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Improved Production of Medium-Chain-Length Polyhydroxyalkanoates in Glucose-Based Fed-Batch Cultivations of Metabolically Engineered *Pseudomonas putida* Strains

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One of the major challenges in metabolic engineering for enhanced synthesis of value-added chemicals is to design and develop new strains that can be translated into well-controlled fermentation processes using bioreactors. The aim of this study was to assess the influence of various fed-batch strategies in the performance of metabolically engineered *Pseudomonas putida* strains, Δ*gcd* and Δ*gcd-pgl*, for improving production of medium-chain-length polyhydroxyalkanoates (mcl-PHAs) using glucose as the only carbon source. First we developed a fed-batch process that comprised an initial phase of biomass accumulation based on an exponential feeding carbon-limited strategy. For the mcl-PHA accumulation stage, three induction techniques were tested under nitrogen limitation. The substrate-pulse feeding was more efficient than the constant-feeding approach to promote the accumulation of the desirable product. Nonetheless, the most efficient approach for maximum PHA synthesis was the application of a dissolved-oxygen-stat feeding strategy (DO-stat), where *P. putida* Δ*gcd* mutant strain showed a final PHA content and specific PHA productivity of 67% and $0.83 \text{ g·l}^{-1}\text{·h}^{-1}$, respectively. To our knowledge, this mcl-PHA titer is the highest value that has been ever reported using glucose as the sole carbon and energy source. Our results also highlighted the effect of different fed-batch strategies upon the extent of realization of the intended metabolic modification of the mutant strains.

Keywords: Dissolved-oxygen-stat, fed-batch process, glucose, medium-chain-length polyhydroxyalkanoates, metabolic engineering, *Pseudomonas putida*

Introduction

Medium-chain-length polyhydroxyalkanoates (mcl-PHAs) are industrially valuable polymers owing to their similar properties to petroleum-based plastics and the possibility to produce them from renewable resources. Beyond initial applications as film materials, mcl-PHAs have been found suitable for a large range of biomedical applications such as heart valves [24], medical temporary implants, and chiral monomers [36]. In particular, *Pseudomonas putida* strains are promising candidates to become efficient industrial mcl-PHA producers since high accumulation of the polyester can be promoted even under nonstandard PHA-accumulating conditions [22]. Their development into tailor-made cell factories for those bio-based polymers shows benefits from the wide industrial application of *P. putida* and the excellent knowledge of this species [20]. However, their transfer into production at industrial scale is still limited by the high cost associated with fermentation and downstream processing, which is up to 10 times higher than that of petroleum-based plastics [6]. Thus, successful production of mcl-PHAs requires a fine-tuned well-established production

process. This involves distinct production phases to deal with the complex interrelationship between biomass and PHA formation in the cell. Usually, the first stage of the process aims at high biomass concentration. This is followed by a second stage with the limitation of an inorganic nutrient and an excess of the carbon source in the medium to promote PHA accumulation. A precise process control is enabled by the feeding profile of the nutrients: constant-feeding, pulse-feeding, pH-stat, or dissolved-oxygen-stat (DO-stat), where the feeding is used to control these parameters to a certain set value. Thereby, the level of the nutrients has to be fine-tuned to avoid cell lysis or inhibition by high substrate levels. In addition, another complication arises since oxygen demand is very high in high-cell-density cultures. This phenomenon is particularly enhanced when fatty acids are used as feedstocks, and hence the use of alternative carbon sources seems to be an excellent option for obtaining more cost-efficient production processes. Recently, different cheap waste materials have been tested for the synthesis of both poly-3-hydroxybutyrate (PHB) and mcl-PHAs. In this regard, PHB has been produced from molasses (sugar industry), xylose (cellulosic industry), whey (manufacturing of cheese), waste glycerol (biodiesel industry), residual oils, and waste water (treatment plants) [12], whereas synthesis of mcl-PHAs has been limited to the use of waste lipids [16, 17, 25]. This is due to the chosen biocatalysts – *Pseudomonas* species –, which have a low yield of mcl-PHAs on sugars [5, 9, 21]. Instead, these microorganisms have evolved to rather prefer other carbon sources such as organic acids like succinate, and aromatics compounds [18, 26]. Today, sugars such as glucose, fructose, and sucrose are the most used carbon substrate for PHA production [34]. In this regard, research efforts have been directed towards the development of mcl-PHA hyperproducing *P. putida* strains *via* metabolic engineering using carbohydrates as carbon substrates [11, 21]. Recently, we were able to generate a genetically engineered *P. putida* strain that was able to produce 100% more mcl-PHA as compared with the wild-type strain with glucose as the sole carbon source [21]. In this study, these promising strains were transferred into a high-cell-density fed-batch process. A carbon-limiting exponential feeding was set up for biomass biosynthesis, which was followed by three different feeding approaches during the PHA accumulation phase (nitrogen-limitation) in the presence of glucose. This enabled the titer of mcl-PHA to increase by more than 23-fold in comparison with the titer previously reported in batch cultivations. Thus, *P. putida* Δ*gcd* has

proven to be suitable for fed-batch cultivations, where a DO-stat feed strategy shows to be the most appropriate approach for mcl-PHA production on glucose.

Materials and Methods

Strains

The wild-type *Pseudomonas putida* KT2440 (DSMZ collection, Germany) and its deletion mutants *P. putida* Δ*gcd* and Δ*gcd-pgl* [21] were used in this study.

Culture Conditions

All strains were kept as frozen stocks in 25% glycerol at –80ºC. To obtain single colonies, they were plated onto Luria-Bertani agar plates after 1 day incubation at 30ºC. Inocula were prepared by picking up a single colony from the plate and inoculating it into a 50 ml shake flask containing 10 ml of the defined minimal medium (M9) consisting of (per liter) 12.8 g $Na_2HPO_4 \cdot 7H_2O$, 3 g KH_2PO_4, 4.7 g $(NH_4)_2SO_4$, and 0.5 g NaCl. This medium was autoclaved and subsequently supplemented with 0.12 g of $MgSO_4 \cdot 7H_2O$, trace elements (6.0 $FeSO_4 \cdot 7H_2O$, 2.7 $CaCO_3$, 2.0 $ZnSO_4 \cdot H_2O$, 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/l)), and 3 g/l of glucose (all filter-sterilized) as the unique carbon source. The cells were grown under aerobic conditions at 30ºC in an Innova incubator shaker (New Brunswik, USA) set at 180 rpm. By taking a calculated volume of the overnight-grown cell suspension, the cells were then inoculated into 1,000 ml baffled Erlenmeyer flasks with 300 ml of culture medium and cultivated in a rotary shaker as described above. This second pre-culture was used as a seed for the fed-batch process. The starting medium (M9) in the fed-batch process was supplemented with 3 g/l glucose, 0.12 g/l $MgSO_4 \cdot 7H_2O$, and 8 ml of the trace element solution. Nitrogen source was supplied by using 14% (w/v) NH_4OH to control the pH for the batch and exponential feeding phase. During the PHA accumulation phase, NH_4OH was replaced by NaOH 10% (w/v). Each liter of feed medium contained 564 g glucose monohydrate and 12 g $MgSO_4 \cdot 7H_2O$. This same medium was used to feed the bioreactor at each stage along the process.

Fed-Batch Cultivations

Fermentations were carried out in a 10 L B10 stirred tank bioreactor (Biologische Verfahrenstechnik, Basel, Switzerland). The starting working volume was 4 L in all experiments. The temperature was controlled at 30ºC, and the pH was set at 6.8 by controlled addition of H_3PO_4 4% (w/v) or [NH_4OH or NaOH (see above)]. Tego Antifoam (Evonik, Germany) was added on demand. The airflow was set to 10 l/min (mixture of air and pure oxygen to a ratio of 10:1). To ensure a dissolved oxygen (DO) level above 10%, the agitation speed was adjusted automatically up to 900 rpm. A gas analyzer (BlueSense, BCpreFerm, Germany) to record online the concentrations of carbon dioxide and oxygen in the course of the process was coupled to the gas outlet of the bioreactor.

Analytical Procedures

Cell growth was recorded as optical density (OD) at 600_{nm} (Ultraspec 2000; Hitachi, Japan). The cell dry weight was determined gravimetrically after collection of 10 ml of culture broth for 10 min at 4°C and 9,000 ×g (Eppendorf 5810 R, Germany) in pre-weighed tubes, including a washing step with distilled water, and drying of the obtained pellet at 100°C until constant weight. The ammonium concentration in cell-free supernatant was measured by a photometric test (LCK 303 kit; Hach Lange, USA). The glucose concentration in cultivation supernatant was analyzed after appropriate dilution by HPLC Agilent 1260 (Agilent, Krefeld, Germany) equipped with an 8 mm Rezex ROA-organic acid H column (Phenomenex, USA) at 65°C, with 0.013 N H_2SO_4 as the mobile phase (0.5 ml/min) followed by detection using a RID detector (Agilent serie1260). Gluconate was quantified by HPLC as previously described [21].

PHA Characterization and Quantification

PHA compositions of the polymer produced, as well as the cellular PHA content concentration, were determined by gas chromatography (GC) and mass spectrometry (MS) of the

methanolyzed polyester. First, 10 ml of the culture broth was placed in a falcon tube and centrifuged for 10 min at 4°C and 9,000 ×g (Eppendorf 5810 R, Hamburg, Germany), followed by a washing step with distilled water. The supernatant was poured away by pipetting and the cell pellet kept at –20°C for further process. Methanolysis was carried out by suspending 5–10 mg of lyophilized aliquots in 2 ml of chloroform and 2 ml of methanol containing 15% sulfuric acid and 0.5 mg/ml 3-methylbenzoic acid as internal standard, respectively, followed by incubation at 100°C for 4 h. After cooling, 1 ml of demineralized water was added and the organic phase containing the resulting methyl esters of monomers was analyzed by GC-MS. Analysis was performed in a Varian GC-MS system 450GC/240MS ion trap mass spectrometer (Varian Inc., Agilent Technologies) and operated by the software MS Workstation 6.9.3 (Varian Inc., Agilent Technologies). An aliquot (1 ml) of the organic phase was injected into the gas chromatograph at a split ratio of 1:10. Separation of the analytes of interest (*i.e.*, the methyl esters of 3-hydroxyexanoate, 3-hydroxyoctanoate, 3-hydroxydecanoate, 3-hydroxydodecanoate, 3-hydroxy-5-*cis*-dodecanoate, 3-hydroxytetradecanoate) was achieved by a FactorFour VF-5ms capillary column (30 m × 0.25 mm i.d. × 0.25 mm film thickness). Helium was used as carrier gas at a flow rate of 0.9 ml/min. The injector and transfer line temperature were 275°C and 300°C, respectively. The oven temperature program was initial temperature 40°C for 2 min, then from 40°C up 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 ionization at 70 eV and the mass spectra were generated by scanning ions of *m/z* 50 to *m/z* 650. The PHA content (wt%) was defined as the percentage of the cell dry weight (CDW) represented by the polyhydroxyalkanoate.

Calculations

In the C-limited exponential phase of fed-batch cultures, the volumetric feed rate of glucose was calculated using the following equation:

$$F(t) = \left(\frac{\mu_{set}}{Y_{x/s}} + m\right)\frac{V_0 X_0 \cdot e^{\mu_{set}(t-t_0)}}{S_0} \tag{1}$$

The values for yield and maintenance coefficients were 0.39 g/g and 0.031 g·g^{-1}·h^{-1}, respectively [33].

Results

Constant-Feeding Approach Under Nitrogen Limitation to Synthesize mcl-PHAs from Glucose

P. putida KT2440. The process started with a batch phase where after 5.5 h the initial amount of glucose (3 g/l) was totally consumed. During this phase (stage I), *P. putida* KT2440 grew at a maximum specific growth rate of 0.56 h^{-1}. To develop a robust carbon-limited fed-batch fermentation process, an exponential feeding strategy was then applied, where the specific growth rate was set to 0.23 h^{-1}. This stage
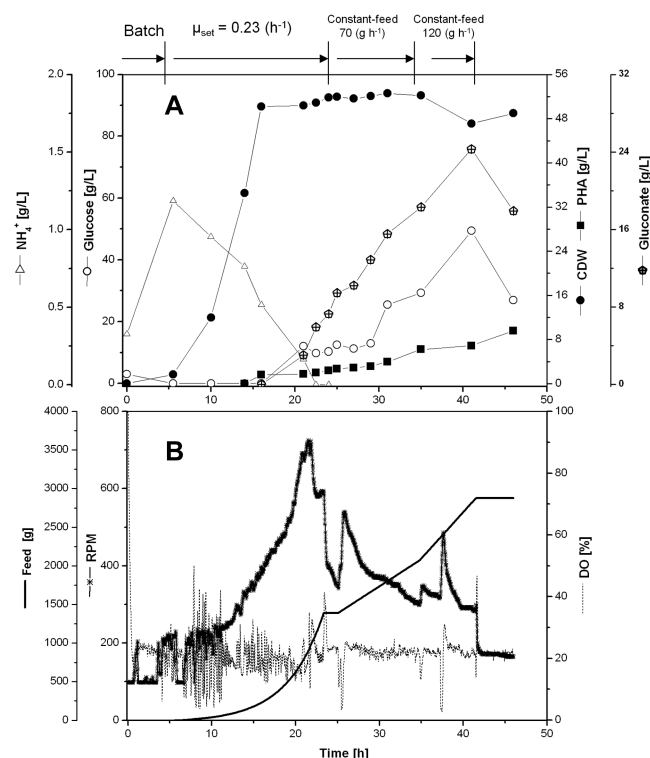

Fig. 1. Fermentation profile of *P. putida* KT2440 during the fed-batch process.
The PHA accumulation phase was performed by a constant-feed strategy. (**A**) Concentration profiles of ammonium, cell dry weight (CDW), PHA, and gluconate. (**B**) Profiles of feed, stirrer speed (RPM), and dissolved oxygen (DO) over time.

(stage II) aimed to produce a high biomass concentration. After 11.5 h of feeding, the process reached a biomass concentration of 45.2 g/l, where glucose did not accumulate in the culture (Fig. 1A). Subsequently, however, the glucose added could only be partially used by the cells owing to overflow metabolism. This also promoted the accumulation of gluconate. At this time point, PHA synthesis was not completely inhibited, which accounted for 4% of the total biomass concentration (Fig. 1A). Intracellular PHA accumulation is normally stimulated by the limitation of an inorganic nutrient while the carbon source is in excess [8]. As soon as glucose was detected in the culture broth, NH_4OH was replaced by NaOH. This promoted rapid consumption of ammonia – below the detection level (2 mg/l). Then glucose was supplied to the fermentor to a constant-feeding rate of 70 g/h. After 9 h of feeding, little PHA accumulation was found and gluconate was excreted by the cells, reaching a titer of 20 g/l. At 35 h of cultivation, the feeding rate was then increased to 120 g/h, but this change did not induce an increase in PHA synthesis. Instead, more gluconate was produced by the cell. The final PHA and total biomass concentration were 8.0 and 58 g/l, respectively.

P. putida Δgcd **mutant strain.** Driven by a systems metabolic engineering approach, several potential genetic targets were identified, which aimed at improving the PHA yield on glucose in *P. putida* KT2440 [21]. Glucose dehydrogenase (encoded by *gcd*) led the ranking of target genes to be deleted from the metabolic network. This enzyme is responsible for carrying out the oxidation of glucose to gluconate in the periplasmatic space. Gluconate can be either secreted to the external medium or further oxidized to 2-ketogluconate by gluconate dehydrogenase (encoded by *gad*) [2]. Deletion of the *gcd* gene in the *P. putida* strain prevented the formation of gluconate and 2-ketogluconate, and most important, increased the PHA yield on glucose by 100% in comparison with its parental strain KT2440 in batch fermentation [21].

In a first evaluation of the knockout mutant Δgcd, it was subjected to a similar process as described for the wild-type strain (Figs. 2A and 2B). During the batch phase (stage I), the mutant strain grew slightly lower than the parent strain (μ_{max} of 0.44 h^{-1}). After depletion of glucose, the exponential feeding (stage II) was set at μ 0.23 h^{-1}. In contrast to the wild-type, however, glucose accumulation began already after 15 h of feeding; at that time 34 g/l of CDW was obtained. As the μ_{set} was closer to μ_{max} for the mutant than the wild-type, it seemed no longer possible to keep the carbon-limited fed-batch. Once glucose had reached 10 g/l,
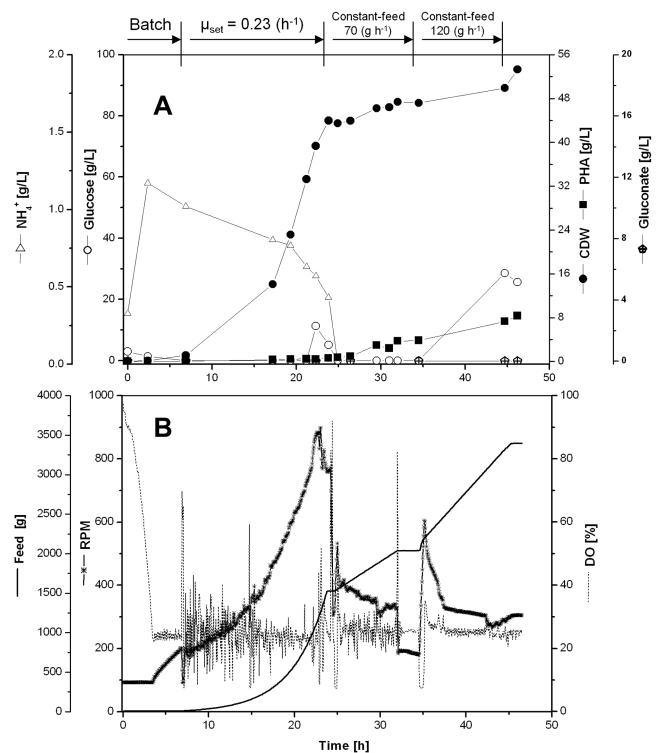

Fig. 2. Fermentation profile of Δ*gcd* mutant strains during the fed-batch process.

PHA accumulation phase was performed by a constant-feed strategy. (**A**) Concentration profiles of ammonium, cell dry weight (CDW), PHA, and gluconate. (**B**) Profiles of feed, stirrer speed (RPM), and dissolved oxygen (DO) over time.

the exponential-feed was stopped. At 25 h, when glucose was consumed, the constant-feeding of glucose began with a rate of 70 g/h. Unexpectedly, the mutant Δgcd consumed all the supplied glucose during the following 10 h. At 35 h, the constant-feed was increased to 120 g/h. After 10 h, the glucose concentration reached a value of 30 g/l. As performed for the wild type, the feed pump was switched off to let the cell consume the remaining glucose. Overall, 16% of the CDW was synthesized as PHAs, resulting in a final PHA and biomass concentration of 8.5 and 53.3 g/l, respectively.

P. putida Δgcd-pgl **mutant strain.** Recently, a genome-scale metabolic model of *P. putida* KT2440 was developed [23]. Using the OptKnock approach, several pairs of genes were selected in order to ensure a higher carbon flux towards PHA synthesis. We constructed the double-mutant engineered strain, where the enzymes glucose dehydrogenase (gcd, PP_1444) and 6-phosphoglucolactonase (pgl, PP_1023) were no longer active. In terms of PHA synthesis, *P. putida Δgcd-pgl* mutant strain produced 33% more PHA in comparison with the wild type KT2440 [21]. This shows
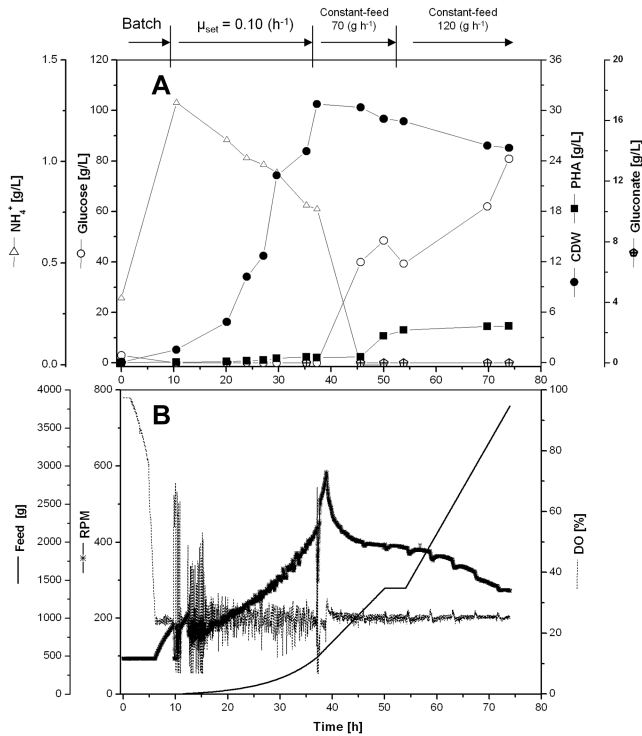
Fig. 3. Fermentation profile of Δ*gcd-pgl* mutant strains during the fed-batch process.
The PHA accumulation phase was performed by a constant-feed strategy. (**A**) Concentration profiles of ammonium, cell dry weight (CDW), PHA, and gluconate. (**B**) Profiles of feed, stirrer speed (RPM), and dissolved oxygen (DO) over time.

Fig. 4. Fermentation profile of *P. putida* KT2440 during the fed-batch process.
The PHA accumulation phase was performed by a pulse-feed strategy. (**A**) Concentration profiles of ammonium, cell dry weight (CDW), PHA, and gluconate. (**B**) Profiles of feed, stirrer speed (RPM), and dissolved oxygen (DO) over time.

that this model-driven strategy is a powerful tool to design efficient engineered strains for biopolymer synthesis. The next step is to test the *P. putida* Δ*gcd-pgl* mutant strain in the process of choice for industrial production of PHA, the fed-batch processes.

The Δ*gcd-pgl* mutant strain showed an even lower maximum specific growth rate of 0.33 h⁻¹ in the batch phase (Fig. 3A). For the exponential-feeding phase, it was challenged to grow at a set μ of 0.23 h⁻¹, as we performed for the other strains. The culture could not cope with the imposed specific growth rate, showing glucose accumulation and low biomass production (data not shown). Therefore, the whole process was repeated to a set μ of 0.10 h⁻¹. This was much better, as the culture reached a biomass concentration of 33 g/l, and glucose accumulation was not observed. At 37 h, a constant feeding rate of 70 g/h of the carbohydrate began (Fig. 2B). This strategy did not promote PHA synthesis within the cell, resulting in glucose accumulation up to 49 g/l. By increasing the feeding rate to 120 g/h, PHA concentration was not altered; instead, the total biomass
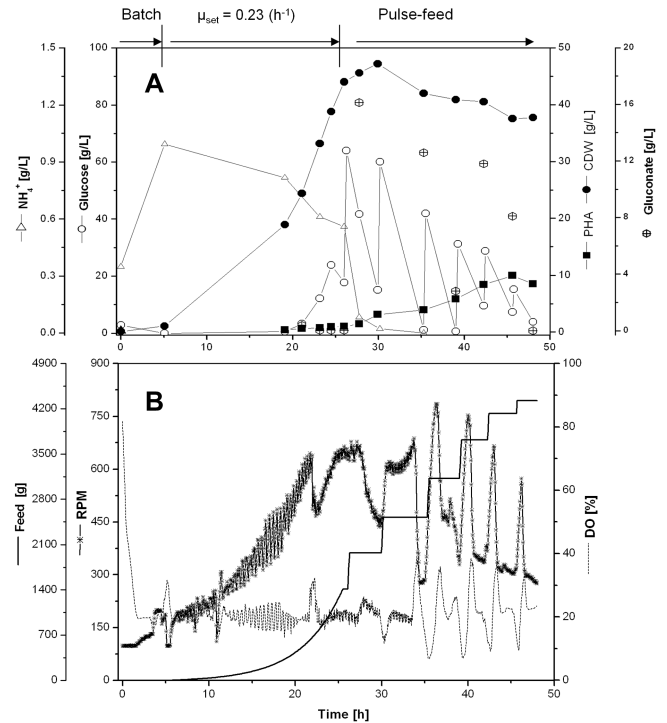
concentration diminished, most likely due to the stress provoked by the glucose concentration (80 g/l) reached at the end of the process. The overall PHA productivity at 74 h was 0.07 g·l⁻¹·h⁻¹. As a conclusion, the double mutant is the least suitable strain to synthesize PHAs among all strains tested using a constant-feeding strategy.

Substrate-Pulse Feeding Approach Under Nitrogen Limitation

P. putida **KT2440.** To further test the PHA production capacity of KT2440, the cells were challenged to a different feeding approach in the PHA accumulation phase. The wild type was initially subjected to the same carbon-limited growth regime described above (Fig. 3A). Once glucose was detected in the culture broth (*i.e.* at 26 h), the pulse-feeding was initiated, which consisted of adding glucose to the bioreactor to a certain concentration (< 60 g/l). This was achieved by monitoring the glucose concentration off-line. As shown in Fig. 4A, this strategy reduced the total biomass produced as compared with the constant-feeding strategy (Fig. 1A). In addition, as can be seen in Fig. 4B, there

Table 1. Summary of PHA production by *P. putida* strains in fed-batch process in the presence of glucose.

Strain	Production strategy	μ^a (h^{-1})	CDW (g/l)	PHA (g/l)	PHA content (%)	Productivity^b (g·l^{-1}·h^{-1})
KT2440	Linear	0.23	57.9	8.0	13.8	0.17
Δgcd	Linear	0.23	54.0	8.5	15.7	0.18
Δgcd-pgl	Linear	0.10	28.9	3.44	11.9	0.07
KT2440	Pulses	0.23	37.2	8.2	22.0	0.17
Δgcd	Pulses	0.23	51.9	23.9	46.0	0.48
Δgcd	DO-stat	0.20	61.8	41.5	67.1	0.83

[a]Imposed specific growth rate within the exponential feeding phase.

[b]The specific PHA productivity was calculated based on the entire time of the fermentation process.

was good correlation between the depletion of glucose and the rise of the DO concentration. The PHA content increased linearly along the process as soon as nitrogen was completely taken up by the cells (at 30 h). Moreover, a high concentration of gluconate − the main by-product − accumulated to a high concentration of 16 g/l. This was further consumed, but as soon as glucose was fed to the

Fig. 5. Fermentation profile of *Δgcd* during the fed-batch process.

The PHA accumulation phase was performed by a pulse-feed strategy. (**A**) Concentration profiles of ammonium, cell dry weight (CDW), PHA, and gluconate. (**B**) Profiles of feed, stirrer speed (RPM), and dissolved oxygen (DO) over time.

bioreactor, gluconate titer rose (Fig. 4A). The final PHA concentration and specific productivity in this process were 8.2 g/l and 0.17 g·l^{-1}·h^{-1}, respectively, which are almost the same as the values achieved by the constant-feeding approach. The most remarkable difference between the two was the PHA content, which was 2-folds higher for the pulse-feeding as compared with the constant-feeding (Table 1).

P. putida Δgcd **mutant strain.** Fig. 5B shows the time profiles of *Δgcd* mutant strain, where the substrate-pulse feeding approach was applied in the accumulation phase. This pulse-feeding strategy began when the total CDW had a concentration of 40 g/l. The PHA titer was already 5 g/l, although nitrogen was still present in the broth medium (at 24 h, Fig. 5A). At this point, glucose concentration was accumulated to a high titer of 90 g/l. When nitrogen limitation was achieved (at 30 h), product accumulation increased while applying pulses of glucose to the process (Fig. 5A). In contrast to the wild type KT2440, *Δgcd* did not produce any by-product (Fig. 4A). The DO concentration showed a stable trend, whereas the stirring speed decreased until the end of the process (Fig. 5B). The mutant strain *Δgcd* had a final PHA titer of 24 g/l, and a PHA content of 46%. The specific PHA productivity was almost 3-folds higher than the one shown by the constant-feeding approach using the same strain and was similarly higher than the one from the wild type by the same substrate-pulse feeding approach (Table 1).
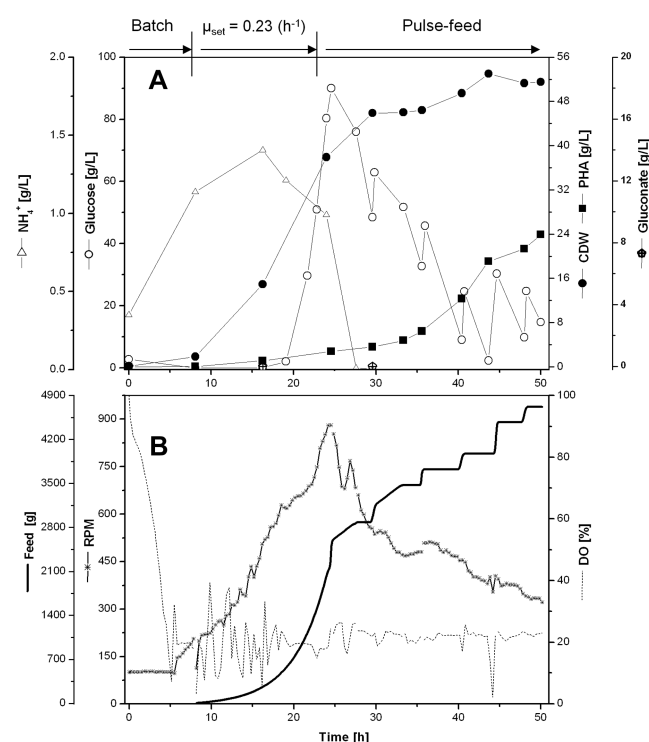
DO-Stat Feeding Approach in *P. putida* Δ*gcd* Mutant Strain

As we found that *P. putida Δgcd* mutant strain produces three times more mcl-PHA in comparison with *P. putida* KT2440 using the pulse-feed approach, mainly due to the avoidance of by-product synthesis by the cell throughout the process, we next proceeded to evaluate the application
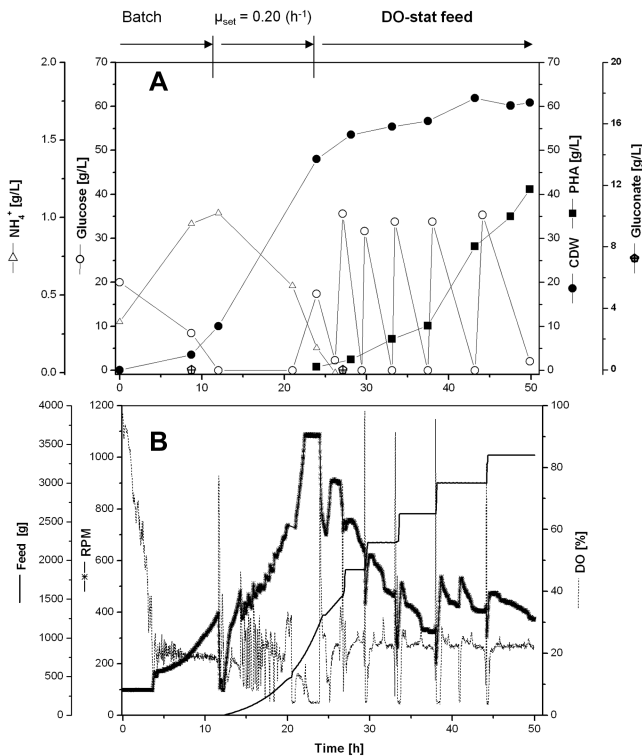
Fig. 6. Fermentation profile of Δ*gcd* during the fed-batch process.
The PHA accumulation phase was performed by a DO-stat feeding strategy. (**A**) Concentration profiles of ammonium, cell dry weight (CDW), PHA, and gluconate. (**B**) Profiles of feed, stirrer speed (RPM), and dissolved oxygen (DO) over time.

of a DO-stat feed strategy to fully exploit the PHA production capacity of the engineered *P. putida* Δ*gcd* strain under a well controlled environment. In this process, glucose was increased from 3 to 20 g/l within the batch phase. Another important modification was the increase in the final glucose concentration in the feeding solution, which was of 775 g/l. Once glucose was completely depleted (at 12 h), the biomass formation phase was initiated by subjecting the cell to grow to a specific growth rate of $0.20\ h^{-1}$. Glucose limitation could be maintained until 21 h. A high total biomass concentration of 50 g/l was achieved (Fig. 6A). At 24 h, glucose was detected in the process owing to oxygen limitation (Fig. 6B), so the glucose feeding to the bioreactor was stopped. This allowed the cells to start to consume the remaining glucose and nitrogen, as well as alleviating the limitation of oxygen. It was observed that the DO concentration rose when glucose was completely taken up. Thus, we programmed the unit controller for a DO-stat strategy. This means as soon as the DO value was higher

than 50%, glucose was automatically fed to the fermentor for a period of 15 min, which aimed to reach a glucose concentration of ~30-35 g/l in the fermentation. By applying this approach, the mutant strain Δ*gcd* began to accumulate mcl-PHAs rapidly. The total biomass concentration was stable along the process (Fig. 6A). The feeding strategy worked well until the end of the fermentation, where a PHA accumulation of 67% of the total CDW was achieved, resulting in a final PHA productivity of $0.83\ g \cdot l^{-1} \cdot h^{-1}$ using glucose as the solely carbon source.

Discussion

Feeding Strategies and the Effects of Genetically Engineered *P. putida* strains

Commercialized PHAs (Mirel, Biomer) are currently produced using fed-batch processes [1]; thus fed-batch is still the most employed technique to obtain both high cell and PHA concentration [1, 32]. These processes normally comprise two phases. First, high biomass formation is desired, since PHA productivity is highly connected to the amount of synthesized cells in the process. Once the desired cell density is attained, the PHA accumulation phase begins by limiting an inorganic nutrient, such as nitrogen. Sun *et al.* [32] have wonderfully reviewed several feeding strategies in fed-batch processes using different *P. putida* strains. High PHA productivity has been achieved in *P. putida* using alkanoates as substrates, where a constant-rate feeding ($0.91\ g \cdot l^{-1} \cdot h^{-1}$) [31], pH-stat ($1.91\ g \cdot l^{-1} \cdot h^{-1}$) [14], and exponential-feeding ($1.44\ g \cdot l^{-1} \cdot h^{-1}$) [29] were applied. In an attempt to combine different carbon sources, several works have been carried out with glucose as the carbon source for the biomass accumulation phase, and as soon as nitrogen is the limiting nutrient, glucose was replaced by fatty acids to synthesize mcl-PHAs [10, 29, 30].

By applying genetic engineering in *P. putida*, this has yielded remarkable results in the material properties of the novel PHAs [4, 15, 19]. On the other hand, the total PHA productivity is normally diminished as a result of detrimental effects provoked by the mutation, thus altering key physiological parameters such as specific growth rate and product yield. In order to achieve high PHA productivity using available and cost-efficient carbon sources, we thus aimed to evaluate different *P. putida* strains as microbial cell factories for the production of mcl-PHA using glucose as the carbon source for both biomass and PHA-production phases. As shown recently, *P. putida* Δ*gcd* and Δ*gcd-pgl* mutant strains could double the PHA titer in comparison with the wild-type strain in batch cultivations [21]. To

assess whether this result can be translated into high-cell-density cultures in fermentors, we challenged these strains using different feeding strategies in fed-batch cultivations. As the attainment of high biomass concentration in a short period of time has been reported in *P. putida* KT2440 [28], the next challenge was to choose a proper feeding approach to fully stimulate the accumulation of PHAs using glucose. We first applied a constant-feeding approach using the wild type KT2440 in the presence of glucose (Figs. 1A and 1B). This strategy was shown to be very promising for organisms that can accumulate PHAs even when all nutrients are in excess [14, 31]. As *P. putida* has been shown to be one of them [22], we investigated in this study to see whether it could highly promote PHA synthesis or not. Nevertheless, that was not the case since only a little rise on PHA concentration was observed, thus confirming that a constant feeding regime is not as efficient as when fatty acid is used as the carbon source [31].

Comparing *P. putida* KT2440 against the Δ*gcd* mutant strain, the latter did not accumulate more PHAs under the same constant feeding rate of glucose (Figs. 1A and 2A). Although the total biomass concentration was quite the same value for both strains at 17 h (Figs. 1A and 2A), there was a delay in the induction of PHA synthesis for the Δ*gcd* mutant strain, which finally affected the overall productivity. Thus, we concluded that instead of applying the same feeding approach to compare the PHA production performance among genetically modified strains, in order to substantially boost their PHA synthesis capacity, the process must be fine-tuned to fulfill the metabolic requirements of each specific strain. It is interesting also to see in the PHA-accumulation phase that when both strains had highly excess glucose, PHA accumulation was inhibited, but as soon as glucose feeding was stopped, PHA accumulation was rapidly triggered by such environmental condition (Figs. 1A, 1B, 2A, and 2B). This phenomenon led us to consider the substrate-pulse approach as a suitable feeding strategy within the PHA-synthesis phase. Concerning the double-deletion mutant (Δ*gcd-pgl*), it was shown as a less efficient producer strain in comparison with the wild type and the single-deletion mutant since the reduced maximum specific growth rate negatively influenced the overall PHA accumulation (Fig. 2B and Table 1).

The pulse-feed approach has been proved as an excellent strategy to induce PHA accumulation in several microorganisms [30]. The *P. putida* KT2440 wild-type strain could also be subjected to such strategy since DO increased when glucose was no longer available in the cultivation broth (Fig. 4B). Thus, it can be taken as a signal to feed glucose

automatically through the process. As the behavior of the *P. putida* Δ*gcd* strain was different from the wild-type strain, the mutant was subjected to a slightly different feeding condition. Apart from the pulses, we also fed glucose linearly for short periods of time (Fig. 5B) owing to the high capacity of the mutant strain to take up glucose. The pulses did not evoke the same responses (DO and stirred speed) as compared with the processes of the wild-type strain (Figs. 4B and 5B). This is due to the fact that glucose was not completely consumed when we added the substrate-pulses to the fermentor. As we did not detect gluconate using *P. putida* Δ*gcd* in the substrate-pulse fed-batch process, we propose that this might be the main reason for the higher accumulation of PHA in comparison with the wild-type strain (Figs. 4A and 5A). By deleting glucose dehydrogenase (PP_1444) in mutant Δ*gcd*, more carbon is available for PHA synthesis, and this modification might lead to a higher carbon flux into the *de novo* fatty acids synthesis instead of biomass formation [21]. Overall, the mutant Δ*gcd* proves to be a better producing strain since the PHA productivity was almost 3-fold higher as compared with the wild-type strain (Table 1). As the Δ*gcd* mutant has superior features in regard to PHA synthesis in pulse-feeding cultures, we subjected it to a DO-stat glucose-based fed-batch process to fully challenge its production features. This strategy provided by far the best environment for PHA accumulation among all tested conditions (Fig. 6A). To our knowledge, this is the highest productivity of mcl-

Table 2. Monomer composition of medium-chain-length PHA produced by metabolically engineered *P. putida* strains in different fed-batch processes.

	C6	C8	C10	C12	C12:1	C14
Constant-fed						
KT2440	n.d.	9.74	68.33	6.42	13.69	1.82
Δ*gcd*	n.d.	10.55	69.61	6.22	12.58	1.04
Δgcd-*pgl*	n.d	9.29	70.03	6.06	13.01	1.61
Pulse-fed						
KT2440	n.d.	10.02	68.59	6.02	13.26	2.10
Δ*gcd*	n.d.	10.82	69.30	6.42	12.31	1.15
DO-stat						
Δgcd	n.d.	9.79	66.68	5.93	12.56	4.94

The data were determined by GC/MS and are given as relative molar fraction (%). C6: 3-hydroxyexanoate; C8: 3-hydroxyoctanoate; C10: 3-hydroxydecanoate, C12: 3-hydroxydodecanoate; C12:1: 3-hydroxy-5-*cis*-dodecanoate, and C14: 3-hydroxytetradecanoate.
n.d. : not detected, less than 0.2%.

Table 3. Comparison of different mcl-PHA-producing strains using sugars as carbon sources.

Strain	Mode	Substrate	CDW (g/l)	PHA (g/l)	PHA content (%)	Productivity (g·l⁻¹·h⁻¹)	Reference
P. putida Δ*gcd*	Fed-batch	Glucose	61.8	41.5	67.1	0.83	This work
P. putida IPT	Fed-batch	Glu + Fruc[a]	50.0	31.5	63.0	0.80	[3]
P. fluorescens	Batch	Fructose	4.0	1.0	25.5	0.02	[13]
Engineered *E. coli strain*	Batch	Glucose	3.4	0.4	11.9	0.008	[35]
P. corrugata	Batch	Soy molasses	1.5	0.23	17.0	0.003	[27]

$Productivity (g·l^{-1}·h^{-1})$

[a]Glucose + Fructose.

PHA ever reached using glucose as the only carbon source throughout the entire process (Table 3). It is noteworthy also to mention that the *P. putida* Δ*gcd* mutant strain has a high potential to be used as a biocatalyst for the synthesis of other chemicals apart from PHAs, since the production of undesired by-products such as gluconate and 2-ketogluconate – typically synthesized in the presence of glucose – is avoided. Therefore, a more efficient process development, and thus the total productivity in fed-batch processes, could be achieved by maximizing the yield of product when the cells are grown on glucose.

Inspection of the monomer composition by gas chromatography indicated no difference among the synthesized monomers: 3-OH decanoic acid (C10) was always relatively abundant (~70%) in each feeding approach independent of the *P. putida* strain used (Table 2). This is a very important finding, considering that fermentation processes using fatty acids could yield different monomer compositions [8, 29, 30], which have been shown to have a high influence in the final mechanical and physical properties of the biopolymers. Here we have shown that it does not happen when glucose is used as the only carbon source. One of the two enzymes needed (3-hydroxyacyl-ACP dehydratases, FabA) to catalyze the dehydration of 3-hydroxyacyl-ACP is restricted to substrates with 10 carbons [7]. Therefore the nature of the PHA-synthesizing pathway – *de novo* fatty acids synthesis – determines the pool of unsaturated fatty acids, which finally yields the high content of 3-hydroxydecanoic acid produced in the PHA polymerization cycle when glucose is used as the carbon source.

Various engineered *P. putida* strains have been tested in this study in glucose-based fed-batch cultures with different feeding approaches in order to explore their mcl-PHA production capacities when grown on glucose. We have shown here that the combination of both a good producer strain (Δ*gcd*) and a fine-tuned process (DO-stat) is the key to achieving high mcl-PHA productivity, and glucose can be used as the sole carbon source instead of fatty acids,

which have so far been dominantly used in those processes owing to their promotion of high PHA concentration [10, 14, 31]. Thus, this work serves as a base to lead further investigation in metabolically engineered *P. putida* strains, which have very promising features for sustainable production of biopolymers.

Acknowledgments

Nomenclature

F(t) volumetric feeding rate (l/h)
S_0 concentration of the substrate in the feeding solution (g/l)
μ_{set} predetermined specific growth rate (h⁻¹)
$Y_{x/s}$ yield coefficient of biomass from glucose (g/g)
m specific maintenance coefficient (g·g⁻¹·h⁻¹)
V_0 initial volume of the bioreactor (L)
X_0 initial biomass concentration (g/l)
t time (h).

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