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# Biocatalytic Conversion of Lignin to Aromatic Dicarboxylic Acids in *Rhodococcus jostii* RHA1 by Re-Routing Aromatic Degradation Pathways

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Abstract: The heteropolymer lignin represents an untapped resource for production of renewable aromatic chemicals, if efficient depolymerisation methods can be developed. In this work, the metabolic pathways in *Rhodococcus jostii* RHA1 for degradation of aromatic lignin breakdown products are re-routed, in order to generate an aromatic dicarboxylic acid product that could be used for bioplastic synthesis. Protocatechuic acid is normally metabolised via ortho-cleavage to the β-keto-adipate pathway. Insertion of recombinant genes for protocatechuate 4,5-dioxygenase or protocatechuate 2,3-dioxygenase into *R. jostii* RHA1, followed by ammonia cyclisation of the extradiol cleavage products, generates pyridine 2,4-dicarboxylic acid or pyridine 2,5-dicarboxylic acid bioproducts in yields of 80-125 mg/L when grown on minimal media containing 1% wheat straw lignocellulose.

## Introduction

The aromatic lignin heteropolymer found in plant cell walls is a potential source of renewable aromatic chemicals, if efficient chemical or biocatalytic methods can be found for lignin depolymerisation [1]. Lignin has proved to be a very challenging substrate for chemo- and biocatalysis [2], but recent studies suggest that microbial lignin degradation could be harnessed for lignin bioconversion to aromatic chemicals [3]. In *Rhodococcus jostii* RHA1, a bacterial lignin degrader in which extracellular peroxidase enzyme DypB has been shown to oxidise lignin [4], deletion of the *vdh* gene encoding vanillin dehydrogenase gives a gene deletion mutant that, upon growth in minimal media containing wheat straw lignocellulose, generates up to 96 mg/L vanillin after 6 days fermentation [5]. This discovery prompted us to investigate whether the aromatic degradation pathways in *Rhodococcus jostii* RHA1 could be re-routed to produce aromatic chemicals for industrial renewable plastic production.

Aromatic dicarboxylic acids are commonly used for the synthesis of polyester thermoplastic polymers such as polyethylene terephthalate (PET) and polybutyrate adipate terephthalate (PBAT) [6]. The terephthalic acid precursor used for PET and PBAT synthesis is derived from petrochemicals, hence there is considerable interest in the development of biobased plastics derived from biomass [7].

In this work we utilise the aromatic catabolic pathways for lignin degradation in *R. jostii* RHA1 to generate aromatic dicarboxylic acid products for synthesis of renewable bioplastics. It is known that the extradiol ring cleavage products on aromatic degradation pathways can be cyclised with ammonia, either under acidic conditions [8], or in minimal media cultures containing ammonium chloride [9], to generate picolinic acid products. Our aim was to re-route the aromatic degradation pathways in *R. jostii* RHA1 from the normal ortho-cleavage pathway to meta-cleavage, followed by cyclisation of the extradiol ring fission product with ammonia, to generate pyridine dicarboxylic acid products analogous to terephthalic acid. As shown in Figure 1, insertion of the *praA* gene encoding protocatechuate 2,3-dioxygenase, followed by cyclisation, would generate pyridine 2,5-dicarboxylic acid (2,5-PDCA), while insertion of the *ligAB* genes encoding protocatechuate 4,5-dioxygenase, followed by cyclisation, would generate pyridine 2,4-dicarboxylic acid (2,4-PDCA).
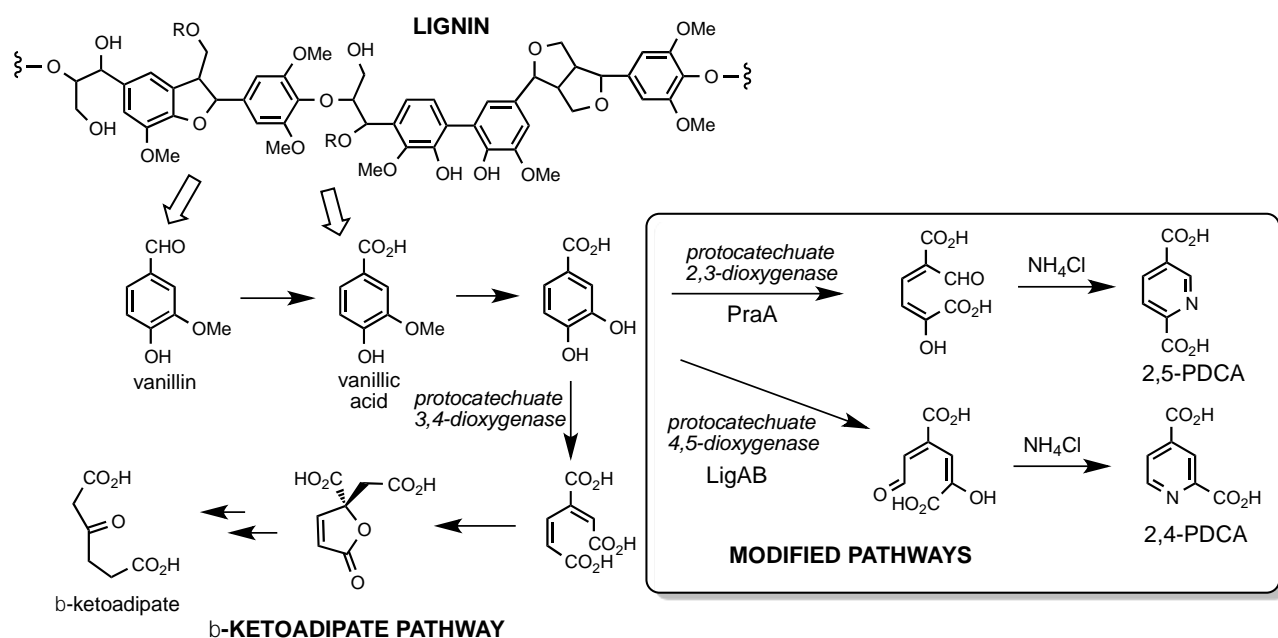


Figure 1: Modified pathways for conversion of lignin to pyridine 2,4-dicarboxylic acid (2,4-PDCA) and pyridine 2,5-dicarboxylic acid (2,5-PDCA), showing the normal β-ketoadipate pathway and the proposed modified pathways.

**Results**

*Insertion of recombinant genes for 4,5-PCD, 2,3-PCD into R. jostii RHA1*

The *ligAB* genes encoding *S. paucimobilis* protocatechuate 4,5-dioxygenase [10] were cloned into expression vector pTipQ2 vector used for inducible gene expression in *Rhodococcus*, using a thiostrepton inducer [11], generating construct pTipQ2-ligAB. The *praA* gene encoding *Paenibacillus* sp. JJ-1b protocatechuate 2,3-dioxygenase [12] was also cloned into pTipQ2 to give construct pTipQ2-praA. The two constructs were each transformed into *Rhodococcus jostii* RHA1 via electroporation.

In order to verify expression of the recombinant genes, cell-free extract was obtained from cultures of *R. jostii* pTipQ2-ligAB and *R. jostii* pTipQ2-praA after induction with 1 µg/ml thiostrepton. Addition of extract to solutions containing protocatechuic acid gave rise to a yellow colour in each case, corresponding to the meta-ring cleavage product, and absorbance changes of 0.2-0.45 absorbance units over 60 min at 410 nm and 350 nm for constructs *R. jostii* pTipQ2-ligAB and *R. jostii* pTipQ2-praA respectively, corresponding to the literature values for the ring cleavage products for 4,5-protocatechuate dioxygenase [10] and 2,3-protocatechuate dioxygenase [12].

*Production of pyridine-dicarboxylic acid metabolites*

Metabolite production was first tested on M9 minimal media containing 0.1% vanillic acid as carbon source and ammonium chloride as nitrogen source. Extracts from *R. jostii* pTipQ2-ligAB grown on M9 media containing 0.1% vanillic acid showed a new peak at retention time 9.8 min with *m/z* 167.7 (MH+) and 189.7 (MNa+) matching the retention time and mass spectrum of authentic 2,4-pyridinedicarboxylic acid, as shown in Figure 2. No metabolite production was observed using wild-type *R. jostii* RHA1 under the same conditions. Maximal metabolite production was observed after 5 and 7 days fermentation, as shown in Figure 3, with no metabolite detected after 3 days, and then metabolite production decreased somewhat after 10-12 days. The yield of pyridine 2,4-dicarboxylic acid was determined by comparison with a standard curve of authentic material to be 112 mg per litre culture media.
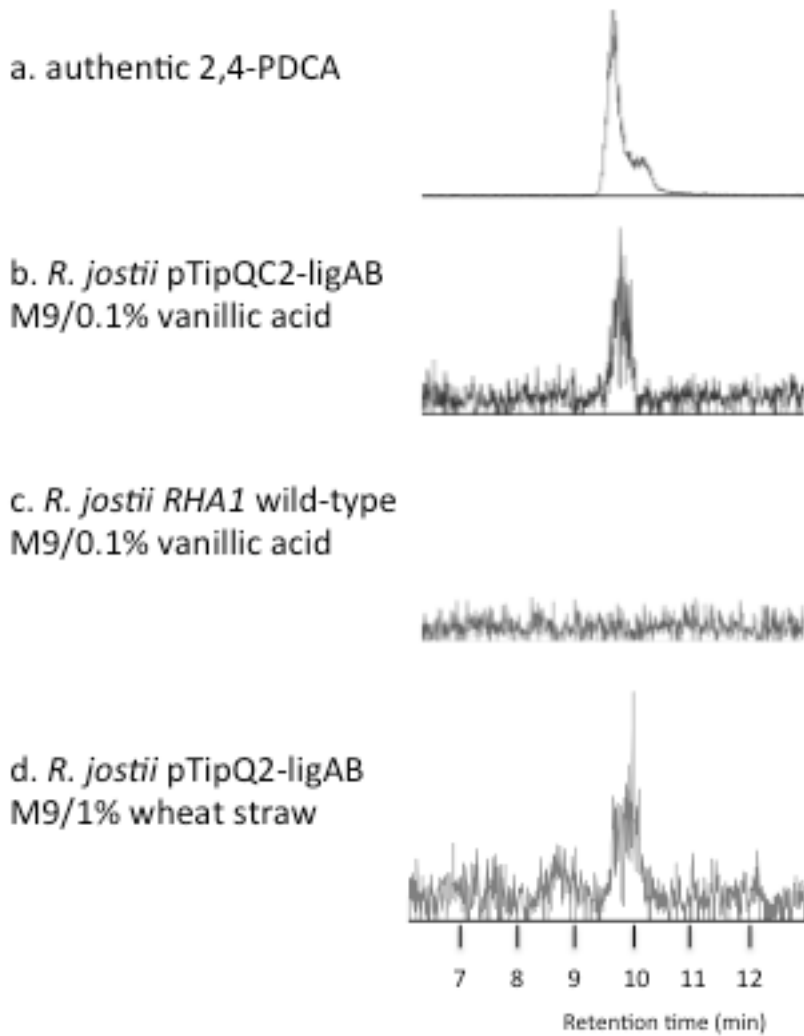
Figure 2. LC-MS data (extracted ion chromatogram at *m/z* 168.0) for production of 2,4-pyridinedicarboxylic acid after 5 days fermentation on M9 minimal media containing 0.1% vanillic acid (b,c) or 1% wheat straw lignocellulose (d) by pTipQ2-ligAB containing *ligAB* genes (b,d) compared with wild-type *R. jostii* (c) & authentic 2,4-PDCA (a)
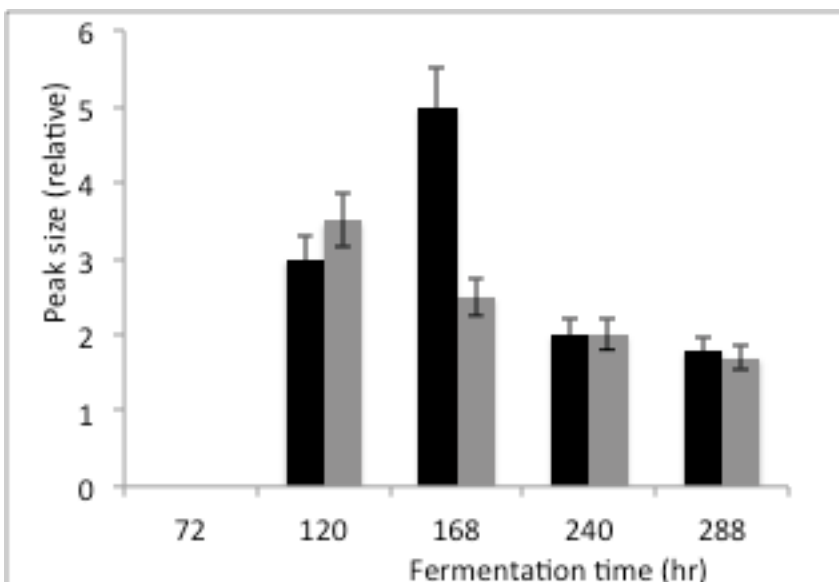
Figure 3. LC-MS peak height for 2,4-PDCA (black) from *R. jostii* pTipQC2-ligAB and 2,5-PDCA (gray) from *R. jostii* pTipQC2-praA grown on M9 minimal media containing 0.1% vanillic acid vs fermentation time

Extracts from *R. jostii* pTipQ2-praA containing recombinant *praA* gene grown for 5 days on M9 media containing 0.1% vanillic acid also generated a new metabolite at retention time 10.0 min with *m/z* 167.7 (MH$^+$) and 189.7 (MNa$^+$) matching the retention time and mass spectrum of authentic 2,5-pyridinedicarboxylic acid, as shown in Figure 4. In this case, peak metabolite production was observed after 5 days. (see Figure 3) The yield was determined to be 80 mg 2,5-pyridinedicarboxylic acid per litre culture from LC-MS analysis.
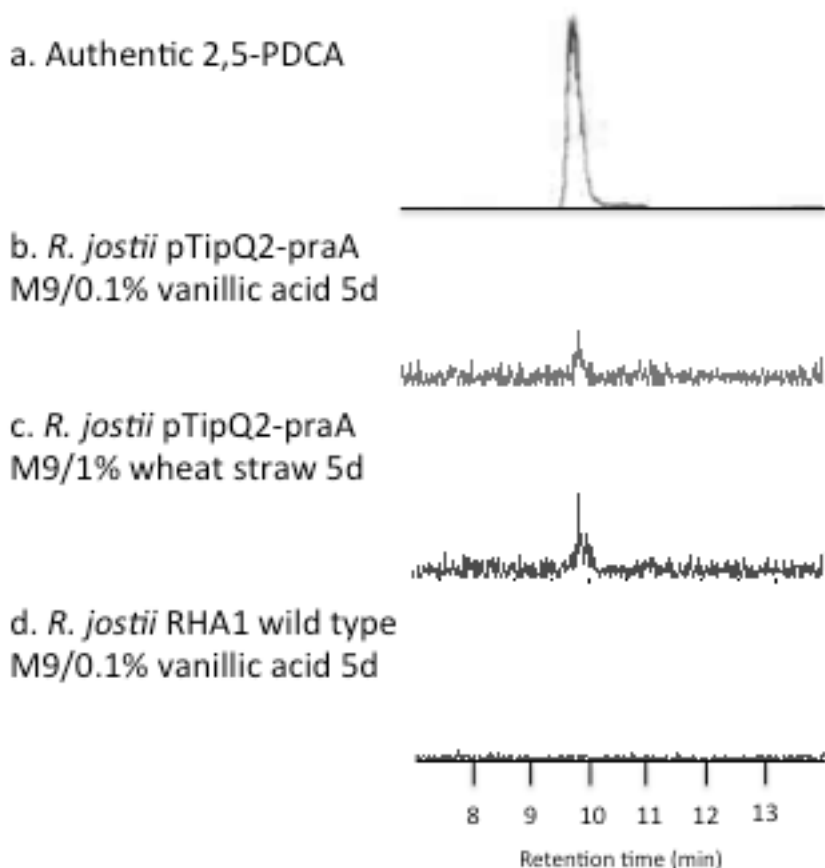


Figure 4. LC-MS data (extracted ion chromatogram at *m/z* 168.0) for production of 2,5-pyridinedicarboxylic acid after 5 days fermentation on M9 minimal media containing 0.1% vanillic acid (b,d) or 1% wheat straw lignocellulose (c) by pTipQ2-praA containing *praA* gene (b,c) compared with wild-type *R. jostii* (d) & authentic 2,4-PDCA (a).

The same constructs were then grown on M9 minimal media containing 1% milled wheat straw lignocellulose. Extracts from *R. jostii* pTipQ2-ligAB gave rise to the LC-MS peak at 9.8 min corresponding to authentic 2,4-PDCA (see Figure 2d). Maximum metabolite

production was observed at 5-7 days, and a yield of 90 mg/litre was determined by comparison with a standard curve for authentic 2,4-PDCA. Extracts from *R. jostii* pTipQ2-praA gave the peak at 10.0 min corresponding to authentic 2,5-PDCA (see Figure 4c). Maximum metabolite production was observed at 5 days, and a yield of 79 mg/litre was determined by comparison with a standard curve for authentic 2,5-PDCA.

*Bioreactor fermentations*. The *R. jostii* pTipQ2-ligAB construct was then grown in a 2.5 litre bioreactor in M9 minimal media containing 1% wheat straw lignocellulose for 9 days, reaching a maximum $OD_{600}$ = 1.08 after 5 days, and $OD_{600}$ = 0.90 after 9 days. After centrifugation of bacterial cells, the supernatant was applied to an Amberlite IRA900 anion exchange column (100 ml volume), washed with water, and then eluted with 0.5 M HCl. The eluted fractions showed absorbance maxima at 273 nm and 230 nm matching those of authentic 2,4-pyridinedicarboxylic acid, which was eluted in 15 x 50 ml fractions, with fractions 5 and 6 showing greatest amount of product (see Supporting Information S4). The amount of 2,4-PDCA present in organic extracts after 9 days fermentation estimated by LC-MS analysis was 125 mg/litre, and the yield of material after ion-exchange chromatography, based on UV-vis analysis, was 102 mg/litre. The material purified by anion exchange chromatography was analysed by C18 reverse phase HPLC, and was found to co-elute with authentic 2,4-PDCA (see Figure 5A).
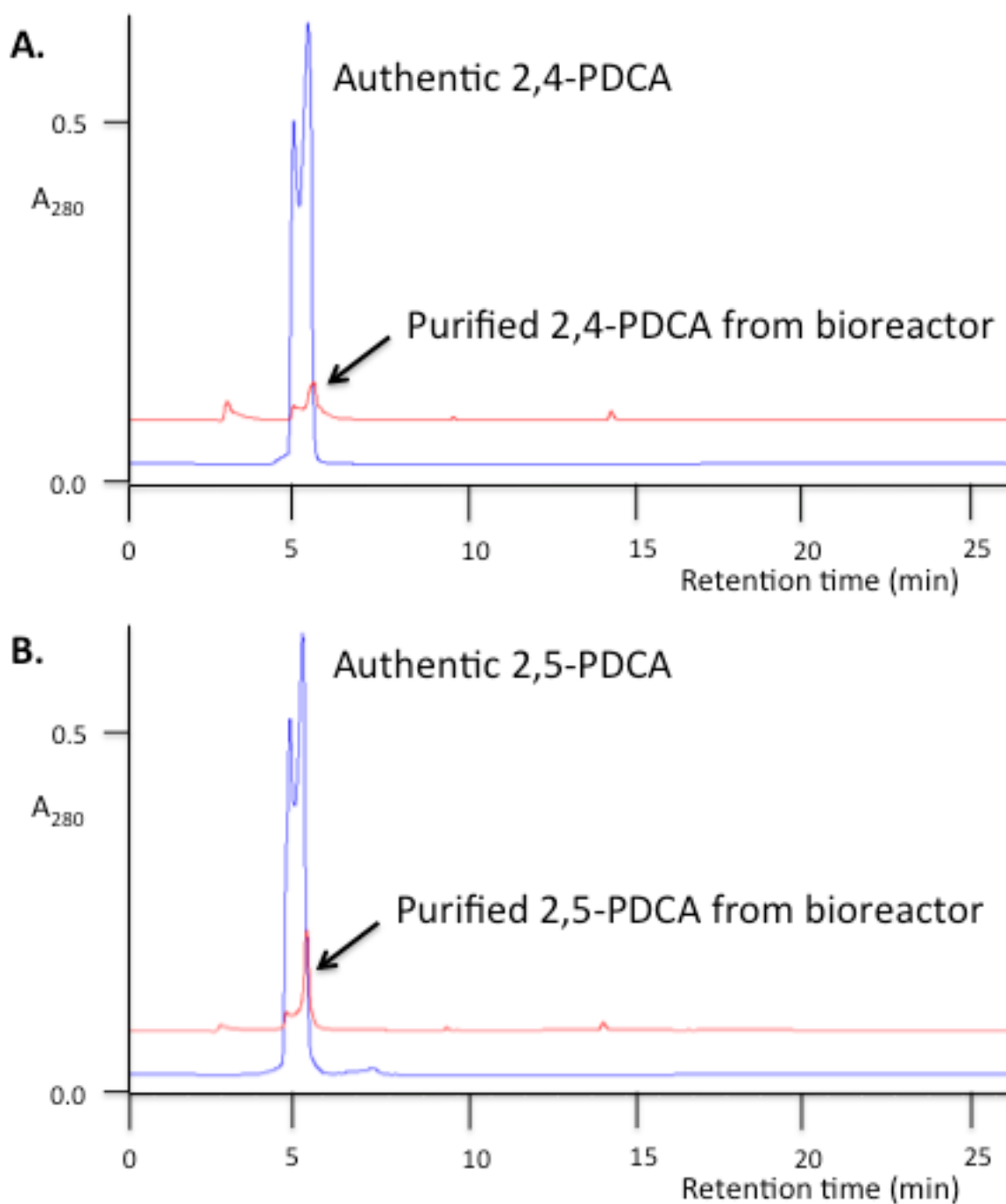
Figure 5. C18 reverse phase HPLC analysis of pyridine dicarboxylic acid products from 2.5 litre bioreactor fermentation, after purification by anion exchange chromatography. A. Purified 2,4-pyridinedicarboxylic acid from fermentation of *R. jostii* pTipQ2-ligAB, compared with authentic 2,4-PDCA. B. Purified 2,5-pyridinedicarboxylic acid from fermentation of *R. jostii* pTipQ2-praA, compared with authentic 2,5-PDCA.

A fermentation of *R. jostii* pTipQ2-ligAB in M9 minimal media containing 0.5% Kraft lignin was also carried out over 4 days, which also produced 2,4-PDCA, confirmed by HPLC analysis. Product isolation via Amberlite IRA900 anion exchange chromatography resulted in a yield of 53 mg/litre 2,4-PDCA.

Growth of the *R. jostii* pTipQ2-praA construct in the 2.5 litre bioreactor in M9 minimal media containing 1% wheat straw lignocellulose over 9 days, followed by product isolation via Amberlite IRA900 anion exchange chromatography, yielded product fractions absorbing at 271 nm matching authentic 2,5-PDCA, with fractions 4 and 5 containing most product (see Supporting Information S9). The amount of 2,5-PDCA present in organic extracts after 9 days fermentation estimated by LC-MS analysis was 106 mg/litre, and the yield of material after ion-exchange chromatography, based on UV-vis analysis, was 65 mg/litre. The material purified by anion exchange chromatography was analysed by C18 reverse phase HPLC, and was found to co-elute with authentic 2,5-PDCA (see Figure 5B). A summary of the yields of pyridinedicarboxylic acid bioproducts is given in Table 1.

Table 1: Yields from different feedstocks, fermentation times

**Conclusions**

Previously we have shown that is possible to accumulate bioproduct vanillin in a gene deletion mutant of *Rhodococcus jostii* RHA1 in which the vanillate dehydrogenase gene was deleted, in a yield of 96 mg/litre culture media [5], hence establishing that manipulation of the lignin breakdown pathways of this organism could be used to generate bioproducts from lignin breakdown. In this study we have used a gene insertion approach to re-route the aromatic degradation pathways downstream from lignin oxidation. Since the conversion of vanillin to protocatechuic acid appears to be a central pathway involved in lignin metabolism [5], we have re-routed the metabolism of key intermediate protocatechuic acid to generate aromatic dicarboxylic acids that could be used to manufacture polyester bioplastics.

Utilising the known transformation of meta-ring fission products with ammonia to generate picolinic acids [8,9], we have inserted genes for protocatechuate 4,5-dioxygenase or protocatechuate 2,3-dioxygenase into *Rhodococcus jostii* RHA1, and have observed the anticipated 2,4-pyridinedicarboxylic acid and 2,5-pyridinedicarboxylic acid bioproducts, using either vanillic acid or wheat straw lignocellulose as carbon source. The yields of 80-125 mg/litre culture media are comparable with the yield of vanillin isolated by gene deletion [5], suggesting that this is the amount of flux that passes through the vanillic acid catabolic pathway from lignin in *R. jostii*. Assuming that 20% of the wheat straw lignocellulose is lignin, then the observed yields correspond to approximately 5% of the lignin present in the fermentation. Improving our understanding of the microbial lignin degradation pathways [13] in the future may allow further enhancement of this yield. The peak production of 2,5-PDCA is

somewhat earlier than that of 2,4-PDCA (see Figure 4), perhaps reflecting a higher specific activity of the PraA enzyme, compared to the LigAB enzyme.

This study provides a novel biocatalytic route to pyridinedicarboxylic acids from a renewable feedstock lignin, and highlights the opportunity for bioconversion of lignin into aromatic products using biotechnology. Linger *et al* have recently shown that *Pseudomonas putida* can also be used to generate polyhydroxyalkanoate biopolyesters from lignin breakdown, via metabolic conversion of lignin breakdown [14]. Pathway engineering has also been used in *Pseudomonas putida* to generate ring cleavage product *cis,cis*-muconic acid, which can be converted via chemocatalysis to adipic acid [15]. Since lignin is generated as a by-product of cellulosic bioethanol production and paper/pulp manufacture, it may be possible to combine this technology with an existing industrial process in order to generate value-added products from the lignin stream. The observation that 2,4-PDCA can be generated from the more condensed Kraft lignin via this approach suggests that valorisation of Kraft lignin, albeit a more challenging substrate, may also be possible using biocatalysis.

**Experimental**

*Plasmids.* The *Sphingobium* sp. SYK-6 *ligAB* genes (1339 bp) encoding the $\alpha$ and $\beta$ subunits of protocatechuate 4,5-dioxygenase were cloned directly into vector pTipQ2 by Genscript to generate pTipQ2-ligAB. The *Paenibacillus* sp. JJ-1b *praA* gene (881 bp) encoding protocatechuate 2,3-dioxygenase was cloned directly into vector pTipQ2 by Genscript to generate pTipQ2-praA. Plasmid DNA was transformed into electrocompetent *R. jostii* cells by electroporation (1.8 kV), and recombinant cells isolated on Luria-Bertani media containing 50 μg/ml chloramphenicol.

*Assay of protocatechuate dioxygenase activity*. Cultures (5 ml) of *R. jostii* pTipQ2-ligAB or *R. jostii* pTipQ2-praA were grown for 24 hr at 30 °C in Luria-Bertani broth containing 50 μg/ml chloramphenicol, then induced with 1 μg/ml thiostrepton, and grown for a further 48 hr. Cell free-extract was prepared by centrifugation (microcentrifuge, 13,000 rpm, 5 min), then resuspension of the cell pellets in 75 μl of 20 mM Tris buffer pH 8.0, addition of lysozyme (5 μl or 5 mg/ml), incubation for 1 hr at 37 °C, then sonication and centrifugation (microcentrifuge). Protocatechuic acid (2.5 mM, 800 μl) was added, and the solutions monitored at 350 nm for *R. jostii* pTipQ2-praA, giving absorbance changes of 0.35 ±0.05 after 60 min and a visible yellow colouration; and at 410 nm for *R. jostii* pTipQ2-ligAB, giving absorbance changes of 0.25 ±0.02 after 60 min and a slight yellow colouration.

*Metabolite production*. Cultures (10 ml) of *R. jostii* pTipQ2-ligAB or *R. jostii* pTipQ2-praA were grown for 24 hr at 30 °C in M9 minimal media (6 g/l $Na_2HPO_4$, 3 g/l $KH_2PO_4$, 0.5 g/l NaCl, 1.0 g/l $NH_4Cl$, 2 mM $MgSO_4$, 0.5 mM $CaCl_2$) containing 50 μg/ml chloramphenicol and either 0.1% (w/v) vanillic acid or 1.0% (w/v) wheat straw lignocellulose, then induced with 1 μg/ml thiostrepton, and then grown for a further 7-11 days at 30 °C, supplementing with 1 μg/ml thiostrepton at 48 hr intervals. Aliquots (1 ml) were removed, centrifuged (13000 rpm, microcentrifuge, 5 min), and the supernatant extracted into ethyl acetate (1 ml). The ethyl acetate extract was injected onto a C18 Zorbax Eclipse plus (Agilent) reverse phase HPLC column on an Agilent 1200 Series system, and analysed by LC-MS using a Bruker HTC-Ultra ESI mass spectrometer. The HPLC solvents were water/0.1% trifluoroacetic acid (solvent A) and methanol/0.1% trifluoroacetic acid (solvent B). The applied gradient was 5% B for 5 min; 5-15% B over 10 min; 15-25% B for 8 min; and 25-100% B for 19 min, at a flow rate of 1.0 ml $min^{-1}$. 2,4-PDCA and 2,5-PDCA were detected by extracted ion analysis for fragment *m/z* 168.0, in positive ion mode, and were compared with authentic standards for 2,4-PDCA and 2,5-PDCA.

*Bioreactor fermentation*. Cultures of *R. jostii* pTipQ2-ligAB or *R. jostii* pTipQ2-praA were grown at 30 °C in 2.5 litres M9 minimal media (6 g/l $Na_2HPO_4$, 3 g/l $KH_2PO_4$, 0.5 g/l NaCl, 1.0 g/l $NH_4Cl$, 2 mM $MgSO_4$, 0.5 mM $CaCl_2$) containing 50 μg/ml chloramphenicol and either 0.1% (w/v) vanillic acid or 1.0% (w/v) wheat straw lignocellulose or 0.5% (w/v) Kraft lignin (from Billerud Ltd, Sweden) in an Electrolab FerMac 3010 bioreactor. Fermentations were induced by addition of 1 μg/ml thiostrepton after 24 hr, and then grown for a further 3-8 days at 30 °C, supplementing with 1 μg/ml thiostrepton at 48 hr intervals. After fermentation, cultures were harvested by centrifugation (6,000 g, 10 min), and the supernatant was applied to an Amberlite IRA900 anion exchange column (100 ml volume), washed with water (200 ml), and then eluted with 0.5 M HCl (800 ml). 15 x 50 ml fractions were collected, and analysed by UV-vis spectroscopy for the presence of 2,4-pyridinedicarboxylic acid ($\lambda_{max}$ 273 nm, $\varepsilon$ = 3.1 x $10^3$ $M^{-1}cm^{-1}$) or 2,5-pyridinedicarboxylic acid ($\lambda_{max}$ 271 nm, $\varepsilon$ = 6.3 x $10^3$ $M^{-1}cm^{-1}$). Fractions containing the desired products were pooled, and analysed by C18 reverse phase HPLC (gradient described above).

**Notes.** A UK patent application involving this work (number TBC) was filed on 15th June 2015.

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Table 1. Yields of pyridine dicarboxylic acids, in mg product/L culture media. a, yield estimated from LC-MS analysis, comparison with authentic standard; b, yield of product after ion exchange chromatography, calculated via UV-vis absorption. Length of fermentation time indicated in brackets.

| Construct | Product | Scale | Carbon source for M9 minimal media | | |
|---|---|---|---|---|---|
| | | | 0.1% vanillic acid | 1% wheat straw | 0.5% Kraft lignin |
| *Rhodococcus jostii* pTipQC2-ligAB | 2,4-pyridine dicarboxylic acid | 50 mL | 112 mg/L (7 days)[a] | [a] 90 mg/L (7 days) | - |
| | | 2.5 L bioreactor | - | [a] 125 mg/L (9 days) [b] 102 mg/L | [b] 53 mg/L (4 days) |
| *Rhodococcus jostii* pTipQC2-praA | 2,5-pyridine dicarboxylic acid | 50 mL | 80 mg/L (5 days)[a] | [a] 79 mg/L (5 days) | - |
| | | 2.5 L bioreactor | - | [a] 106 mg/L (9 days) [b] 65 mg/L | - |