Research Article

Whey as a renewable source for lipid production by *Rhodococcus* strains: Physiology and genomics of lactose and galactose utilization

O. Marisa Herrero[1,2] and Héctor M. Alvarez[1]

[1] Centro Regional de Investigación y Desarrollo Científico Tecnológico, Facultad de Ciencias Naturales, Universidad Nacional de la Patagonia, San Juan Bosco, Comodoro Rivadavia, Chubut, Argentina
[2] Oil m&s, Comodoro Rivadavia, Chubut, Argentina

We investigated biomass and lipid production from whey by different *Rhodococcus* strains. The studied microorganisms exhibited different capability for growing and producing lipids from whey permeate. Strains belonging to *R. opacus* exhibited high yields of biomass (6.1–6.3 g/L) and lipid content (45–48% of CDW), whereas *R. jostii*, *R. erythropolis*, *R. fascians*, and *R. equi* strains produced low biomass (1.8–2.9 g/L) and lipids (lesser than 5% of CDW) from whey. Lactose and galactose, which are main constituents of whey, supported growth of *R. opacus* strains, but not of the other investigated species. A genome-wide bioinformatic analyses demonstrated that some genes coding for transport systems (LacEFGK) and the β-galactosidase (LacB) enzyme for lactose cleavage are lacking in such species, which may explain their inability to utilize lactose, galactose, and whey for an efficient biomass and lipid production. *R. opacus* possesses a complete genetic endowment for degrading lactose, galactose, and whey as well as for lipid biosynthesis from such substrates. Thus, *R. opacus* is a robust candidate for single-cell oil production from whey. The cultivation of *R. opacus* cells on crude whey resulted in an increase of lipid production from 3.0 to 6.4 g/L, in comparison to whey permeate.

Practical application This study demonstrates that the bioconversion of whey to oils by Rhodococcus strains is feasible. However, we found some genetic and physiological differences for whey, lactose and galactose catabolism and assimilation among rhodococcal species. Our results demonstrate that among five different rhodococcal species, Rhodococcus opacus has the more robust genetic endowment for supporting high yields of biomass and lipid production from whey. The use of whey for single cell oil production by rhodococci may serve as platform for developing environmentally friendly biotechnological processes.

Keywords: Galactose / Lactose / Triacylglycerols / Whey

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1 Introduction

The biodiesel is a clean and renewable alternative for replacement of fossil diesel. The raw materials for biodiesel production are triglycerides (TAG) or free fatty acids (FA) from biological sources such as vegetable oils and in a smaller proportion animal fats and recycled oils. Recently, the focus has been directed to microbial oils, namely single cell oils (SCO), produced by oleaginous microorganisms, including bacteria, yeasts, and algae. Microbial lipids are now considered as promising oil sources for biodiesel production, with some advantages over vegetable oils: (i) they do not compete with edible oils; (ii) their production is not restricted to particular geographic regions; (iii) they are not affected by climatic conditions; (iv) they can be produced in bioreactors under controlled conditions; and (v) they usually require shorter production cycles [1, 2]. Another interesting aspect of

Correspondence: Héctor M. Alvarez, Centro Regional de Investigación y Desarrollo Científico Tecnológico (CRIDECIT), Facultad de Ciencias Naturales, Universidad Nacional de la Patagonia San Juan Bosco, Km. 4-Ciudad Universitaria, 9000 Comodoro Rivadavia, Chubut, Argentina
E-mail: halvarez@unpata.edu.ar
Fax: +54 297 4550339

Abbreviations: CDW, cell dry weight; **COD,** chemical oxygen demand; **FA,** fatty acids; **OD,** optical density; **SCO,** single cell oils; **TAG,** triacyglycerols

microbial lipids is that they allow obtaining oils with different chemical compositions suitable for a diversity of industrial applications [3, 4].

The ability to accumulate significant amounts of TAG is a common feature of several actinobacteria, such as *Rhodococcus*, *Nocardia*, *Gordonia*, and *Mycobacterium* genera [5]. Among them, *Rhodococcus* bacteria have been extensively investigated regarding to the accumulation of lipids [6]. Most studies focused on TAG production by rhodococci were performed using single compounds as carbon sources, such as gluconate and glucose, among others [7–10]. Certainly, these substrates are not considered for biotechnological processes of SCO production for economical reasons. In this context, Fernandes Castanha et al. [11] reported that the higher cost of biodiesel production from SCO is due to the carbon source used in the culture, representing nearly 80% of the total culture media cost. For this reason, several studies were focused on the search of alternative and inexpensive raw materials to be used as substrates for microbial oil production, such as wastes from agriculture, forestry, and food industry [12–14]. Whey is a waste of the dairy industry, which is generated worldwide in enormous quantities (1 kg of cheese generated 9 kg of whey) [15]. The major component of whey is lactose (5–7%), plus lesser amounts of glucose and galactose, proteins (0.8–1.2%), and lipids (0.06–3%) [15]. Whey final disposal is a central problem for the dairy industry due to their high values of chemical oxygen demand (COD) (50,000 to 70,000 g/L), which generates significant pollution when wastes are freely disposed in the environment [16], and the high costs of their eventual treatment. The bioconversion of whey to valuable microbial oils is an interesting avenue, which may allow reducing the environmental impact caused by the disposal of this waste in natural environments, and simultaneously generating at low cost, lipids useful for the production of biodiesel, bio-lubricants, oleochemicals, cosmetics, among other bioproducts. Interestingly, the use of whey for oil production has been reported for yeasts [11, 17, 18], but not for bacteria yet.

The use of whey for the production of valuable oils by bacteria demands to enhance our understanding on the physiology and molecular biology for the uptake and degradation of the main components occurring in the waste, such as lactose and galactose. The genetics of lactose and galactose utilization in actinobacteria is still incipient, and remains to be elucidated in detail. Previous studies have suggested that a solute binding protein dependent transport system (SBPT) may be responsible for the transport of lactose and galactosides in *Streptomyces coelicolor* A3(2) [19] and in *Rhodococcus opacus* (PD630 and B4) [16], which is clustered with a probable β-galactosidase involved in lactose degradation.

In this work, the feasibility of converting whey into lipids by different *Rhodococcus* species was evaluated. We combined physiological and genome-wide bioinformatic analyses of diverse strains belonging to *R. opacus*, *R. jostii*, *R. erythropolis*, *R. fascians*, and *R. equi*, in order to integrate their ability to utilize whey, lactose, and galactose as substrates for growth and lipid production, with their genetic endowment for the catabolism and assimilation of these carbon sources. These results allowed us to select more robust rhodococcal species for the efficient conversion of whey to cell biomass and lipids. In addition, some aspects that can be relevant for a biotechnological process, such as pretreatment of whey and aeration for cell cultivation or the kinetics of TAG accumulation during cultivation of cells with whey, were also analyzed in this study.

2 Materials and methods

2.1 Physicochemical characterization of the whey

The sample of whey used in this study, corresponds to residual whey from the industrial production of mozzarella by La Salamandra company in Buenos Aires, Argentina. Chemical oxygen demand and total organic nitrogen were analyzed by standard methods [20]. The total sugar concentration was assayed by phenol-sulfuric acid method [21]. Acidic hydrolysis of sugars of whey and lactose was performed with 2 M trifluoroacetic acid (120°C for 4 h) [22]. Sugar composition of products of acidic hydrolyses and whey were analyzed by ascending paper chromatography, using paper Whatman N° 1 and pyridine/ethyl acetate/acetic acid/water (5:5:1:3, by vol.) as mobile phase. Lactose and glucose were used as standards. Qualitative and quantitative analyses of fatty acids from whey were performed as reported below.

2.2 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. After growth, cells were harvested, washed, and resuspended with sterile saline solution (NaCl 0.85% w/v). In all cases, the cultures were inoculated with cell suspensions to reach an initial OD_{600} of about 0.25 and then incubated at 28°C on a rotary shaker.

In order to investigate the growth and lipid production by different *Rhodococcus* strains from whey, 250 mL erlenmeyer flasks containing 50 mL of pretreated whey were inoculated. The pretreatment applied consisted in whey sterilization by moist

Table 1. Bacterial strains used in this study

Bacterial strains	References
Rhodococcus opacus PD630	DSMZ 44193
Rhodococcus opacus MR22	DSMZ 3346
Rhodococcus jostii RHA1	Seto et al. [23]
Rhodococcus erythropolis	DSMZ 43060
Rhodococcus fascians F7	BNM 542
Rhodococcus equi	ATCC 6939

heat at 1.4 atm for 20 min and subsequent permeation under sterile conditions to remove protein clots. The pH was adjusted to 7 before inoculation. When lactose and galactose were used as carbon sources, 250 mL erlenmeyer flasks containing 50 mL mineral salt medium (MSM) according to Schlegel et al. [24] with 1 gL^{-1} NH_4Cl were used in order to analyze cell growth. To promote accumulation of lipids, the concentration of NH_4Cl in mineral salts medium was reduced to 0.1 gL^{-1} (nitrogen-limiting conditions). Lactose and galactose were used at a final concentration of 1% w/v. Cells were harvested at specific time-points corresponding to stationary phase and washed with a saline solution and dried for chemical analyses.

2.3 Cell biomass and lipid analyses

Cell biomass was harvested in stationary phase by centrifugation of 10 mL of culture, then washed twice with sterile saline solution and dried until constant weight.

The qualitative and semiquantitative analyses of total intracellular lipids were carried out by thin layer chromatography (TLC). For this, 5 mg of lyophilized cells was extracted by means of vigorous shaking with 200 μL chloroform/methanol (2:1, v/v) for 90 min at 4°C. Twenty five 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 [9]. Tripalmitin (Merck) was used as TAG reference substance.

Since in *Rhodococcus* cells TAG accounts for over 98% of the total lipids, the quantification of total FAs is a good estimation of TAG content. For qualitative and quantitative determination of FAs, 5–7 mg of dry cells was subjected to methanolysis in the presence of 15% v/v sulfuric acid [25]. The resulting acyl-methylesters were analyzed by gas chromatography (GC) using an HP 5890 A GC equipped with an InnoWAX capillary column (30 m × 0.53 mm × 1 μm) and a flame ionization detector. The injection volume was 0.5 mL, and hydrogen was used as 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. All determinations of biomass and lipids were performed in triplicate experiments and results expressed as average values with their corresponding standard deviations.

2.4 Treatments applied to remove native microorganisms from whey

In order to reduce the content of native microorganisms from whey for the culture of inoculated rhodococcal cells, different treatments with chemical or physical agents were applied. One of the treatments consisted in the addition of chloroform to the whey (10%, v/v), and agitation at 200 rpm during 1 h with intervals of 10 min. After 24 h of settlement for separating the two phases, the chloroform phase was discarded, and the supernatant was used as culture medium.

Other treatment consisted in the increase of the whey pH from 5 to 9–11 by the addition of sodium hydroxide. Since the native whey microorganisms are usually adapted to low pH, and rhodococcal cells are able to grow in alkaline environments, the increase of pH may significantly reduce the original microbial charge of the whey.

In the case of treatment with physical agents, the effect of moist heat and dry heat was tested. For moist heat treatments, we used two different steam pressures with different time periods (1.4 atm during 20 min and 1.0 atm during 1 h). For dry heat treatments, we used different combinations of temperatures and times. Heat treatments at 90°C or higher caused protein coagulation; thus, the material was filtered to generate whey permeate.

2.5 Electron microscopy analysis

Cells were washed, suspended in 0.1 M potassium phosphate buffer (pH 7.5), and fixed with glutaraldehyde for 24 h. Then, cells were washed with a solution of sucrose 0.32 M in phosphate buffer and embedded in low viscosity resin [26]. Thin sections were contrasted with uranyl acetate and ruthenium red [27]. Images that were obtained utilized a Zeiss 109T electron microscope with a Gatan ES camera.

2.6 Analyses of sequences

Genomes analyzed in this work for genes involved in the transport and degradation of lactose and galactose, correspond to the following strains: *R. jostii* RHA1 [28], *R. opacus* PD630 [10], *R. opacus* B4 [29], *R. erythropolis* PR4 [30], *R. equi* 103S [31], and *R. fascians* F7 [32]. The protein sequences were downloaded from the National Center for Biotechnology Information (NCBI) website, and from Rapid Annotation using Subsystem Technology (RAST) server [33]. To establish gene occurrence, genes involved in each metabolic reaction were identified using pathways presented in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [34], and were also searched by gene annotation or protein names using the NCBI and RAST Internet servers. To find more representative homologs, protein sequences were used as queries for further searches using the BLASTP program. The search was run using the default parameters set by the program, considering as homologous proteins of those with significant alignments (*E*-value $\leq 10^{-50}$). We also used well-characterized protein sequences of different microorganism to find their homologs in different species of the genus *Rhodococcus* analyzed. As example, we used: lactose permease (Lacy) and β-galactosidase (LacZ or LacB) of *Lactobacillus johnsonii*, *Lactobacillus acidophilus*, *Streptococcus thermophilus*, *Lactococcus cremois*, *Lactococcus lactis*, *Escherichia coli*; galactose permease (GalP) and enzymes involved in the Leloir pathway (Galactokinase, Galactose-1-phosphate uridylyltransferase, and UDP-galactose-4-epimerase) of *Lactococcus cremois*, *Lactococcus lactis*,

Streptomyces coelicolor, and *Mycobacterium smegmatis*; specific components PTS^{Lac} of *Lactobacillus casei*, *Lactococus lactis*, *Streptococcus thermophilus*, specific components PTS^{Gal} of *Lactobacillus rhamnosus*, *Lactococcus garaviae*, *Streptococcus thermophilus*, and binding-protein-dependent lactose transport system (LacEFGKZ) of *Agrobacterium radiobacter*. The following criteria were considered for predicting the possible function of proteins: (i) the identity of the considered protein was more than 30% to that of the reference microorganism; (ii) the organization of genes in conserved clusters; and (iii) specific analyses of protein sequences searching conserved domains using diverse bioinformatic tools, such as Conserved Domain Database (CDD), Protein Family Database, and Pfam. Further analyses using the BLASTP program were performed to identify orthologs among the detected homologous proteins, considering the best hit with the highest percentage identity.

3 Results and discussion

3.1 Physicochemical characterization of the whey used in this study

We analyzed the chemical composition of the whey used in this study. The waste possessed high COD values indicating a significant content of organic matter, which is consistent with high levels of nitrogen and total sugars observed (Table 2). Ascending paper chromatography analysis revealed that the main sugar occurring in the whey was lactose (Fig. S1A), in addition to small amounts of glucose (Table 2).

The presence of neutral lipids was also detected in the waste as is shown in Table 2. The predominant FA occurring in lipids contained in the whey was the C8:0 species (78.9% of the total FA), in addition to C6:0 (13.8%), C22:3 (6.3%), and C15:1 (1%) (Fig. S1B). Whey composition determines high C/N ratios (Table 2), which is a favorable condition for lipids accumulation by *Rhodococcus* strains [8].

The waste used in this study exhibited low pH (5.0), which is characteristic of the whey generated during the production of fresh cheese and mozzarella. For this reason, it was necessary to adjust the pH (to pH 7.0) for the optimal cultivation of bacterial cells.

Table 2. Composition of the whey

Physicochemical parameters	Whey	Whey permeate
COD (mg/L)	66,800	66,100
Total nitrogen (mg/L)	1,280	618
C/N ratio	52.2	107
Total sugar (g/L)	66.7	65.4
Glucose (g/L)	0.57	0.59
Total lipids (g/L)	1.6	1.4
Total solids	28.7	12.8
pH	5.0	4.8

3.2 Growth and lipid accumulation by *Rhodococcus* strains during cultivation on whey

The sterilized whey permeate was used as base medium for cultivation of strains belonging to different species of *Rhodococcus* genus, such as *R. opacus*, *R. jostii*, *R. erythropolis*, *R. fascians*, and *R. equi* (Table 1), in order to analyze their ability to grow and produce TAG.

The investigated strains exhibited different capability to produce biomass and lipids from whey permeate. Based in their behavior, they could be divided into two groups: (1) those that showed good growth and lipid accumulation, including *R. opacus* (strains PD630 and MR22), which produced high amounts of cell biomass (6.1–6.3 g/L) and lipids (45–48% of CDM and yields of 2.8–3.0 g/L) after 120 h of incubation (Figs. 1 and 3A); and (2) those exhibiting poor growth on whey and low biomass production (1.8–2.9 g/L) and lipid content (lesser than 5% of CDW), including strains of *R. jostii*, *R. erythropolis*, *R. fascians*, and *R. equi* (Figs. 1 and 3A). The poor performance of these species for growing and producing TAG from whey was striking, especially in the case of *R. jostii* RHA1, since this strain is an oleaginous research model organism closely related to *R. opacus* PD630 [35]. *R. opacus* PD630 was able to produce large amounts of lipid inclusions during cultivation of cells on whey permeate as is shown in Fig. 2. Lipids occurring in the inclusion bodies of *R. opacus* (strains PD630 and MR22) grown on whey permeate consisted predominantly of TAG (Fig. 1B), containing saturated and unsaturated straight long-chain fatty acids as revealed by GC analyses (Table 3). Palmitic acid (C16:0), which amounted approximately 40% of the total FA, was in all cases the predominant FA occurring in the accumulated lipids. The proportion of saturated FA was approximately twofolds higher than that of unsaturated species during growth on whey (Table 3). This is a favorable fatty acid profile for biodiesel production, since biodiesel produced from saturated oils have a better oxidative stability and fewer NOx emissions, in addition to a lower content of monounsaturated fatty acids, which is useful for maintaining the flow of the biofuel at low temperatures [36, 37].

The relative amounts of palmitic acid ($C_{16:0}$) in TAG samples of the whey-grown cells of strain PD630 was approximately 10% higher in comparison to cells cultivated on lactose and galactose as sole carbon sources (Table 3), or on gluconate and other simple sugars as reported in previous studies [7]. In contrast, the relative content of oleic acid ($C_{18:1}$) in TAG from cells grown on whey was lower (Table 3). The occurrence of other compounds in whey such as lipids (Table 3), which can be also used as carbon sources in addition to lactose and glucose by rhodococcal cells, may determine the differential FA composition in TAG accumulated from whey, compared to cells grown on single sugars. These results suggested that the use of whey wastes with different compositions for cultivation of rhodococcal cells may allow the production of oils with different properties.

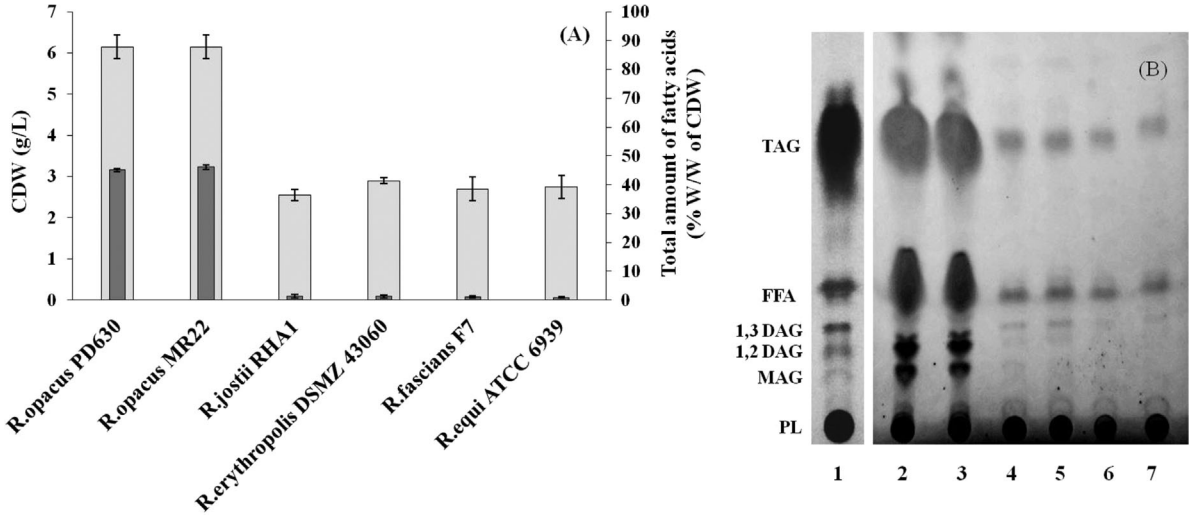

Figure 1. Growth and lipids production by different *Rhodococcus* strains from whey permeate. (A) Yields of biomass and lipids. The light gray bars represent the generated biomass expressed as cellular dry weight (CDW), and dark gray bars the lipid content. Data are expressed as means of triplicate experiments and their standard deviations. (B) TLC of neutral lipids extracted from whey-grown cells. Lanes: (1) Mixture of reference lipids used as control (TAG, triacylglycerol; FFA, free fatty acid; DAG, diacylglycerol; MAG, monoacylglycerol; PL, phospholipid). (2) *R. opacus* PD630. (3) *R. opacus* MR22. (4) *R. jostii* RHA1. (5) *R.erythropolis* DSMZ 43060. (6) *R. fascians* F7. (7) *R. equi* ATCC 6939.

3.3 Lactose and galactose utilization by rhodococci and key genes for degradation

In order to explain the differences in growth and TAG accumulation by different rhodococcal species during cultivation on whey, we investigated the ability of cells to grow on lactose and galactose as sole carbon sources, and performed a comparative genome analysis on the occurrence and distribution of key genes involved in their transport and degradation in rhodococci. Experimental assays showed that *R. opacus* (strains PD630 and MR22) possessed the ability to grow with lactose and galactose as sole carbon sources, whereas *R. jostii* RHA1, *R. erythropolis* DSMZ 43060, *R. fascians* F7, and *R. equi* ATCC 6939 were not able to grow with such substrates (Fig. 3B and C). These results suggested that the poor growth and lipid accumulation exhibited by these microorganisms from whey may be due to their inability to use lactose as carbon source. Additional nutrients occurring in whey, such as glucose and lipids (Table 2), may allow only poor growth of cells as is shown in Fig. 3A.

On the other hand, in order to find the molecular basis to explain the differential abilities to use lactose and galactose as carbon sources by diverse rhodococcal species, we searched the genomes of six strains for genes involved in lactose and galactose transport and metabolism. Uptake of lactose and galactose into bacterial cells can be mediated by several transport mechanisms, such as specific phosphoenolpyruvate-dependent phosphotransferase systems (PTS), ABC transporters, or different specific permeases [38]. When lactose or galactose are incorporated by PTS, the resulting galactose 6-phosphate is usually metabolized by tagastose-6-P pathway; whereas if they are transported by a permease, the lactose is hydrolyzed to galactose and glucose by a β-galactosidase enzyme, and the resulting galactose is degraded via the Leloir pathway [39]. Similar proteins to well-known

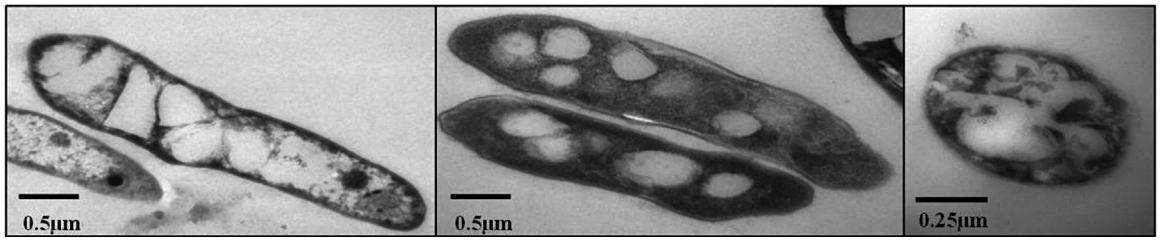

Figure 2. Lipid inclusions in *R. opacus* PD630 grown on whey as revealed by electron microscopy. The length of the bars is indicated in each micrograph.

Table 3. Fatty acid composition of lipids accumulated by *R. opacus* (strains PD630 and MR22) from whey, lactose, and galactose

Bacteria and substrate	Total amount of fatty acids (% CDW)	Relative proportion of fatty acids (% w/w)								Proportion (SFA/UFA)
		C14:0	C15:0	C16:0	C16:1	C17:0	C17:1	C18:0	C18:1	
R. opacus PD630										
Whey	45.1	2.1	5.1	40.8	9.0	12.9	9.9	8.3	11.7	2.2
Lactose	38.0	1.6	6.5	28.5	5.6	14.3	15.5	8.8	19.3	1.5
Galactose	36.7	1.4	6.7	27.0	6.1	15.8	15.2	9.1	18.3	1.5
R. opacus MR22										
Whey	46.1	2.9	5.3	39.5	9.7	11.3	8.8	7.8	14.7	2.0
Lactose	36.5	1.8	6.9	27.7	5.7	13.8	15.3	8.9	19.9	1.5
Galactose	35.2	1.5	6.5	28.2	6.2	14.4	15.3	9.0	18.9	1.4

SFA, saturated fatty acids; UFA, unsaturated fatty acids.

phospho-β-galactosidase, lactose, and galactose specific components of PTS (EIIBC-PTS^{Lac}, EIIA-PTS^{Lac}, EIIBC-PTS^{Gal}, EIIA-PTS^{Gal}) of different *Lactococcus*, *Lactobacillus*, and *Streptococcus* species, were not found in the analyzed rhodococcal genomes. In addition, all the enzymes usually involved in the tagastose-6-P pathway (galactose-6-P isomerase, tagastose-6-P kinase, and tagastose 1,6 bisphosphate aldolase), seemed to be absent in rhodococci. On the other hand, homologous genes coding for putative galactose and lactose permease proteins from

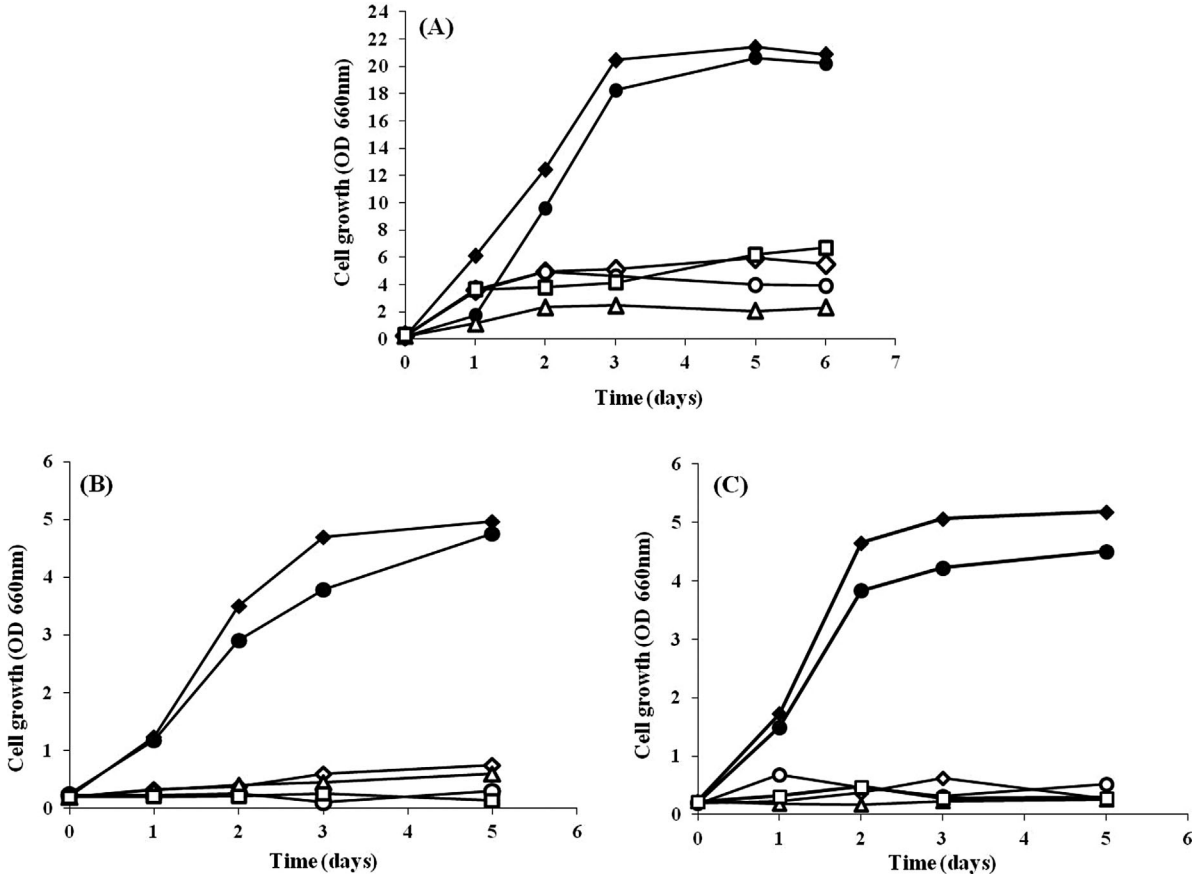

Figure 3. Growth kinetics of different *Rhodococcus* strains cultivated on: (A) whey permeate base media, (B) MSM1 with lactose 1% w/w, and (C) MSM1 with galactose 1% w/w. In dark are represented those strains showing good growth. *R. opacus* PD630 (closed diamond), *R. opacus* MR22 (closed circles), *R. josttii* RHA1 (open diamond), *R. erythropolis* DSMZ 43060 (open circles), *R. fascians* F7 (open triangles), *R. equi* ATCC 6039 (open squares).

different *Lactococcus*, *Lactobacillus*, and *Streptococcus* species, and LacP of *M. smegmatis* and *S. coelicolor* were not found in analyzed genomes.

Since it has been demonstrated in *A. radiobacter* that lactose is transported by a solute binding protein transporter system encoded by a gene cluster (*LacEFGZK*), which also contains the β-galactosidase gene [40, 41], we used these sequences to screen rhodococcal genomes for proteins involved in lactose transport and degradation. The protein sequences of *R. opacus* B4: *ROP_55650*, *ROP_55660*, *ROP_55670*, *ROP_55600*, *ROP_55640*, *ROP_55680*, and their orthologs in *R. opacus* PD630, exhibited 32, 33, 37, 50, 32, and 41% identity to LacE (Lactose binding protein), LacF (solute binding protein transporter), LacG (solute binding protein transporter), and LacK (ATP-binding protein), LacB/LacZ (β-galactosidase) and LacA (α-galactosidase), respectively. These genes are organized together in a large cluster in the genomes of such rhodococci, containing all components of the solute binding protein transporter system (SBPT), a α-galactosidase (LacA), a LacB (β-galactosidase), a DeoR family regulator transcriptional, and also including the enzymes of Leloir pathway (GalK, GalT, and GalE), necessary for degradation of galactose (Figs. 4 and 5). The organization of this gene cluster had been partially reported for *R. opacus* PD630 in a previous study [10]. Interestingly, the genomic region including genes for the putative lactose binding protein transporter system (LacEFGK), LacA, LacB, GalE, and DeoR family regulator, is not present in *R. jostii* RHA1, *R. erythropolis* PR4, *R. fascians* F7, and *R. equi* 103S, which only possess *galK* and *galT* genes in their genomes (Fig. 5). However, all these strains contain the genes coding for the Leloir pathway enzymes (GalK, GalT, and GalE). In this case, the clustered rearrangement of *galK* and *galT* seems to be conserved among such rhodococci, whereas *galE* gene was located separately in genomes (Fig. 5).

Interestingly, in the genome of *R. opacus* B4, we found two genes (*ROP_55540* and *ROP_55560*) coding for potential transposases belonging to the IS605 and IS200 families located downstream of lactose–galactosides gene cluster as shown in Fig. 5. In addition, a gene encoding an IS605 transposase (*ro05492*) was found upstream of *galK*–*galT* genes in the genome of *R. jostii* RHA1. The presence of insertion elements in the vicinity of these gene clusters may suggest that the current genetic endowment and gene organization for lactose and galactose metabolism resulted, to some extent, from genomic rearrangements mediated by insertion elements during evolution of rhodococci.

Our results suggested that strains belonging to *R. jostii*, *R. erythropolis*, *R. fascians*, and *R. equi* were unable to grow on lactose and galactose as sole carbon sources (Fig. 3B and C) because they do not possess in their genomes, the genes coding for the specific transport systems and the β-galactosidase enzyme for lactose cleavage (Fig. 5).

Resuming for supporting an efficient growth and lipid production from whey, lactose, or galactose, it is necessary that the microorganism not only possesses an efficient genetic endowment for the synthesis and accumulation of lipids, like *R. opacus* PD630, and *R. jostii* RHA1 [10, 32, 35], but also that for the transport and catabolism of such substrates, which allows generating energy and metabolic intermediates necessaries for growth and TAG synthesis. In this sense, *R. opacus* demonstrated to possess a robust genetic endowment for the degradation of lactose and galactose as well as for the

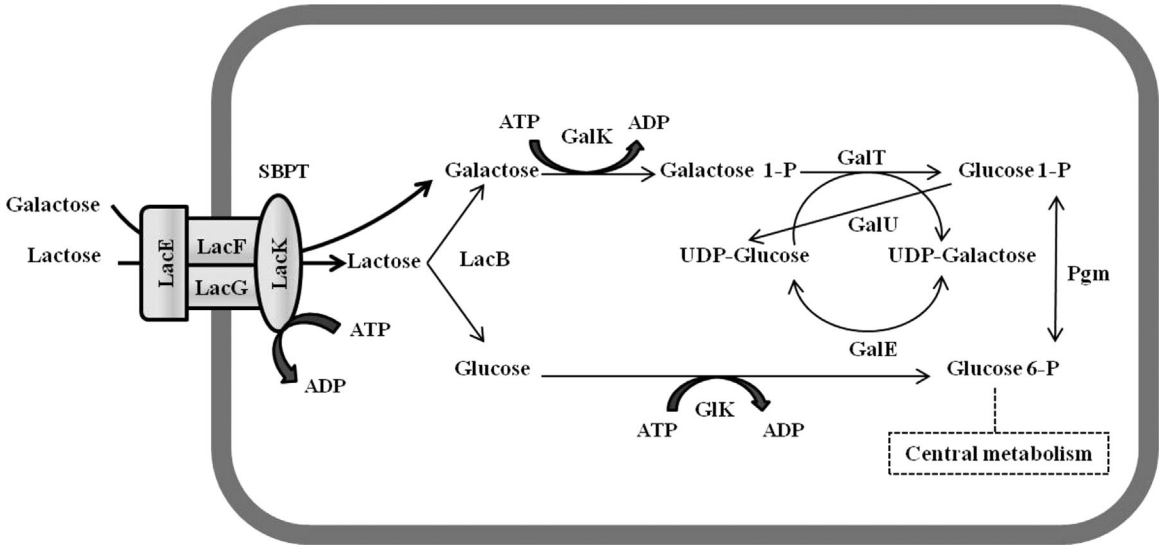

Figure 4. Schematic representation of the possible transporter and degradation pathways of lactose and galactose occurring in *Rhodococcus* strains. LacE, lactose binding protein of SBPT; lacF and LacG, integral membrane proteins of SBPT; LacK, ATP-binding protein of SBPT; LacB, β-galactosidase; HP, hypothetical protein; GalK, galactokinase; GalT, galactose-1-phosphate uridylyltransferase; GalE, UDP-glucose 4-epimerase; GalU, UTP-glucose-1-phosphate uridylyltransferase; SBPT, solute binding protein transporter system.

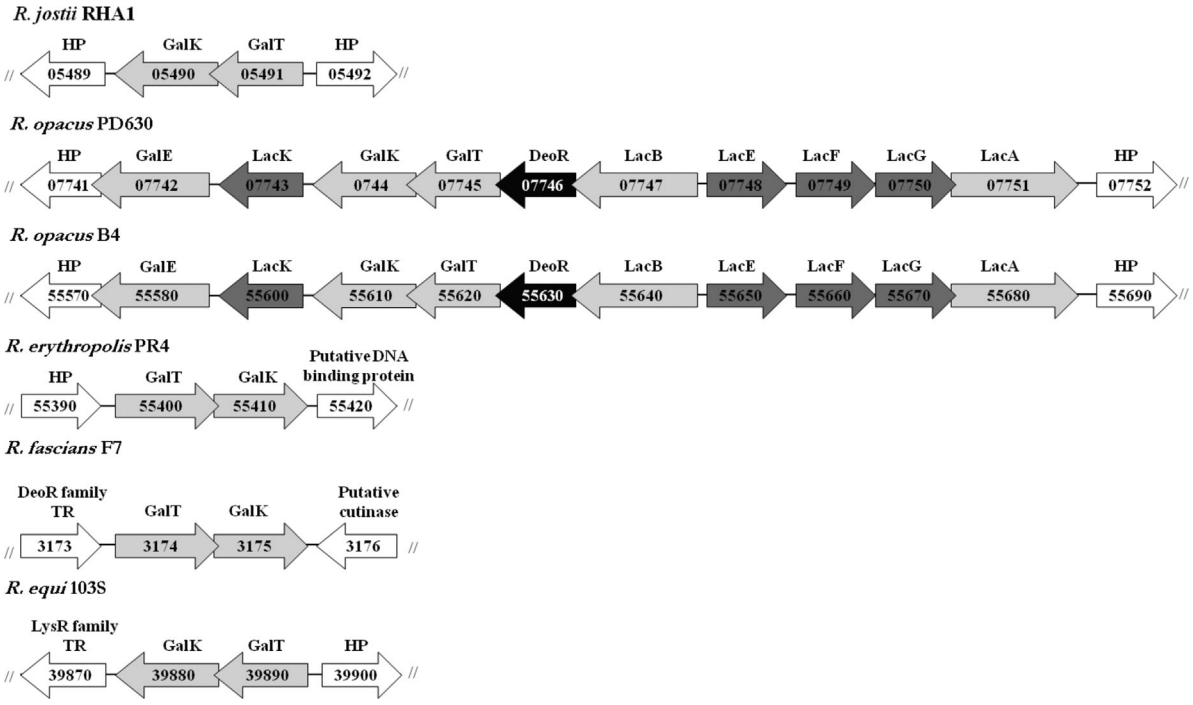

Figure 5. Organization of genes putatively involved in lactose catabolism in rhodococci. The arrows indicate the lengths and transcriptional orientations of genes. Genes encoding lactose transport system are depicted in dark gray, lactose catabolic genes are colored in light gray, and regulatory genes probably related to lactose metabolism are highlighted in black. Genes are shown by their number and their corresponding prefixes: RHA1_ro, *R. jostii* RHA1; OPAG_, *R. opacus* PD630; ROP_, *R. opacus* B4; RER_, *R. erythropolis* PR4; F7_, *R. fascians* F7; and REQ_, *R. equi* 103S. The names on the arrows represent the proteins encoded by each gene. GalE, lactose binding protein SBPT; GalF and GalG, integral membrane proteins of SBPT; LacK, ATP-binding protein of SBPT; LacB, β-galactosidase; LacA, α-galactosidase; HP, hypothetical protein; GalK, galactokinase; GalT, galactose-1-phosphate uridylyltransferase; GalE, UDP-glucose 4-epimerase; GalU, UTP-glucose-1-phosphate uridylyltransferase; TR, transcriptional regulator.

synthesis of TAG, which allows producing simultaneously high yields of cell biomass and lipids. These properties make *R. opacus* strains good candidates for single cell oil production from whey wastes.

3.4 Culture conditions for lipid production by *R. opacus* PD630 from whey

Since *R. opacus* PD630 demonstrated to be an efficient microorganism to produce cell biomass and lipids from whey, we investigated some culture variables which may influence production yields, such as preliminary treatment of whey, agitation of cultures, or times for lipid accumulation.

3.4.1 Effect of time and agitation on the yields of biomass and lipids

The accumulation of TAG by rhodococci occurs predominantly in stationary growth phase; however, after reaching the maximum cell density and TAG content, cells usually start the mobilization of the stored lipids [8]. In order to determine the optimum time for harvesting cells, we analyzed the time course of biomass and TAG production by *R. opacus* PD630 after entering in stationary growth phase (72 h of cultivation) during growth on whey. The maximum values of cell biomass (6.7 g/L) and TAG content (47.2% of CDW) from whey permeate occurred after 120 h of cultivation, which were maintained until 168 h, whereas those parameters declined later (Fig. 6A).

On the other hand, aeration of cell cultures promoted biomass and TAG production by *R. opacus* PD630 (Fig. 6B). The agitation of cultures at 150/200 rpm promoted an approximately twofold increase in biomass (6.4–6.6 g/L) and fivefold increase in TAG production (45.5–45.9% of CDW) in comparison to cells cultivated without agitation (3.1 g/L of biomass and 9.1% of lipids by CDW). Thus, lipid yield (g/L) obtained with agitation of cultures was approximately 11-fold higher than those without agitation.

3.4.2 Treatment to remove native microorganisms from whey

We investigated diverse treatments for removing the native microbial population of whey, which may negatively affect

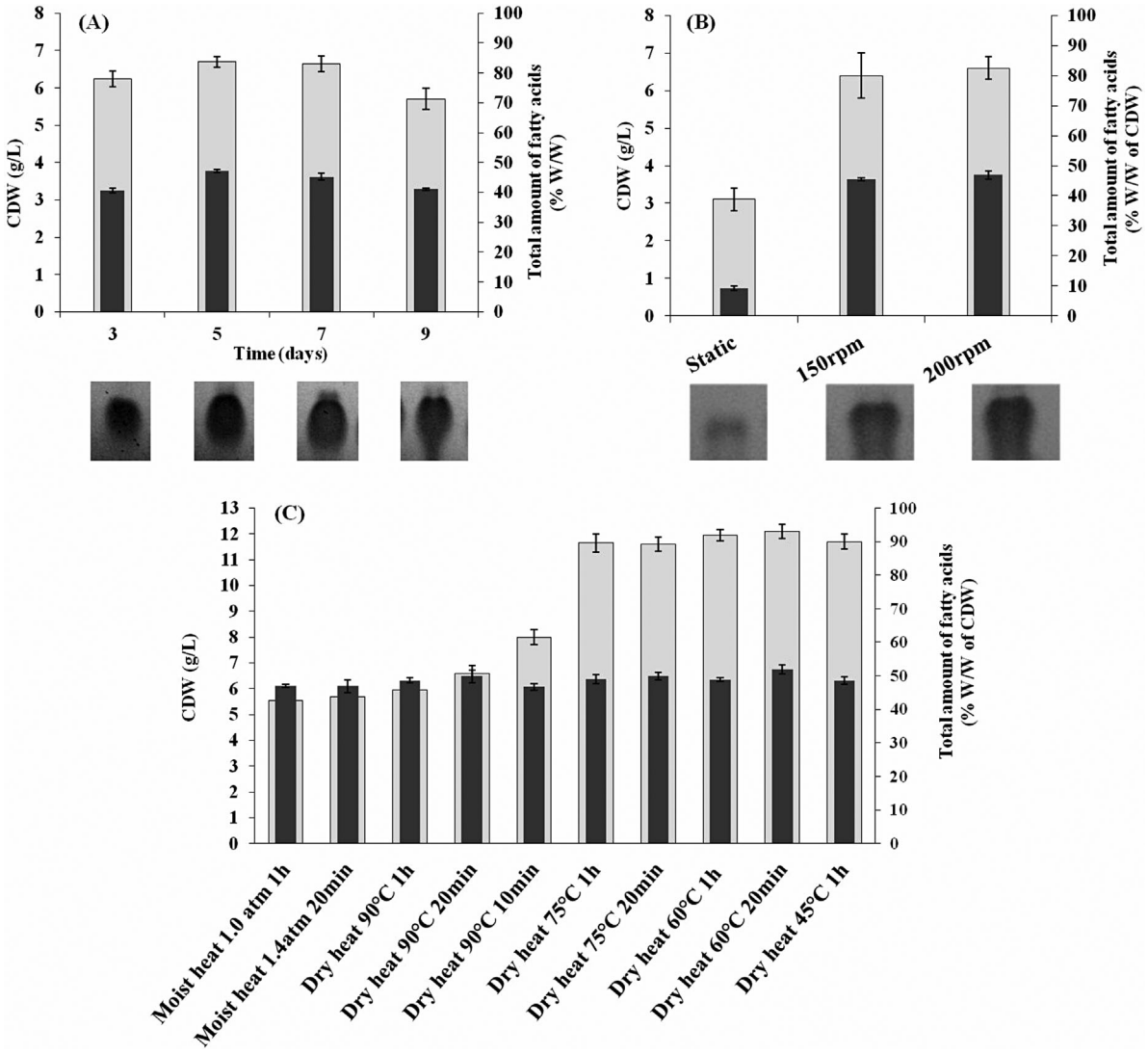

Figure 6. Effect of different culture parameters on biomass and lipid production by *R. opacus* PD630 from whey. Effect of the time (A), agitation of cultures (B), and different treatments applied to remove native whey microorganisms (C). The light gray bars represent the generated biomass expressed as cellular dry weight (CDW), and dark gray bars the lipid content. Dates are expressed as means of triplicate experiments and their standard deviations.

growth and lipid production by rhodococcal strains (Fig. S2). For each treatment, we analyzed the efficiency for removing native microorganisms of whey, the presence and purity of the inoculated strain, and the yields of biomass and TAG accumulation after cultivation of cells.

The chemical treatment of the whey by the addition of chloroform or alkali to the medium (pH 9 and 11) was not efficient enough to eliminate completely the native microbial population of the residue.

In contrast, heat treatments, either moist heat (heating with high pressure steam) and dry heat (heating without steam) showed to be effective in eliminating whey native microorganisms, obtaining pure cultures of rhodococcal cells after incubation.

Different combinations of temperatures and time of dry heat treatment were analyzed in this study (Fig. 6C). The minimum condition that resulted effective to remove whey native microorganisms was heating at 45°C for 1 h. When the temperature reached 90°C or higher, in both, moist heat and dry heat treatments, the coagulation of proteins contained in whey occurred. Protein clots interferes with the measurement of some culture parameters such as optical density, or with the harvest of cells. For these reasons, in this case, we decided to remove protein clots and work with whey permeate.

We analyzed the chemical composition of the whey without and after the applied different treatments. When whey was treated by heat at temperatures under 90°C for avoiding protein coagulation, the treated waste showed almost identical chemical composition than untreated whey (Table 2). In contrast, the whey permeate exhibited a decrease of approximately twofold in the nitrogen content compared to crude whey, whereas the rest of the components, such as total sugars, glucose, or lipids remained constant; thus, the C/N ratio of the whey permeate increased accordingly. Similar chemical composition was observed for all whey permeate samples obtained after different treatments at temperatures above 90°C, with nitrogen values between 580 and 620 mg/L in Table 2. The chemical composition of one of these whey permeated samples is shown in Table 2.

The exception to the mentioned behavior resulted after treatment with moist heat at 90°C for 10 min, which showed a partial precipitation protein and nitrogen values higher than the rest of whey permeates (784 mg/L).

On the other hand, we analyzed the yields of biomass and TAG production after cultivation of rhodococcal cells on the treated whey, using *R. opacus* PD630 for culture assays. Figure 6C shows that the intensity of whey treatments affected growth of cells and in minor proportion TAG accumulation. Biomass production by cells of *R. opacus* PD630 was significantly higher when the temperature of the whey treatment was maintained below 75°C (11.7–12.1 g/L), whereas TAG accumulation exhibited minor dependence on the treatment (47–52% of CDW) (Fig. 6C). Thus, when proteins were removed from whey by treatment at high temperatures (coagulation and filtration of proteins), biomass production by strain PD630 only reached 5.8–6.8 g/L (Fig. 6C). Interestingly, after treatment of whey with moist heat at 90°C for 10 min, which promoted partial protein coagulation, cells of strain PD630 produced intermediate values of biomass production (8.0 g/L) (Fig. 6C).

Altogether, these results indicated that the use of whey for cell cultivation allows increasing twofold cellular biomass production, which results in an increase of lipid production from 3.0 to 6.4 g/L, compared with the use of whey permeate. The higher biomass production during growth on whey in comparison to whey permeate may be a result of the higher nitrogen content in the first case (Table 2). Although the optimal C/N ratio for lipid production by *R. opacus* PD630 from whey should be determined in further studies, it is clear that the C/N ratio (approximately 52.2) of raw whey used in this study supported good growth and TAG accumulation by cells. In a previous study, Ykema et al. [18] reported that the optimal C/N ratio for lipid production by the oleaginous yeast *Apiotrichum curvatum* from whey was approximately of 30–35. These results suggested that the production of cell biomass by *R. opacus* may be enhanced, resulting in improved yields of lipids production, by an increase of the nitrogen content in the whey, to some extent.

4 Conclusions

This study demonstrated that the bioconversion of whey to microbial lipids by *Rhodococcus* strains is possible. However, not all species of this genus are appropriate for this purpose. Among five different rhodococcal species used in this work, only *R. opacus* possesses a robust genetic endowment for the degradation of lactose and galactose, and the physiological ability to produce high yields of cell biomass and lipids from such sugars and whey. These results suggested that the cultivation of *R. opacus* on residual whey could be applied to the biotechnological production of interesting single cell oils as platform for biofuels and other derived products.

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The authors have declared no conflict of interest.

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