Growth-(un)coupled biotransformation of D-xylose to D-xylonic acid by *Pseudomonas putida* EM42.

by

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Summary

The ability of genome-edited strain *Psudomonas putida* EM42 to valorize D-xylose - one of the major lignocellulosic carbohydrates - by converting this pentose into D-xylonic acid has been investigated. Genetic and biochemical experiments show that xylonate can be efficiently generated by promiscuous periplasmic glucose oxidation route (formed by the products of *gcd* and *gnl*) in recyclable *P. putida* resting cells. Alternatively, xylose oxidation can be coupled to growth of *P. putida* with cytoplasmic β-glucosidase BglC from *Thermobifida fusca* on D-cellobiose. Cellobiose turned out to be a better co-substrate for xylose-to-xylonate biotransformation than monomeric glucose. This is because unlike glucose, cellobiose does not block oxidation of the pentose by periplasmic glucose dehydrogenase Gcd. The resting cell strategy allowed higher xylonate yield from xylose (up to 0.97 g g^{-1}) than growth-coupled biotransformation. At the same time, oxidation of pentose by cellobiose-grown *P. putida* offered the benefit of simultaneous valorization of the two (hemi)cellulosic sugars with an optional production of disaccharide-born polyhydroxyalkanoates.

Introduction

Up to 220 million tonnes of lignocellulosic and cellulosic waste are available for biotechnological purposes only in 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). The soil bacterium and growingly used robust platform strain *P. putida* KT2440 can naturally assimilate a spectrum of aromatic compounds and organic acids but from carbohydrates only glucose and fructose (Linger *et al.*, 2014; Belda *et al.*, 2016; Nikel and de Lorenzo, 2018; Jayakody *et al.*, 2018). Its metabolism can be nonetheless engineered to reach out other sugars, including carbohydrates typically produced upon (hemi)cellulose hydrolysis or pyrolysis (Meijnen *et al.*, 2008; Linger *et al.*, 2016; Löwe *et al.*, 2018).

In our recent work, *P. putida* EM42, a *P. putida* KT2440-derived strain with streamlined genome and better physiological properties (Martínez-García *et al.*, 2014), was empowered with a xylose transporter and isomerase pathway from *Escherichia coli* along with a cytoplasmic β-glucosidase BglC from *Thermobifida fusca* (Dvořák and de Lorenzo, 2018). This allowed the resulting strain to co-utilise and grow on mixtures of D-glucose, D-cellobiose, and D-xylose. Alas, degradation does not mean valorisation and the mix of carbohydrates was metabolized and converted into CO_2 and biomass without any other return. Yet, the same study identified the ability of *P. putida* EM42 to oxidize D-xylose to D-xylonic acid (Dvořák and de Lorenzo, 2018). D-xylonate is a platform molecule of considerable biotechnological interest (Werpy and Petersen, 2004; Toivari *et al.*, 2012; Mehtiö *et al.*, 2016).

It was reported to be used as a complexing agent or chelator, as a precursor of polyesters, 1,2,4-butanetriol, ethylene glycol or glycolate, and it can serve as a cheap, non-food derived alternative for D-gluconic acid (Toivari *et al.*, 2012). 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 *Gluconobacter oxydans*, *Pseudomonas fragi*, or *Klebsiella pneumoniae* (Buchert *et al.*, 1988; 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; M. Toivari *et al.*, 2012). Certain limitations such as broad substrate specificity of periplasmic dehydrogenases in natural xylonate producers, low productivity of recombinant hosts due to the intracellular accumulation of the product, undesired assimilation of xylose and xylonate for biomass formation, or a high cost of co-substrates and nutrients (*e.g.*, peptone, yeast extract, ethanol, vitamins) and sensitivity of the hosts to hydrolysate inhibitors nonetheless prevent development of an efficient bioprocess and new solutions are sought (M. H. Toivari *et al.*, 2012; Mehtiö *et al.*, 2016).

In this context, we present below our efforts to merge the advantages of *P. putida* EM42 natural xylonate producer with the ability of an engineered variant to grow on the cheap cellulose-derived substrate cellobiose. Our results show that *P. putida* EM42 can convert xylose to xylonate with a high yield in a non-growing mode with its periplasmic glucose oxidative pathway (**Fig. 1**). Production of xylonate is inhibited when the cells are cultured on glucose but occurs in cellobiose-grown recombinant (**Fig. 1**). We also demonstrate that the biotransformation of xylose to xylonate in *P. putida* EM42 grown on cellobiose can be accompanied by parallel accumulation of PHA in the cells. This study reveals the potential of *P. putida* EM42 as a microbial platform for production of D-xylonic acid and proposes a fresh

strategy for simultaneous valorisation of xylose and cellobiose, two important components of hemicellulosic and cellulosic wastes.

Results and Discussion

Biotransformation of xylose to xylonate by P. putida EM42 resting cells

Periplasmic xylose conversion to xylonate was previously identified as a competing reaction for xylose assimilation by recombinant *P. putida* EM42 with the isomerase pathway from *E. coli* (Dvořák and de Lorenzo, 2018). Here, we initially tested whether xylose can be oxidized to xylonate by non-growing *P. putida* cells. Biotransformations with whole resting, but metabolically active, cells gain considerable interest from academia and industry because they allow achieving high product yields without accumulation of biomass. Moreover, non-growing microorganisms are less susceptible to inhibitory effects of toxic chemicals that occur in many biotechnologically relevant reactions, including fermentations of lignocellulosic hydrolysates (Buchert *et al.*, 1988; Julsing *et al.*, 2012; Dvorak *et al.*, 2015; Tiso *et al.*, 2015; Zhou *et al.*, 2017).

We incubated resting cells of *P. putida* EM42 (strains and plasmids used in this study are listed in Table S1), pre-cultured in lysogeny broth (LB) rich medium, for 48 h in M9 minimal medium with 5 g L^{-1} xylose (Fig. 2A). Under conditions defined in the figure legend and in Supplementary information, xylose was converted to xylonate which was detected in reaction supernatants. The yield at the end of the incubation was 0.95 g g^{-1} which was 85% of the theoretical maximum 1.12 g g^{-1}. Detected dehydrogenase activity measured with whole cells reached 2.87 ± 0.21 U per gram of dry biomass. Release of sugar acid was accompanied by pH drop in the medium from initial 7.00 ± 0.00 to 6.15 ± 0.04 at the end of the reaction. No growth was observed suggesting that *P. putida* EM42 utilises neither xylose nor xylonate for biomass

formation. This observation was consistent with our former study in which *E. coli* xylose transporter and isomerase pathway had to be introduced into *P. putida* EM42 in order to allow its growth on xylose (Dvořák and de Lorenzo, 2018).

Periplasmic glucose dehydrogenase was shown to be a crucial component for xylose oxidation in several xylonate producing bacteria including *Klebsiella pneumoniae* and some pseudomonads (Hardy *et al.*, 1993; Meijnen *et al.*, 2008; Wang *et al.*, 2016; Dvořák and de Lorenzo, 2018). The enzyme has a broad substrate specificity and can oxidise both hexoses and pentoses (Duine *et al.*, 1986). Glucose dehydrogenase oxidises xylose to xylonolactone with pyrroloquinoline quinol (PQQ) as a cofactor. Lactone then opens spontaneously in presence of water molecule or is converted to xylonate with the help of lactonase (Buchert and Viikari, 1988). In *P. putida* KT2440, and correspondingly also in strain EM42, membrane-bound glucose dehydrogenase Gcd (PP1444), gluconolactonase Gnl (PP1170), and gluconate dehydrogenase (PP3382-PP3384) form one of two major routes for glucose utilisation (del Castillo *et al.*, 2007). We employed *P. putida* EM42 Δ*gcd* mutant to test the effect of absent dehydrogenase in incubation of resting cells with xylose. Indeed, xylose was not converted to xylonate, neither xylonolactone was detected in the medium. (Fig. 2B). This intermediate was discerned in small quantities in the reaction with EM42 cells during the initial phase of fast xylose conversion (Fig. 2A). Lactone presence in the medium correlated with the rapid drop in optical density of the cells and appearance of flocks – cell aggregates – in flasks. These flocks might correspond to a biofilm formation which is a common *P. putida* stress response mechanism observed *e.g.*, in cells with overexpressed porins or other membrane proteins (Wagner *et al.*, 2007; Löwe *et al.*, 2018). As the initial optical density drop as well as the formation of flocks and their subsequent re-solubilisation were observed also during the experiment with Δ*gcd* mutant, we assume that the stress response might be attributed rather to the osmolarity change due to the transfer of the cells from rich LB medium to the minimal

medium than to the presence of small amount of inhibitory intermediate (Buchert and Viikari, 1988). To conclude, these experiments proved the importance of Gcd for D-xylose oxidation to xylonate in *P. putida* EM42 (Fig. 1) and showed that xylonolactone intermediate is converted rapidly to xylonic acid which is released into the medium rather than utilised by the cells. In contrast, a study with *P. fragi*, which is the best described pseudomonad in term 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). *P. putida* may thus represent an attractive alternative to *P. fragi* with higher lactone hydrolysing activity.

Xylose biotransformation to xylonate by reused P. putida EM42 resting cells, effects of shaking and pH

We next tested whether the resting *P. putida* cells can be recycled and used repeatedly for xylose oxidation to xylonic acid. Cells of the optical density (A_{600}) of 1.0 were incubated in M9 minimal medium with 5 g L^{-1} xylose for 24 h. After this time interval, cells were separated from the reaction mixture by centrifugation and re-suspended in fresh medium with xylose. This procedure was repeated four times and xylose conversion to xylonate (g g^{-1}) was determined for each of the five cycles (Fig. 3). The conversion reached 94 % in the first cycle, then decreased and reached 60 % in the last fifth cycle. As optical density of the cells measured at the end of the experiment was only about 0.3, the decrease can be attributed mainly to the loss of the biomass in the reactions due to the centrifugation/re-suspension cycles and cell lysis. Medium pH drop detected at the end of each cycle corresponded with the level of xylose-to-xylonate conversion (Fig. 3). Importantly, the data confirmed that the xylonic acid production from xylose in *P. putida* EM42 is not growth-dependent and the resting cells can be recycled. This finding is inconsistent with previous reports on *P. fragi* for which xylonate production was described as growth associated (Buchert *et al.*, 1986; Buchert and Viikari, 1988). Also a

vast majority of other studies on microbial xylonate production has worked with hosts growing on xylose, xylonic acid or a co-substrate (Toivari *et al.*, 2012; Wang *et al.*, 2016). Zhou and co-workers (2017) described recently a strategy comprising of fed-batch addition of xylose and recycling of *G. oxydans* cells in bioreactor with improved oxygen delivery system (Zhou *et al.*, 2017). As *G. oxydans*, similarly to *P. putida*, does not metabolize xylonic acid formed through the action of periplasmic PQQ-dependent dehydrogenase and no co-substrate was added, the authors concluded that all oxygen delivered to the system was used solely for xylose oxidation with water being the only by-product. The ease of PQQ recycling in presence of molecular oxygen O_2 and catalase, which neutralises resulting hydrogen peroxide, is known also from *in vitro* experiments (Mukai *et al.*, 2016). We thus assume, that recycling of PQQ in periplasm of *P. putida* resting cells is growth independent and is allowed by the oxygen dissolved in the medium.

We examined the effect of improved aeration through increased agitation on xylose-to-xylonate conversion by *P. putida* resting cells in a following shake flask experiment. In the aforementioned study of Zhou *et al.* (2017), increase in agitation speed in bioreactor from 300 to 500 rpm improved accumulation of xylonate by 25%. We incubated resting cells in minimal medium with 10 g L^{-1} xylose at agitation of 170 or 300 rpm and the level of xylose conversion to xylonate was determined after 48 h (Fig. 3B). Xylose oxidation to xylonate was about 10% more efficient in flasks agitated at higher speed. The increase was nonetheless not statistically significant ($P > 0.05$). Another variable that can affect production of xylonate in *P. putida* is pH. Xylonate accumulation results in acidification of the medium and low pH can inhibit activity of glucose dehydrogenase as shown previously for *P. fragi* (Buchert *et al.*, 1986). To verify the effect of pH, we increased buffering capacity of M9 medium by mixing it with 100 mM sodium phosphate buffer. EM42 cells ($A_{600} = 1.0$) in buffered medium were able to convert 10 g L^{-1} of xylose to 9.67 ± 0.39 g L^{-1} of xylonate during 48 h time interval while only 8.50 ±

0.43 g L^{-1} of xylonic acid were detected in non-buffered culture supernatants (Fig. 3C). The final pH determined in buffered and non-buffered cultures proved that the sodium phosphate buffer of used concentration could efficiently prevent excessive pH drop due to the formed xylonic acid. Taken together, we show that culture parameters such as agitation speed and pH have an effect on efficiency of xylose conversion to xylonate by resting *P. putida* cells. These parameters can be further altered to achieve higher product titres and productivities, which is, however, not the purpose of this study.

Xylose biotransformation to xylonate by P. putida EM42 growing on glucose or cellobiose

Xylose oxidation to xylonate was not accompanied by cellular growth in any of the aforementioned experiments. To obtain sufficient cell biomass for the bioconversions, *P. putida* cells were pre-grown in LB medium containing amino-acid rich yeast extract and tryptone. Media containing yeast extract, tryptone, peptone or combinations of these components have been used for biomass propagation of other xylonate producers, both natural ones such as *G. oxydans* or *K. pneumoniae* and recombinants of yeasts *S. cerevisiae* and *K. lactis* (Nygård *et al.*, 2011; M. Toivari *et al.*, 2012; Wang *et al.*, 2016; Zhou *et al.*, 2017). These substrates are nonetheless expensive, often poorly defined and thus unsuitable for large-scale bioprocesses. Hence, growth of xylonate producing microorganism in low cost medium with carbon source derived, in ideal scenario, directly from the lignocellulosic material is desirable. D-glucose is the most abundant monomeric sugar in lignocellulosic hydrolysates prepared by using commercial enzyme cocktails with endoglucanase, exoglucanase, and β-glucosidase (Taha *et al.*, 2016) and it is also a natural growth substrate of many microorganisms including *P. putida* (del Castillo *et al.*, 2007). However, glucose is typically a preferred substrate for glucose dehydrogenase and might thus inhibit xylose oxidation by this enzyme. Indeed, *P. putida* EM42 cultured in minimal medium with 10 g L^{-1} glucose did not oxidise xylose to

xylonate during the whole 72 h time interval when hexose was present in the medium (Fig. 4A).

We attempted to bypass this bottleneck by employing D-cellobiose as an alternative growth substrate for *P. putida*. D-cellobiose is a disaccharide composed of two β-glucose monomers linked by a β(1→4) bond. It is a by-product of cellulose saccharification with standard commercial mixtures of cellulases but becomes a predominant product when β-glucosidase is omitted from the cocktail (Chen, 2015). Well-defined microbial hosts capable of efficient cellobiose utilisation 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).

Our laboratory recently reported recombinant *P. putida* EM42 which grows rapidly on D-cellobiose ($\mu = 0.35\ h^{-1}$) used as a sole carbon source by virtue of β-glucosidase BglC from *T. fusca* expressed from low copy pSEVA2213 plasmid with EM7 constitutive promoter (Fig. 1) (Dvořák and de Lorenzo, 2018). We showed that cellobiose enters *P. putida* cells through the glucose ABC transporter and is then cleaved by BglC to two glucose molecules which are further processed in the cytoplasm. The putative role of peripheral glucose oxidative pathway in cellobiose uptake was also discussed but not confirmed. Hence, we presumed that cellobiose could be used instead of glucose as a growth substrate for *P. putida* while xylose would be oxidised 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 initial 24 h of the culture under conditions described in the legend of Figure 4. No glucose was detected in the medium. During the same time interval, 2.75 ± 0.42 g L^{-1} of xylonate were produced from xylose with average volumetric productivity of 114 mg L^{-1} h^{-1}. Xylose oxidation was fastest during initial 32 h of exponential growth phase and then

slowed down in stationary phase. Xylonate yield at the end of the three-day experiment was 0.59 ± 0.02 g g^{-1}. Minor quantities of lactone were detected in supernatant during the whole course of the culture (Fig. 4B). Xylonate production and propagation of cells were accompanied by acidification of the medium. The pH decreased from 7.00 ± 0.00 to 6.16 ± 0.06, 5.19 ± 0.03, and 4.55 ± 0.06 after 24, 48, and 72 h of culture, respectively.

The amount of xylonate formed after initial 24 h of exponential growth (3.46 ± 0.19 g L^{-1}) increased by 21 % when *P. putida* EM42 pSEVA2213_*bglC* was cultured in modified conditions tested previously with resting cells, *i.e.*, in minimal medium with 100 mM sodium phosphate buffer and at higher agitation of 300 rpm (Fig. S1). Then, the cells entered stationary growth phase and improvement of xylonate production during additional 24 h of culture was almost negligible when compared with former experiment encompassing cells grown in standard M9 medium at 170 rpm. The difference between the amount of xylonate produced after 48 h of culture in modified (4.91 ± 0.13 g L^{-1}) and non-modified (4.46 ± 0.62 g L^{-1}) conditions was only 9 % though buffering of the medium was more efficient in former case (pH decreased only to 6.00 ± 0.04). We thus argue that the low supply of oxygen, which is consumed by rapidly growing cells in the exponential culture phase, might be the remaining limiting factor preventing faster xylose oxidation in stationary period.

Importantly, our data show that cellobiose, potentially abundant cellulosic carbohydrate, does not inhibit xylose oxidation to xylonate in *P. putida* and can thus be used as a cheap growth substrate for cells performing this biotransformation.

Co-production of xylonate and PHA by P. putida EM42 grown on cellobiose

The verified ability of *P. putida* to utilise cellulosic substrate, cellobiose, with its cytoplasmic metabolic machinery and convert hemicellulosic xylose to desirable chemical with the periplasmic oxidative pathway opens up opportunities for parallel co-production of two

biotechnologically relevant compounds. Such co-fermentation strategy can facilitate industrial viability of a bioprocess (Dumon *et al.*, 2012; Larroude *et al.*, 2018). Possibilities to use carbon from cellobiose for VAC manufacturing in *P. putida* are, as in case of glucose, many (Poblete-Castro *et al.*, 2012; Loeschcke and Thies, 2015). *P. putida* KT2440 has been traditionally employed as a model organism for production of PHA, biodegradable polyesters with high application potential (Prieto *et al.*, 2016). Synthesis of biopolymer in *P. putida* was demonstrated from fatty acids and unrelated substrates such as acetate, ethanol, glycerol, or some sugars including glucose (Prieto *et al.*, 2016). Glucose gives rise to PHA under nitrogen limitation *via* acetyl-CoA and *de novo* fatty acid biosynthesis. Production of PHA in *P. putida* from cellodextrins such as cellobiose has not yet been reported.

We aimed at examining the capacity of *P. putida* EM42 pSEVA2213_*bglC* for intracellular accumulation of cellobiose-born PHA and parallel release of xylose-born xylonate into the medium. For that purpose, we first probed formation of PHA granules in cellobiose-grown *P. putida* cells. We identified PHA production from cellobiose in *P. putida* by flow cytometry and checked it visually with confocal microscopy (Experimental procedures, Results, and Fig. S2 in Supporting information). Once it was proven that recombinant *P. putida* EM42 metabolizes cellobiose, accordingly to the monomeric glucose, to acetyl-CoA and can utilize the shortest cellodextrin for formation of biopolymer, we tested also co-production of PHA with xylonate in single culture. EM42 pSEVA2213_*bglC* strain was grown in nitrogen limited minimal medium with 100 mM sodium phosphate buffer, 5 g L^{-1} cellobiose, and 10 g L^{-1} xylose. Sugar and xylonic acid concentrations were determined in culture supernatants while formation of PHA within the cells was followed by flow cytometry and confocal microscopy (Fig. 5). Sugar acid and biopolymer were simultaneously produced during initial 48 h of the three-day experiment. Nile Red fluorescence in the cells increased during the first two days and then decreased in the bacteria collected at the end of the culture (Figs. 5B and 5C). This

trend correlated with the presence of carbon source – cellobiose and glucose – in the culture medium (Fig. 5A). As in former experiments, cellobiose was consumed rapidly within 24 h of the culture. However, cellobiose uptake was this time accompanied by secretion of glucose in the medium. Glucose concentration reached its maximum of 1.61 ± 0.37 g L^{-1} at 12 h of the culture and then slowly decreased. The secreted glucose affected efficiency of xylose oxidation by Gcd, hence, only about 25 % of the pentose was converted to xylonate at the end of the experiment. We presume that glucose accumulation and secretion out of the cells might be caused by imbalance between heterologous BglC β-glucosidase and innate Glk glucokinase (PP1011) activities. While *bglC* gene is expressed continuously in EM42 recombinant from constitutive EM7 promoter also during pre-culture in LB medium (cells in co-production experiment were pre-cultured in lysogeny broth and not in M9 medium with cellobiose to avoid formation of PHA granules before start of the experiment), expression of *glk*, forming part of *edd*/*glk*/*gltR-II* operon, depends on native *P. putida* regulatory mechanisms and is induced later by presence of glucose metabolism intermediates (Udaondo *et al.*, 2018). Slower biomass production and protein biosynthesis under nitrogen limitation might result in lower amount of Glk in cells and secretion of unprocessed non-phosphorylated glucose. This bottleneck could be possibly removed by overexpressing *glk* or by reducing amount of BglC in cells. Despite the suboptimal xylonate yield, the presented foundational experiment points to the possible way for co-production of VAC from two major lignocellulosic sugars in *P. putida*.

Conclusion

In this work we have investigated the capacity of *P. putida* EM42, a robust derivative of *P. putida* KT2440, to transform D-xylose to desirable platform chemical, D-xylonic acid. Xylonate production was first assayed with *P. putida* resting cells. Periplasmic glucose oxidative pathway and Gcd glucose dehydrogenase were shown to play a major role in xylose

transformation to xylonate, which was secreted into the medium. When xylonate is a target chemical, such periplasmic production and secretion are advantageous over intracellular xylose oxidation as intermediates and the product do not cross-talk with host's metabolism and acid can be purified with high yield from the medium (Wang *et al.*, 2016). *P. putida* resting cells could convert xylose to xylonate even after five rounds of recycling with average yield of 0.81 g g^{-1} in the whole course of the shake flask experiment. Medium pH and agitation speed were two culture parameters shown to have effect on xylonate production with resting cells and could become the basis for designing a bioprocess.

In the second part of the study, we demonstrated that *P. putida* biomass for xylose biotransformation to xylonate can be efficiently generated from another lignocellulosic sugar, D-cellobiose. In contrast to monomeric glucose, which is a preferred substrate for glucose dehydrogenase in *P. putida* and in some other natural hosts that have been shown to employ this enzyme for xylose oxidation (Toivari *et al.*, 2012; Wang *et al.*, 2016; Dvořák and de Lorenzo, 2018), disaccharide does not compete with xylose for Gcd active site and can be used as a carbon source for growth-associated xylonate production. Bioprocesses based on microbial hosts capable of intracellular utilisation of cellodextrins from partial cellulose hydrolysis are recently drawing more attention (Ha *et al.*, 2011; Chen, 2015; Parisutham *et al.*, 2017; Dvořák and de Lorenzo, 2018). The strategy demonstrated here on example of recombinant *P. putida* EM42 with cytoplasmic β-glucosidase can thus represent a promising alternative to the xylonate bioproductions reported thus far (M. H. Toivari *et al.*, 2012; Mehtiö *et al.*, 2016). Xylonate yield and productivity reached with growing bacteria were lower when compared with resting cells used in similar experimental conditions. However, this situation might change when the cells are cultured in a bioreactor with efficient oxygen supply which would support PQQ recycling (Zhou *et al.*, 2017).

As identified in this study, the growth-associated xylonate production by *P. putida* could also benefit from parallel intracellular accumulation of valuable PHA formed from cellobiose. Such co-fermentation approach will nonetheless require tailoring of reaction conditions and medium composition and activity balancing of BglC β-glucosidase and innate glycolytic enzymes, namely glucokinase, in order to prevent secretion of accumulated glucose out of the cells.

Beyond the transformations described in this work, the wealth of genetic tools available for *P. putida* can facilitate introduction of downstream pathways for intracellular xylonate conversion to ethylene glycol, glycolic acid, 1,4-butanediol or other higher value chemicals (Valdehuesa *et al.*, 2018). This will be the subject of future endeavours in our laboratory.

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Conflict of interest

Authors declare that they have no conflict of interest.

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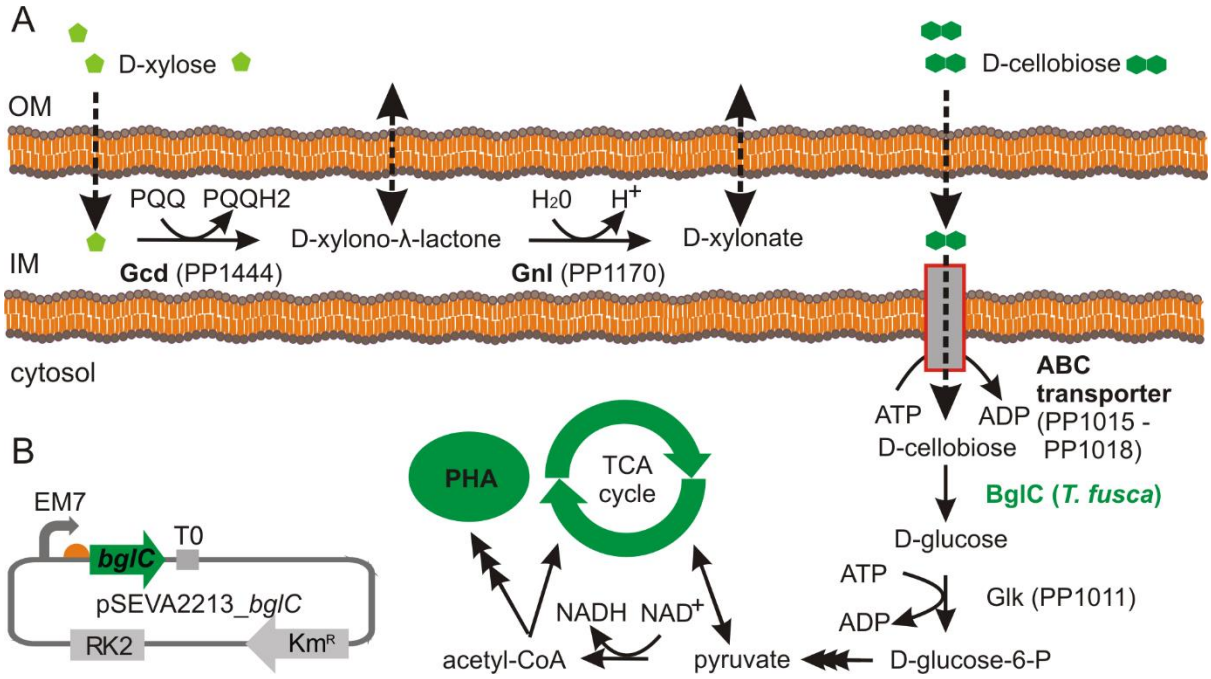
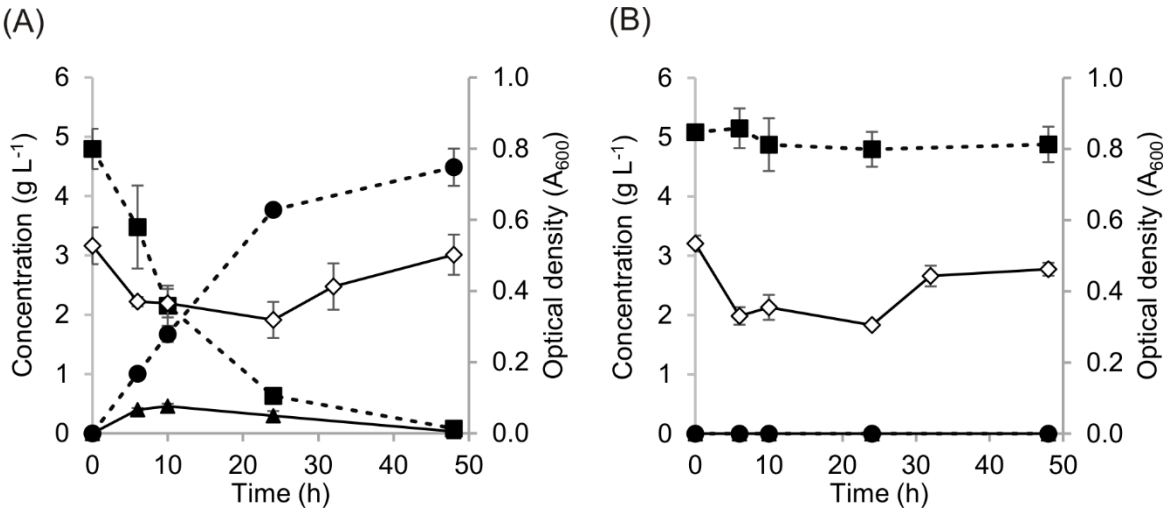
Figures

Figure 1. Metabolic fate of xylose and cellobiose in *bglC*+ *P. putida* EM42.



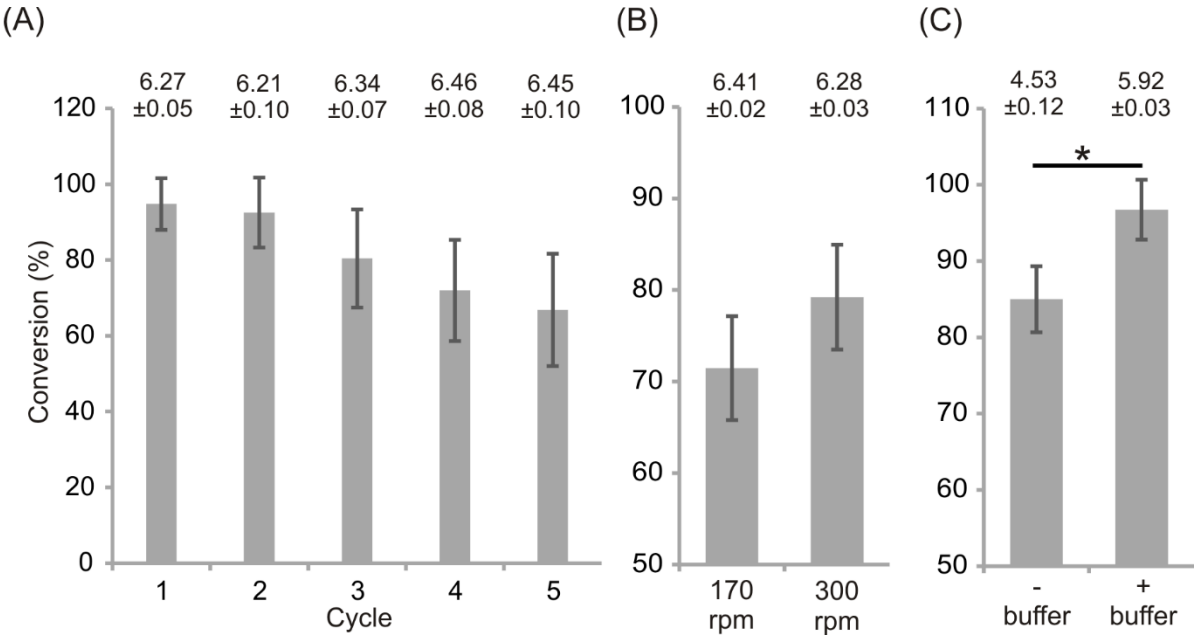
(A) Innate periplasmic route for D-xylose oxidation and introduced pathway for D-cellobiose utilisation in *Pseudomonas putida* EM42. (B) Graphical representation of low copy plasmid pSEVA2213 with constitutive promoter EM7 used for expression of *bglC* gene encoding β-glucosidase from *Thermobifida fusca*. Abbreviations: Gcd, glucose dehydrogenase; Gnl, gluconolactonase; Glk, glucokinase; PQQ and PQQH, pyrroloquinoline quinone and its reduced form, respectively; OM, outer membrane; IM, inner membrane; TCA cycle, tricarboxylic acid cycle; PHA, polyhydroxyalkanoates.

Figure 2. Role of *gcd* in oxidation of D-xylose to D-xylonate in *P. putida* EM42.



Incubation of resting cells of (A) *Pseudomonas putida* EM42 and (B) its deletion mutant *P. putida* EM42 Δ*gcd* in minimal medium with 5 g L^{-1} D-xylose. Experiments were carried out in 25 mL of minimal medium in flasks shaken at 170 rpm and 30 °C. Minimal medium was inoculated to the initial A_{600} of 0.5 using cells obtained from overnight culture in lysogeny broth. D-xylose, filled squares (■); D-xylonate, filled circles (●); D-xylono-λ-lactone, filled triangles (▲); cell biomass, open diamonds (◊). Data points shown as mean ± SD from three biological replicates.

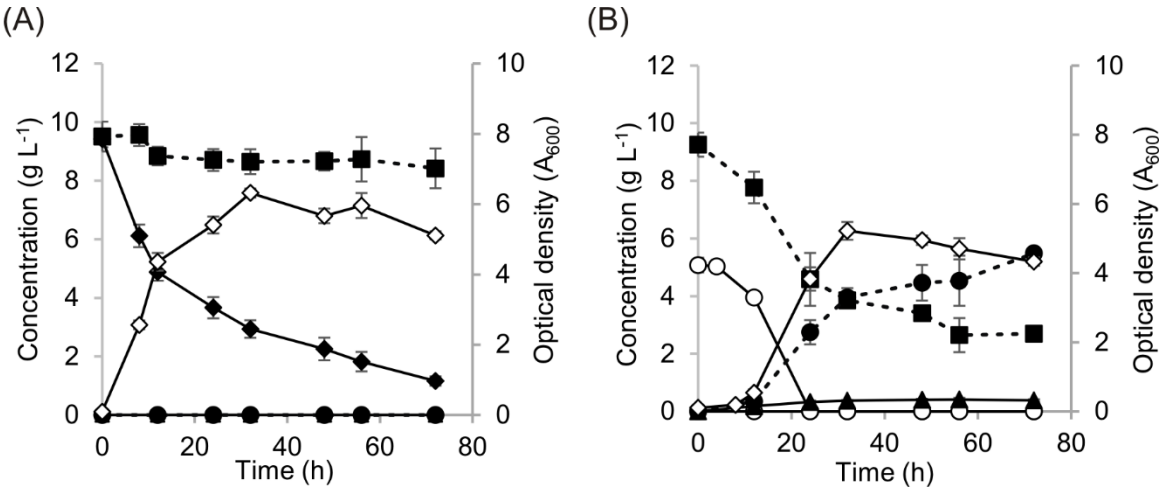
Figure 3. Growth-uncoupled biotransformation of D-xylose to D-xylonate by *P. putida* EM42 resting cells.



(A) Recycling of *Pseudomonas putida* EM42 whole-cell biocatalyst for conversion (g g^{-1}) of D-xylose to D-xylonic acid. Medium with 5 g L^{-1} D-xylose was inoculated to the initial A_{600} of 1.0 and cells were incubated at 170 rpm and 30 °C. After 24 h, cells were centrifuged (4000 RPM, 15 min. at room temperature), pellets were re-suspended in fresh medium with xylose and supernatants were used for further quantifications. (B) Effect of agitation speed on conversion of xylose to xylonic acid. Cells of A_{600} = 0.2 were incubated in medium with 5 g L^{-1} xylose with agitation of 170 or 300 rpm and the level of xylose conversion to xylonate was determined after 48 h. (C) Effect of pH on conversion of xylose to xylonic acid. Cells of A_{600} = 1.0 were incubated in medium with or without 100 mM sodium phosphate buffer, with 10 g L^{-1} xylose and at agitation of 170, the level of xylose conversion to xylonate was determined after 48 h. Numbers above the columns show pH of the medium measured at the end of each cycle or experiment. All experiments were carried out with resting cells collected from overnight cultures in LB medium, washed and re-suspended in shake flasks with 20 mL of M9 minimal medium. Columns represent means ± SD from at least four biological replicates from

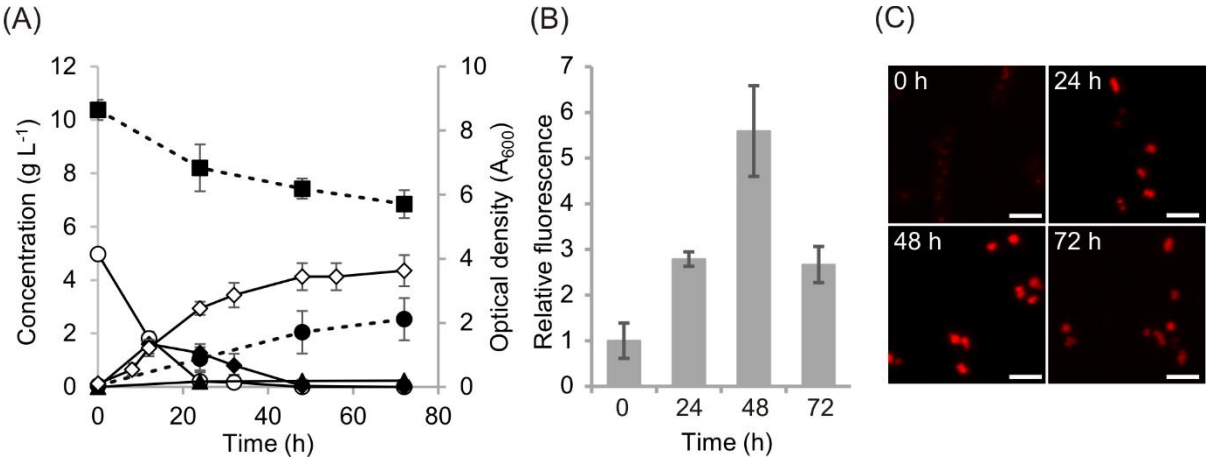
two independent experiments. Asterisk denotes significance in difference in between two means at $P < 0.01$ as evaluated by Student's *t* test.

Figure 4. Growth-coupled biotransformation of D-xylose to D-xylonate by *P. putida* EM42.



Three-day culture of (A) *Pseudomonas putida* EM42 and (B) *Pseudomonas putida* EM42 pSEVA2213_*bglC* in minimal medium with 10 g L^{-1} D-xylose and 10 g L^{-1} D-glucose or 5 g L^{-1} D-cellobiose, respectively, used as a sole carbon source for growth. Experiments were carried out in 25 mL of minimal medium in flasks shaken at 170 rpm and 30 °C. Minimal medium was inoculated to the initial A_{600} of 0.1 using cells obtained from overnight culture in lysogeny broth. D-xylose, filled squares (■); D-xylonate, filled circles (●); D-xylono-λ-lactone, filled triangles (▲); D-glucose, filled diamonds (♦); D-cellobiose, open circles (○); cell biomass, open diamonds (◊). Data points shown as mean ± SD from three biological replicates.

Figure 5. Co-production of D-xylonic acid and PHA from D-xylose and D-cellobiose, respectively, by *P. putida* EM42 pSEVA2213_*bglC*.



Cultures (A) were carried out in 25 mL of nitrogen limited M9 minimal medium with 100 mM sodium phosphate buffer, 5 g L^{-1} cellobiose, and 10 g L^{-1} xylose in flasks shaken at 300 rpm and 30 °C. Minimal medium was inoculated to the initial A_{600} of 0.1 using cells pre-cultured overnight in lysogeny broth. (B) Relative fluorescence of bacterial populations analysed by flow cytometry every 24 h during the three-day culture. Cells were stained by Nile Red and processed as described in Supplementary Information. (C) Confocal microscopy of *P. putida* EM42 pSEVA2213_*bglC* cells at denoted time intervals. Stained bacteria were processed as described in Supplementary Information. White scale bars show 2 μm distance. D-xylose, filled squares (■); D-xylonate, filled circles (●); D-xylono-λ-lactone, filled triangles (▲); D-glucose, filled diamonds (♦); D-cellobiose, open circles (○); cell biomass, open diamonds (◇). Data points and columns in (A) and (B), respectively, show mean ± SD from three biological replicates.