Exploring the Selective Demethylation of Aryl Methyl Ethers with a *Pseudomonas* Rieske Monooxygenase

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Biocatalytic dealkylation of aryl methyl ethers is an attractive reaction for valorization of lignin components, as well as for deprotection of hydroxy functionalities in synthetic chemistry. We explored the demethylation of various aryl methyl ethers by using an oxidative demethylease from *Pseudomonas* sp. HR199. The Rieske monooxygenase VanA and its partner electron transfer protein VanB were recombinantly coexpressed in *Escherichia coli* and they constituted at least 25% of the total protein content. Enzymatic transformations showed that VanB accepts NADH and NADPH as electron donors. The VanA–VanB system demethylates a number of aromatic substrates, the presence of a carboxylic acid moiety is essential, and the catalysis occurs selectively at the *meta* position to this carboxylic acid in the aromatic ring. The reaction is inhibited by the byproduct formaldehyde. Therefore, we tested three different cascade/tandem reactions for cofactor regeneration and formaldehyde elimination; in particular, conversion was improved by addition of formaldehyde dehydrogenase and formate dehydrogenase. Finally, the biocatalyst was applied for the preparation of protocatechuic acid from vanillic acid, giving a 77% yield of the desired product. The described reaction may find application in the conversion of lignin components into diverse hydroxyaromatic building blocks and generally offers potential for new, mild methods for efficient unmasking of phenols.

Introduction

Phenolic groups occur in numerous natural bioactive compounds and are important functionalities in the synthesis of specialty chemicals. However, because of their high reactivity, protection of phenolic groups is often required prior to further synthetic steps, through the preparation, for example, of stable and unreactive methyl ethers. Although such methylation strategies are highly effective for the protection of phenols, the subsequent deprotection of the inert methyl ethers generally requires harsh reaction conditions such as the use of strong acids or bases. Alternative approaches that allow more facile demethylation in mild environments are therefore desired.

As well as in synthetic chemistry, new demethylation protocols might also be exploited in the valorization of compounds derived from lignin. This complex natural polymer is rich in aryl methyl ether groups deriving from the precursors coniferyl and sinapyl alcohol. In the past decade, great progress has been made with regard to the development of catalytic strategies for lignin depolymerization to produce fine chemicals. Independently of the method used, the majority of these processes result in aromatic compounds that contain the guaiacyl or syringyl moiety. O-Demethylation reactions would generate hydroxylated aromatics that might serve as building blocks for the production of value-added chemicals through diverse synthetic strategies. The demethylation of guaiacyl derivatives, for example, leads to catechols, which are present in numerous pharmaceuticals, pesticides, and flavors. Alternatively, catechols are converted by oxidation into muconic acid, which can be hydrogenated to adipic acid derivatives to be used in polymer synthesis.

Green and environmentally friendly solutions to O-demethylation can in principle be addressed by biocatalysis. However, although demethylases are widely present in nature, they have barely been explored for biocatalytic applications. Reasons for this might be the complex architecture of some of these multicomponent systems, as well as the requirement for expensive and/or delicate cofactors (e.g., cobalamin- and/or tetrahydrofolate-dependent enzymes). An example of such a demethylation system is cytochrome P450 CYP199A4 from *Rhodopseudomonas palustris*, a heme-dependent monooxygenase that catalyzes the oxidative demethylation of 4-methoxybenzo-
ic acid to 4-hydroxybenzoic acid.\textsuperscript{13–14} The enzyme was recombinantly expressed in \textit{Escherichia coli} together with electron transfer proteins, and whole cells were employed for the transformation of veratric acid into vanillic acid.\textsuperscript{13} CYP199A4 only demethylates methoxy groups at the para position (to a carboxylic acid) of the phenyl ring. It tolerates other substituents at the ortho and meta positions, whereas the carboxylate moiety is critical for substrate recognition.\textsuperscript{15} Interestingly, the single Ser244Asp mutation in the active site of the enzyme abolished the carboxylate dependence and extended the substrate scope to benzene derivatives.\textsuperscript{16}

Lignin harbors most of its O-methyl groups at the 3- or the 3- and 5-positions (meta) on the aromatic ring, and accordingly demethylases with different regiospecificity have raised interest. The tetrahydrofolic acid (THF)-dependent O-demethylation LigM from \textit{Sphingobium} sp. SYK-6 was recently characterized and its crystal structure was solved.\textsuperscript{17–20} This enzyme (MetE) requires THF cofactor in tenfold molar excess over substrate in order to achieve good conversion.\textsuperscript{18} Rosini and co-workers proposed a tandem reaction involving a methionine synthase for cofactor recycling.\textsuperscript{19} This enzyme (MetE) converts 5-methyl-THF back into THF by transfer of the methyl group to l-homocysteine, forming l-methionine. With this developed biotransformation, the authors were able to obtain 5 mM proteocatechic acid (PCA, 1b, Table 2, below) with only 0.1 mM THF added. Nevertheless, in view of the high cost of the cofactor, the availability of THF-dependent \textit{meta}-demethylation systems could offer an attractive alternative. One example is the newly discovered cytochrome P450 from \textit{Amycolatopsis} sp. ATCC 39116, which catalyzes the \textit{O}-demethylation of guaiacol and its analogues at the \textit{meta} position of the aromatic ring.\textsuperscript{21}

Early genetic studies indicated that bacteria that degrade lignin-derived components and dimeric model compounds express an additional type of demethylases to LigM, with demethylation of vanillic acid (1a, Table 2, below) to PCA being catalyzed by a two-component system consisting of VanA and VanB.\textsuperscript{22–24} Sequence similarities suggest that VanA belongs to the Rieske non-heme iron-dependent monooxygenases and that it is a homologue of a demethylating dicamba (3,6-dichloro-2-methoxybenzoic acid) monooxygenase (DdmC), the structure and catalytic mechanism of which are known.\textsuperscript{25–27} Rieske proteins are distinguished by the presence of an iron-sulfur cluster [2Fe–2S] that functions as an electron-transfer and electron-storage organometallic complex. The active site contains a single free iron ion that is involved in the binding and activation of molecular oxygen, allowing substrate hydroxylation.\textsuperscript{28,29}

One example of such a two-component system is the \textit{O}-demethylase from \textit{Streptomyces} (StVanA) partially characterized by Nishimura and co-workers.\textsuperscript{30} They explored the substrate scope of StVanA after recombinant protein expression, using whole-cell biocoversions with \textit{E. coli}. The work showed the potential value of StVanA–VanB as a whole-cell biocatalyst, but also underlined the need to improve expression of soluble protein. Difficulties with heterologous production were also reported for other iron–sulfur proteins; these, together with protein instability, constitute major limitations for the exploitation of Rieske oxygenases (ROs).\textsuperscript{31}

A demethylase system that is potentially attractive for applied biocatalysis is present in \textit{Pseudomonas} sp. HR199 (DSM 7063), a strain capable of degrading lignin-derived compounds to products of lower molecular weight.\textsuperscript{32} It transforms eugenol into vanillin, which is further oxidized to vanillic acid and then converted into PCA, and finally metabolized by dioxygenase-mediated cleavage of the aromatic ring.\textsuperscript{33} The gene sequence of the monooxygenase component of the vanillate demethylation system from \textit{Pseudomonas} HR199 [Ps(HR199)VanA] was identified in the late 1990s, and the enzymatic activity was confirmed after recombinant expression in \textit{E. coli}.\textsuperscript{34} However, the properties of the protein and the selectivity of the enzyme have never been thoroughly investigated.

Here we describe the optimization of recombinant coexpression of \textit{Pseudomonas} HR199 VanA and VanB in \textit{E. coli}. Subsequently, the catalytic properties of the two-component system were determined with lyophilized cells and its suitability for biocatalytic applications was explored. Finally, VanA–VanB was employed in multienzyme cascade reactions for effective cofactor regeneration and by-product removal.

**Results and Discussion**

**Amino acid sequence analysis**

A multiple sequence alignment of the monooxygenase VanA from \textit{Pseudomonas} HR199 (UniprotKB O05616) with StVanA and DdmC was performed (Figure S5 in the Supporting Information). DdmC was the only appreciable hit obtained after a Blast search of Ps(HR199)VanA against the PDB database. Ps(HR199)VanA has 36% identity with StVanA and 34% with DdmC. Key residues and motifs were pinpointed in the amino acid sequence by comparison to DdmC as reference sequence. Rieske domains are characterized by two conserved His and two conserved Cys residues that coordinate the [2Fe–2S] cluster. Cys47–His49 and Cys66–His69 are the first and second ligand pairs distinguishable as the typical C\textit{XH} and C\textit{OXH} motifs. With regard to the active site, the putative residues involved in non-heme Fe ion coordination are His156, His161, and Asp298. The so called bridging Asp residue (Asp153) is also conserved.\textsuperscript{25,35} This observation suggests a multimeric structure of Ps(HR199)VanA and, similarly to other RO enzymes, that the electron transport chain acts \textit{intermolecularly} from the [2Fe–2S] cluster of monomer A to the active site of monomer B. From the results of D’Ordine and co-workers relating to DdmC, the proposed electron-transfer chain in Ps(HR199)VanA involves \textit{cHis49, cHis69, bAsp153, bHis156, and bHis161}.\textsuperscript{25} The multiple sequence alignment suggests a different architecture of the active site and substrate coordination between DdmC and vanillate oxygenase. The key residues His251, Asn230, and Trp285, for the coordination of the carboxylic moiety in DdmC, for example, are not conserved in Ps(HR199)VanA, consistently with what has been reported by Dumitru and co-workers.\textsuperscript{24} This is explainable in terms of the different substrates and dealkylation position of the two en-
zymes (DdmC catalyzes demethylation at the position ortho to the carboxylic acid of the aromatic ring).

VanB (UniprotKB O05617) is the partner reductase enzyme, indispensable for the supply of electrons to the Rieske domain of VanA. A protein Blast search of its amino acid sequence against the PDB database retrieved only one hit with a high score (37% identity and 100% query coverage): the phthalate reductase from Burkholderia cepacia. A pairwise alignment of the two amino acid sequences was performed (Figure S6). A conserved domain search (CDD database) identified a flavin- and a nicotinamide-cofactor-binding domain, consistently with a role in hydride transfer from NAD(P)H to the flavin cofactor during catalysis. Additionally, the sequence alignment showed a conserved ferredoxin domain recognizable by the four conserved Cys residues responsible for the coordination of the [2Fe–2S] cluster (Cys266, Cys271, Cys274, and Cys304). These results suggested that, similarly to other non-heme oxygenases enzymes, the partner reductase Ps(HR199)VanB reduces the flavin cofactor by oxidation of NAD(P)H and then transfers the electrons to the ferredoxin domain, which likely interacts with the Rieske domain of VanA and provides electrons necessary for the catalysis.

Coexpression of VanA and VanB in E. coli

To obtain appreciable amounts of the enzymatic demethylation system, the VanA and VanB proteins were coexpressed in E. coli BL21 Star (DE3) by using a pET-derived vector named pE2T_Ps(HR199)VanA_VanB. To boost transcription, each coding DNA sequence was inserted downstream of an independent T7 promoter. Preliminary protein purification experiments by affinity chromatography failed, due to the partial loss of the [2Fe–2S] complexes and low yields. Additionally, a detrimental effect of an N-terminally fused His$_6$ tag on the activity of the purified enzymes was observed (data not shown). Therefore, the two proteins were coexpressed in their native form, without any affinity tag. Various cultivation conditions for iso-propyl-$eta$-d-thiogalactopyranoside (IPTG)-induced protein production were examined; this revealed that high-level soluble overexpression of both proteins was best achieved when additional iron and sulfur sources were added, suggesting that maturation of the iron–sulfur clusters is a critical step in protein synthesis (Experimental Section). After optimization, the two enzymes were well expressed and constituted approximately 25% of the total soluble protein in cell lysates, as indicated by two prominent bands at 39 kDa (VanA) and 35 kDa (VanB) that were observed upon SDS-PAGE analysis (Figure S1).

The high-level recombinant coproduction made it possible to avoid protein isolation and to prepare a stable biocatalyst by lyophilization of whole cells. After centrifugation and washing, 14.2 g wet cell weight (WCW) was obtained per liter of culture, which corresponded to 3.0 g dry cell weight (DCW) per liter of culture after freeze-drying. The obtained powder containing the biocatalyst VanA–VanB was used for all following experiments.

Biocatalytic reaction characterization

Because information on the biochemical properties of vanillate O-demethylases is scarce, we examined the biocatalytic activity of the Pseudomonas HR199 VanA–VanB system under various sets of reaction conditions. In these experiments, 5 mM vanillic acid (1a) was chosen as the model substrate and the formation of protocatechuic acid (PCA, 1b) was studied. The conversion was carried out over 24 h and both substrate and product concentrations were determined by HPLC (Tables 1 and S1).

![Scheme 1. Proposed cascade for O-demethylation of aryl methyl ethers catalyzed by the Rieske non-heme monoxygenase coupled with a cofactor regeneration system.](image)

**Table 1. Initial screening of demethylation reaction conditions with 7.5 mg mL$^{-1}$ of freeze-dried cells after 4 h of incubation.**

<table>
<thead>
<tr>
<th>Reaction conditions</th>
<th>PCA analytical yield [%]$^\text{a}$</th>
</tr>
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<tbody>
<tr>
<td>1 0.1 mM NADH, 0.1 mM FeSO$_4$, 10% DMSO, regeneration system</td>
<td>58.8 ± 2.6</td>
</tr>
<tr>
<td>2 0.1 mM NADPH, 0.1 mM FeSO$_4$, 10% DMSO, regeneration system</td>
<td>44.4 ± 0.6</td>
</tr>
<tr>
<td>3 0.1 mM FeSO$_4$, 10% DMSO, regeneration system</td>
<td>20.5 ± 0.2</td>
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<tr>
<td>4 0.5 mM NADH, 0.1 mM FeSO$_4$, 10% DMSO, regeneration system</td>
<td>30.7 ± 0.2</td>
</tr>
<tr>
<td>5 0.1 mM NADH, 0.1 mM FeSO$_4$, 10% DMSO</td>
<td>7.1 ± 0.8</td>
</tr>
<tr>
<td>6 0.1 mM NADH, 10% DMSO, regeneration system</td>
<td>46.7 ± 2.0</td>
</tr>
<tr>
<td>7 0.1 mM NADH, 1 mM FeSO$_4$, 10% DMSO, regeneration system</td>
<td>60.2 ± 0.3</td>
</tr>
<tr>
<td>8 0.1 mM NADH, 0.1 mM FeSO$_4$, regeneration system</td>
<td>53.3 ± 1.9</td>
</tr>
<tr>
<td>9 control 1: no cells (conditions as in 1)</td>
<td>≤ 2</td>
</tr>
<tr>
<td>10 control 2: E. coli BL21 (DE3) Star (conditions as in 1)</td>
<td>≤ 2</td>
</tr>
<tr>
<td>11 control 3: PCA instead of vanillic acid (conditions as in 1)</td>
<td>80.5</td>
</tr>
<tr>
<td>12 control 4: no cells, PCA instead of vanillic acid (conditions as in 1)</td>
<td>80.6</td>
</tr>
<tr>
<td>13 control 5: E. coli BL21 (DE3) Star, PCA instead of vanillic acid (conditions as in 1)</td>
<td>92</td>
</tr>
</tbody>
</table>

*a* Determined by HPLC. *b* Regeneration system: 10 μM PTDH and 10 mM phosphate. *c* Measured in triplicate. *d* Single experiment.

Initial experiments were performed by adding rehydrated freeze-dried cells containing VanA–VanB, NAD(P)H, and the phosphite dehydrogenase (PTDH) cofactor regeneration system to the reaction mixture (Scheme 1, reaction conditions: Table 1, entries 1, 2, and 4). The oxidoreductase VanB accept-
ed both NADPH and NADH as reducing electron donors, with a preference for the latter. This dual cofactor acceptance is in accordance with what was previously reported for other *Pseudomonas* species. [38] Because omission of NADH and NADPH from reaction mixtures drastically reduced conversion of vanillic acid (Table 1, entry 3), we conclude that addition of the reduced nicotinamide cofactor is essential to obtain high yields of the demethylated product PCA and that both NADH and NADPH are incorporated by rehydrated freeze-dried cells. In situ cofactor regeneration is commonly adopted for biocatalytic applications of nicotinamide-cofactor-dependent oxidoreductases and avoids the need for stoichiometric addition of costly NAD(P)H (Scheme 1). As expected, O-demethylation coupled with PTDH for cofactor regeneration allowed the reaction to proceed beyond stoichiometric consumption of the reduced cofactor, whereas when cofactor regeneration was omitted the level of formation of PCA was much lower (Table 1, entry 5).

The addition of an excess of iron to the reaction mixture showed a beneficial effect on the overall conversion, in agreement with the involvement of the metal in the catalysis through oxygen activation. Finally, VanA–VanB tolerated 10% DMSO, which is advantageous in cases of poorly soluble substrates. On these bases, we used 0.1 mM NADH, 0.1 mM FeSO₄, 10% DMSO, and the PTDH-based cofactor regeneration system for further characterization experiments (Table 1, entry 1).

The described transformations showed a molar imbalance between the consumption of the substrate and the production of PCA (Table S1), suggesting partial conversion of vanillic acid by unknown *E. coli* enzyme(s) or partial loss of the catechol due to its instability or further enzymatic degradation. To examine these possibilities, a series of controls were designed. Initially, the reaction mixture containing 5 mM vanillic acid was incubated without the biocatalyst, either by omission of cells or by using the *E. coli* strain lacking VanA–VanB (Table 1, entries 9 and 10). The latter reaction showed 37% substrate depletion but no specific product was identified (Table S1). Additionally, partial consumption of the product was observed when 5 mM PCA was added to the mixture instead of vanillic acid (Table 1, entries 11, 12, and 13; Table S2), thus confirming the occurrence of some product loss through the action of non-biocatalytic processes by endogenous *E. coli* enzymes and compound instability.

**Oxygen sensitivity**

Anaerobic conditions might represent an optimal environment for the stability of iron–sulfur proteins, but the demethylation reaction uses molecular oxygen as a reactant that obviously cannot be left out. To examine the possibility that the enzymatic demethylation might be restricted by detrimental effects of molecular oxygen on the activity and stability of the VanA–VanB system, we examined the biocatalytic formation of PCA in the presence of antioxidants at various concentrations (Figure 1).

In the absence of an antioxidant, a dramatic loss of enzyme activity was observed after 2 h of preincubation at 30 °C, with only 29% of the initial activity retained and less than 1 mM PCA was detected. The biocatalyst was essentially inactive after 4 h of preincubation (Figure 1A). The addition of dithiothreitol (DTT) substantially improved the stability for the first 2 h, and more than 80% of the initial activity was retained (Figure 1A). The beneficial effect of the antioxidant was also visible on examination of the effective conversion (Figure 1B). Higher product formation was achieved when glutathione (GSH) or DTT were added. The best result was recorded in the presence of 5 mM DTT, and 80% analytical yield was obtained (Figure 1B). These results suggested that oxygen has a large effect on protein stability.

**Substrate scope**

To investigate the substrate specificity of VanA, a panel of 19 compounds was selected (Scheme S1). Each biocatalytic re-
action was set up with cofactor regeneration as outlined in Scheme 1 and incubations were carried out for 7 h.

At first, the biocatalyst was tested for the conversion of vanillin, vanillyl alcohol, acetovanillone, and methyl vanillate into their corresponding catechols. No activity was detected; this suggests the requirement for a carboxylic moiety for substrate recognition. This result is in accordance with previous in vivo studies on Acinetobacter, Streptomyces, and Pseudomonas fluorescens vanillate demethylases. The presence of longer aliphatic chains in bulkier compounds was unfavorable as well: homovanillic acid, ferulic acid, dihydroferulic acid, sinapyl acid, and vanilpyruvic acid were not accepted and no product formation or substrate consumption was observed.

Table 2 reports the obtained analytical yields of a series of methoxy aromatic acids and related products observed by HPLC. VanA showed high activity for 3-methoxy-4-methylbenzoic acid (2a), veratric acid (3a), and m-anisic acid (4a), with ≥85% of each substrate being converted into the corresponding phenol. Depletion of veratric acid was comparable with that of vanillic acid, but the amount of product was increased by about 20% (Table S3). This improvement can be explained in terms of the higher stability of iso vanillic acid (3b) vs. the catechol functionality, resulting in better product recovery. Additionally, a control reaction showed that 3a is not significantly subjected to breakdown by endogenous E. coli enzymes, whereas up to 32% consumption of vanillic acid was observed in the absence of the biocatalyst VanA–VanB (Tables S1 and S4).

In spite of the presence of two distinct methoxy functionalities, the conversion of 3a led to only one product, thus implying that the oxidative demethylation occurs regiospecifically at position 3 of the aromatic ring (meta). To confirm the high regioselectivity of VanA, meta-, ortho-, and para-anisic acid (4a, 5a, and 6a) were assayed. No product formation was observed after incubation with 5a and 6a, whereas 4a showed 86.8% analytical yield. Interestingly, when both positions 3 and 5 bore a methoxy substituent (7a), 3,4-dihydroxy-5-methoxybenzoic acid (8a) was obtained in 60% yield, but the reaction did not go any further and 8a was not accepted as substrate. This result distinguishes Ps(HR199)VanA from both AcVanA and StVanA. The first cannot convert 7a, whereas StVanA is less specific and generates a mixture of 8a and gallate.

Biocatalytic production of PCA
To investigate the applicability of the biocatalytic reactions described here, a semipreparative bioconversion was set up as depicted in Scheme 1. An amount of 10 mg of vanillic acid was added to 12 mL reaction mixture with 360 mg of freeze-dried cells (Experimental Section). After the vanillic acid was completely consumed, the product was isolated and purified, and 7.1 mg of PCA (77% yield) was obtained as white crystals; the product identity was confirmed by NMR (Figures S13 and S14).

Multienzymatic cascades
The by-product of oxidative demethylation is formaldehyde, which is formed in stoichiometric amounts. Hibi and co-workers showed by deletion studies in E. coli that formaldehyde can be toxic to the cells, thus suggesting the necessity of a rapid detoxification machinery for efficient biotransformation. This raises the question of whether the formaldehyde is toxic to the overall cell machinery or specifically affects VanA–VanB ac-
tivity. To investigate its possible impact on enzymatic activity, 2 mM formaldehyde was added to the reaction mixture together with 5 mM vanillic acid. As a result, the multienzymatic system was inhibited and conversion dropped by \( \approx 50\% \) (Figure 2A).

A molecule regeneration concomitant with elimination of formaldehyde. The cascade could be further extended by introduction of formate dehydrogenase (FDH), driving the reaction to completion by converting the formaldehyde into \( \text{CO}_2 \). This system would also favor the reaction workup and purification of the phenolic product.

These proposed enzymatic cascades in which PTDH-driven cofactor regeneration was omitted were tested for the synthesis of PCA from 5 mM vanillic acid and compared with the standard VanA–VanB system with NADH regeneration by PTDH (Figure 2). Good conversion was achieved with both of the designed cascades without the need for extensive optimization (one-step cascade with FADH 56.5 ± 1.9% analytical yield, two-step cascade with FADH + FDH 84.4 ± 3.3% analytical yield after 4 h reaction time). However, a five times higher concentration (than the previously adopted 0.1 mM) of NADH was required. This can be explained in terms of the different nature of the cofactor recycling machinery: FADH and FDH activity are dependent on formaldehyde formation by VanA–VanB, whereas PTDH reduces the cofactor by phosphite oxidation that is present in excess from the beginning. Conversions stopped after 2 h, probably due to VanA–VanB instability. Whereas VanA–VanB–FADH seems comparable to VanA–VanB–PTDH, a significant improvement was observed for the two-step aldehyde oxidation (VanA–VanB–FADH–FDH), and 4.1 mM PCA was obtained (Figure 2C).

Conclusion

The two-component enzymatic system VanA–VanB from *Pseudomonas* sp. HR199 was recombinantly coexpressed in *E. coli* in good amounts. The resulting biocatalyst promoted oxidative demethylation of methoxy ethers, showing high conversion and high selectivity for small aromatic acids with methoxy groups at the meta position to a carboxylic acid moiety on the aromatic ring. VanA–VanB conveniently accepts both NADH and NADPH as cofactor. After a semipreparative-scale reaction, it was possible to isolate PCA in good yields. Finally, as proof of concept, multienzymatic cascades were established, demonstrating in situ cofactor regeneration coupled with simultaneous by-product removal from the reaction mixture.

The results suggest that VanA–VanB has good potential as biocatalyst for the removal of methoxy groups from lignin degradation products and other compounds, offering a valuable green tool for lignin valorization and synthetic chemistry. For future practical applications, structural studies would help enzyme engineering for the improvement of stability and substrate specificity of the biocatalyst.

Experimental Section

General: Solvents and organic substrates were purchased from Sigma–Aldrich, TCI Europe, and Abcr, GmbH unless otherwise specified. NAD\(^+\) and NADH were acquired from Carl Roth. Molecular biology reagents were purchased from New England Biolabs or ThermoFischer Scientific. DNA isolation and purification kits were obtained from Qiagen. Primers were obtained from Sigma.

Figure 2. Formaldehyde inhibition and cascade reaction. A) Time courses of enzymatic demethylation of 5 mM vanillic acid under standard conditions (green) and with addition of 2 mM formaldehyde (white). Dashed lines indicate vanillic acid depletion and solid lines product formation. B) Cascade reaction for removal of formaldehyde together with cofactor regeneration. C) Bioconversion of 5 mM vanillic acid with three different NADH regeneration systems and 15 mg mL\(^{-1}\) of freeze-dried cells. Standard conditions with PTDH (green), one-step cascade with 6 U mL\(^{-1}\) FADH (blue), and two-step cascade with 6 U mL\(^{-1}\) FADH and 10 U mL\(^{-1}\) FDH (purple); 0.5 mM NADH was employed for the conversion with FADH and FDH. Dashed lines indicate vanillic acid depletion and solid lines product formation.
fragments were purchased from Integrated DNA Technology (gBlock) and Thermofisher Scientific (GeneArt strings). Phosphite dehydrogenase was recombinantly expressed and purified as previously described.[41]

Cloning: The synthetic genes of VanA (UniProtKB O05616) and VanB (UniProtKB O05617) optimized for E. coli were purchased from GenScript. BsaI sites suitable for Golden Gate cloning were inserted up and downstream of the coding sequence (CDS) by PCR. pET28(b)·GG was developed as a universal and versatile vector for easy and highly efficient cloning of multiple fragments by the Golden Gate method. The vector was prepared by Gibson cloning with pET28(b)·as template and a stuffer fragment was inserted. The two CDSs flanked by BsaI sites and a designed synthetic fragment containing an additional T7 promoter were cloned into the pET28·GG vector in a one-pot reaction by use of Golden Gate cloning. The final product pET2_Ps(HR199)VanA·VanB was transformed in E. coli NFIG 10 Beta and positive clones were selected on lysogeny broth (LB) supplemented with kanamycin (50 mg mL⁻¹). Details are reported in the Supporting Information.

Coexpression of VanA and VanB and biocatalyst preparation: A previously reported protocol was optimized for the heterologous coexpression of VanA and VanB in E. coli.[31] Competent cells of E. coli BL21 Star (DE3) were transformed with the pET2_Ps(HR199)VanA·VanB vector. A sample of regenerated culture (200 mL) was immediately added to LB medium (50 mL) supplemented with kanamycin (50 mg mL⁻¹) of culture) followed by overnight growth at 37 °C. Then, pre-culture (5 mL) was transferred to terrific broth (TB) medium (0.5 L) prepared with a double amount of glycerol (0.8%, v/v), and cultivation was done with kanamycin selection at 37 °C under strong agitation. At OD₆₀₀ = 1, cells were cooled at 20 °C for 20–30 min and then protein expression was induced by addition of IPTG (0.5 mM), FeSO₄ (0.1 mg mL⁻¹), Fe⁺₆ ammonium citrate (0.1 mg mL⁻¹), and cytochrome (1 mM). Protein production was performed at 20°C for 20 h with moderate shaking. At the end of the cultivation, cells were harvested by centrifugation (2975 g, 4 °C, 10 min) and washed once with Tris·HCl (pH 7.5, 50 mM). Next, the cell pellet was resuspended in Tris·HCl (pH 7.5, 100 mM, 250 mL) and shock frozen drop by drop in liquid nitrogen. Finally, frozen cell drops were dried for 48 h (ice condenser CHRIST Alpha 2–4 LDplus). The obtained fluffy powder (0.5 g of dried cells g⁻¹ of powder) was kept at −20°C or at −80°C for longer storage.

Biocatalytic reaction: Stock solutions of substrate (50 mM) in DMSO were prepared and stored at 4 °C. Reaction mixtures (2 mL) were set up as follows unless otherwise indicated. Freeze-dried cells expressing VanA–VanB (15, 30, or 60 mg, corresponding to 30, 60, or 120 mg of powder) were weighed into a 15 mL glass vial and allowed to rehydrate in Tris-HCl (pH 7.5, 100 mM) containing DTT (10 mM) for 15–20 min at 30 °C and 100 rpm (shaker: Innova 40, New Brunswick). Afterwards, FeSO₄ (0.1 mM), phosphate (10 mM), PTDH (0.01 mM), and methoxy substrate (5 mM) were added to the mixture. The enzy reaction was initiated by the addition of NADH (0.1 mM) and incubated at 30 °C at 100 rpm. Each experiment was performed in duplicate.

HPLC analysis: At different time points 200 μL of the reaction mixture was quenched with acetonitrile (Boom, B.V., 100%, 200 μL). The samples were centrifuged for 10 min at 16000 g at 4 °C, and supernatants (200 μL) were transferred to an HPLC vial. The analysis was performed with Jasco 980-31 HPLC apparatus, a Jasco 2075 Plus UV detector, and a Jasco MD-2010 Plus PDA detector. An Altima HP C₁₈ 5 μm length 250 mm i.d. 4.6 mm column was used for sample separation. Mobile phase eluent A: sodium acetate (2 mM), formic acid (0.05%); mobile phase eluent B: acetonitrile (100%). Method: 0.5 mL min⁻¹ flow; 5–10 μL injection; 5 min isocratic 95% A/5% B; 15 min gradient to 5% A/95% B; 5 min isocratic 5% A/ 95% B; 5 min gradient to 95% A/5% B; 10 min isocratic 95% A/ 5% B. Substrates and products were detected at 254 nm and 280 nm.

Preparative scale and product isolation: A 12 mL reaction mixture was set up in a 50 mL bottle. Biocatalyst powder (720 mg, 30 mg of freeze-dried cells expressing VanA–VanB per mL of reaction mixture) was rehydrated in Tris-HCl (pH 7.5, 100 mM, 7.31 mL) and DTT (10 mM, 0.120 mL of 1 M stock) at 30 °C and 100 rpm. Next, the mixture was supplemented with FeSO₄ (0.1 mM), phosphate (10 mM), and PTDH (0.025 mM). Finally, the reaction was initiated with NADH (0.2 mM) and vanillic acid (10 mg). The mixture was incubated overnight at 30 °C and 100 rpm. Complete substrate consumption was confirmed by HPLC. The reaction mixture was then quenched by addition of an aqueous solution of citric acid (10%), adjusted to pH 3, and extracted with ethyl acetate (three times). The combined organic extracts were washed with brine, dried with anhydrous MgSO₄, filtered, and concentrated under reduced pressure. The crude product was purified by flash chromatography on silica gel (SiliaFlash P60 40–63 μm, 230–400 mesh, SiO₂, dichloromethane/methanol 95:5). NMR spectra were recorded with an Agilent Technologies 400 NMR (¹H NMR at 400 MHz, ¹3C NMR at 100 MHz).

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Conflict of Interest

The authors declare no conflict of interest.

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