# Sustainable production of fine chemicals by the solvent-tolerant *Pseudomonas putida* S12 using lignocellulosic feedstock

**Jean-Paul Meijnen [1, 2, 3, A *], Johannes H. de Winde [1, 2, 3] and Harald J. Ruijssenaars [1, 2, 3, 4]**

[1] Department of Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands.
[2] B-Basic, Julianalaan 67, 2628 BC Delft, The Netherlands.
[3] Kluyver Centre for Genomics of Industrial Fermentation, P.O. Box 5057, 2600 GA Delft, The Netherlands.
[4] BIRD Engineering BV, Westfrankelandsedijk 1, 3115 HG Schiedam, The Netherlands.

[*] Contact author: Laboratory of Molecular Cell Biology, Institute of Botany and Microbiology, Katholieke Universiteit Leuven and Department of Molecular Microbiology, Flanders Institute of Biotechnology (VIB), Kasteelpark Arenberg 31, B-3001 Leuven-Heverlee, Flanders, Belgium.
Tel: +32 16 329 615 Fax: +32 16 321 979 Email: JeanPaul.Meijnen@mmbio.vib-kuleuven.be

## abstract

**The rising price of oil and impending deficit of fossil resources stimulate the development of alternative processes for the production of chemicals. The production of chemicals from lignocellulosic biomass is a promising alternative. Lignocellulosic biomass consists of a mixture of sugars that can be converted into valuable products or chemicals by means of bioconversion. It is essential that, in order to establish an economically sound process, the feedstock is utilized as close to completion as possible. However, due to its heterogeneous nature, lignocellulosic feedstock is often metabolized incompletely. This situation is also encountered during the production of aromatic compounds by engineered strains of the solvent-tolerant micro-organism *Pseudomonas putida* S12. This bacterial strain is not able to use all sugars from biomass, most notably the pentose fraction. Therefore, strategies were explored to engineer D-xylose metabolic pathways in *P. putida* S12, to enable the consumption of the most abundant pentose sugar present in lignocellulosic biomass, thereby lowering production costs of commodity chemicals.**

Keywords: **arabinose, bio-based chemicals, metabolic engineering, renewable feedstocks, sustainability, xylose**

### Producción sustentable de sustancias químicas finas mediante *Pseudomonas putida* S12 tolerante a solventes utilizando materia prima lignocelulósica

El precio en aumento del petróleo y el alarmante déficit de recursos fósiles estimulan el desarrollo de procesos alternativos para la producción de sustancias químicas. La producción de éstas a partir de biomasa lignocelulósica es una alternativa prometedora. La biomasa lignocelulósica consiste en una mezcla de azúcares que pueden convertirse en productos valiosos o en sustancias químicas mediante la bioconversión. Es esencial que, con el objeto de establecer un proceso económicamente viable, la materia prima sea utilizada en la forma más completa posible. No obstante, dado lo heterogéneo de su índole, la materia prima lignocelulósica es, frecuentemente, metabolizada incompletamente. Esta situación también se encuentra durante la producción de compuestos aromáticos mediante cepas de ingeniería del microorganismo *Pseudomonas putida* S12 tolerante a solventes. Esta cepa bacteriana no puede utilizar todos los azúcares de la biomasa, notablemente la fracción de pentosas. En consecuencia, se exploraron estrategias para introducir por ingeniería vías metabólicas de la d-xilosa en *Pseudomonas putida* S12 para permitir el consumo del azúcar más abundante en la biomasa lignocelulósica bajando, por lo tanto, los costos de fabricación de los productos químicos.

### A produção sustentável de produtos químicos de síntese pela *Pseudomonas putida* S12 tolerante a solventes utilizando matéria-prima lignocelulósica

O aumento do preço do petróleo e o déficit iminente dos recursos fósseis estimulam o desenvolvimento de processos alternativos para a produção de produtos químicos. A produção de produtos químicos a partir da biomassa lignocelulósica é uma alternativa promissora. A biomassa lignocelulósica é composta por uma mistura de açúcares que podem ser convertida em produtos de valor ou em produtos químicos por meio de bioconversão. É essencial que, a fim de estabelecer um processo economicamente viável, a matéria-prima utilizada esteja o mais perto do acabamento possível. No entanto, devido à sua natureza heterogênea, a matéria-prima lignocelulósica é freqüentemente metabolizada de forma incompleta. Esta situação também ocorre durante a produção de compostos aromáticos por linhagens de engenharia do micro-organismo *Pseudomonas putida* S12 tolerante a solventes. Esta linhagem de bactéria não é capaz de usar todos os açúcares da biomassa, sobretudo a fração das pentoses. Portanto, estratégias foram exploradas para criar rotas metabólicas de d-xilose na *P. putida* S12, para permitir o consumo do açúcar pentose mais abundante na biomassa lignocelulósica, diminuindo assim os custos de produção de produtos químicos de base.

## Introduction

Our world is addicted to oil. Since the Industrial Revolution in the 19th century, the demand for crude oil is ever rising, making the petroleum industry the world's largest industry in terms of monetary value. In the last 50 years alone, the driven-by-demand annual global oil production increased 4-fold to 4.2 trillion litres in 2009 (Fig. 1) (http://www.eia.doe.gov/emeu/aer/txt/ptb1105.html), and although the global economic crisis tempers the increase, still 75.7 million barrels of oil per day were produced in the 3rd quarter of 2010 (http://omrpublic.iea.org).

This increasing demand for oil, and in addition for natural gas and coal (collectively called fossil resources), comes with drawbacks. It is common knowledge that the supply of oil is

**Figure 1.** Plot of world oil production from 1960 to 2009

The graph was generated using data from the Energy Information Administration of the U.S. Department of Energy

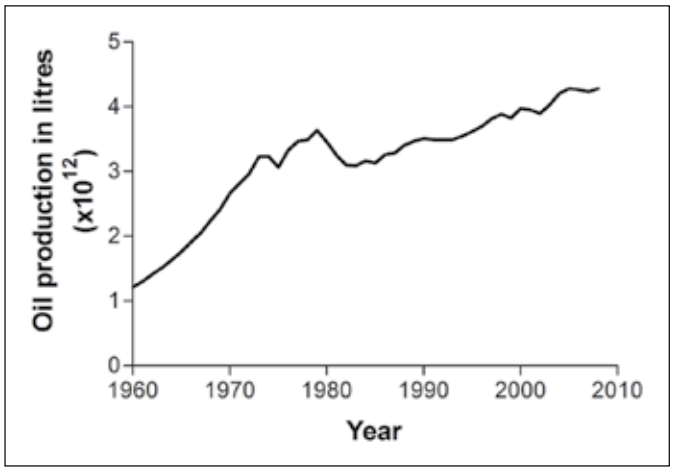
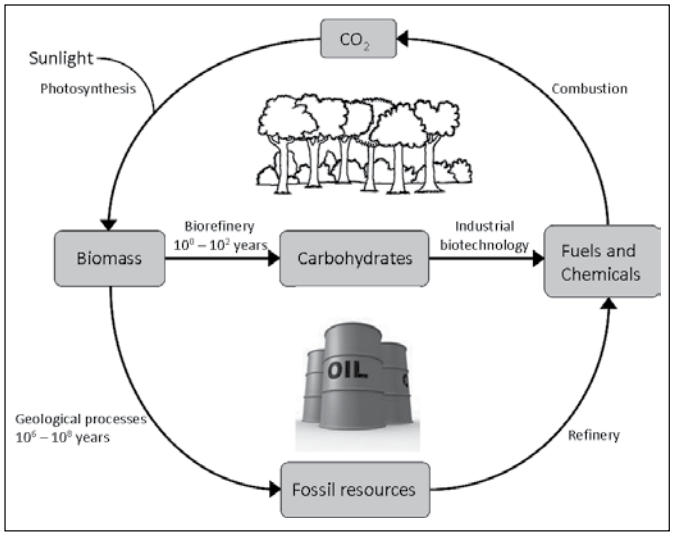



**Figure 2.** Schematic overview of the carbon cycle for the production of fuels and chemicals from industrial biotechnological processes (upper half) and fossil resource based processes (lower half) (after Van Maris *et al*.[37])




finite and although the peak in global oil production may not be imminent, it is nevertheless foreseeable[17]. When production of oil and other fossil resources can no longer fulfil the global need, an explosive increase in the price of crude oil becomes inevitable. In addition, carbon emissions as a result of fossil resource consumption lead to the accumulation of greenhouse gases in the atmosphere, causing global warming. Taking all these issues into account, it is imperative to become less dependent on fossil feedstock. A shift from fossil-based to bio-based raw materials is therefore necessary to (I) prepare the world for a decrease in oil availability, and (II) to stop man-made global warming.

## The shift to bio-based materials

Industrial chemicals produced from petroleum-based feedstock are currently valued at over €1871 billion[9]. However, for the reasons described above, it is crucial to develop production processes that rely on renewable feedstock. Nowadays, a wide range of chemicals is being produced by biotechnological processes, using micro-organisms as biocatalyst and utilizing biomass-derived compounds as feedstock. Some well-known examples of such processes are the production of ethanol by the yeast *Saccharomyces cerevisiae*, citric acid by the fungus *Aspergillus niger* and lactic acid by various lactic acid bacteria. The development of molecular genetics and the concomitant ability to genetically modify micro-organisms has boosted the possibilities to produce chemicals from biological materials. For example, insulin and chymosin are currently produced with, amongst others, genetically engineered *Escherichia coli*. The preferred feedstock for production of bulk chemicals would be biomass from, e.g. agricultural residues, since it presents a solution for the major problems encountered with fossil feedstock: It is virtually inexhaustible, it is cheap and, above all, its use can close the carbon cycle on a much shorter term.

The process of converting organic materials into useful products or energy carriers using biological systems is referred to as bioconversion. For an efficient and economically sound bioconversion process it is essential that the feedstock and the host organism match as closely as possible. Depending on the compound to be produced or the feedstock to be used, the natural metabolic capacity of micro-organisms may require engineering by genetic modification.

## Lignocellulosic materials as feedstock

The sugars and starch used in the bioconversion process are currently derived from sugarcane and cereal crops, mainly corn and wheat. The controversy surrounding food vs fuel debate has resulted in policymakers shifting interest in the use of complex but fairly cheap feedstocks, namely crop residues to be exploited for biofuels production.

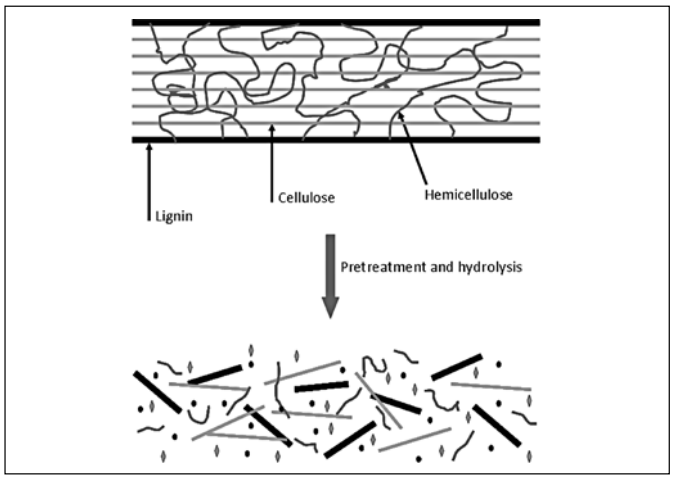
The 'second generation' feedstocks, which are to succeed the first-generation feedstocks described above, comprise lignocellulosic materials that make up the fibrous and woody structural components of plants. The estimated annual production of these lignocellulosic materials is 1.5 trillion tonnes, making it an essentially inexhaustible source of raw material[1, 19]. Potentially useful lignocellulosic raw materials include wheat straw, rice straw, palm, corncobs, corn stems and husk. Lignocellulose typically consists of three major polymers: cellulose, hemicellulose and lignin (Table 1). Depending on the source, the exact composition and cellulosic content vary. Some feedstocks furthermore contain pectin, which is mainly found in waste streams like citrus peels and sugar beet pulp[8].

**Table 1.** Polymer composition of lignocellulose[2, 8, 21]

| Polymer | Amount in lignocellulose | Major monomers |
|---|---|---|
| Cellulose | 40 - 65% | Glucose |
| Hemicellulose | 25 - 35% | Mannose, glucose, galactose, xylose, arabinose, sugar acids |
| Lignin | 10 - 25% | Aromatic alcohols, ethers |
| Pectin | 2 - 20% | Galacturonic acid, rhamnose |

**Figure 3.** Schematic representation of the composition of lignocellulose. During the pretreatment, the structure is destroyed and the polymeric components are released. Subsequent hydrolysis depolymerises the released components, resulting in fermentable monomeric sugars

(Figure adapted from Mosier *et al* [27])




## Liberating sugars from lignocellulose

In order to serve as a substrate for bioconversion purposes, lignocellulose must be degraded into fermentable monomeric units. Monomeric sugars can be released from lignocellulose by various strategies that all involve a pre-treatment and hydrolysis step (Fig. 3). Pre-treatment is aimed at attacking the quaternary structure of lignocellulose. The major polymeric fractions described above are disentangled and, thus, made more accessible to the hydrolyzing agents (acid or enzymes) that are able to release the monomeric sugars. A multitude of pre-treatment methods has been designed over the years, which can be divided into physical, chemical, physicochemical and biological methods. Often a combination of methods is used for maximum effectivity [7, 37]. After pre-treatment, the lignin fraction is separated from the cellulose and hemicellulose fractions. Cellulose and hemicellulose are subsequently depolymerised by chemical or enzymatic hydrolysis. At present, enzymatic hydrolysis is preferred as it is more environmentally friendly [23]. For enzymatic hydrolysis of the cellulose and hemicellulose fractions a variety of enzymes is required. For example, the degradation of xylan, the most abundant polysaccharide in hardwood after cellulose, is achieved with a mixture of endo-1,4-β-xylanase, β-xylosidase, α-glucuronidase, α-L-arabinofuranosidase, acetylxylan esterase, feruloyl esterase and *p*-coumaroyl esterase [2]. Also the homopolymeric cellulose requires a multitude of enzymes for efficient and complete hydrolysis consisting of endoglucanases, exoglucanases and cellobiohydrolases. Pectin, if present, can be hydrolysed by using a mixture of enzymes consisting of various esterases, lyases and hydrolyases [37]. The final broth that results from these procedures is referred to as lignocellulosic hydrolysate.

The ill-fermentable lignin fraction is typically considered a low-value residue because it contains no sugars. The irregular structure of this polymer makes it difficult to design a general approach for the conversion into a value-added product and it is therefore commonly incinerated to produce heat and electricity [7].

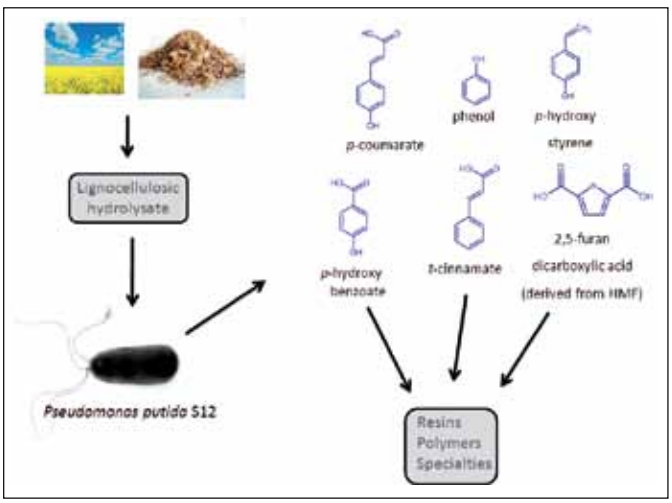
## *Pseudomonas putida* S12

The Gram-negative bacterium *Pseudomonas putida* is a bacterial species that is of great environmental and biotechnological interest. Characteristic for this group of bacteria is the large metabolic capacity and adaptability, which is illustrated by their ability to adapt to diverse and challenging environments. Furthermore, they can degrade recalcitrant compounds and synthesize a variety of low-molecular-weight compounds like siderophores and polyketides, as well as biopolymers such as polyhydroxyalkanoates and alginate [28, 33, 41]. A subgroup of this species is extremely tolerant to organic solvents [14, 18, 31].

*Pseudomonas putida* S12 is an example of such a micro-organism with a highly flexible metabolism, a wide substrate range and an exceptionally high tolerance to organic solvents [11, 40]. Various mechanisms allow this bacterium to withstand several types of solvents at concentrations that are lethal to most micro-organisms [40]. The most important mechanisms for solvent-tolerance have been extensively reviewed [12, 14, 18, 31-32, 42]. Compounds can be produced that are too toxic for other hosts, and these compounds can be produced to high titres without provoking harmful effects [5]. Furthermore, *in-situ* product extraction can be applied in fermentations by adding a second phase of a water-immiscible solvent. This prevents the accumulation of product to inhibitory concentrations, improving the productivity of the chemical [13] and facilitating downstream processing.

The potential of *P. putida* S12 as a host for bio-based chemicals production has been demonstrated in recent years. *P. putida* S12 has been developed as a platform host for the production of diverse compounds of varying toxicity from renewable substrates. The general approach to biochemicals production was to convert central metabolites into the desired product via a heterologous conversion. The main focus was on substituted aromatic compounds derived from aromatic amino acids. These industrially relevant compounds are applied in

**Figure 4.** The *Pseudomonas putida* S12 platform for the production of commodity chemicals. The sugar fractions in lignocellulosic hydrolysate are converted by *P. putida* S12 into a variety of chemicals, which can subsequently serve as the starting material for the production of resins and polymers



the production of, e.g. resins and polymers. However, their hydrophobic nature prohibits efficient production in non-solvent tolerant microbial host systems. Lab-scale production processes have been developed for *t*-cinnamate via L-phenylalanine [29] and *p*-coumarate, phenol, *p*-hydroxybenzoate and *p*-hydroxystyrene via L-tyrosine [30, 38-39, 45]. *P. putida* S12 has also been employed for the one-step conversion of hydroxymethylfurfural into 2,5-furan-dicarboxylic acid [20], a chemical which is listed in the top twelve of most promising biological compounds for the production of bio-based chemicals [44].

In order to establish an economically sound production process of biochemicals, the feedstock must be utilized as completely as possible. However, due to its heterogeneous nature, lignocellulosic feedstock is often metabolized incompletely. The same problem is encountered with the production of substituted aromatic compounds by *P. putida* S12. This bacterial strain is able to use D-glucose, but not D-xylose and L-arabinose. As D-xylose is the most abundant pentose sugar, strategies were explored to engineer D-xylose metabolic pathways in *P. putida* S12.

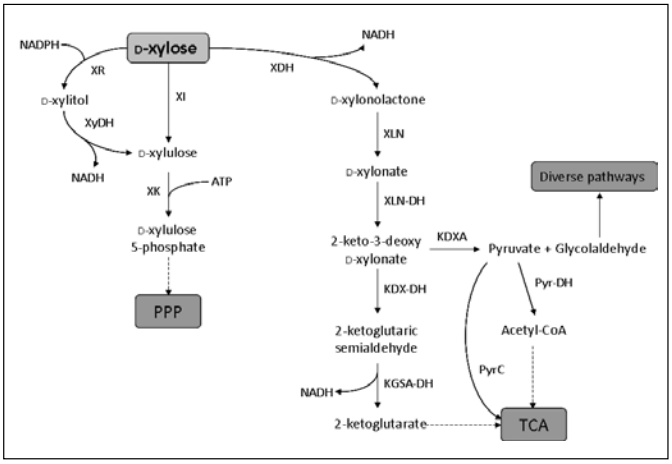
## Engineering D-xylose metabolism in *Pseudomonas putida* S12

In order to establish D-xylose utilization by *P. putida* S12, D-xylose has to be converted into an intermediate of the central carbon metabolism. To achieve this, multiple strategies can be employed. Figure 5 shows an overview of pathways currently known to dissimilate D-xylose. The D-xylose dissimilative pathways commonly found in bacteria involve the isomerization of D-xylose into D-xylulose and subsequent phosphorylation by D-xylulokinase [22]. This pathway is commonly employed to engineer D-xylose metabolism into bacteria [16, 46-47] and therefore, this pathway was chosen to engineer *P. putida* S12 for D-xylose utilization.

In order to construct the bacterial phosphorylative pathway described above, genes from *Escherichia coli* were expressed coding for the enzymes D-xylose isomerase (XylA) and D-xylulokinase (XylB). These enzymes convert D-xylose into D-xylulose-5-phosphate, which can subsequently be processed by the pentose phosphate (PP) pathway. This approach resulted in utilization of D-xylose, albeit at a low efficiency. The initial yield on D-xylose was low (14 Cmol %) and the growth rate was poor (0.01 $h^{-1}$). In order to optimize the growth performance of the engineered strain *P. putida* S12xylAB, a laboratory evolution approach was applied. This approach yielded an efficient D-xylose utilizing phenotype, showing a biomass-to-substrate yield of 67 Cmol% at a considerably improved growth rate of 0.35 $h^{-1}$. Subsequent experiments on D-glucose consumption revealed that the ability of *P. putida* S12 to utilize D-glucose was not affected by the evolutionary engineering procedure. Thus, a *P. putida* S12 strain was constructed that was able to efficiently utilize D-xylose and D-glucose. However, for optimal utilization of lignocelluloses-derived feedstock, L-arabinose should also be metabolized. Although strain S12xylAB2 was not specifically engineered for L-arabinose utilization, the introduced D-xylose metabolic enzymes may show nonspecific activity towards L-arabinose, a C4 epimer of D-xylose [19, 21]. Surprisingly, L-arabinose was utilized as a carbon source at an efficiency similar to that

**Figure 5.** Four pathways for dissimilation of D-xylose.

In the phosphorylative pathways (left-hand part) D-xylose is converted into D-xylulose either directly (bacterial pathway) [15, 22] or via D-xylitol (eukaryotic route) [10, 15]. D-xylulose is subsequently phosphorylated at the expense of ATP. In the oxidative route (right-hand part), D-xylose is oxidized to D-xylonate and further converted into 2-ketoglutarate via two dehydrations and one oxidation step [1, 3, 35, 43]. The route leading from 2-keto-3-deoxy-D-xylonate towards pyruvate was reported for an undefined Pseudomonas strain [4]. Cofactors involved in the pyruvate-route are omitted for clarity. XI, D-xylose isomerase; XR, D-xylose reductase; XyDH, D-xylitol dehydrogenase; XK, D-xylulokinase; XDH, D-xylose dehydrogenase; XLN, D-xylonolactonase; XLN-DH, D-xylonate dehydratase; KDX-DH, 2-keto-3-deoxy-D-xylonate dehydratase; KGSA-DH, 2-ketoglutaric semialdehyde dehydrogenase; KDXA, 2-keto-3-deoxy-D-xylonate aldolase; Pyr-DH, pyruvate dehydrogenase; PyrC, pyruvate carboxylase; PPP, Pentose Phosphate Pathway; TCA, TriCarboxylic Acid cycle.




of growth on D-xylose. Furthermore, growth experiments performed with the evolved *P. putida* S12xylAB2 on mixtures of D-glucose, D-xylose and L-arabinose showed full utilization of all three sugars. It was additionally shown that the evolved D-xylose-utilizing strain *P. putida* S12xylAB2 can be deployed in simultaneous saccharification and fermentation of steam-treated wheat-straw, efficiently utilizing the sugars D-glucose, D-xylose and L-arabinose that were liberated during the process (unpublished results). Thus, genetic engineering in combination with the evolutionary selection procedure yielded a *P. putida* S12 derived strain that efficiently utilizes the three main sugars present in lignocellulosic hydrolysate: D-glucose, D-xylose and L-arabinose [25].

In an alternative approach, the oxidative D-xylose metabolic pathway from *Caulobacter crescentus* was expressed in *P. putida* S12 [26]. It was expected that this pathway was more suitable for engineering a D-xylose catabolism in *P. putida* S12, as this bacterium employs an oxidative sugar metabolism. Introduction of the genes *xylXABCD* resulted in utilization of D-xylose with a biomass yield of 66 Cmol% and a maximum growth rate of 0.21 $h^{-1}$. Additional research furthermore showed that endogenous enzyme activities were able to cooperate with heterologous enzyme activities, confirming the suitability of this metabolic pathway to engineer *P. putida* S12 for D-xylose utilization. As the final product of the oxidative D-xylose pathway is α-ketoglutarate (Fig. 5), it may be expected that this approach can be employed to use pentoses as a source for the production of TCA-cycle derived C4 building blocks.

## Production of fine chemicals with *P. putida* S12

The production of substituted aromatic compounds by *P. putida* S12 has been well established and extensively reported [29-30, 38-39, 45]. The general approach for the production of these compounds is to convert aromatic amino acids into the desired product via a heterologous conversion, e.g. the one-step conversion of L-tyrosine into phenol. It was furthermore shown that an optimized phenol-producing *P. putida* S12 strain [45] can be deployed as a platform organism, as the range of compounds produced with this host organism can be broadened with relatively simple interventions. For example, the phenol-producing *P. putida* S12 strain was altered to produce *p*-hydroxybenzoate by removing the gene *tpl* and introducing the gene *pal/tal* [38], or for the formation of *p*-hydroxystyrene by expressing *pal/tal* and *pdc* [39]. However, previous research on the production of aromatic compounds by *P. putida* S12 assigned the limited availability of the PP pathway intermediate erythrose-4-phosphate (E4P) as a possible bottleneck for efficient aromatics production. It was therefore expected that the E4P pool could be increased by feeding pentoses directly into the PP pathway. A *p*-hydroxybenzoate-producing *P. putida* S12 strain was engineered with the D-xylose isomerase pathway to demonstrate the possibility to produce aromatic compounds from sugar mixtures. Chemostat experiments performed with the resulting strain showed that *p*-hydroxybenzoate can indeed be produced from D-xylose. Even more, when mixtures of D-glucose and D-xylose were used as feed, the product-to-substrate yield increased 1.6-fold. The results from this case study not only showed that aromatic compounds can be produced from D-xylose, but that mixed substrate feeding has a stimulating effect on the productivity. This implies that lignocellulose, containing roughly about 50% D-glucose and 20% D-xylose, is an excellent substrate for the biological production of aromatic compounds by *P. putida* S12 (24).

## Future perspectives

The construction of a pentose-utilizing aromatics-producing *Pseudomonas putida* S12 is an important step towards the efficient utilization of lignocellulosic feedstock for bio-based chemicals production. The prospects for these kind of processes are highly promising; a preliminary economic evaluation study (unpublished data) indicated that the pricing gap between chemically produced and bio-based *p*-hydroxybenzoate is rapidly closing as a result of the described developments with regard to mixed-substrate feeding (Koen Meesters, personal communication). However, additional issues must be addressed to make bio-based chemicals production truly economically feasible. A major issue are the product titres that can presently be achieved. To this end, the production host may be additionally engineered to increase the availability of the aromatic precursors, erythrose-4-phosphate (E4P) and phospho-enol pyruvate (PEP). Increased availability of E4P was apparently achieved by co-feeding D-xylose, but the availability of PEP may also have to be improved. This can be achieved by constructing a glycolytic *P. putida* S12 strain (Pseudomonads lack a functional glycolysis), so that two molecules of PEP can be produced from D-glucose, instead of the single PEP molecule that is derived from D-glucose via the Entner-Doudoroff pathway that is normally employed in Pseudomonads. In addition, PEP-dependent transporters can be replaced by non-PEP-dependent transporters, rendering more PEP available for aromatics production.

A second issue to further increase bio-based chemicals productivity is the appearance of diauxy when using multiple carbon sources as feedstock. Although this problem can be circumvented by applying carbon-limited growth conditions, it poses possible drawbacks for the efficient production of biochemicals. It was reported previously that a switch in metabolism is accompanied with lower metabolic fluxes and a downregulation of proteins associated with the biosynthetic machinery [6]. A diauxic shift therefore probably conflicts with fast and complete utilization of sugars, and presents a new target for increasing the efficiency of utilizing sugar mixtures. Preventing diauxy makes it possible to set up a production process in fed-batch mode. Fed-batch production process generally show lower product yields, but the product titres are higher. Higher product titres will furthermore lower downstream process costs, as highly diluted products often require expensive recovery techniques.

Although the production host *P. putida* S12 is solvent-tolerant, even this micro-organism suffers from high product concentrations. Lowering product concentrations in the culture will therefore also stimulate biochemicals production, preferably by physically separating the aqueous and organic phases. The development of *in-situ* product removal strategies like membrane extraction [34] or solvent-impregnated resins [36] then results in a virtually endless product sink, and can therefore be a major step towards industrial production of biochemicals from lignocellulosic feedstock. Also, *in-situ* product extraction can be applied in fermentations by adding a second phase of a water-immiscible solvent, thereby preventing the accumulation of product to inhibitory concentrations. A fine example of this strategy is the application of a second phase of decanol to extract *p*-hydroxystyrene from fed-batch cultivation, resulting in an increase in product titre from 4.5 to 21 mM [39].

## Conclusions

The increasing price of oil and imminent shortage of fossil fuels necessitates the development of biochemicals production from renewable resources. Lignocellulose provides a promising alternative feedstock for bio-based chemicals production; however, lignocellulose conversion processes are still expensive. Cost reduction is therefore imperative for lignocellulose to replace oil as starting material. Expanding the substrate range of the biochemical-producing *Pseudomonas putida* S12 is yet another step in constructing a fully-fledged metabolic engineering platform for the cost-effective production of chemicals. It enables the production of commodity compounds with substantially lower energy consumption and carbon dioxide emissions compared to petroleum-based processes, and paves the way to a more efficient and cost-effective use of bio-based resources.

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