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A One-Pot, Whole-Cell Biocatalysis Approach for Vanillin Production using Lignin Oil

Ivana Marić,[+a] Yiming Guo,[+a] Maximilian J. L. J. Fürst,[a] Korneel Van Aelst,[b] Sander Van den Bosch,[b] Mario De Simone,[c] Lígia O. Martins,[c] Bert F. Sels,[b] and Marco W. Fraaije[a,*]

[a] Molecular Enzymology, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands
E-mail: m.w.fraaije@rug.nl
[b] Center for Sustainable Catalysis and Engineering, Dept. of Microbial and Molecular Systems, KU Leuven, Celestijnenlaan 200F, 3001 Leuven, Belgium
[c] Instituto de Tecnologia Química e Biológica António Xavier, Universidade NOVA de Lisboa, Av. da República, 2780-157 Oeiras, Portugal

[+] These authors contributed equally.

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Abstract: Vanillin is a popular and versatile flavor compound, almost entirely produced from petroleum-derived phenol by a multi-step chemical synthesis. The process is hazardous to the environment and unsustainable for its fossil oil usage. Therefore, developing environmentally friendly, efficient, and sustainable routes to biobased vanillin is essential. Here, we report on vanillin production from 4-*n*-propylguaiacol (4PG), one of the main components in lignin oil obtained through reductive catalytic fractionation (RCF) of soft wood, by employing recombinant *Escherichia coli* cells. Conversion is based on the expression of two engineered oxidative enzymes: a 4-*n*-propylguaiacol oxidase and an isoeugenol dioxygenase. A high yield of vanillin, 66% from 4PG in RCF lignin oil was achieved through rounds of optimisation of the whole-cell conversion process. This high-performance strategy was readily scaled up to produce vanillin at an unprecedented 18% and 3% yield based on lignin oil and spruce wood respectively. The whole-cell bioconversion process shows good tolerance even at high loadings of starting material, showcasing the robustness and applicability of the employed biocatalysts. This work paves the way for further development towards the efficient production of high-titer biobased vanillin using depolymerised lignin as the feedstock.

Keywords: vanillin; lignin; biocatalysis; oxidase; dioxygenase

Introduction

Vanillin (4-hydroxy-3-methoxy benzaldehyde) is among the most "tasteful" and universally appreciated aromatic chemicals worldwide. It is extensively used as a flavor for food and beverages and a fragrance ingredient in perfumes and cosmetics. It is even seen as an appealing platform chemical for synthesising materials, fine chemicals, and pharmaceutical products.[1,2] Natural vanillin (1200–4000 USD/kg)[1] is extracted from cured vanilla beans harvested from the plant *Vanilla plantifolia.* Vanillin produced from cured vanilla beans is costly and covers less than 1% of the global vanillin market (Figure 1).[1–4] In contrast, synthetic vanillin obtained from petroleum-derived guaiacol satisfies more than 85% of the total demand at a highly accessible price (10–20 USD/kg).[1] The remaining part is met by fermentative production or by

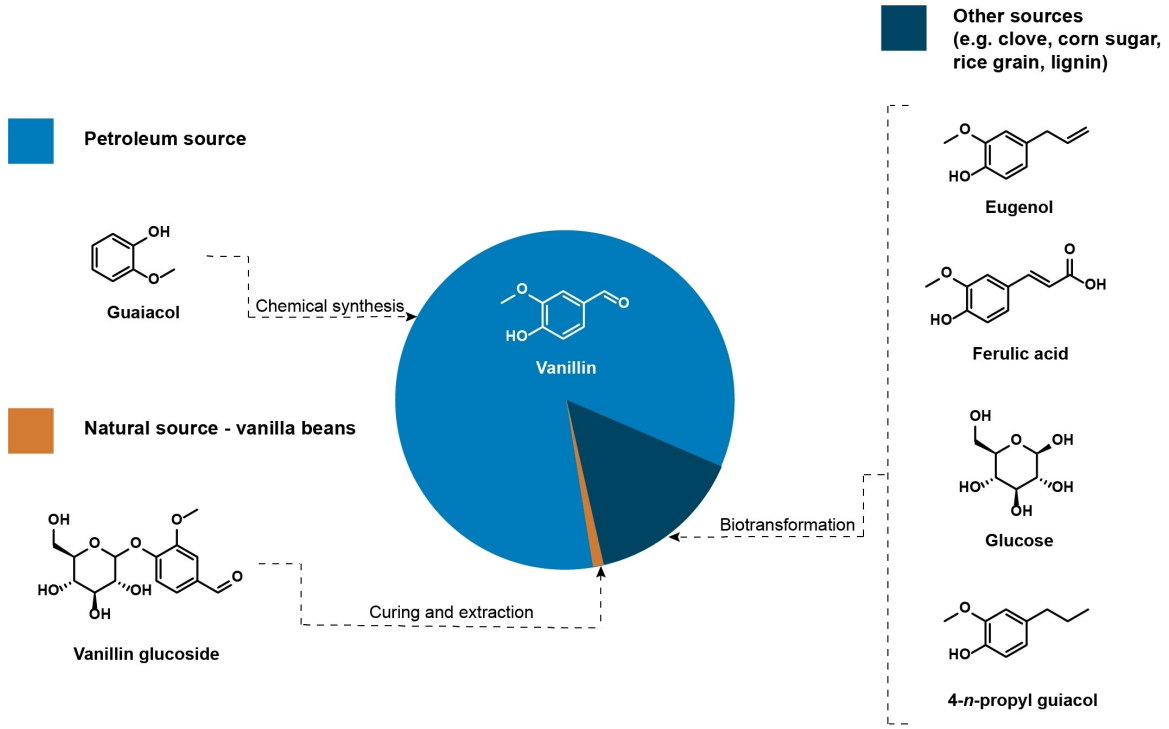


Figure 1. Distribution of global vanillin production from different sources.

chemical and/or microbial conversion of kraft lignin.[2] Although the cost of petroleum-based vanillin is relatively low, the environmental effect of the production process and strict regulations in the food and pharmaceutical industries limit its appeal.[4] Furthermore, the increasing costs for CO_2 make it attractive to develop a more sustainable production process from renewable carbon resources.

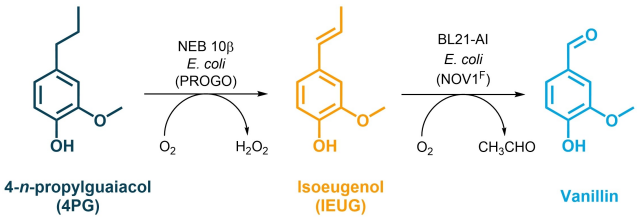
Lignin is the most abundant source of natural aromatics and, thus, is a promising renewable starting material for producing biobased vanillin. Indeed, vanillin has been produced from kraft lignin for many years through various oxidative depolymerisation processes.[5] Yet, the chemical process from kraft lignin involves harsh conditions, limiting a further increase in production capacity, hence asking for a more environmentally friendly and efficient process. Recent developments in lignin depolymerisation approaches may offer new opportunities for an alternative vanillin production process. In particular, the emerging lignin-first biorefining approach that employs reductive catalytic fractionation (RCF), results in low-molecular-weight oligomers and, importantly, phenolic monomers in high yield and selectivity (21–50%).[6,7] The generated lignin-derived monomers, 4-alkylguaiacols and syringols, can be valorised into high-value end-products,[8] and we envision that vanillin could be among them. Moreover the global warming potential (GWP) of RCF lignin oil as alternative substrate for the here investigated enzymatic vanillin production has the potential to outperform the GWP of phenol as substrate for today's dominant multi-step petrobased vanillin route, via catechol, guaiacol and vanillyl alcohol as intermediates.[8b]

For converting an RCF-produced lignin-derived monomer, such as 4-*n*-propylguaiacol, into vanillin, the alkyl moiety should be modified. Previously reported chemical approaches demonstrate the possibility of activating the side chain through the formation of *p*-quinone methide from benzyl alcohols under harsh conditions.[9] Interestingly, similar to these chemical reactions, a group of flavin-containing oxidases discovered in fungi and bacteria, represented by vanillyl alcohol oxidase (VAO),[10] catalyse the hydroxylation and dehydrogenation of 4-alkylphenols via such *p*-quinone methide intermediate. Recently, we have engineered eugenol oxidase, a bacterial VAO, into a 4-*n*-propylguaiacol oxidase (PROGO) for the selective production of isoeugenol from 4-n-propylguaiacol (4PG).[11] This affords a biocatalyst by which a dominant product obtained by RCF of lignocellulose can be converted into a vanillin precursor. Isoeugenol can be converted into vanillin by a non-heme-dependent dioxygenase, NOV1.[12] Wild-type NOV1 is involved in catalysing the cleavage of double bonds of stilbenes, yielding various phenolic aldehydes. With the aim to develop a biocatalytic cascade with PROGO to produce vanillin from 4PG, we have recently carried out enzyme redesign study of NOV1 to improve its ability to convert isoeugenol into vanillin. This has

resulted in the NOV1-S283F mutant (NOV1^F).[12] For each engineered enzyme, PROGO and NOV1^F, we could demonstrate that the respective conversion could be efficiently performed using recombinant cells, expressing the respective biocatalyst. Whole-cell biocatalysis has been drawing the strong interest of industrial application, due to the cost contribution of cell lysis and enzyme purifi-cation.[13,14] Furthermore, the use of whole cells can shield the expressed enzymes from potentially harm-ful surroundings and remain in the natural enzymatic environment for efficient conversion.[15]

With both biocatalysts available, PROGO and NOV1^F, we set out to develop a biocatalytic process to convert lignin-derived 4PG into vanillin. Specifically, we developed a whole-cell cascade to produce vanillin from 4PG using two types of *Escherichia coli* cells expressing PROGO and NOV1^F, respectively (Scheme 1). We opted for two separate types of cells expressing the corresponding biocatalysts, rather than a single *E. coli* strain expressing both enzymes. This makes it easy to find the best ratio for the two biocatalysts that makes full use of their catalytic potential. It also allows to use the best host and growth conditions for each biocatalyst in terms of expression levels. Furthermore, we employed one-pot approach having in mind further process scale up, and related downstream processing. In general, cascade reactions performed in one pot have a potential to minimize downstream processing steps and significantly reduce produced waste.[16]

The reaction parameters such as the amounts of cells, pH, temperature, and timing of addition of biocatalysts were optimised employing commercially available 4PG. To illustrate the full potential of our approach, RCF lignin oil was used in bioconversion under optimised conditions. The resulting vanillin was isolated and characterised to confirm the utility of the developed process in the valorisation of lignin.



Scheme 1. The developed whole-cell cascade for vanillin production from 4PG.

Results and Discussion

Activity of Individual Whole-Cells Toward 4PG and Isoeugenol

In separate and previously reported studies, we have demonstrated that *E. coli* NEB 10β cells harboring PROGO[11] (P cells) and *E. coli* BL21-AI cells containing a NOV1^F[12] (N cells) catalyse the conversion of 4PG to isoeugenol, and isoeugenol to vanillin, respectively. This study investigated the feasibility of a one-pot conversion of 4PG to vanillin, utilising P cells and N cells (Scheme 1). Before combining the two types of cells in one reaction mixture, we probed the performance of the individual cell types on their corresponding substrates at 25 °C and in the presence of 10% (v/v) DMSO as a cosolvent. It was shown that DMSO could enhance the activity of eugenol oxidase[17] (the oxidase used to engineer PROGO), while the effect on NOV1 had not been previously investigated. The quantity of cells (expressed in OD_{600}) was varied to determine the optimal amount for reaching a complete or close to complete conversion. After 24 h, a >95% conversion of 50 mM 4PG was achieved using P cells at an OD_{600} of 75 (Figure 2a, Figure S3a). Lower amounts of P cells resulted in less efficient yet considerable conversions, indicating that lowering the OD_{600}, alongside optimisation of other reaction conditions (temperature, pH, reaction time), could lead to satisfactory yields. The N cells showed activity on commercially available, E-isoeugenol already at OD_{600} 2. Production of vanillin yielded approximately 85% when the OD_{600} value was above 10 (Figure 2b, Figure S3b). Despite increasing the amounts of N cells to OD_{600} 50, the vanillin yield remained at around 85%.

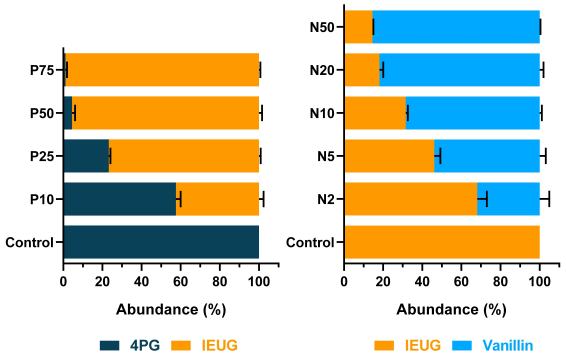


Figure 2. Conversions of 50 mM substrate of a) 4PG by P cells at different OD_{600} values (10, 25, 50, and 75); and b) isoeugenol (IEUG) by N cells at different OD_{600} values (2, 5, 10, 20, and 50). Reactions were performed in 50 mM KPi buffer, pH 7 in the presence of 10% (v/v) DMSO at 25 °C for 24 h. All experiments were performed in duplicate.

Previous reports indicate that P cells catalyse the formation of a mixture of *E*- and *Z*-isoeugenol.[11] To anticipate the actual reaction conditions of the cascade, we followed the depletion of a mixture of isoeugenol isomers (*E*/*Z* = 75/25) catalysed by N cells over time. We hypothesised that the ratio of unreacted isoeugenol, remaining after the cascade, should remain constant if the N cells are equally active on both isomers. The NMR analysis of the crude reaction mixture at different time intervals points toward the preference of NOV1^F for the E- isomer, as the ratio of *E*/*Z* changes from 3 to 0.8 after 24 h (Figure 3a, Figure S14). To further support this evidence, a docking study using the crystal structure of NOV1^F revealed a minimal clashing between the CH_3 moiety of the alkyl chain of the *Z*-isomer and F283 (Figure 3b). It appears that *Z*-isoeugenol can adapt within the active site of the enzyme, thus being still a possible substrate but with hampered reactivity when compared to the *E*-isomer. Thus, we expected that the conversion of both isomers would take place, however, more readily on *E*-isoeugenol, which might have an effect on the final vanillin yield.

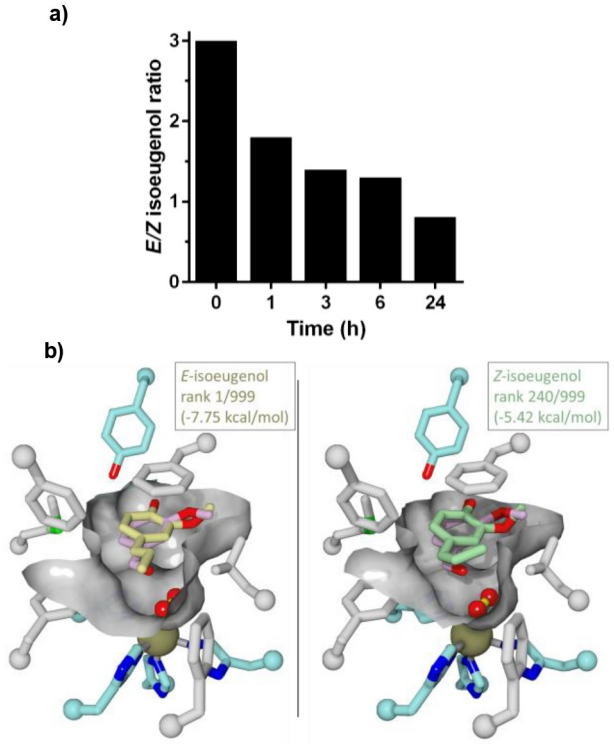


Figure 3. Substrate preference of NOV1^F. a) NMR analysis of the whole-cell conversion of 50 mM *E*/*Z*-isoeugenol (starting ratio *E*/*Z* = 75/25). N cells (OD_{600} 10) were used in 50 mM KPi buffer, pH 7, with 10% (v/v) DMSO as a cosolvent, at 25 °C; b) Docking result for NOV1^F with the *E*- (pale yellow left) and *Z*- (pale green, right) isomers of isoeugenol. Active site residues that were flexible during docking are white, frozen residues cyan, and the catalytic iron and oxygen are in gold and red, respectively. For comparison, the active site-bound vanillin from PDB:5 J55 is shown in purple. While *E*-isoeugenol docked readily into the active site and with −7.75 kcal/mol binding energy is the highest ranking docking result out of 999 VINA runs, the *Z*-isomer docks mostly in non-catalytic positions (where the ligand's alcohol oxygen atom was more than 3.5 Å from the catalytic oxygen of Y101). The best (< 3.5 Å O–O distance) catalytic pose of *Z*-isoeugenol binds with 5.42 kcal/mol as rank 240 – significantly less than the top-ranked (non-catalytic) pose, which binds with −7.17 kcal/mol.

Optimising the Productivity

For optimal performance, each step of the cascade must be tuned while keeping an eye on its compatibility. Different temperatures, buffer systems, and pH values for the two individual biocatalysts were analysed and compared to identify the most suitable reaction conditions to perform the two-step cascade process in one pot. Temperature and compartmentalisation of enzymes within the cell are relevant factors in whole-cell conversions. As temperature increases, the reaction rate increases until the stability limit of the biocatalyst, improving production rate and yield. The cellular confinement protects enzymes from the surrounding environment, stabilising the enzyme's catalytic performance.[18,19] To use these advantages, we determined isoeugenol and vanillin productivity in whole-cell conversions at higher tem-peratures. It has been reported that isolated eugenol oxidase exhibits a relatively low conversion rate at 25 °C.[17] Thus, as expected, isoeugenol productivity was significantly improved at temperatures increasing from 25 °C to 37 °C, to an almost complete conversion of 4PG at 37 °C (Figure 4a, Figure S5a). In contrast, varying the temperature of the whole-cell conversions of isoeugenol using N cells revealed that the highest vanillin yield was obtained at 25 °C (Figure 4b, Figure S5b).

Conversions of 4PG and isoeugenol were also performed in TRIS buffers with different pH values (pH 7 to 9). Both conversions showed a higher yield of the corresponding products at higher pH values. The pH effect on the isoeugenol conversion resulted in a 24% improvement when increasing the pH from 7 to 9 (Figure 4c, Figure S6a), while a 17% enhancement of 4PG conversion was observed (Figure 4d, Figure S6b). However, the conversions performed in 50 mM KPi buffer at pH 7 afforded a similar yield as conversions using a TRIS buffer at pH 9 (Figure 4c, 4d, compare bottom and top bars). To avoid or reduce to minimum the cell lysis which could hamper sample manipulation and considering the resemblance of the yields at pH 7 (KPi) and pH 9 (TRIS), we continued our study using KPi buffer (50 mM, pH 7) at 25 °C.

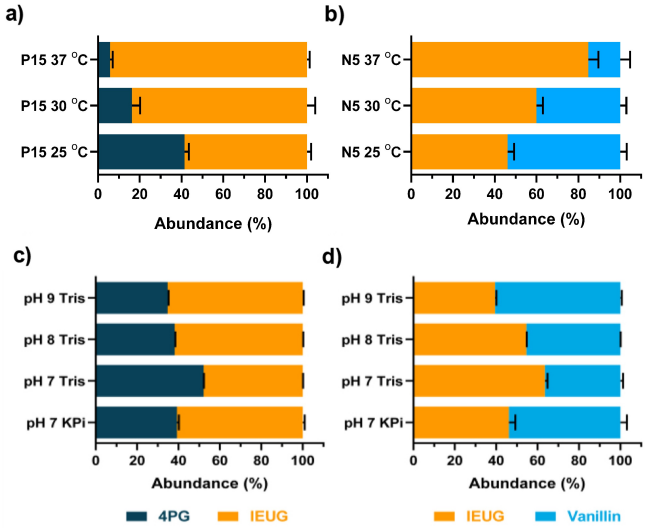


Figure 4. Temperature and pH profiles. Conversions of 50 mM solutions of a) 4PG by P cells at OD_{600} 15; and b) conversions of isoeugenol by N cells at OD_{600} 5 in 50 mM KPi buffer, pH 7, at three different temperatures: 25 °C, 30 °C, and 37 °C. Conversions of 50 mM solutions of c) 4PG by P cells at OD_{600} 15; and d) conversions of isoeugenol by N cells at OD_{600} 5 at 25 °C, in four buffer systems at 50 mM concentration: pH 7 KPi, pH 7 TRIS, pH 8 TRIS and pH 9 TRIS. Reactions were performed in the presence of 10% (v/v) DMSO for 24 h. All experiments were performed in duplicate.

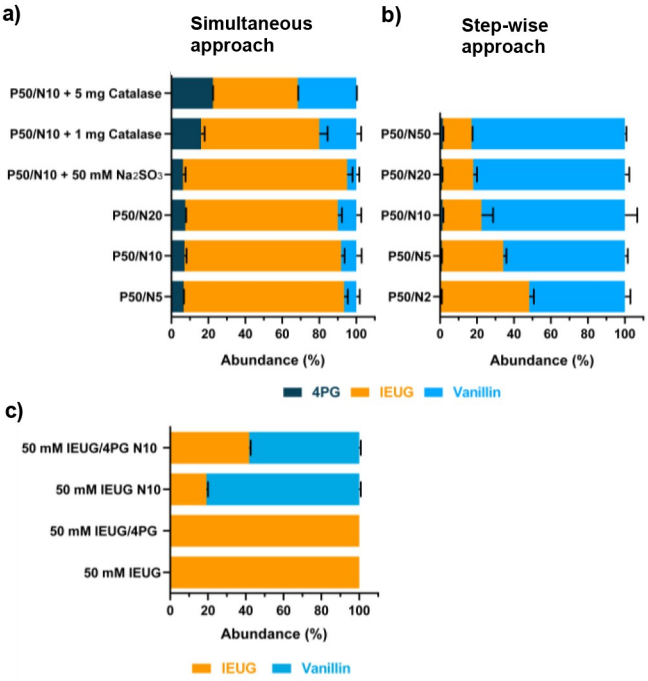


Figure 5. Biocatalytic cascade reactions using P and N cells in one pot. a) One-pot conversions of 4PG to vanillin using a whole-cell multi-enzyme cascade in a simultaneous approach. Regardless of the ratio between the two cell types, vanillin production did not exceed 10%. Sodium sulfite and catalase were supplied to quench the excess of H_2O_2. b) One-pot conversions of 4PG to vanillin using a whole-cell multi-enzyme cascade in a step-wise fashion. Different amounts of N cells were added after almost full conversion of 50 mM 4PG was reached utilising P cells at OD_{600} 50. Reactions were shaken at 150 rpm at 25 °C for 24 h for a simultaneous approach, and at 37 °C for 24 h, followed by the change to 25 °C for 24 h for the step-wise method. c) Effect of 4PG on the conversions of 50 mM isoeugenol by N cells: conversions of 50 mM isoeugenol in presence and absence of 4PG were performed by N cells (OD_{600} 10) in 50 mM KPi buffer, pH 7, with 10% (v/v) DMSO as a cosolvent, at 25 °C for 24 h. All experiments were performed in duplicate.

Cascade Conversions: Simultaneous vs. Stepwise Addition of Whole Cell Catalysts

Analysis of the individual catalysts, temperature, and pH/buffer preferences, was followed by putting the two cell types together in one pot to catalyse the desired cascade reaction. To enhance the efficiency of the multi-enzyme process, we aimed to optimise the stoichiometry of the biocatalysts by keeping the concentration of substrate, 4PG (50 mM), and amount of P cells (OD_{600} 50) constant while varying the quantity of N cells (OD_{600} 5, 10 and 20). Under the given experimental conditions (50 mM KPi buffer, pH 7, 10% (v/v) DMSO, 25 °C), combining the two cell types in different ratios slightly influenced the oxidation of 4PG (Figure 5a, Figure S7a) compared with conversions where only P cells were used. However, the vanillin yield significantly decreased in the cascade reaction, compared to an 85% yield in the transformation of isoeugenol by N cells alone. Given that sufficient dioxygen was supplied, we speculated that the by-product, hydrogen peroxide, may inhibit the performance of the N cells. Theoretically, the biocatalytic oxidation of 50 mM 4PG affords 50 mM of hydrogen peroxide, which might exceed the quenching ability of naturally present catalase in cells.[19,20] Therefore, 50 mM sodium sulfite was added to the reaction mixture containing P and N cells at OD_{600} 50 and 10 to diminish the potentially damaging effect of hydrogen peroxide. Introducing the chemical quencher boosted the oxidation of 4PG, but no improvement in vanillin production was observed. The acceleration of the first step could be caused by protecting cells against oxidative stress[21] and/or providing a relatively rapid dioxygen regeneration from the decomposition of H_2O_2. The significant decrease in vanillin production could be related to the oxidation of sodium sulfite, which can inhibit the activity of $NOV1^F$, an iron-containing dioxygenase.[22,23] Following this hypothesis, the biological alternative, 1 mg of catalase (corresponding to the decomposition of 2–5 mM H_2O_2 per minute, at pH 7 and 25 °C), indeed improved the yield of vanillin, confirming the toxicity of excess hydrogen

peroxide accumulating in the reaction. However, when 5 mg catalase was introduced, the amount of vanillin produced increased only slightly compared to the expected five times higher yield. This demonstrates that formed hydrogen peroxide plays only a minor role in limiting the conversion of isoeugenol to vanillin. Another possible explanation for the inefficiency of the one-pot approach is (partial) substrate inhibition of $NOV1^F$ by 4PG (Figure 5c, Figure S8). Isoeugenol and 4PG are very similar compounds, only differing in the propyl moiety. Therefore, 4PG may also bind in the active site of $NOV1^F$ as a substrate analogue, competing with isoeugenol binding.[24] Thus, in the absence of a sufficient amount of isoeugenol at the beginning of the cascade, 4PG can act as a strong competitive inhibitor resulting in poor transformation.

As the results of simultaneous cascade reactions indicate that the two types of cells interfere with each other's activity, a step-by-step strategy was used to achieve a one-pot biocatalytic production of vanillin. In such an approach, all reaction steps of the cascade are still performed in one pot but are separated in time. Furthermore, separating the consecutive reactions in time allowed us to apply different temperatures in each step to match the optimal conditions of the independent biocatalysts, thus, maximising the production of the desired product. To start the cascade, P cells were added to a 50 mM 4PG. The N cells were introduced in an equal volume after 24 h. At the same time, the reaction temperature was decreased from 37 °C to 25 °C, while shaking was continued. The newly added biocatalyst was allowed to react for another 24 h before analysis. Gratifyingly, without extra elimination of hydrogen peroxide, a high yield of vanillin (80%) was obtained when using P cells at an OD_{600} of 50 and N cells at an OD_{600} of 10, while only a small portion of isoeugenol remained unreacted and all 4PG has been converted (Figure 5b, Figure S7b). Clearly, the stepwise approach was successful in boosting the conversion of 4PG in vanillin, probably by eliminating the inhibiting effect of 4PG on $NOV1^F$ activity.

In general, economically viable biotechnological production of any compound requires high substrate loading while maintaining the biotransformation capability of the system. Therefore, we investigated the feasibility of our biocatalytic cascade reaction at higher substrate concentrations. This revealed that when using optimised reaction conditions, 92% of 100 mM 4PG was converted, while with 260 mM 4PG, only 69% was converted to give a mixture of isoeugenol and vanillin (Figure 6, Figure S9). Despite the difference in yields, the obtained vanillin production was 9.9 g/L (for 100 mM) and 8.7 g/L (for 260 mM), values that are similar to that obtained for 50 mM 4PG conversions (6.1 g/L). The obtained values are within the average for previously reported procedures using isoeugenol as substrate, while other biotechnological approaches and substrates remain inferior to our process.[25] To improve the production at high substrate concentrations, one could consider using higher biocatalyst loading. Yet, this may also add complications such as very viscous reaction mixtures, hampered oxygen exchange, and arduous agitation. It will complicate the production process and increase the downstream processing costs.[26]

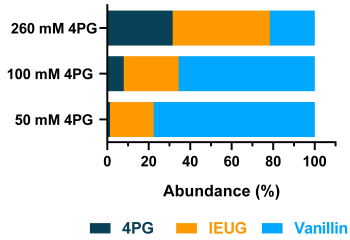


Figure 6. One-pot conversions of 50, 100, and 260 mM 4PG in KPi buffer (50 mM, pH 7) with 10% (v/v) DMSO using a whole-cell, multi-enzyme cascade in a step-wise fashion. To catalyse the first step of the process, P cells (OD_{600} 50) were employed, and N cells (OD_{600} 10) were used in the second step. The reaction was shaken at 150 rpm at 37 °C for 24 h, followed by the change to 25 °C for 24 h.

One-Pot Conversion of Lignin Oil

After optimising the reaction conditions on a model system, pure 4PG, we shifted our attention to a more complex starting material: a raw depolymerised lignin oil obtained by RCF on spruce wood. Using the established procedure, half a gram of RCF lignin oil, containing 27.1% (wt) 4PG, resuspended in KPi buffer (50 mM, pH 7) and 10% (v/v) DMSO, was first reacted with P cells (OD_{600} 25) for 24 h at 37 °C, followed by a conversion by N cells (OD_{600} 10) for 24 h at 25 °C. In parallel, the conversion of another half gram of oil was tested under similar conditions without the cosolvent DMSO. DMSO was originally used to promote the solubility of 4PG. Nevertheless, we hypothesised that the relevant component of the lignin oil, 4PG, is soluble enough for effective conversion by P cells. Indeed, regardless of the cosolvent's presence, 4PG in the mixture was converted to yield 15% of isoeugenol and 66% of vanillin within 48 h. Although there is no evident effect of the cosolvent on the conversion, we note that the use of DMSO was more practical in handling lignin-derived oil. The one-pot conversion results in a pre-purification vanillin yield of 18% based on RCF lignin oil and 3% based on the initial spruce feedstock (see ESI), an estimated 4-fold higher yield compared to current industrial wood-based vanillin production processes. The relative increase for the post-purification vanillin yield is expected to be even larger due to the lack of a homogeneous metal-catalyst

in the here presented vanillin product stream. It is worth noting that the one-pot conversion of pure 4PG using the previously optimised settings for the conversion on a small scale resulted in an approximate 90% yield of vanillin (Figure 7, Figure S10).

Notably, the obtained conversion yields using oil produced by RCF as a starting material were lower than those obtained using pure and commercially available 4PG. However, it should be taken into account that the applied lignin oil comprises about 32% phenolic monomers (27.1% being 4PG), alongside lignin-derived dimers and small oligomers (Mw < 1000 g/mol).[27] Other structurally similar monomers such as 4-ethylguaiacol or 4-propanolguaiacol can be potential enzyme inhibitors, while the higher oligomers are possibly toxic to the cells. Yet, despite the complexity of the reaction mixture, the biocatalysts remained remarkably efficient showing promise to valorise the lignin oil from lignocellulose into a value-added chemical with a satisfactory yield.

Finally, we aimed to simplify the process of bioconversion. To do so, cells, after the growth phase and in their native media were considered ready-to-use biocatalysts. Following a previously devised procedure for the step-wise production of vanillin, half a gram of lignin oil in the presence of DMSO as a cosolvent was mixed with P cells in LB medium for 24 h at 37 °C.

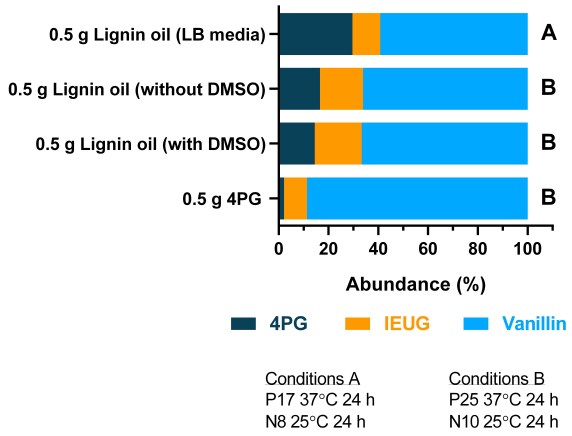


Figure 7. One-pot conversions of commercial 4PG and lignin-derived oils using a whole-cell, multi-enzyme cascade in a step-wise fashion under different experimental conditions. One-pot conversions of 0.5 g lignin oil or 4PG in KPi buffer (50 mM, pH 7). For conversions of 0.5 g lignin oil, the effect of 10% (v/v) DMSO as cosolvent was tested. To catalyse the first step of the process, P cells at OD_{600} 25, and N cells at OD_{600} 10 were used in the second step. A conversion of 0.5 g RCF oil was performed using P and N cells at OD_{600} 17 and OD_{600} 8, respectively, resuspended in the growth media, i.e. LB media. All the reactions were shaken at 150 rpm at 37 °C for 24 h, followed by the change to 25 °C for 24 h.

After 24 h, N cells, also in LB medium, were added and conversion was continued for 24 h at 25 °C. Both types of cells were used at their final OD_{600} values: OD_{600} 17 for P cells and OD_{600} 8 for N cells, which are lower than what was found to be the optimum for the highest vanillin production. Nonetheless, vanillin was produced in 60% yield, which is comparable to results obtained for KPi buffered systems (Figure 7). The successful conversion using cells expressing the corresponding enzymes in growth media demonstrates the robustness and utility of this process and encourages the translation of this biobased laboratory protocol into a viable industrial process.

To isolate and fully characterise vanillin produced through the biocatalytic cascade, 1 gram of RCF lignin oil containing 27.1% (wt) of 4PG (279 mg, 1.7 mmol) was reacted for 48 h in one pot to produce the desired product, vanillin. The reaction mixture was quenched with ethyl acetate, and the organic residue was purified by column chromatography, followed by recrystallisation. The first purification step yielded 40% (99 mg, 0.7 mmol) of vanillin, while the NMR analysis indicated that the isolated product contained traces of impurities (Figure S11a, Figure S12 top). Then, the same sample was recrystallised, which significantly reduced the yield of vanillin (10%, 26 mg, 0.2 mmol), while the purity increased (Figure S11b, Figure S12 bottom). We propose that an interplay of factors determines the efficacy of the employed isolation procedure: (i) retention of the product in the cells or cell debris; (ii) suitability of the extraction solvent to dissolve the desired product, and omitting the other components of lignin oil; and (iii) efficiency of the purification method. Note that this protocol was in no way optimized and should purely be regarded as a first proof-of-concept. Further development of the purification process is required, building further upon industrially existing best practices.

At the same time, the NMR analysis of unreacted isoeugenol, obtained through column chromatography of the ethyl-acetate extract, sheds some light on the reasons behind its incomplete conversion to vanillin. The integration of corresponding CH_3 signals of isoeugenol isomers indicates that the *Z*-isoeugenol is the more dominant component of the mixture (Figure S11c, Figure S13), which is in agreement with NMR and docking studies on the activity of N cells towards *E*/*Z*-isoeugenol (Figure 3). To achieve a full conversion of isoeugenol generated in the first step of the cascade, most likely, an improved, engineered variant of NOV1 is needed.

Conclusion

Lignin is an abundant natural polymer and thus an attractive renewable starting material. Recent developments on reductive catalytic fractionation (RCF) of

lignocellulose biomass offers relatively uniform phenolic monomers at a high yield and selectivity, supported by positive TEA and LCA, hence providing opportunities for follow-up chemistry to high-value chemicals.[8] In this study, a biocatalytic cascade for the production of vanillin from RCF lignin-derived 4-*n*-propylguaiacol (4PG) was developed using a combination of *E. coli* cells expressing an engineered 4-*n*-propylguaiacol oxidase and a vanillin-forming isoeugenol dioxygenase. The conditions and strategy of the whole-cell based biocatalytic process were optimised concerning the amounts of cells, temperature, pH, and order of biocatalyst addition. A high vanillin yield of 90% was achieved starting from synthetically pure 4-*n*-propylguaiacol, while a 66% vanillin yield was achieved when starting from a real lignin oil, comprising 27.1% 4PG, that was obtained by RCF biorefining spruce wood. This whole-cell, multi-enzyme cascade results in a pre-purification vanillin yield of 18% based on RCF lignin oil and 3% based on the initial spruce feedstock (see ESI), an estimated 4-fold higher yield compared to current industrial wood-based vanillin production processes.[28] The relative increase for the post-purification vanillin yield is expected to be even larger due to the lack of a homogeneous metal-catalyst in the here presented vanillin product stream. Furthermore, we showed that cells in growth medium could be used as ready-to-use biocatalysts, making this one-pot conversion of lignin oil into vanillin a rather facile process. The presented lignin refining strategy paves the way for further development to sustainably produce biobased vanillin using lignin as the feedstock.

Experimental Section

Materials

Isoeugenol, 4PG, and vanillin were purchased from Sigma Aldrich and Fluorochem Limited. *E*/*Z*-isoeugenol (75/25) was generated by converting 4PG with purified PROGO, followed by column chromatography. Norway spruce, *Picea abies*, was obtained from Borregaard (Sarpsborg, Norway). All chemicals related to medium and antibiotics were obtained from Fisher Scientific chemicals. The products were purified by column chromatography using Merck 60 Å 230–400 mesh silica gel. Solvents used for purification and analysis were purchased from commercial sources and used as received.

Procedures

Details on biocatalyst and lignin oil preparation, conditions for biocatalytic conversions, and chemical analyses can be found in the Supporting Information.

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