060, *R. fascians* F7 and *R. equi* ATCC6939 exhibited poor growth (producing 1–1.6 g/l of cell biomass) and lipid production (lesser than 15% of CDW) (Fig. 3). Interestingly, the group composed by *R. opacus, R. wratilaviensis* and *R. jostii* possesses larger genomes (between 9.42 and 10.40 Mb) compared to the other species, comprising *R. erythropolis, R. fascians* and *R. equi* (between 5.04 and 6.89 Mb). The first rhodococcal group possesses more redundancy of genes coding for transporters and enzymes involved in lipid metabolism; thus, the broader genetic endowment of *R. opacus–R. wratilaviensis-R. jostii* group may determine an increased ability of cells to generate metabolic intermediates, reduced equivalents (NADPH) and energy from OMW, required for supporting growth and lipid biosynthesis. The heterogeneous metabolic and genetic architecture of *rhodococci* determine a differential potential for the bioconversion of industrial wastes into cellular biomass and lipids. Thus, *R. opacus* but not *R. jostii* was the most robust rhodococcal species for lipid production from whey (Herrero and Alvarez 2016); whereas *R. fascians* and *R. erythropolis* the best candidates for bioconversion of glycerol into lipids (Herrero et al. 2016). Previous studies demonstrated that lipid production by *rhodococci* can be improved by genetic engineering (Herrero et al. 2016; Hernández et al. 2013, 2015; Kurosawa et al. 2014; MacEachran and Sinskey 2013). Since OMW is rich in FA-related compounds, we decided to explore the possibility to use the induced expression of a gene encoding a FA importer protein identified in a previous study (Villalba and Alvarez 2014) for improving productivity in *R. jostii*. The overexpression of *ltp*1 gene in *R. jostii* RHA1 (RHA1-pJAM2/*ltp*1) during cultivation in OMW base medium promoted an increase of 3.4 fold in lipid production values, at expenses of an increase in the cellular biomass production (2.2 fold) and improved lipid accumulation in recombinant cells (1.2 fold). The induced expression of the FA importer in strain RHA1 may improve the uptake of FA derived from the OMW and probably the efficiency of carbon processing to some extent, resulting in an increase in cell growth. From a biotechnological point of view, it would be interesting to consider the insertion the *ltp*1 gene into the bacterial genome under the control of a native promoter to optimize yields of lipid production in the recombinant strain, avoiding the partial inhibitory effect of the inducer and the antibiotic on cell growth. Lipids accumulated by the investigated strains as well as by the recombinant RHA1-pJAM2/*ltp*1 strain were composed by saturated and monounsaturated straight FA of chain lengths between 14 and 18 carbon atoms, with palmitic acid and oleic acid as predominant components. This fatty acid composition of rhodococcal lipids is compatible with biodiesel production (Ramos et al. 2009; Kumar et al. 2011). In addition, the metabolism of *R. opacus, R. wratilaviensis* and *R. jostii*, which showed to be the most robust *rhodococci* for producing oils from OMW, is flexible enough to incorporate and integrate heterologous reactions and pathways to produce new lipid derivatives with commercial interest. In a previous study we engineered *R. opacus* PD630 to produce wax esters from gluconate and industrial diary wastes by heterologous expression of a

fatty acyl-CoA reductase from a marine bacterium (Lanfranconi and Alvarez 2017). In this context, the production of wax esters or other lipidic derivatives from OMW using engineered *rhodococci* could be predicted.

Altogether, this study demonstrated that strains belonging to the *R. opacus–R. wratilaviensis–R. jostii* group are the most robust candidates for lipid production from OMW, and that the productivity of this process can be improved by genetic engineering. Under optimized conditions, OMW can be considered an appropriate low-cost substrate for oil production by *rhodococci*. The conversion of OWM into industrial oils might contribute to reduce the negative environmental impact generated by the disposal of this residual material in the environment.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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