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Supplementary Information

Efficient synthesis of 2,6-bis(hydroxymethyl)pyridine using whole-cell biocatalysis

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1. Experimental procedures

1.1 General methods

Chemical vendors are specified in Table S1. Chemically-competent *E. coli* NEBxpress were obtained from New England Biolabs. *E. coli* RARE strain was obtained from Addgene. Plasmid pSPZ3 has been described previously[1] and was available in the in-house plasmid collection.

1.2 Media Preparation

For 1 L of medium, components A and B were prepared using the stock solutions in Table S2. Component A consists of 200 mL Salts (5x) and 750 mL ddH_2O mixed sterilized by autoclaving. Component B was prepared by aseptically mixing 20 mL glucose or glycerol (500 g L^{-1} stock), 2 mL magnesium sulfate (1 M stock), 2 mL thiamine (1000x stock), 1 mL trace elements (1000x stock), 1 mL kanamycin (1000x stock), 0.37 mL calcium chloride (1 M stock) and 23.63 mL ddH_2O. Component B (50 mL) is then added to sterilized component A (950 mL) when the temperature of component B had decreased below 35°C to obtain 1 L of ready-to-use medium. The medium was used in shake flasks or in a bioreactor. In the bioreactor, neutral pH was maintained by adding 30% ammonia water.

1.3 Transformation of *E. coli* NEBxpress and cell banking

Cells were transformed with plasmid pSPZ3 (pBR322, KanR) following the manufacturer's recommendations. For long-term storage, an overnight (200 rpm, 37°C, 14 – 16 h) culture from a single colony grown in LB medium (10 g L^{-1} peptone, 10 g L^{-1} NaCl, 5 g L^{-1} yeast extract) was diluted twofold with 50% glycerol in a 1.8 mL cryovial. The vial was stored at -80°C until needed.

1.4 Pre-culture preparation

E. coli NEBxpress/ pSPZ3 were streaked from a glycerol stock on LB agar supplemented with 50 µg mL^{-1} kanamycin. The plate was grown for 12 - 16 h at 37°C until visible colonies were present and was stored in the fridge for no more than another 5 - 7 days prior to further use. Overnight cultures (14 - 16 h) were prepared in 50 mL Erlenmeyer flasks filled with 10 mL of LB medium. The flask was inoculated from a single colony picked from fresh agar plate and grow at 200 rpm, 37°C. In 1 L Erlenmeyer flasks, 100 mL of growth medium as in Table S2 was inoculated with a starting inoculum corresponding to 0.04 grams cell dry weight per liter (g_{CDW} L^{-1}) from the overnight culture in LB. The flask was incubated at 200 rpm, 37°C until a cell biomass concentration corresponding to 1.5 - 1.8 g_{CDW} L^{-1} was reached.

1.5 Shake flask biotransformations

Shake-flask biotransformations were performed in 100 mL Erlenmeyer flasks by innoculating 10 mL of medium supplemented with glucose (see Section 1.2) with an overnight pre-culture (Section 1.4) to a starting OD_{600} of 0.01 (0.0035 g_{CDW} L^{-1}). The cells were propagated at 30°C, 200 rpm OD_{600} of ~0.75 (0.26 g_{CDW} L^{-1}) was reached. At this point the inducer (0.02% dicyclopropyl ketone, DCPK) was added and the bacterial culture was incubated further (70 – 90 min) until an OD_{600} of 1 (0.35 g_{CDW} L^{-1}) was reached. At this point the biotransformation was initiated by adding substrate (between 0 - 5 mM)

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and cultivation proceeded for 6- 8 hours during which samples were collected intermittently and analysed immediately by HPLC.

1.6 Whole-cell biotransformation of 2,6-lutidine in a bioreactor using glucose as carbon source

Bioreactor experiments were carried out on 1 L scale in Minifors Bioreactors (Infors) equipped with 2.5 L glass vessel. In the pilot fermentations, glucose was used as a carbon source. A starting concentration of 10 g L^{-1} was supplied to the medium (see section 1.2) prior to inoculation. An *E. coli* NEB pre-culture (see section 1.4) was added to a final OD_{600} of 0.025 (0.004 g_{CDW} L^{-1}). When inoculated at the said opitical density, the batch phase of the fermentation took roughly 13 hours until complete consumption of the initially provided carbon source. Sterile air was provided at 3 liters per minute (lpm) at an initial stirring speed of 300 rpm. The stirring speed was increased during the batch phase such that every time the dissolved oxygen dropped below 30%, the stirring speed was increase by 50 rpm. Depletion of carbon source was indicated by a sharp increase of dissolved oxygen level in the bioreactor. At this point the stirring speed was set to 1200 rpm. When dissolved oxygen exceeded 80%, continuous glucose feed was intiated at a rate of 5.5 g L^{-1} h^{-1} for one hour and then increased to 11 g L^{-1} h^{-1}. After an additional 90 min, the glucose feed rate was changed to 17.5 g L^{-1} h^{-1} until the end of the biotransformation. Recombinant protein expression was initiated by the addition of 0.02 % DCPK when biomass corresponding to 12.5 g_{CDW} L^{-1} had been formed (approximately 3 hours after start of fed-batch). Once the biomass has reached approximately 17 g_{CDW} L^{-1}, feeding of 2,6-lutidine ***1*** was initiated, using a syringe pump and applying a rate of 1 mL min^{-1} for 1 minute followed by 0.085 ml min^{-1} for an additional 59 min corresponding to a total substrate load of 5.5 g L^{-1}. Samples were taken in regular intervals and the product profile and residual substrate were quantified by HPLC (see section 1.8). The biotransformation was stopped approximately 7 hours after initiation of 2,6-lutidine ***1*** feed at which point the reaction approached completion (i.e. no starting material and only a minimal amount of intermediates were detected in the fermentation broth).

1.7 Whole-cell biotransformation of 2,6-lutidine in a bioreactor using glycerol as a carbon source

When glycerol was used as a carbon source instead of glucose in the bioreactor experiments, the medium (section 1.2) was supplemented with an initial concentration of 5 g L^{-1} glycerol. The reactor was seeded from a pre-culture to achieve a starting OD_{600} of 0.125 (0.04 g_{CDW} L^{-1}). It took roughly 12 hours for the cells to completely consume the initial carbon source. Sterile air was provided at 3.5 lpm and the stirring speed was intitally adjusted on 300 rpm. Stirrer speed ramping was applied during the batch phase such that every time dissolved oxygen dropped below 30%, the stirring speed was increase by 50 rpm. Carbon source depletion was indicated by a sharp increase of dissolved oxygen in the bioreactor. At this point the stirring rate was set to 1200 rpm and when dissolved oxygen exceeded 80%, the fed-batch phase of the fermentation was initiated by feeding glycerol at a rate of 4.8 g L^{-1} h^{-1} for 90 min and then increased to 10 g L^{-1} h^{-1} . When OD_{600} reached 10 (3.5 g_{CDW} L^{-1}), 0.02% (v/v) DCPK was added to the reactor. About 90 min later or when OD_{600} of ~ 14 (5 g_{CDW} L^{-1}) was reached, the biotransformation phase was initiated by feeding of 2,6-lutidine ***1*** using a syringe pump at rate of 1 mL min^{-1} for 1 minute followed by 0.085 mL min^{-1} for an additional 59 min. The reactor was sampled in regular intervals and the product profile was immediately analysed by HPLC (see 1.8). Based on the most recent HPLC measurement, a decision was made on whether a new dose of substrate should be added (approximately every 3 h). In total four substate additions were carried out corresponding to a substrate load of roughly 11.5 g L^{-1}. The biotransformation was stopped approximately 12 hours after initiation of 2,6-lutidine ***1*** feed at which point the reaction approached completion (i.e. no starting material and a minimal amount of intermediates were detected in the fermentation broth). The glycerol feed (which was kept at a constant rate of 10 g L^{-1} h^{-1} since the start of the biotransformation) was intermittently paused and re-started as soon as a sharp increase of dissolved oxygen of more than 5% was observed. Because low whole-cell catalyst load was employed, the dissolved oxygen throughout the whole process was maintained above 50% in the course of the biotransformation.

1.8 HPLC analysis of products and overflow metabolites

Samples were withdrawn at different time points and centrifuged at 15,000 g for 15 minutes to pellet cells. The supernatants were diluted 5- to 50-fold in ddH_2O and analysed by HPLC. 2,6-Lutidine, 2,6-pyridine dimethanol, 6-methyl-2-pyridine methanol, 6-methyl-2-pyridine carboxaldehyde, 6-(hydroxymethyl)pyridine-2-carbaldehyde, 6-methyl-2-pyridinecarboxylic acid were quantified using an Agilent 1200 Infinity HPLC with a diode array detector, equipped with Prontosil 120-5-C18aq column (Bischoff Chromatography) and a Prontosil 120-5-C18aq 10 x 4 mm cartridge (Bischoff Chromatography). Distilled water supplemented with 1% (v/v) formic acid was used as mobile phase. Detection was carried out at 25°C at 270 nm with a flow of 1 mL min^{-1} for 15 minutes. Each injection had a volume of 2 µl. Calibration curves were constructed for all pyridine derivatives using analytical standards.

For the determination of overflow metabolites (acetate, lactate) and residual glycerol over time, samples were clarified by centrifugation (15,000 g for 15 minutes) and the supernatant was used for further analysis in undiluted form. For analysis, Agilent 1200 Infinity HPLC with a refractive index detector was equipped with Metab ACC 300 x 7 mm column (ISERA) with Metab ACC Guard Cartridge. Analysis was carried out using 5 mM H_2SO_4 as mobile phase with injection volume of 20 µL and

a flow of 1 mL min^{-1} for 25 minutes. Calibration curves were constructed for all compounds of interest using analytical standards.

Table S1 Stock medium components

Chemical name	CAS Number	Supplier
2,6-Lutidine, 99%	108-48-5	Merck
2,6-Pyridine dimethanol, 99%	1195-59-1	VioChemicals AG
6-Methyl-2-pyridine methanol, 99%	1122-71-0	Alfa Aesar
6-Methyl-2-pyridine carboxyaldehyde, 98%	1122-72-1	Merck
6-(Hydroxymethyl)pyridine-2-carbaldehyde, 99%	39621-11-9	Merck
6-Methyl-2-pyridinecarboxylic acid, 98%	934-60-1	Merck
Agar, Bacteriology grade	9002-18-0	Applichem
Ammonium chloride (NH_4Cl), 99%	12125-02-9	Roth
Ammonium hydroxide, 30%	1336-21-6	Merck
BD Difco™ LB Broth Powder	n/a	Becton Dickinson
Calcium chloride dihydrate ($CaCl_2*H_2O$), 99%	10035-04-8	Merck
Casamino acids	n/a	BD BactoTM
D(+)-glucose, 99%	50-99-7	Merck
Dicyclopropylketone (DCPK), 95%	1121-37-5	Merck
Dipotassium hydrogen phosphate (K_2HPO_4), 99%	7758-11-4	Roth
Disodium hydrogen phosphate (Na_2HPO_4), 99%	7558-79-4	Roth
Ethylenediaminetetraacetic acid disodium salt (EDTA), 99%	6381-92-6	Chemie Brunschwig AG
Glycerol, 99%	56-81-5	Applichem
Kanamycin sulfate, 99%	25389-94-0	Merck
Magnesium sulfate anhydrous ($MgSO_4$), 99%	10034-99-8	Roth
Potassium dihydrogen phosphate (KH_2PO_4), 99%	7778-77-0	Roth

Sodium chloride (NaCl), 99%	7647-14-5	Merck
Sodium acetate, 99%	127-09-3	Merck
Sodium L-lactate, 99%	867-56-1	Merck
Thiamine hydrochloride, 99%	67-03-8	Merck

Table S2 Stock medium components

Stock	Stock Composition
Calcium chloride	1 M in H_2O, sterile filtration
Magnesium sulfate	1 M in H_2O, sterile filtration
Salts (5x)	22.5 g L^{-1} KH_2PO_4
	31.5 g L^{-1} Na_2HPO_4
	11.5 g L^{-1} $(NH_4)_2SO_4$
	9 g L^{-1} NH_4Cl
	5 g L^{-1} citric acid in H_2O, pH 7.0 with NaOH, autoclaved
Trace elements (1000x)	0.5 g L^{-1} $CaCl_2$. $2H_2O$;
	0.18 g L^{-1} $ZnSO_4$. $7H_2O$,
	0.1 g L^{-1} $MnSO_4$,
	20.1 g L^{-1} Na_2-EDTA,
	16.7 g L^{-1} $FeCl_3$ $6H_2O$,
	0.16 g L^{-1} $CuSO_4$. $5H_2O$ in H_2O
	sterile filtered
Thiamine (1000x)	10 g L^{-1} in H_2O, sterile filtered
Kanamycin (1000x)	50 mg mL in H_2O
Glucose/Glycerol	500 g L^{-1} and 50% - 70% (w/w) in H_2O
Dicyclopropylketone (DCPK)	Use undiluted (working conc. 0.01 – 0.05% (v/v))
Ammonium hydroxide solution, 30%	Use undiluted

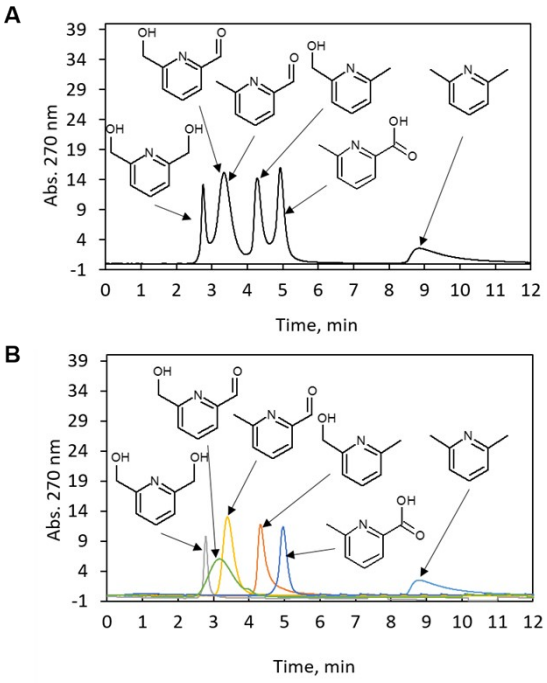
A
B
Abs. 270 nm
Time, min
39
34
29
24
19
14
9
4
-1
0 1 2 3 4 5 6 7 8 9 10 11 12
OH
N
O

Figure S1 HPLC chromatograms of analytical standards analysed as a mixture (A) and individually (B).

Figure S2 Time course of the bioconversions of 2,6-lutidine ***1*** (A) and 6-methyl-2-pyridinemethanol ***2*** (B).

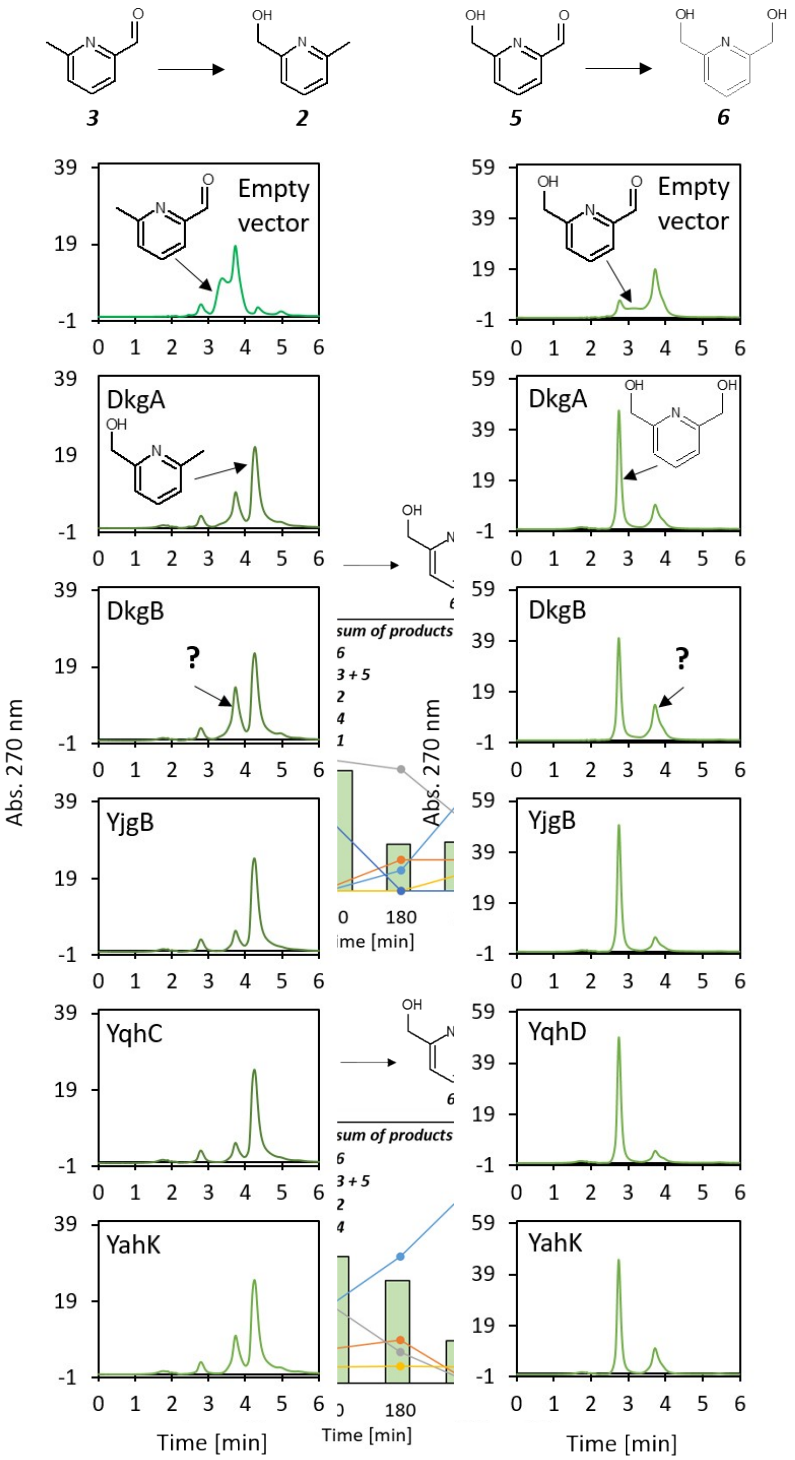

Figure S3 HPLC chromatograms of 2 h incubation of *E. coli* RARE strain harboring an empty vector or expressing reductases DkgA, DkgB, YjgB, YqhC and YahK. The ability of the reductase-deficient strain *E. coli* RARE to reduce aromatic aldehydes ***3*** and ***5*** is restrored by any of the five proteins.

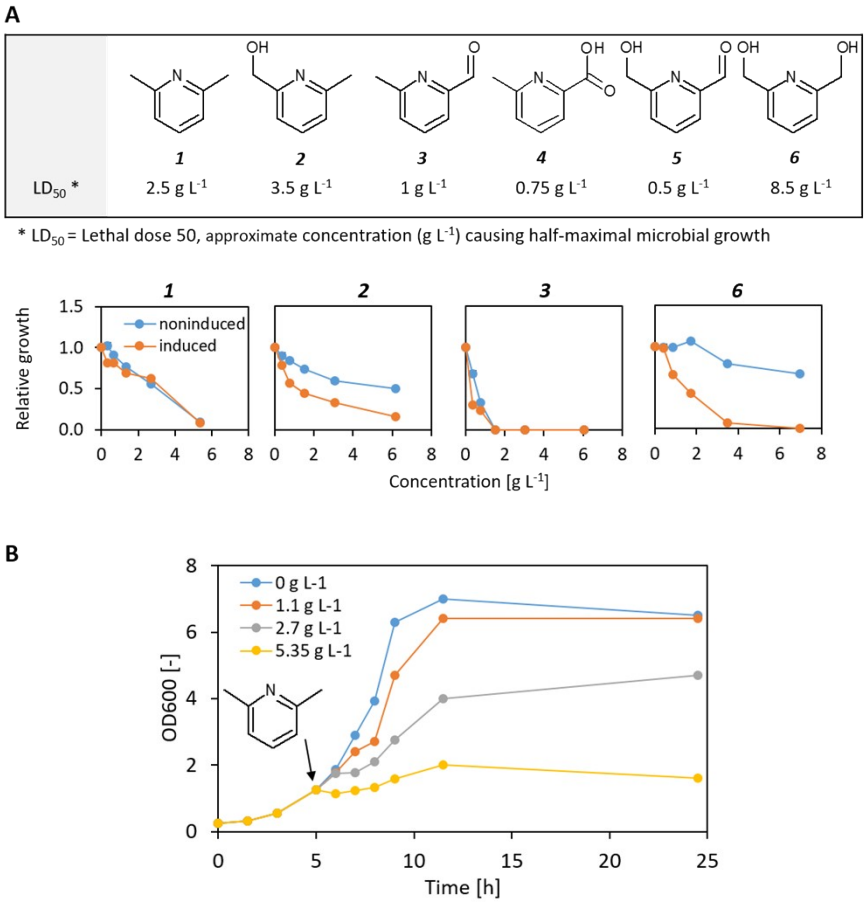

Figure S4 Toxicity of compounds ***1*** – ***6*** to *E. coli*.

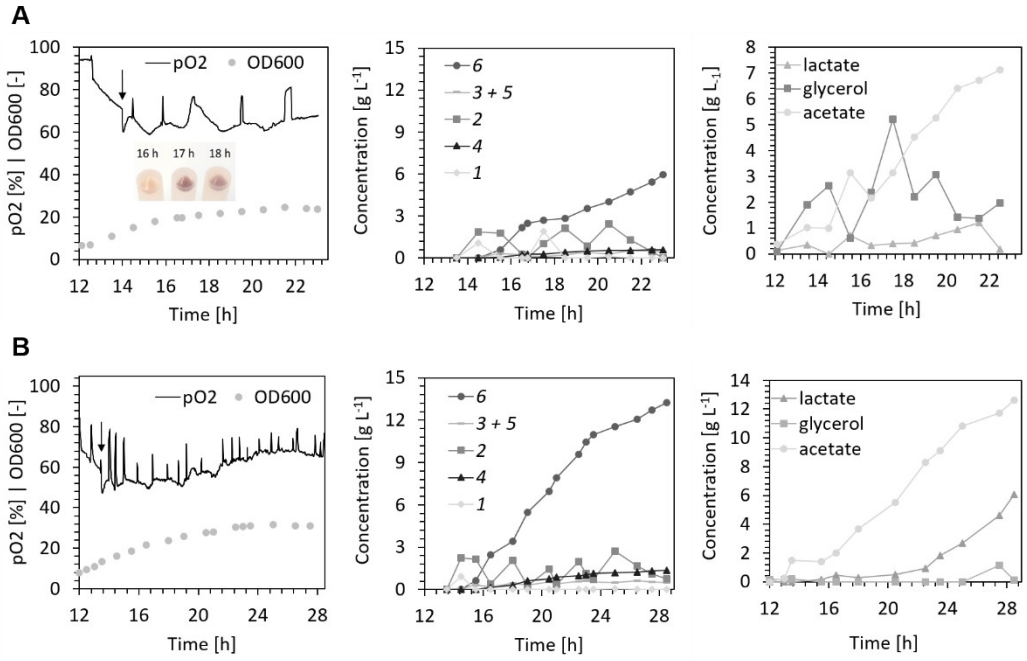

Figure S5 Dissolved oxygen (pO_2), optical density (left panel) and concentrations of reactant and products (middle panel) and overflow metabolites (right panel) of a biotransformation with (**A**) suboptimal and (**B**) optimised substrate supply scheme.

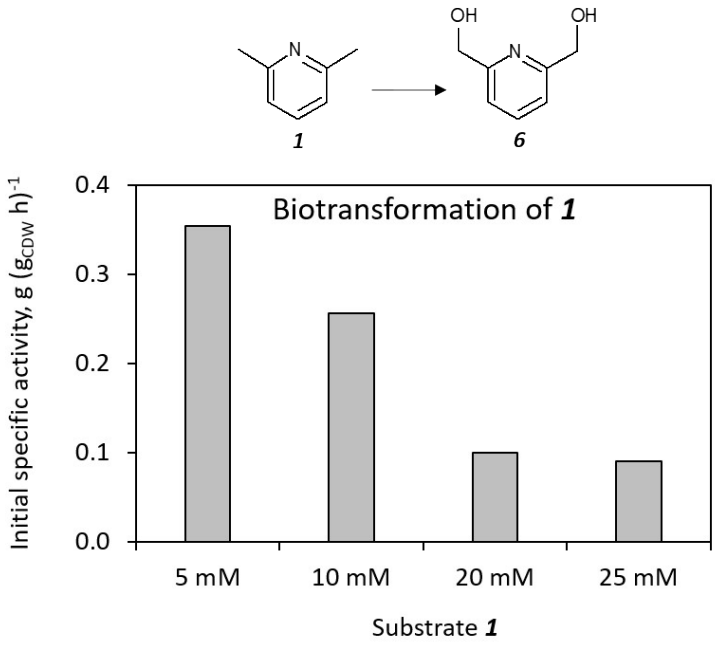

Figure S6 Initial (60 min) conversion rate of lutidine ***1*** at increasing initial substrate concentrations.

Figure S7 HPLC chromatograms of conversion of 6-methyl-2-pyridinecarboxilic acid ***4*** at the beginning of an XMO catalysed biotransformation (0 min) and after long term incubation (1020 min).