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Enhanced synthesis of medium-chain-length poly(3-hydroxyalkanoates) by inactivating the tricarboxylate transport system of *Pseudomonas putida* KT2440 and process development using waste vegetable oil

José Manuel Borrero-de Acuña[1,2], Carla Aravena-Carrasco[1], Izabook Gutierrez-Urrutia, Daniela Duchens, Ignacio Poblete-Castro[*]

Biosystems Engineering Laboratory, Center for Bioinformatics and Integrative Biology (CBIB), Facultad de Ciencias de la Vida, Universidad Andres Bello, Republica 330, Santiago, Chile

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ABSTRACT

The use of waste materials as feedstock for biosynthesis of valuable compounds has been an intensive area of research aiming at diminishing the consumption of non-renewable materials. In this study, *P. putida* KT2440 was employed as a cell factory for the bioconversion of waste vegetable oil into medium-chain-length Polyhydroxyalkanoates. In the presence of the waste oil this environmental strain is capable of secreting enzymes with lipase activities that enhance the bioavailability of this hydrophobic carbon substrate. It was also found that the oxygen transfer coefficient is directly correlated with high PHA levels in KT2440 cells when metabolizing the waste frying oil. By knocking out the *tctA* gene, encoding for an enzyme of the tripartite carboxylate transport system, an enhanced intracellular level of *mcl*-PHA was found in the engineered strain when grown on fatty acids. Batch bioreactors showed that the KT2440 strain produced 1.01 ($g{\cdot}L^{-1}$) of PHA whereas the engineered Δ*tctA P. putida* strain synthesized 1.91 ($g{\cdot}L^{-1}$) after 72 h cultivation on 20 ($g{\cdot}L^{-1}$) of waste oil, resulting in a nearly 2-fold increment in the PHA volumetric productivity. Taken together, this work contributes to accelerate the pace of development for efficient bioconversion of waste vegetable oils into sustainable biopolymers.

1. Introduction

It is estimated that the ever-increasing amount of plastic waste that is deposited in nature is about 79% of the total worldwide production [1], thus negatively impacting wildlife, natural environments, and human health. Given that the majority of monomers used to synthesize synthetic plastics are from non-renewable materials such as petroleum, they are extremely challenging to degrade once deposited in the environment [2,3]. Efforts have been made by the public and the private sector to promote sustainable alternatives to the polymer manufacturing industry. Microbial production of biopolymers has been growing in the last decades resulting in materials that meet the technical prerequisites of oil-based plastics [4]. Polyhydroxyalkanoates (PHAs), degradable polyoxoesters with similar mechanical and physical properties as compared to polypropylene, are currently being produced at industrial scale [5,6]. One of the most proficient genera of natural PHA-producing microorganisms is *Pseudomonas* [7], which display a high metabolic versatility and thrive under harsh conditions [8,9]. *Pseudomonas putida* strains naturally produce medium-chain-length PHAs (*mcl*-PHAs), a more elastic biopolymer than their counterpart Poly(3-hydroxybutyrate) (PHB) and Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) [10,11]. They accumulate high levels of *mcl*-PHAs as intracellular inclusion bodies from a wide variety of carbon substrates such as glucose [12], glycerol [13], fatty acids [14], aromatic compounds [15], and waste materials [16,17]. Engineering of these environmental microorganisms has yielded specific PHAs with defined monomer compositions when grown on fatty acids [18]. In addition, various metabolic engineering and synthetic biology strategies have been applied to *Pseudomonas putida* KT2440 for enhanced PHA synthesis and recovery [6] using non-related carbon sources [12,19], which are cheaper substrates than pure fatty acids but result in lower yields of PHAs [20,21]. A sustainable and economical alternative to pure fatty acid is the use of oil waste materials [16]. Currently, millions of tons of waste frying oil (WFO) are being discarded to the environment by the food industry and households [22]. Several studies have shown that bacterial strains, like

[*] Corresponding author.
E-mail address: Ignacio.poblete@unab.cl (I. Poblete-Castro).
[1] These authors contributed equally to this work.
[2] Current address: Technische Universität Braunschweig, Institute of Microbiology, Spielmannstraße, 7, D-38106 Braunschweig, Germany.

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Cupriavidius and *Pseudomonas*, are able to metabolize WFO and produce PHAs under nitrogen and phosphorous limiting conditions [23–27]. Some of these works started the batch culture with a high biomass concentration in order to improve the rate of consumption of the available oil, which is low due to its high degree of hydrophobicity. The synthesis of this initial biomass to begin the fermentation process is time-consuming and has not been included in the total PHA productivity and process economics. On the other hand, little is known concerning the bioavailability process of the waste vegetable oils and what are the means of environmental bacteria for degrading these highly hydrophobic carbon substrates. Another important aspect to fully exploit waste frying oils as feedstock for microbial synthesis of biopolymers is to find new genetic targets in the biocatalyst towards elevated PHA synthesis [28]. It is well reported that protein synthesis by the cells is very costly in terms of energy and metabolic resources [29,30]. As most of the PHA-producing strains are environmental microorganisms, they have evolved various molecular mechanisms to constantly scavenge for nutrients in the changing environment where they thrive [31], which allow them to prevail in soil, where *Pseudomonas* bacteria are predominantly encountered in contrast to other species of organisms—1.6% of the total population [32]. *P. putida* KT2440 prefers organic acids and aromatics as carbon substrates over sugars during growth [33], in a process governed by a carbon catabolic repression (CCR) system [34]. As WFO is metabolized trough the β-oxidation pathway in bacteria, we hypothesize that the inactivation of transport systems for preferred carbon sources such as the tripartite tricarboxylate transport (TTT) would have a positive influence on PHA synthesis in *P. putida* KT2440. In this study, we knocked out the *tctA* gene encoding for a key transport enzymes of carboxylic acids located in the periplasm of the cell and evaluated the production of *mcl*-PHA in KT2440 cells growing on WVO as the unique carbon substrate. We also assessed how the bacterial supernatant influence the bioavailability of waste vegetable oils and its impact on *mcl*-PHA productivity in bioreactors in this natural PHA-producing microorganism. Taking together, we show that the combination of process development and genetic engineering of the work-horse enhanced the biopolymer synthesis in batch culture establishing the basis towards the efficient bioconversion of waste frying oil into medium-chain-length Poly(3-hydroxyalkanoates).

2. Material and methods

2.1. Bacteria and growth conditions

The wild-type *P. putida* KT2440 (DSM 6125, DSMZ, Germany) was used in this study. Cells were kept as frozen stocks in 25% glycerol at −80 °C. To obtain single colonies, it was plated onto Luria Bertani agar plates after one-day incubation at 30 °C. The first pre-inoculum was prepared by picking up a single colony from the plate and inoculating into a 50 mL shake flask containing 10 mL of Luria Bertani medium. Afterwards, 5 mL of culture were inoculated in 100 mL shake flask containing 20 mL of defined minimal medium (M9): 12.8 ($g{\cdot}L^{-1}$) $Na_2HPO_4{\cdot}7H_2O$, 3 ($g{\cdot}L^{-1}$) KH_2PO4, 1 ($g{\cdot}L^{-1}$) NH_4Cl, 0.5 ($g{\cdot}L^{-1}$) NaCl. This medium was autoclaved and subsequently supplemented with 0.12 ($g{\cdot}L^{-1}$) of $MgSO_4{\cdot}7H_2O$, trace elements (6.0 $FeSO_4{\cdot}7H_2O$, 2.7 $CaCO_3$, 2.0 $ZnSO_4{\cdot}H2O$, 1.16 $MnSO_4{\cdot}H_2O$, 0.37 $CoSO_4{\cdot}7H_2O$, 0.33 $CuSO_4{\cdot}5H_2O$, 0.08 H_3BO_3) ($mg{\cdot}L^{-1}$) (filter-sterilized) and adjusted to a pH of 6.9.

The second preinoculum was prepared in M9 minimal medium under the same conditions as described above supplemented with 3 ($g{\cdot}L^{-1}$) of waste frying oil. The cells were grown under aerobic conditions at 30 °C in an Ecotron incubator shaker (INFORS HT, Switzerland) set at 180 rpm for 7 days.

To evaluate the biomass and PHA synthesis in the *Pseudomonas* strains in flask cultures, a calculated volume of the second preinoculum cell suspension (less than 5% of the final volume), where the cells were then inoculated into 500 mL baffled Erlenmeyer flasks with 100 mL of culture, containing M9 minimal medium, trace elements and magnesium sulfate supplemented with 20 ($g{\cdot}L^{-1}$) of waste frying oil and cultivated for 72 h in a Ecotron rotary shaker set at 30 °C, 180 rpm.

2.2. Construction of the KT2440 ΔtctA knockout mutant strain

Bacterial strains, plasmids and oligos used in this work are listed in Table 1. For the generation of a *P. putida* KT2440 *tctA* knockout mutant a deletion system was harnessed as described previously [35] based on the homologous recombination of gene flanking regions followed by I-SceI mediated double strand breaks and the subsequent DNA repair. For that

Table 1
Bacterial strains, plasmids and oligos employed throughout this study.

Bacterial strains	Relevant features [a]	Source/Reference
Pseudomonas putida		
KT2440	Wild-type strain originated from *P. putida* mt-2, cured of the pWW0 TOL plasmid	(DSM 6125, DSMZ, Germany)
Δ*tctA*	KT2440 knockout mutant lacking the *tctA* gene	This study
Escherichia coli		
DH5α	F– Φ80*lacZ*ΔM15 Δ(*lacZYA-arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	ThermoFisher, Scientific, Darmstadt, Germany
DH5αλpir	*sup* E44, Δ*lacU169* (Φ*lacZ*ΔM15), *recA1*, *endA1*, *hsdR17*, *thi*-1, *gyrA96*, *relA1*, λpir phage lysogen	Biomedal, Seville, Spain
HB101	Helper strain; F– λ– *hsdS20*(rB– mB–) *recA13 leuB6*(Am) *araC14* Δ(gpt- proA)62 *lacY1 galK2*(Oc) *xyl*-5 *mtl*-1 *thiE1 rpsL20* (Sm^R) *glnX44*(AS)	[78]
Plasmids		
pJET1.2	Ap^R; *oriV* (pMB1) Plasmid employed for subcloning steps	ThermoFisher, Scientific, Darmstadt, Germany
pRK600	Cm^R; *oriV* (ColE1), tra^+ mob^+ conjugational functions from plasmid RK2	[78]
pSEVA212	Km^R; *oriV* (R6K), Sce-I RS. Suicide vector	[79]
pSEVA628	Gm^R; *oriV* (RK2), *xylS*-Pm – > SceI	[79]
pJET1.2-*tct*AUPDW	Ap^R; pJET1.2 plasmid bearing the fused up- and downstream fragments of *tctA* gene	This study
pSEVA212-*tct*AUPDW	Km^R; pSEVA212 harboring the fused up- and downstream regions of *tctA* gene	This study
Oligos (5´ → 3´)		
*tct*AUpFw	GAATTCCACAGCCGATACGATGCTT	Fermelo Biotec, Santiago, Chile
*tct*AUpRv	CGAACAATGGCAACGACCGATTCAGTTCTCCAGTACGCTC	Fermelo Biotec, Santiago, Chile
*tct*ADwFw	TCGGTCGTTGCCATTGTTCG	Fermelo Biotec, Santiago, Chile
*tct*ADwRv	GGATCCCTGCCAGAGCAAGGCAAC	Fermelo Biotec, Santiago, Chile
*tct*AKOFw	AGCTCGATCCTGCTGAACAT	Fermelo Biotec, Santiago, Chile
*tct*AKORv	CAGACGATATCTGGCTGCTG	Fermelo Biotec, Santiago, Chile

[a] Abbreviations employed in this table: Cm^R = chloramphenicol resistance; Km^R = kanamycin resistance; Gm^R = gentamycin resistance; Ap^R = ampicillin resistance; I-SceI = SceI endonuclease; *xylS*-Pm = genetic expression system inducible by 3MB. EcoRI and BamHI restriction sites are underlined.

purpose, approximately 600 bp of the up- and downstream regions of the *tctA* gene from the KT2440 genome was amplified via single colony PCR with the pairs of oligos tctAUpFw/tctAUpRv and tctADwFw/tctADwRv, respectively. The resulting up- and downstream flanks of the gene were fused through splicing by overlap extension (SOEing)-PCR and cloned into the blunt-end commercial plasmid pJET1.2. The *Escherichia coli* DH10b strain was used to replicate the pJET1.2 construct. Subsequently, the spliced DNA module was EcoRI-BamHI digested and ligated into the suicide vector pSEVA212 resulting in the pSEVA212-*tct*AUPDW construct [19]. The DH5αλpir suitable host was the vehicle for further proliferation of this vector. Afterwards, the vector was transferred into *P. putida* KT2440 by triparental mating using the helper strain *E. coli* HB101/pRK600. Integration events were monitored by PCR employing the primers tctAUpFw/tctADwRv. Triparental mating was exploited again for the introduction of the pSEVA628 plasmid harboring the I-SceI restriction enzyme gene precursor. The expression of the I-SceI endonuclease was triggered by addition of 5 mM 3-methyl benzoate (3MB) and colonies sensitive to kanamycin were screened. Kanamycin intolerant colonies were further tested for the loss of the gene of interest via PCR using the primers tctAKOFw/tctAKORv. The pSEVA628 plasmid-curing was accomplished through several passages on Luria Bertani medium in the absence of antibiotics. When required, antibiotics were supplemented at the following concentrations: 50 ($\mu g \cdot mL^{-1}$) kanamycin, 30 ($\mu g \cdot mL^{-1}$) chloramphenicol, 150 ($\mu g \cdot mL^{-1}$) ampicillin and 15 ($\mu g \cdot mL^{-1}$) gentamycin for *E. coli* strains; 500 ($\mu g \cdot mL^{-1}$) kanamycin and 30 ($\mu g \cdot mL^{-1}$) gentamycin for *P. putida* KT2440.

2.3. Determination of k_La coefficients in flask experiments

For the determination of k_La coefficients at flask scale, the correlation estimated by [36] was used:

$$K_La = 0.143 \cdot N^{0.86} \cdot (V_L \cdot V_0{}^{-1})^{-0.80} \ (R^2 = 0.99)$$

Where N is the shaker speed (min^{-1}), V_L the liquid volume in flask, and V_0 the flask volume and k_La is expressed in h^{-1}. The constants were obtained from the linear regression of the data with $\ln k_La = \ln k + a \ln N + b \ln (V_L \cdot V_0{}^{-1})$.

2.4. Biomass quantification

The cell dry mass was determined gravimetrically after collection of 10 mL culture broth for 10 min at 4 °C and 9000 × g (Eppendorf 5810 R, Hamburg, Germany) in pre-weighed tubes, including two washing step with 10 mL mixture of pure hexane: ethanol at a ratio of 1:4, and a final washing step with a NaCl solution (0.9%). Then the final pellet was dried at 100 °C in an oven (Labtech, Korea) until constant weight. To fully corroborate these values, in another round of experiments, the cell pellet obtained from 10 mL culture, after centrifugation and washing steps as described above, was kept at -20 °C for 24 h. Subsequently, it was subjected to freeze-drying for 12 h. The obtained cell dry pellet was then placed on an analytical balance to record its weight.

2.5. Ammonium quantification

Cell-free supernatants obtained from the centrifugation step for biomass quantification were filtered (0.22 μm) and used to monitor concentrations of ammonium ions using the Spectroquant® ammonium test following the manufacture instructions (Merck, Germany). The detection limit of the test is 2.6 ($mg \cdot L^{-1}$).

2.6. PHA and oil characterization and quantification

Monomeric composition of PHA was determined by gas chromatography mass spectrometry (GC/MS). For this purpose, methanolysis was carried out by re-suspending 5–10 mg of lyophilized cell dry mass (previously obtained from the biomass quantification) in 2 mL chloroform and 2 mL methanol, containing 15% ($v \cdot v^{-1}$) sulfuric acid and 0.5 ($mg \cdot mL^{-1}$) 3-methylbenzoic acid as internal standard, respectively, followed by incubation at 100 °C for 4 h in a thermoblocker. After cooling to room temperature, 1 mL of demineralized water was added and the organic phase, containing the resulting methyl esters of the PHA monomers. Analysis was performed in a gas chromatographer coupled with a mass spectrometer model YL6900 (Young Instruments, Korea). An aliquot (2 μL) of the organic phase was injected into the gas chromatograph at a split ratio of 1:10. Separation of the methyl esters of 3-hydroxyacid compounds was achieved by a FactorFour VF-5 ms capillary column (30 m · 0.25 mm i.d. · 0.25 mm film thickness, Varian Inc., Agilent Technologies). Helium was used as carrier gas at a flow rate of 0.9 ($mL \cdot min^{-1}$). Injector and transfer line temperatures were 275 °C and 300 °C, respectively. The oven temperature program was: initial temperature 40 °C for 2 min, then from 40 °C up to 150 °C at a rate of 5 °C min and finally up to 280 °C at a rate of 10 °C min. Positive ions were obtained using electron impact ionization at 70 eV and mass spectra were generated by scanning ions from *m/z* 50 to *m/z* 650. The PHA concentration was determined by the method described by Lageveen and co-workers [37]. Briefly, quantification of PHA was carried out by using a calibration curve generated from 0.5 to 2 mg of a purified Poly(3-hydroxyhexanoate-*co*-3-hydroxyoctanoate-*co*-3-hydroxydecanoate), obtained from a previous study [38] to interpolate sample data. The PHA content (wt%) was defined as the percentage of the CDM represented by the *mcl*-PHA.

The fatty acid composition of the used waste frying oil was characterized after transesterification of the lipids to the corresponding methyl esters (FAME) in a GC/MS [39]. For this, 1 mL of the waste vegetable oil was added to 4 mL of sodium hydroxide in methanol and heated at 80 °C for 1 h. 5 mL of (15%) boron trifluoride in methanol was then added to methylate the samples. After 10 min, the solution was removed from the heat blocker. Then, 15 ml of heptane was added to the extract and the mixture was shaken vigorously for 5 min and the heptane phase separated phase via centrifugation. GC/MS analysis was performed by injecting 1 μL of the solution with an injection port at 250 °C, split ratio 1:100, and helium as carrier gas. The temperature program was 80 °C/5 min, 250 °C/20 min, and 250 °C/5 min. The fatty acid methyl esters of the WFO was determined as palmitic acid (16:0) 11.3%; stearic acid (18:0) 5.4%; oleic acid (18:1) 51.7%; linoleic acid (18:2) 31.6% using a NIST mass spectral library.

2.7. Transmission electron microscopy

Bacteria were fixed by chilling the cultures to 4 °C and adding of glutaraldehyde (2%) and formaldehyde (5%). They were then washed with cacodylate buffer (0.01 M cacodylate, 0.01 M $CaCl_2$, 0.01 M $MgCl_2$ 6H2O, 0.09 M sucrose, pH 6.9) and stained with 1% aqueous OsO_4 for 1 h at room temperature. Samples were then dehydrated with a graded series of alcohol (10, 30, 50, 70, 90 and 100%) with incubation for 30 min at each concentration, except for the 70% alcohol, which contained 2% uranyl acetate and was cultivated overnight. Samples were infiltrated with an epoxy resin, according to the Spurr formula for hard resin, for several days with pure resin. Ultrathin sections were cut with a diamond knife, counterstained with uranyl acetate and lead citrate and examined in a TEM910 transmission electron microscope (Carl Zeiss, Oberkochen, Germany) at an acceleration voltage of 80 kV. Images were taken at calibrated magnifications using a line replica. Images were recorded digitally with a Slow-Scan CCD-Camera (ProScan, 1024·1024, Scheuring, Germany) with ITEM-Software (Olympus Soft Imaging Solutions, Munster, Germany). Contrast and brightness were adjusted with Adobe Photoshop CS3.

2.8. Bioreactor fermentations

P. putida strains were grown in M9 medium supplemented with 20 ($g \cdot L^{-1}$) of waste vegetable oil as the only C source and 0.5 ($mL \cdot L^{-1}$) of

TEGO antifoam (Evonik, Germany). 20% ($v{\cdot}v^{-1}$) of filtered supernatant was added to the bioreactor before inoculation and mixed for 5 h. Then 100 mL (5% $v{\cdot}v^{-1}$) from the second preinoculum were added to the bioreactor to begin the culture, and the length if the fermentation was 85 h. Bioreactor batch fermentations were carried out in a 3 L top-bench LiFlux GX bioreactor (Biotron, Korea) with a working volume of 2 L, at 30 °C. The aeration rate was set to 1 vvm using a mass flow controller. The dissolved oxygen level was kept above 20% air saturation by controlling of the agitation speed up to a maximum of 900 rpm. The pH was maintained at 7.0 by automatic pH controlled addition of 0.5 M H_2SO_4 or 1 M of KOH.

2.9. Lipase activity

At the end of the fermentation process in bioreactors, cell-free supernatant of the mutant and wild-type *P. putida* strains were assessed for lipase activity using the *para*-nitrophenyl palmitate (*p*-NPP) method described by [40]. Briefly, 1 mL of isopropanol containing 3 mg of p-nitrophenyl palmitate was mixed with 9 mL of 0.05 M Tris–HCl pH 8.0, containing 40 mg Triton X-100. The substrate solution mixture was stirred until all the constituents were dissolved completely. A total amount of 2.4 mL of freshly prepared substrate solution was dispensed into each test tube. Thereafter, 0.1 mL of cell-free supernatant was added to initiate hydrolysis. After 10 min of incubation at 30 °C, the reaction mixture was rapidly placed in ice to stop the hydrolysis, and subsequently centrifuged at 2000*g* for 3 min at 4 °C. The optical density of the obtained supernatant was measured at 410 nm against an enzyme free control. One lipase unit (U) was defined as the amount of enzyme that liberated 1 μmoL p-nitrophenol per milliliter per minute under the standard assay conditions. All the enzyme assays were carried out in triplicates and the mean values were calculated.

3. Results and discussion

3.1. Addition of cell-free supernatant in the culture broth enhances the biomass and Polyhydroxyalkanoate synthesis

P. putida KT2440 was grown in minimal medium supplemented with (20 $g{\cdot}L^{-1}$) of waste frying oil as the only carbon source using shaking flask. After 72 h of cultivation, we observed that most of the available oil remained on the surface of the culture with apparent low degradation of the WFO by the cell. We next quantified biomass and PHA formation and found that only 0.56 ($g{\cdot}L^{-1}$) of cell dry mass (CDM) was produced with a biopolymer accumulation of approximately 30% of the CDM as PHA (Fig. 1A). The PHA level obtained in this experiment was very similar to the one reported by Song and coworkers in *Pseudomonas* sp. DR2 growing on WFO [24]. Exploring strategies for elevating the PHA synthesis in KT2440, we have previously observed that the pre-inoculum, which was grown using 3 ($g{\cdot}L^{-1}$) of WFO and 7 days incubation, had a whitish color and no apparent remaining oil within the culture. Therefore, we were prompted to take a portion of the filtered supernatant of the pre-inoculum and mix it with the final culture for 5 h before inoculating KT2440 cells into the final flask culture. Fig. 1A shows the effect of using various ratios of supernatant when *P. putida* KT2440 was underwent to PHA-producing conditions, where a 4-fold increase in PHA accumulation was found for KT2440 when 20% ($v{\cdot}v^{-1}$) of the supernatant was used. Increasing the supernatant to a 30% ($v{\cdot}v^{-1}$) of the culture, no further improvement in PHA accumulation (0.8 $g{\cdot}L^{-1}$) was observed as compared to cultures with 20% ($v{\cdot}v^{-1}$) of supernatant (Fig. 1A).

3.2. The oxygen transfer coefficient is key for efficient waste oil degradation

As the degradation of WFO occurs trough the β-oxidation pathway of the cell —a highly oxygen-demanding metabolic route [21]— we evaluated the impact of different oxygen transfer coefficients (k_La) on PHA synthesis in shaking flask experiments by adjusting the reaction volume of the culture. It is clear that by decreasing the k_La in flask cultures, lower amount of PHA was synthesized by the cell where no PHA production occurred with a K_La value of 16.5 (h^{-1}) (Fig. 1B). It is noteworthy to point out that all experiments were carried out with 20% ($v{\cdot}v^{-1}$) of supernatant. Surprisingly, the culture in which no PHA was present, it also coagulated as defined spheres after 72 h cultivation (Fig. 2). They were very viscous and no planktonic cells were found in the culture (Fig. 2). Electron microscopy revealed that the spheres were comprised of WFO, where the cells were entrapped within the waste oil (Fig. 2). Micrographs of PHA-producing cells with 20% ($v{\cdot}v^{-1}$) of supernatant were likewise obtained. Under this condition PHA granules accumulated in the cytoplasm of the cells where the number of inclusion bodies ranged from one to three granules (Fig. 2). Traces of the WFO can be also visualized on the micrographs but no cells were attached or trapped into the oil feedstock. Microbial degradation of hydrophobic compounds like fat oil and polycyclic aromatics is restricted since the biocatalyst has no access to the target chemical compound [41]. It becomes clearer that *P. putida* KT2440 might secrete enzymes or biosurfactant agents that improve the bioavailability, in a more soluble fashion, of the WFO. Artificial surfactants and emulsifiers have been used to improve the bioavailability of fatty substrates for mcl-PHA biosynthesis by *Pseudomonas* sp. [42,43]. On the other hand, *Pseudomonas* strains have been described as natural biosurfactant producers including rhamnolipids [44–46], glycolipids [47], and lipopeptides [48]. All of them use 3-hydroxyacids as precursors for their synthesis [49,50], thus competing against the PHA biosynthetic pathway [51]. As *P. putida* KT2440 neither produces rhamnolipids nor lipopeptides the synthesis of lipases or estereases appears to be implicated in this process.

3.3. Disruption of the tricarboxylate transport system leads to improved biopolymer synthesis of KT2440 cells growing on fatty acids

We next sought for genetic targets that could lead to elevated PHA synthesis in *P. putida* KT2440. It is well reported that *P. putida* strains prefer organic acids *e.g.* succinic or citric acids, over sugars or fatty acids as carbon substrates [34]. This carbon catabolic repression phenomenon endows *Pseudomonas* species with an evolutionary advantage as compared to other soil bacteria, where the concentration of carboxylic acids and aromatic compounds are constantly fluctuating in their ecological niches [52]. Another crucial molecular mechanism displayed by *Pseudomonas* bacteria that allow them to prevail among bacterial communities in soil is the secretion of binding proteins, which are very specific for sequestering carboxylic acids into the cell [53]. The genes encoding for the enzymes of the tripartite tricarboxylate transport system of environmental bacteria have been reported to be constantly expressed [54,55], thus expending unnecessary energy when cells are growing on non-related substrates such as fatty acids. As PHA is an energy storage compound, we inactivated the transport capability of carboxylic acids in *P. putida* KT2440 by knocking out the *tctA* gene expecting that more energy to be stored by the cell as PHA granules. First, we corroborated that the Δ*tctA* mutant strain showed almost completely growth arrest when cultured in M9 minimal medium using citrate (2 $g{\cdot}L^{-1}$) as C substrate (data not shown). Subsequently the Δ*tctA* mutant and KT2440 strain were subjected to accumulate PHAs on decanoate and octanoate (20 mM) in M9 minimal medium (Table 2). We used these fatty acids as they are metabolized via the β–oxidation pathway, serving as model compounds for WFO. After 48 h of cultivation in batch cultures, the Δ*tctA* mutant strain accumulated 54.1 and 18.5% more PHA ($g{\cdot}L^{-1}$) in comparison to the wild type strain KT2440 on decanoate and octanoate, respectively (Table 2). It appears that the inactivation of the Δ*tctA* gene in KT2440 has a greater impact on PHA production when using longer chain-lengths of fatty acids. Furthermore, we analyzed the monomer composition of the synthesized PHA, where no difference can be observed by comparing the obtained PHA of the Δ*tctA* mutant and the

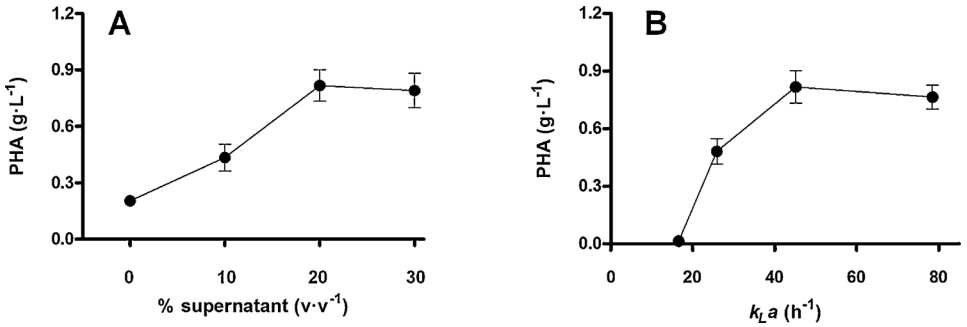

Fig. 1. A. PHA synthesis using various ratios of supernatant of the wild-type KT2440 strain when growing on 20 ($g{\cdot}L^{-1}$) of WVO. B. Effect of different oxygen transfers coefficients on PHA synthesis in *P. putida* KT2440 after 72 h of cultivation.

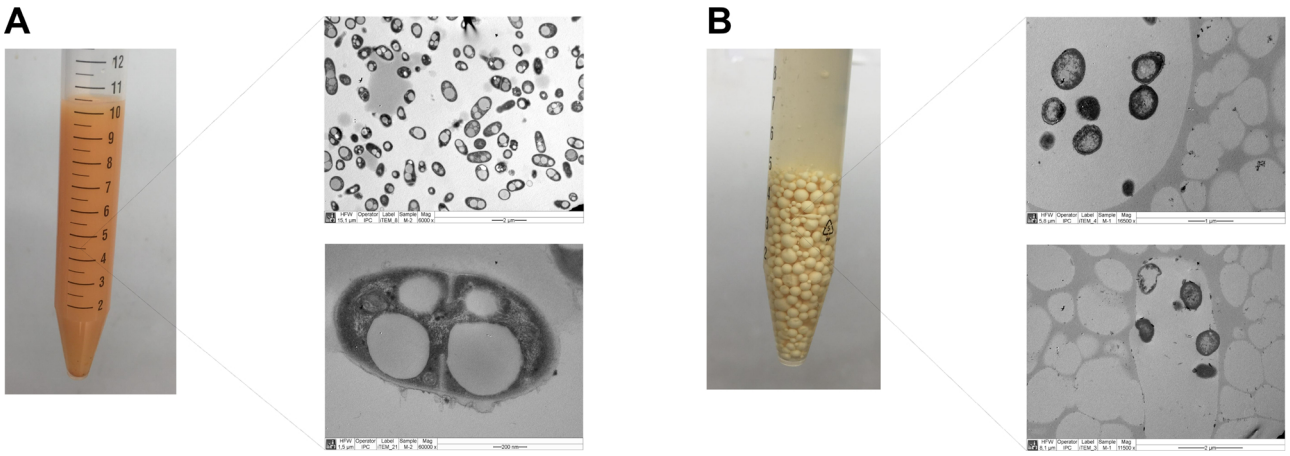

Fig. 2. Micrographs of *P. putida* KT2440 cells grown on 20 ($g{\cdot}L^{-1}$) of WVO with a high (A) and low (B) k_La value.

Table 2
Monomer composition, *mcl*-PHA and biomass synthesis by *P. putida* KT2440 and Δ*tctA* mutant strain using fatty acids as carbon substrate (20 mM).

Strain	Carbon source	CDM ($g{\cdot}L^{-1}$)	PHA[a] (%wt)	PHA ($g{\cdot}L^{-1}$)	Composition[b] (%)		
					C6	C8	C10
KT2440	Octanoate	1.89 ± 0.03	29.31	0.56 ± 0.09	13.23	86.77	–
Δ*tctA*	Octanoate	1.66 ± 0.12	38.37	0.64 ± 0.12	16.36	83.64	–
KT2440	Decanoate	2.30 ± 0.05	29.75	0.68 ± 0.07	3.02	39.44	57.54
Δ*tctA*	Decanoate	2.40 ± 0.06	43.71	1.05 ± 0.12	3.21	38.90	57.88

Standard deviation (±) from three independent experiments (biological).
[a] PHA content relative to cell dry mass (CDM) after 48 h of cultivation.
[b] C6: 3-hydroxyhexanoate, C8: 3-hydroxyoctanoate, C10: 3-hydroxydecanoate.

parental strain when grown on both substrates (Table 1). To date, researchers have inactivated genes of the β-oxidation pathway to enhance the PHA synthesis in *Pseudomonas* strains on fatty acids [56]. By inactivating the *fadB* and *fadA* gene in KT2442, the PHA synthesis was improved in batch cultures using decanoate or octanoate as C source [57,58]. Deletion of the *phaZ* gene in *P. putida* KT2442, encoding for a PHA depolymerase enzyme, has been one of the few cases where the inactivation of genes that do not belong to the β-oxidation pathway led to an increased production of mcl-PHA on fatty acids [59]. Metabolic engineering and synthetic biology strategies aiming at improving PHA synthesis in whole-cell biocatalysts have been mainly applied for redirecting the carbon flux towards PHA precursors, acceleration of cell growth, and overexpression of genes belonging to the PHA biosynthetic operon [28]. Other molecular mechanisms involved on enhanced PHA synthesis in bacteria are still elusive. As presented in this work, inactivation of protein synthesis that is not beneficial for the biocatalyst when producing target chemical compounds appears as a suitable approach for the microbial synthesis of PHAs.

3.4. Combination of process development with strain engineering enhances the Polyhydroxyalkanoate productivity in batch bioreactors

As we have developed both a superior PHA-producing strain and an efficient bioconversion of waste frying oil in batch culture, we now compared the PHA production capacity of the Δ*tctA* mutant and the wild-type KT2440 strain in well-controlled bioreactor. Fig. 3 shows the time course of biomass and PHA synthesis of both *P. putida* strains when metabolizing the waste frying oil (20 $g{\cdot}L^{-1}$) in batch cultures. As the β–oxidation pathway generates direct precursors for PHA synthesis, PHA accumulation begins without the need of nitrogen limitation in both bacterial strains (Fig. 3). *P. putida* KT2440 accumulated ~30% of its CDM as PHA showing a PHA productivity of 0.34 ($g\ (L{\cdot}day)^{-1}$) after 72 h. On the other hand the Δ*tctA* mutant strain amassed ~38% of its CDM as PHA and almost double the PHA productivity 0.65 ($g\ (L{\cdot}day)^{-1}$) as compared to KT2440 due to a higher total biomass production (residual biomass + PHA). The mutant strain developed in this work has the highest *mcl*-PHA productivity so far achieved using WFO

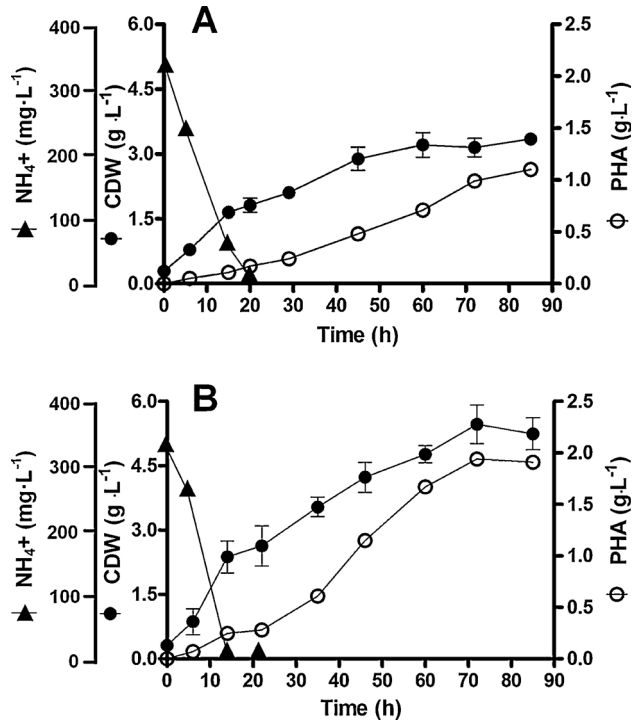

Fig. 3. Time course of biomass and PHA synthesis in *P. putida* KT2440 (A) and Δ*tctA* mutant (B) strain in M9 minimal supplemented with 20 ($g{\cdot}L^{-1}$) of WVO in bioreactors.

in batch cultures. Table 3 summarizes the production parameters of the Δ*tctA* mutant, *P. putida* KT2440 strain, and other *Pseudomonas* bacteria using WFO as C source for PHA synthesis. The best reported natural producer of *mcl*-PHA on WFO is *P. resinovorans* in batch cultures, showing a PHA productivity of 0.50 ($g\ (L{\cdot}day)^{-1}$) [60]. We did not take into consideration any *Pseudomonas aeruginosa* strain in this comparison since they are classified as pathogens and are not suitable for industrial biotechnology [61,62]. We also inspected the monomer composition of the produced *mcl*-PHA at the end of the fermentation process. As previously reported for *mcl*-PHAs obtained using waste frying oil in other *Pseudomonas* species [24,60], the main monomers of the polymeric chain of the synthesized *mcl*-PHA in this study are 3-hydroxyoctanoate and 3-hydroxydecanoate, which together account for more than 90% of the total mol% (Table 2). Similar monomer composition of produced *mcl*-PHAs in *Pseudomonas* species has been attributed to the given similarity between PHA synthases (PhaC) that they possess [63]. Unfortunately, none of the *Pseudomonas* strains reported for the conversion of WVO into *mcl*-PHA are sequenced, so no further analysis can be conducted concerning the homology among the PHA synthases. Taking into consideration that the lipid composition of the used WFO has a high content of unsaturated fatty acids (see material and methods), one could expect that it would be reflected in the final monomer composition of the produced *mcl*-PHA, which is not the case for any work reported so far in *Pseudomonas* bacteria (Table 2). As the waste oils are degraded trough the β-oxidation pathway in bacteria, two carbons are lost for each round within the cycle. It is surprising that no 3-hydroxytetradecanoate was found as one of the monomers of the polymeric chain. It has been previously reported that the PHA synthase of KT2440 has substrate preference towards 3-hydroxydecanoyl-CoA (C10) and 3-hydroxyoctanoyl-CoA (C8) [64], thus explaining the enhanced proportion of these monomers within the polymeric chain when *P. putida* cells metabolized the WFO (Table 2).

3.5. *P. putida strains show lipase activities in the presence of waste frying oil during Polyhydroxyalkanoate synthesis*

We searched then for secreted enzymes that might act on the waste frying oil to increase its bioavailability. One of the best-described enzymes to be produced and secreted by microbes in the presence of fats belongs to the family of lipases [65], being bacteria of the genus *Pseudomonas* good lipase producers [66,67], which are used for various biotechnological applications. High-level synthesis of microbial lipases is promoted by environmental conditions specially in the presence of certain carbon compounds in the media like polysaccharides and fats, high temperature, and cell density [66]. In this study, the lipase activity was measured in cell-free supernatants at the end of the fermentation period in bioreactors via the *para*-nitrophenyl palmitate method (Fig. 4). The Δ*tctA* mutant strain shows an enzyme activity of 0.99 ($U{\cdot}mL^{-1}$) whereas the wild type KT2440 displays a value of 0.45 ($U{\cdot}mL^{-1}$) at 30 °C. This difference in lipase activity might explain why the engineered *P. putida* strain reached a higher total biomass than its parental KT2440 strain in bioreactors (Fig. 3). We also carried out the enzymatic assay at 40° and 50 °C, where the lipase activity clearly increased at elevated temperatures, reaching its maximum value at 40 °C for both *P. putida* strains (Fig. 4). Lipases produced by environmental bacteria have also been described to increment their activity at higher temperatures [68]. Inspection of the genome of *Pseudomonas putida* KT2440 revealed that this versatile microorganisms harbours three genes encoding for lipase enzymes, namely *lip* (PP_4844), PP_3812, and PP_2083 [69]. Its genetic organization differs from the one found in *P. aeruginosa* where the *lip* gene forms an operon with the gene *lif*, the latter a lipase-specific foldase [66]. This genetic conformation is necessary in *P. aeruginosa* since the translocation system of the mature lipase occurs trough the Xcp machinery, which is classified as a secreton-mediated system type II [70]. There are other secretion systems of lipolityc enzymes in *Pseudomonas* bacteria, like the one shown by *P. fluorescens*, where its translocation to the extracellular medium occurs

Table 3
Composition and production of mcl-PHAs from waste cooking oil by *P. putida* in KT2440, Δ*tctA* mutant strain and various *Pseudomonas* species in batch cultures.

Strain	$Y_{PHA/Xr}$[a] ($g{\cdot}g^{-1}$)	PHA (%wt)[b]	Composition[c] (mol%)					PHA productivity $g{\cdot}(L{\cdot}day)^{-1}$	Reference
			C6	C8	C10	C12	C14		
***P. putida* KT2440**	0.45 ± 0.1	30.5 ± 2.4	7.7	54.6	34.8	2.9	N.D	0.34 ± 0.03	This work
Δ*tctA*	0.62 ± 0.1	38.3 ± 3.1	6.3	51.3	39.2	3.2	N.D	0.65 ± 0.05	This work
P. resinovorans	0.39	28	11	43	33	12	N.D	0.50	[60]
***Pseudomonas* sp.GI06**	0.21	20	27	51	10	12	N.D	0.43	[26]
***Pseudomonas* sp.**	0.31	23.5	5.9	45.7	34.9	5.2	8.4	0.04	[24]

Standard deviation (±) from two independent bioreactors.
N.D: Not detected.
[a] Xr: residual biomass (PHA-free).
[b] PHA content relative to cell dry mass (CDM).
[c] C6: 3-hydroxyhexanoate, C8: 3-hydroxyoctanoate, C10: 3-hydroxydecanoate, C12:3-hydroxydodecanoate.

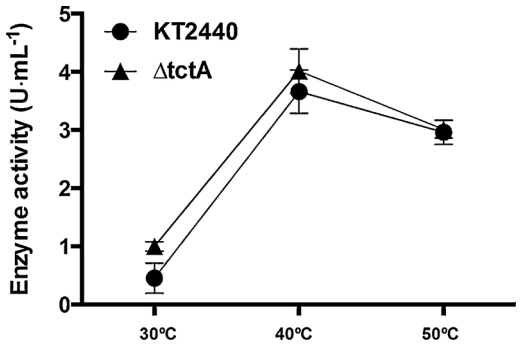

Fig. 4. Lipase activities of cell-free supernatant of *P. putida* KT2440 and the Δ*tctA* mutant strain obtained at the end of the batch fermentation (72 h). One lipase unit (U) was defined as the amount of enzyme that liberated 1 μmoL p-nitrophenol per milliliter per minute.

via a ABC-transporter system [71]. Although *P. putida* KT2440 possesses the Xcp secretion system, this has not been proven to be involved in the secretion of lipases [72]. More studies are required to elucidate the translocation mechanism of lipase enzymes in KT2440 to fully exploit the lipase-producing capacity of this versatile cell factory for various biotechnological applications. Concerning process development, there is still a big challenge to produce PHAs at high titer using substrates with low solubility such as waste vegetable oils [73]. The use of emulsifiers or surfactants *e.g.* gum arabic and Tween 80, has proven to increase the PHA yield with *C. necator* strains as cell factories using fatty feedstocks [74,75] in the process of choice for the industrial production of PHA, the fed-batch process. The use of lipase synthesized by the natural PHA producer, as the one shown in this study, brings about advantages in terms of costs and time over employing chemical emulsifying agents. The next challenge is to develop a fed-batch process for the production of *mcl*-PHAs using WVO as the only carbon substrate, which so far has resulted in low levels of the biopolymer by *Pseudomonas* bacteria [76] in comparison to processes carried out with pure fatty acids [77]. Future research should be focused on optimizing the mixing time and the concentrations of the used lipase with the feeding solution at different temperatures as well as tailoring the monomer composition of the produced PHA in novel engineered bacterial strains using low-cost waste vegetable oils to expand the portfolio of degradable biopolymers.

4. Conclusions

The bioconversion of waste oils into biopolymers offers a more sustainable route as compared to the use of renewable substrates obtained from arable soils for food production. In this study the lipase activity of KT2440 was investigated. The addition of cell-free supernatant to the final culture resulted in a higher bioavailability of the WFO, enabling improved yields of biomass and *mcl*-PHA in comparison with the untreated culture. In addition, inactivation of the TTT system of KT2440 led to an enhanced *mcl*-PHA accumulation on WFO, demonstrating that the combination of process development and strain engineering is imperative towards biopolymer synthesis.

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