Biotransformation of D-xylose to D-xylonate coupled to medium-chain-length polyhydroxyalkanoate production in cellobiose-grown Pseudomonas putida EM42

Pavel Dvořák1* Jozef Kovác1 and Victor de Lorenzo2 1Department of Experimental Biology (Section of Microbiology), Faculty of Science, Masaryk University, Kamenice 753/5, 62500, Brno, Czech Republic. 2Systems and Synthetic Biology Program, Centro Nacional de Biotecnología CNB-CSIC, Cantoblanco, Darwin 3, 28049, Madrid, Spain.

Summary

Co-production of two or more desirable compounds from low-cost substrates by a single microbial catalyst could greatly improve the economic competitiveness of many biotechnological processes. However, reports demonstrating the adoption of such co-production strategy are still scarce. In this study, the ability of genome-edited strain Pseudomonas putida EM42 to simultaneously valorize α-xylose and α-cellobiose – two important lignocellulosic carbohydrates – by converting them into the platform chemical D-xylonate and medium-chain-length polyhydroxyalkanoates, respectively, was investigated. Biotransformation experiments performed with P. putida resting cells showed that promiscuous periplasmic glucose oxidation route can efficiently generate extracellular xylonate with a high yield. Xylose oxidation was subsequently coupled to the growth of P. putida with cytoplasmic β-glucosidase BglC from Thermobiflida fusca on α-cellobiose. This disaccharide turned out to be a better co-substrate for xylose-to-xylonate biotransformation than monomeric glucose. This was because unlike glucose, cellobiose did not block oxidation of the pentose by periplasmic glucose dehydrogenase Gcd, but, similarly to glucose, it was a suitable substrate for polyhydroxyalkanoate formation in P. putida. Co-production of extracellular xylose-born xylonate and intracellular cellobiose-born medium-chain-length polyhydroxyalkanoates was established in proof-of-concept experiments with P. putida grown on the disaccharide. This study highlights the potential of P. putida EM42 as a microbial platform for the production of xylonate, identifies cellobiose as a new substrate for mcl-PHA production, and proposes a fresh strategy for the simultaneous valorization of xylose and cellobiose.

Introduction

Up to 220 million tonnes of lignocellulosic and cellulosic waste are potentially available for biotechnological purposes only in the EU every year (Searles and Malins, 2013). Lignocellulose can be decomposed by physical or chemical pre-treatment to cellulose, hemicellulose and lignin, and these fractions can be further hydrolysed enzymatically to monomeric sugars and lignin-derived aromatics serving as cheap substrates for microbial fermentations and biosynthesis of value-added chemicals (VAC; Mosier et al., 2005; Kawaguchi et al., 2016). Economics of these bioprocesses is regrettably still often unsatisfactory but can be significantly improved by parallel valorization of two or more lignocellulosic substrates. This is allowed by co-streaming of carbon from several sources into a single-valued compound or by simultaneous production of two or more VAC (Dumon et al., 2012; Li et al., 2017; Larroude et al., 2018; Baral et al., 2019; Wang et al., 2019). Co-production of extracellular and intracellular biochemicals is desirable for facilitated downstream processing (Wang et al., 2019). However, studies reporting such parallel biomanufacturing of two...
VAC from the second-generation carbon sources are infrequent and well-defined cell factories that could efficiently perform these tasks are scarce.

The soil bacterium and growingly used robust platform strain *P. putida* KT2440 can naturally assimilate a spectrum of aromatic compounds and organic acids but only a few plant biomass-derived carbohydrates: glucose, mannose and fructose (Linger et al., 2014; Belda et al., 2016; Nikel and de Lorenzo, 2018; Jayakody et al., 2018). Its metabolism was engineered to reach out to other sugars, including carbohydrates typically produced upon (hemi)cellulose hydrolysis or pyrolysis (Meijnen 2018). Its metabolism was engineered to reach out to a cheap, non-food-derived alternative for D-gluconic acid or chelator, as a precursor of polyesters, 1,2,4-butanolate was reported to be used as a complexing agent of *P. putida* ethanol, glycerol or some sugars including glucose (Prieto et al., 2009). Synthesis of mcl-PHA was demonstrated from *Cupriavidus necator* (Poblete-Castro et al., 2012; Loeschcke and Thies, 2015). The mcl-PHA have better elastomeric properties and broader application potential than short-chain-length PHA produced by *Cupriavidus necator* or recombinant *E. coli* (Chen, 2009). Synthesis of mcl-PHA was demonstrated from fatty acids and unrelated substrates such as acetate, ethanol, glycerol or some sugars including glucose (Prieto et al., 2016) but never from cellobextrins such as cellobiose. In a previous study, we also identified the ability of *P. putida* EM42 to oxidize D-xylose to D-xylonate, a platform molecule of considerable biotechnological interest (Werpy and Petersen, 2004; Toivari et al., 2012a,b; Mehtó et al., 2016; Dvorák and de Lorenzo, 2018). D-xylonate was reported to be used as a complexing agent or chelator, as a precursor of polyesters, 1,2,4-butanetniol, ethylene glycol or glycolate, and it can serve as a cheap, non-food-derived alternative for D-gluconic acid (Toivari et al., 2012a,b). Xylonate is naturally formed in the first step of oxidative metabolism of xylose by some archaea, bacteria and fungi via the action of D-xylose or D-glucose dehydrogenases. Production of xylonate was reported for instance in *Glucobacter oxydans*, in several *Pseudomonas* strains including *P. fragi*, *P. taiwanensis* or *P. putida* S12, or in *Klebsiella pneumoniae* (Buchert et al., 1988; Meijnen et al., 2008; Köhler et al., 2015; Wang et al., 2016). Several other microorganisms including *Escherichia coli* or *Saccharomyces cerevisiae* were engineered for xylonate production from xylose (Nygård et al., 2011; Liu et al., 2012; Toivari et al., 2012a,b; Gao et al., 2019). High production costs nonetheless hinder commercialization of both xylonate and mcl-PHA, and new solutions are appealing for easing the biomanufacture of these chemicals (Chen, 2009; Toivari et al., 2012a,b; Mehtó et al., 2016; Li et al., 2017). Their co-production from the second-generation carbon sources can thus be a promising approach in this context.

We present below our efforts to merge the advantages of *P. putida* EM42 as a natural xylionate producer with the ability of an engineered variant to grow on cellulose-derived substrate. Our results confirm that *P. putida* EM42 can convert xylose to xylionate with a high yield with its periplasmic glucose oxidative pathway and release the acid in the medium (Fig. 1). Furthermore, we show that xylionate production is inhibited in the presence of glucose but does occur in the cellobiose-grown recombinant strain. Most importantly, we demonstrate that periplasmic production and release of xylionate by cellobiose-grown *P. putida* EM42 are accompanied by parallel accumulation of mcl-PHA in the cells.

**Results and discussion**

**Biotransformation of xylose to xylionate by *P. putida* EM42 resting cells**

Periplasmic xylionate conversion to xylionate was previously identified as a competing reaction for xylose assimilation by recombinant *P. putida* EM42 during a five-day cultivation experiment (Dvorák and de Lorenzo, 2018). Periplasmic glucose dehydrogenase was shown to be a crucial component for xylose oxidation in our strain as well as in several xylionate-producing bacteria including *Klebsiella pneumoniae* and some other pseudomonads (Hardy et al., 1993; Meijnen et al., 2008; Köhler et al., 2015; Wang et al., 2016; Dvorák and de Lorenzo, 2018; Bator et al., 2020). In *P. putida* KT2440, and correspondingly also in strain EM42, membrane-bound glucose dehydrogenase Gcd (PP1444) oxidizes xylose to xylonolactone with pyrroloquinoline quinone (PQQ) as a cofactor. Lactone can then open spontaneously in the presence of water or might be converted to xylionate with the help of gluconolactonase Gnl (PP1170; Fig. 1). Neither xylose nor xylionate is utilized for biomass formation (Dvorák and de Lorenzo, 2018; Bator et al., 2020).

Here, we initially tested whether xylose can be oxidized to xylionate in a short time interval and with a high...
yield by *P. putida* resting cells of defined optical density. Xylonolactone concentrations were newly determined in culture supernatants using the hydroxamate method (Lien, 1959), which allowed more precise quantification of xylonate than in our previous work (Dvořák and de Lorenzo, 2018). *P. putida* EM42 cells (strains and plasmids used in this study are listed in Table S1), pre-cultured in lysogeny broth (LB), washed and diluted to a starting OD$_{600}$ ~ 0.5, were incubated for 48 h in M9 minimal medium with 5 g l$^{-1}$ xylose (Fig. 2A). The yield of extracellular xylonate detected in the medium at the end of the incubation was 0.95 g per g of xylose which was 86% of the theoretical maximum 1.11 g g$^{-1}$ (molar mass of D-xylose and D-xylonate is 150.13 and 166.13 g mol$^{-1}$, respectively). Lactone accumulated in small quantities (up to 0.45 g l$^{-1}$) in the medium during the initial phase of fast xylose conversion, but its concentration then declined to zero at the end of the experiment. The release of sugar acid was accompanied by a pH drop in the medium from the initial 7.00 $\pm$ 0.00 to 6.15 $\pm$ 0.04 at the end of the reaction. Neither lactone nor xylactone was detected in the identical experiment repeated with *P. putida* EM42 Δgcd mutant lacking glucose dehydrogenase (Fig. 2B). These experiments confirmed the importance of Gcd for D-xylose oxidation to xylonate in *P. putida* EM42 and showed that xylonolactone intermediate is converted rapidly to xylonate which is released into the medium rather than utilized by the cells. In contrast, a study with *P. fragi* (the best-described pseudomonad in terms of xylonate production thus far) reported slow spontaneous hydrolysis and accumulation of inhibitory xylonolactone in this bacterium during the early phases of fermentation experiments (Buchert *et al.*, 1986; Buchert and Viikari, 1988). Another well-characterized xylose-oxidizing pseudomonad, *P. taiwanensis* VLB120, uses xylonate for biomass formation (Köhler *et al.*, 2015). *P. putida* thus represents an attractive addition to these strains for fast high-yield production of extracellular xylonate.

It is worth noting that the resting *P. putida* cells could be recycled and used repeatedly in five cycles of xylose (5 g l$^{-1}$) oxidation to xylonate (Fig. S1). The conversion reached 94% in the first cycle, then decreased and reached 60% in the last fifth cycle. As the optical density of the cells measured at the end of each cycle continuously decreased, the decline in productivity can be attributed mainly to the loss of the biomass in the reactions due to the centrifugation/re-suspension cycles and cell lysis (Fig. S1). Medium pH drop detected at the end of each cycle corresponded with the level of xylose-to-xylonate conversion (Fig. S1). This result indicates that the xylitol oxidation in *P. putida* EM42 is not necessarily growth-dependent as reported with *P. fragi* (Buchert *et al.*, 1986; Buchert and Viikari, 1988). It is noteworthy that a number of studies on microbial xylonate production have reported the association of xylitol oxidation to
a host's growth (Toivari et al., 2012a,b; Köhler et al., 2015; Wang et al., 2016) but some have not. One example of the latter is recent work by Zhou et al. (2017) on *G. oxydans*, which could be used repeatedly for xylonate production in a bioreactor with an improved oxygen delivery system. Such cell recycling can be a promising strategy offering high xylonate yield and reduced process costs.

Since oxygen availability may become a bottleneck for the xylose-to-xylonate conversion, we next examined the effect of improved aeration through increased agitation of *P. putida* resting cells. In Zhou et al. (2017), the increase in agitation speed from 300 to 500 rpm enhanced the accumulation of xylonate by 25%. To check whether we could observe the same trend, we incubated resting cells in minimal medium with 5 g l\(^{-1}\) xylose at agitation of 170 or 300 r.p.m. and the level of xylose conversion to xylonate was determined after 48 h (Fig. S2A). Xylose oxidation to lactone and xylonate was 8% more efficient in flasks agitated at higher speed, but the increase was only marginal.

Another variable tested was pH. Xylonate accumulation results in acidification of the medium, and low pH can inhibit the activity of glucose dehydrogenase, as shown previously for *P. fragi* (Buchert et al., 1986). To inspect the effect of pH, we increased the buffering capacity of the M9 medium by mixing it with 100 mM sodium phosphate buffer while escalating xylose concentration to 10 g l\(^{-1}\) to intensify acidification. In these conditions, EM42 cells gave rise to \(\sim 12\%\) more oxidized product after 48 h than cells in non-buffered cultures (Fig. S2B). The final pH values determined in buffered and non-buffered cultures (5.92 ± 0.03 and 4.53 ± 0.12, respectively) proved that the sodium phosphate buffer of used 100 mM concentration could efficiently prevent excessive pH drop. The joint effect of modified reaction conditions is shown in Figure 2C and D which depict time courses of xylose conversion to xylonate by *P. putida* EM42 resting cells incubated in non-buffered minimal medium in flasks shaken at 170 r.p.m. (Fig. 2C) and in buffered medium in flasks shaken at 300 r.p.m. (Fig. 2D). In the latter case, the xylonate yield reached 0.85 ± 0.06 g g\(^{-1}\) xylose...
Xylose biotransformation to xylolactone by P. putida EM42 growing on glucose or cellubiose

In none of the experiments mentioned above, xylose oxidation to xylolactone was tested during growth. Instead, the production of whole-cell catalyst biomass. Similarly to other transformation experiments were preceded by the production of xylose-to-xylonate was tested during growth. Instead, the xylonolactone and faster xylose-to-xylonate conversion especially during the last 24 h of the reaction in which a pronounced pH drop was prevented (the final pH values in buffered and non-buffered cultures were 4.45 ± 0.01 and 5.99 ± 0.08, respectively). These observations on culture conditions were considered for increasing the efficiency of xylose conversion to xylolactone also in subsequent experiments with growing P. putida cells.

Table 1. Parameters determined in the cultures with Pseudomonas putida EM42 resting cells and in the cultures with P. putida EM42 or P. putida EM42 pSEVA2213_bglC grown on d-glucose or d-cellubiose, respectively, and transforming d-xylose to d-xylonate.

| P. putida strain and culture conditions | \( \mu \) (h\(^{-1}\)) | Xylose yield (g l\(^{-1}\)) | Xylophilic productivity (mg l\(^{-1}\) h\(^{-1}\)) | CDW (g) | pH |
|----------------------------------------|----------------|-----------------|---------------------|-------|-----|
| EM42 resting cells opt\(^a\)           | n.a.           | 0.56 ± 0.06/0.85 ± 0.06 | 217 ± 15/164 ± 11   | 0.15 ± 0.01/0.16 ± 0.01 | 6.30 ± 0.01/5.99 ± 0.08 |
| EM42 glucose                           | 0.58 ± 0.02     | 0.18 ± 0.02/0.17 ± 0.03 | 69 ± 6/32 ± 6       | 1.73 ± 0.12/1.60 ± 0.06 | 6.26 ± 0.01/5.81 ± 0.03 |
| bglC EM42 cellubiose                   | 0.27 ± 0.03     | 0.30 ± 0.06/0.48 ± 0.09 | 114 ± 18/93 ± 13    | 1.46 ± 0.13/1.88 ± 0.05 | 6.16 ± 0.06/5.19 ± 0.03 |
| bglC EM42 cellubiose optA\(^b\)        | 0.28 ± 0.05     | 0.34 ± 0.05/0.54 ± 0.10 | 138 ± 21/107 ± 17   | 2.06 ± 0.07/1.85 ± 0.03 | 6.23 ± 0.07/5.88 ± 0.05 |
| bglC EM42 cellubiose optB\(^c\)        | 0.30 ± 0.02     | 0.35 ± 0.02/0.50 ± 0.01 | 144 ± 8/102 ± 3     | 2.41 ± 0.11/2.18 ± 0.07 | 6.27 ± 0.08/6.00 ± 0.04 |
| bglC EM42 cellubiose PHA\(^d\)         | 0.24 ± 0.01     | 0.41 ± 0.09/0.52 ± 0.08 | 156 ± 32/99 ± 13    | 0.86 ± 0.02/1.24 ± 0.14 | 6.24 ± 0.02/5.90 ± 0.01 |

Values represent the mean ± standard deviation of three biological replicates. Parameters (except for \( \mu \)) were determined after 24 h/48 h of the culture. CDW, cell dry weight; n.a., not applicable.

a. Resting cells, pre-cultured in LB medium, were incubated in flasks with M9 minimal medium buffered with 100 mM sodium phosphate buffer and shaken at 300 r.p.m.

b. Cultures, inoculated from pre-cultures grown in LB medium, were carried out in flasks with M9 minimal medium buffered with 100 mM sodium phosphate buffer and shaken at 300 r.p.m.

c. Cultures, inoculated from pre-cultures grown in M9 medium with d-cellubiose, were carried out in flasks with M9 minimal medium buffered with 100 mM sodium phosphate buffer and shaken at 300 r.p.m.

d. Cultures were carried out in flasks with M9 minimal medium with reduced content of nitrogen, buffered with 100 mM sodium phosphate buffer and shaken at 300 r.p.m.

e. The specific growth rate \( (\mu) \) was determined during exponential growth.

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the cocktail (Chen, 2015). Well-defined microbial hosts capable of efficient cellobiose utilization are therefore desirable because they can be applied in simultaneous saccharification and fermentation of cellulose for production of VAC while the process cost is reduced as addition of expensive β-glucosidase is not needed (Ha et al., 2011; Chen, 2015; Parisutham et al., 2017).

Previous work revealed that a recombinant P. putida EM42 derivative which expressed β-glucosidase gene bglC from T. fusca grew rapidly on D-cellobiose as a sole carbon source (Fig. 1; Dvorák and de Lorenzo, 2018). In this case, cellobiose enters P. putida cells through the glucose ABC transporter and it is then cleaved by BglC to two glucose molecules which are further processed in the cytoplasm. The peripheral glucose oxidative pathway probably does not play a role in cellobiose uptake. Hence, it was presumed that cellobiose could be used instead of glucose as a growth substrate for P. putida while xylose would be oxidized by non-occupied Gcd (Fig. 1). To test this hypothesis, we cultured P. putida EM42 pSEVA2213_bglC in minimal medium with 5 g l⁻¹ cellobiose and 10 g l⁻¹ xylose. Cellobiose was consumed within the initial 24 h of the culture under conditions described in the legend of Figure 3. No glucose was detected in the medium. During the same time interval, 2.75 ± 0.42 g l⁻¹ xylonate was produced from xylose with average volumetric productivity 114 mg l⁻¹ h⁻¹ which was 65% higher than in the culture on glucose (Table 1). Xylose oxidation was fastest during the initial 32 h of the exponential growth phase and then slowed down in the stationary phase. Xylonate yield at the end of the two-day experiment was 0.48 ± 0.09 g g⁻¹ xylose. Minor quantities of xylono-lactone were detected in supernatant during the whole

Fig. 3. Biotransformation of D-xylose to D-xylonate by P. putida EM42 growing on D-glucose or D-cellobiose. Two-day cultures of (A) Pseudomonas putida EM42 in minimal medium with 10 g l⁻¹ D-xylose and 5 g l⁻¹ D-glucose used as a sole carbon source for growth. (B,C,D) Cultures of Pseudomonas putida EM42 pSEVA2213_bglC in minimal medium with 10 g l⁻¹ D-xylose and 5 g l⁻¹ D-cellobiose used as a sole carbon source. Experiments (A) and (B) were carried out in 25 ml of minimal medium in flasks shaken at 170 r.p.m. and 30°C. Minimal medium was inoculated to the initial A600 of 0.1 using cells obtained from an overnight culture in lysogeny broth. Experiments (C) and (D) were performed in flask with 25 ml of minimal medium buffered with 100 mM sodium phosphate buffer and shaken at 300 r.p.m. (30°C). Cells used for inoculation of the main culture to the initial A600 of 0.1 were pre-grown overnight in lysogeny broth (C) or in minimal medium with 5 g l⁻¹ D-cellobiose (D). D-xylose, filled squares (■); D-xylonate, filled circles (●); D-xylono-lactone, filled triangles (▲); D-glucose, filled diamonds (♦); D-cellobiose, open circles (○); cell biomass, open diamonds (◊). Data points shown as mean ± SD of three biological replicates. Please note that the elevated xylonate concentrations detected after 4 and 8 h in the culture (A) do not reflect the real levels of the xylose oxidation product. Hydroxamate method (Lien, 1959) used here for xylonate quantification was originally designed for the detection of gluconate and its lactone, which temporally accumulated in the culture medium during glucose utilization in (A). Accumulation of gluconate at the times 4 and 8 h was verified also by the specific D-Gluconic Acid/D-Glucono-δ-lactone Assay Kit (Megazyme, data not shown).
The xylonate productivity after initial 24 h of the exponential growth further increased 1.21-fold (to 138 mg \(\text{L}^{-1}\) h\(^{-1}\)) when the \(bglC^+\) \textit{P. putida} EM42 strain was cultured in the modified conditions used previously with resting cells (100 mM sodium phosphate buffer and 300 r.p.m.; Fig. 3C and Table 1). Then, the cells entered the stationary growth phase and xylonate production during the additional 24 h of culture was comparable with the former experiment with cells grown in standard M9 medium at 170 r.p.m. Pre-growing the cells in M9 minimal medium with 5 g \(\text{L}^{-1}\) cellobiose reduced lag phase of the main culture and improved biomass yield but did not help with increasing the xylonate yield and productivity (Fig. 3D, Table 1). We argue that suboptimal oxygen supply in shake flasks might be the limiting factor preventing efficient xyllose oxidation by dense culture in the stationary period. In any case, these experiments indicate that cellobiose, an abundant cellulosic carbohydrate, does not inhibit xyllose oxidation to xylonate in \textit{P. putida} and can thus be used as a growth substrate for cells performing this biotransformation.

**Co-production of xylonate and PHA by \textit{P. putida} EM42 grown on cellobiose**

The ability of \textit{P. putida} to both metabolize cellobiose in the cytoplasm and oxidize xyllose by the periplasmic pathway paved the way for parallel co-production of the two biotechnologically relevant compounds – xylonate and mcl-PHA. The mcl-PHA have been reported to be co-produced with alginate oligosaccharides from glucose or glycerol (Guo et al., 2011; Licciardello et al., 2017) or with rhamnolipids from fatty acids (Hori et al., 2011). Also, \(\alpha\)-xylonate was generated simultaneously with xylitol or bioethanol from xyllose and glucose (Wiebe et al., 2015; Zhu et al., 2019). However, the synthesis of mcl-PHA along with the release of xylonate has not yet been reported. To this end, we first examined the formation of PHA granules in cellobiose-grown \textit{P. putida} cells. As shown in Figure S4, flow cytometry and confocal microscopy identified PHA in the bacteria (Experimental procedures and Results and discussion in Supporting information).

This simple test indicated that \textit{P. putida} EM42 \(bglC^+\) metabolized cellobiose to the monomeric glucose, then to acetyl-CoA and next channelled this metabolic intermediate towards the formation of the polymer. In order to verify that PHA could be generated along with xylonate production, the \(bglC^+\) strain was pre-cultured in nitrogen-rich LB medium (to avoid any PHA accumulation) and then grown in nitrogen-limited M9 medium with 100 mM sodium phosphate buffer, 5 g \(\text{L}^{-1}\) cellobiose and 10 g \(\text{L}^{-1}\) xylose (Fig. 4). Sugar and xylonate concentrations were determined in culture supernatants while intracellular PHA formation was followed by flow cytometry and confocal microscopy. As shown in Figure 4A–C, xylonate and PHA were produced simultaneously during the initial 48 h of the three-day experiment. Cellular polymer content increased during the first two days and then declined towards the end of the experiment (Fig. 4B and C). This trend correlated with the presence of the carbon source (cellobiose and glucose) in the medium (Fig. 4A).

As in previous experiments, cellobiose was almost completely consumed within the initial 24 h. However, uptake of the disaccharide was this time accompanied by the appearance of glucose in the medium, which reached its maximum concentration (1.61 ± 0.37 g \(\text{L}^{-1}\)) at 12 h of the culture. Minute quantities of extracellular gluconate were detected as well (≤ 0.1 g \(\text{L}^{-1}\), data not shown). Under these circumstances, it became apparent that the secreted glucose affected xyllose oxidation by Gcd; only ~ 25% of the pentose was converted to xylonate at the end of the experiment. Although we do not have a trivial explanation for such unexpected release of glucose, we speculate that it could be due to [i] slower growth (\(\mu = 0.19 \pm 0.01 \text{ h}^{-1}\)) under nitrogen limitation as compared to the standard M9 medium (\(\mu = 0.30 \pm 0.02 \text{ h}^{-1}\); Fig. 3C) and/or [ii] an imbalance between the knocked-in \(\beta\)-glucosidase and the innate Glk glucokinase (PP1011) activities stemming from the difference in composition of pre-culture (LB) and culture (M9 with cellobiose) medium (see a scrutiny of these possibilities in supplementary Results and discussion, Experimental procedures and Fig. S5).

To overcome this bottleneck, \textit{P. putida} EM42 \(bglC^+\) cells were pre-grown overnight in standard M9 medium with 5 g \(\text{L}^{-1}\) cellobiose instead of LB. Faster growth of the main cultures (\(\mu = 0.24 \pm 0.01 \text{ h}^{-1}\)) in the nitrogen-limited M9 medium with cellobiose and xyllose was then indeed observed, and only minute concentrations of glucose (up to 0.12 g \(\text{L}^{-1}\)) were detected in the supernatants during the first 24 h (Fig. 4D). As a consequence, the volumetric productivity of xylonate during this period increased 3.5-fold (from 44 ± 18 mg \(\text{L}^{-1} \text{h}^{-1}\) to 156 ± 32 mg \(\text{L}^{-1} \text{h}^{-1}\)) when compared with the previous experiment shown in Fig. 4A. Xylonate yield was 2.4 times higher and reached 0.52 ± 0.08 g \(\text{g}^{-1}\) xyllose after 48 h of the culture (Table 1). Interestingly, the xylonate yield per gram of cell dry weight was 1.7-fold higher compared to the cells growing faster and reaching higher OD\text{600} in M9 medium with standard nitrogen content (Fig. 3C, Table 1).

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The same cultures were stopped after 48 h to quantify also PHA content within the cells which turned out to be 21 % (w/w) of cell dry weight. The biopolymer yield was 0.05 g/g C60.1 g of cellobiose. These values are close to those reported for P. putida KT2440 grown on glucose (Huijberts et al., 1992; Poblete-Castro et al., 2013). The PHA titre in the shake flasks reached 0.26 ± 0.03 g l⁻¹. The monomer composition of the analysed biopolymer was also consistent with the previous reports on mcl-PHA production from glucose (Fig. 4E). The major fraction (> 75%) was formed by 3-hydroxydecanoate, followed by 3-hydroxyoctanoate, 3-hydroxydodecanoate and small amount of 3-hydroxyhexanoate. Taken together, the above experiments confirmed the co-production of two value-added molecules (xylonate and mcl-PHA) out of xylose and cellobiose in P. putida.

**Conclusion**

In this work, we have exploited the metabolic versatility of P. putida EM42, a robust derivative of P. putida KT2440, for prototyping the simultaneous conversion of xylose and cellobiose into xylonate and mcl-PHA. Periplasmic oxidation of D-xylose to D-xylonate was first assayed with recyclable P. putida EM42 resting cells. Rapid transformation of pentose into free xylonate with only minor accumulation of xylonolactone intermediate was observed. Such extracytoplasmic production and secretion are advantageous over intracellular xylose oxidation: cytoplasm acidification is avoided, the reaction of interest does not cross-interfere with the host’s metabolism, and xylonate can be purified directly from the culture medium (Wang et al., 2016).
We then demonstrated that xylose conversion to xylonate can be efficiently catalysed also by recombinant *P. putida EM42 bglC* growing on d-cellobiose. In contrast to monomeric glucose, which is a preferred substrate for glucose dehydrogenase in *P. putida*, the disaccharide did not compete with xylose for Gcd and was a better carbon source for growth-associated xylonate production. Importantly, cellobiose-grown *P. putida* was able to stream the carbon from disaccharide into the intracellular mcl-PHA and concomitantly oxidize xylose to xylonate. Resting cell system can be a preferable option for shake flask set-up if xylonate is the only product of interest because it offers higher productivity and yields than growing cells (Table 1). However, the benefit of co-valorization of the two substrates can be exploited only with growing cells. Both xylonate and PHA yields could be further increased not only through bioprocess design but also by additional genetic interventions in the host that are known to improve the two bioproductions separately. This includes, e.g., overexpression of gcd and PQP biosynthesis genes which would deprive them of their natural regulation (An and Moe, 2016; Yu et al., 2018) and/or overexpression of pyruvate dehydrogenase subunit gene *acoA* (Borrero-de Acuña et al., 2014). These optimization efforts will be the subject of our further work. Bioprocesses based on microbial hosts capable of parallel production of two or more VAC from cheap abundant substrates are drawing considerable attention (Dumon et al., 2012; Li et al., 2017; Larroute et al., 2018; Baral et al., 2019; Wang et al., 2019). We argue that the study with recombinant *P. putida* EM42 expressing cytoplasmic β-glucosidase reported here represents a promising route for valorization of (hemi)cellulosic residues and an attractive alternative to the xylonate and mcl-PHA bioproductions reported thus far.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Experimental procedures

Results and discussion

Table S1. Strains and plasmids used in this study.

Fig. S1. Recycling of P. putida EM42 resting cells converting D-xylose to D-xylonate.

Fig. S2. Effect of agitation speed and medium pH on conversion of xylose to xylonate by P. putida EM42 resting cells.

Fig. S3. Two-day culture of P. putida EM42 in minimal medium with 10 g l⁻¹ D-xylose and 10 g l⁻¹ D-glucose used as a sole carbon source for growth.

Fig. S4. Polyhydroxyalkanoate (PHA) accumulation in P. putida EM42 cells grown on diverse carbon sources including D-cellobiose.

Fig. S5. Activities of β-glucosidase BglC and glucokinase Glk measured in cell-free extracts prepared from bglC mutation strain of P. putida EM42 grown in several different conditions.