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Research Article

Whole-Cell Bioconversion of Renewable Biomasses-Related Aromatics to *cis,cis*-Muconic Acid

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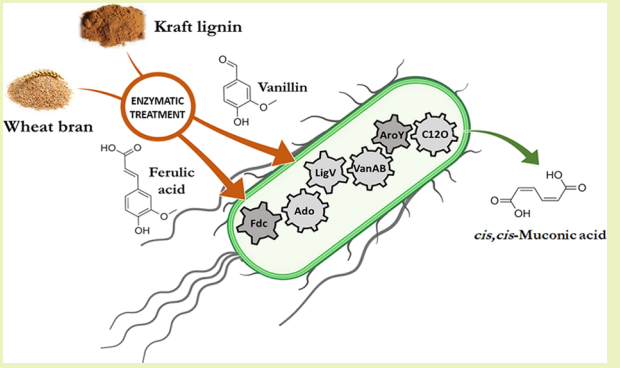
ABSTRACT: Lignin and wheat bran represent renewable feedstocks for generation of useful and value-added compounds such as vanillin (a popular flavoring agent) and *cis,cis*-muconic acid (ccMA, a building block for the synthesis of plastic materials). In the present work, we report on the setup of an efficient and green process for producing such valuable compounds based on (a) the optimization of the extraction procedures for vanillin from lignin and ferulic acid from wheat bran and (b) the genetic engineering of an *Escherichia coli* strain with up to three plasmids differing in copy numbers to modulate the expression of up to seven recombinant enzymes. In detail, we used two sequential reactions catalyzed by the decarboxylase Fdc and the dioxygenase Ado to convert wheat bran-derived ferulic acid into vanillin: nature-identical vanillin was produced in one pot with a >85% yield in 20 h. Next, the dehydrogenase LigV, the demethylase VanAB, the decarboxylase AroY, and the dioxygenase C12O converted lignin-derived vanillin into ccMA with a >95% conversion yield and a productivity of 4.2 mg of ccMA/g of Kraft lignin in 30 min. Finally, when the optimized *E. coli* strain expressing all the abovementioned enzymes was used, ccMA was produced with a >95% conversion yield starting from ferulic acid in 10 h following product isolation, corresponding to 0.73 g of ccMA/g of ferulic acid, 1.4 g of ccMA/L, and 2.2 g of ccMA/g of wheat bran biomass. The optimized whole-cell system represents a sustainable and cost-competitive process for producing high value-added products from renewable resources.



KEYWORDS: *biotransformation, cascade reaction, renewable biomasses, wheat bran, lignin valorization, vanillin, system biocatalysis, engineered E. coli*

INTRODUCTION

Lignin accounts for 15−25% of plant cell wall lignocellulose and represents the largest renewable source of aromatic compounds in the biosphere.[1,2] Massive amounts of technical lignins are generated industrially during biomass fractionation in lignocellulose processing (e.g., 50 million metric tons of lignin is generated from the pulp and paper industry every year).[3] At present, lignin is largely burnt for energy instead to be valorized into useful chemical products.[4−6] Furthermore, the wheat grain milling process generates wheat bran, usually used as livestock feed or as a fiber-rich ingredient in food products.[7] Wheat bran is rich in phytochemicals; among them, ferulic acid represents the most abundant phenolic compound.

Lignin and wheat bran represent renewable feedstocks for generation of useful and value-added compounds. On this side, vanillin (4-hydroxy-3-methoxybenzaldehyde), the second most popular flavoring agent after saffron used in the food and cosmetics industry, is of main industrial interest. Three types of vanillin—namely, natural, nature-identical (biotechnologically produced from ferulic acid), and synthetic—are available on the market: only natural and nature-identical vanillins are considered as food-grade additives. Due to the high cost and small amount produced (≈6500 tons/year), the worldwide production of natural vanillin does not fulfill the market demands.[8] Vanillin is also widely used as a building block in pharmaceutical synthesis and for the production of biopolymers.[9] Furthermore, a well-suited starting building block for the synthesis of relevant chemicals (adipic acid and terephthalic acid) and precursors of commercial plastics (such as nylon 6,6-polytrimethylene terephthalate) is represented by *cis,cis*-muconic acid (*cis,cis*-2,4-hexadienedioic acid, ccMA).

One strategy for biocatalytic valorization of lignin and wheat bran is to funnel a heterogeneous mixture of lignin-derived compounds into few, selected value-added compounds, also

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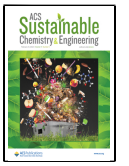
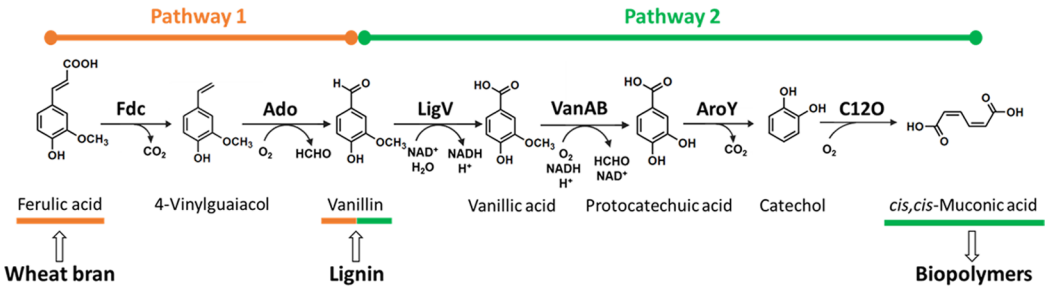


Scheme 1. Artificial Biosynthetic Pathway to Produce *cis,cis*-Muconic Acid from Ferulic Acid[a]



[a]Pathway 1: production of vanillin from ferulic acid by a cofactor-independent decarboxylase/oxygenase two-step process catalyzed by Fdc and Ado. Pathway 2: bioconversion of vanillin to *cis,cis*-muconic acid catalyzed by the multi-enzymatic system comprising the vanillin dehydrogenase LigV, the vanillic acid *O*-demethylase VanAB, the protocatechuate decarboxylase AroY, and the catechol 1,2-dioxygenase C12O. The alternative oxygenase enzyme Cso2 was also evaluated for the whole-cell biotransformation.

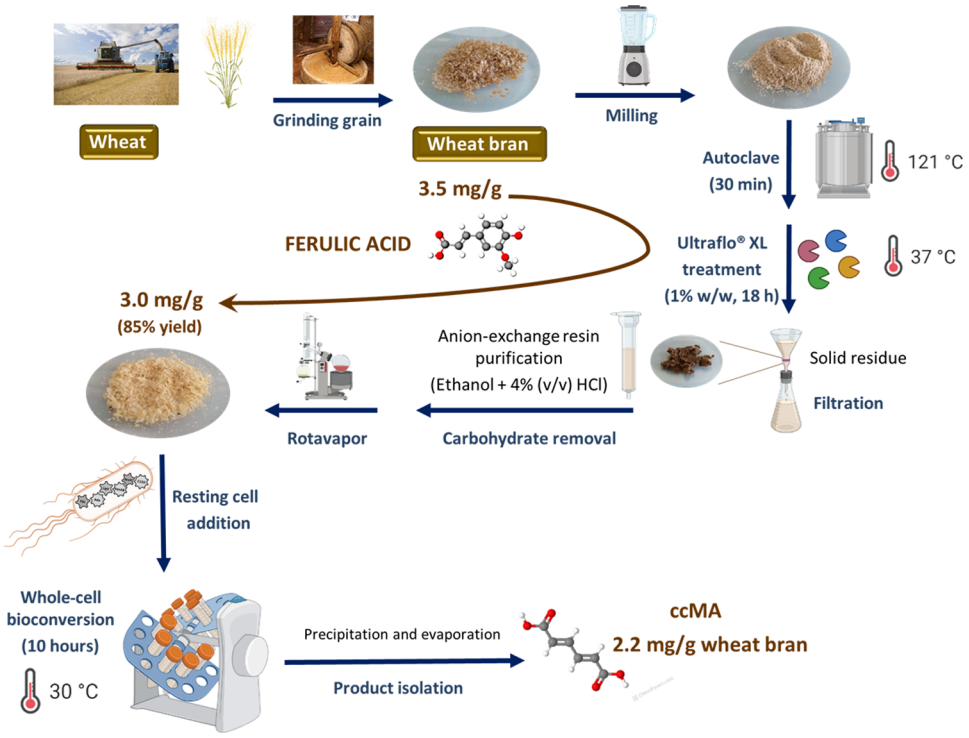


Figure 1. Schematic representation of the optimized protocol for recovery and purification of ferulic acid from wheat bran (see treatment iv; Table 2) followed by its bioconversion into ccMA.

taking advantage of engineered microorganisms, in the so-called "biological funneling" approach.[6]

Whole-cell biocatalysts offer several advantages, such as multi-step reactions with cofactor regeneration, high catalytic efficiency, and mild conditions, but require tailoring by means of protein and metabolic engineering approaches[10] and a critical evaluation of the results in comparison to cell-free systems.[11] Recently, we used a system biocatalysis approach to develop the first multi-enzymatic, one-pot bioconversion process of vanillin into ccMA based on four sequential reactions catalyzed by the commercial enzyme xanthine oxidase, the recombinant enzyme *O*-demethylase LigM in the presence of tetrahydrofolate (THF) and of the cofactor-regeneration enzyme methyl transferase MetE, and *Escherichia coli* cells expressing the decarboxylase AroY and the recombinant enzyme catechol 1,2-dioxygenase C12O.[12] The optimized lab-scale procedure converted 5 mM vanillin into ccMA in ≈30 h with a 95% yield.

Here, we focused on the generation of an engineered *E. coli* strain able to convert in one-pot both vanillin arising from lignin and ferulic acid isolated from wheat bran into ccMA (Scheme 1). The previous aromatic compound was isolated from Kraft lignin using the enzymatic procedure described by Vignali et al.,[13] the latter from wheat bran using an optimized thermo-enzymatic process. The engineered cell system efficiently generated ccMA, a useful metabolite of main industrial relevance: the global market of the ccMA derivatives is estimated at $22 billion,[14] with a market for ccMA alone at more than $100 million.[15]

MATERIALS AND METHODS

Reagents. Methanol (ACS Grade, ≥99%), formic acid (ACS Grade, ≥98%), softwood Kraft lignin, 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO), ethyl acetate, and analytical grade standards of ferulic acid (*trans*-hydroxy-3-methoxycinnamic acid), 4-vinylguaiacol (2-methoxy-4-vinylphenol), vanillin (4-hydroxy-3-methoxybenzalde-

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Table 1. Synthetic Genes Used in this Study

gene	accession number	enzyme	source	encoded protein (amino acids)	reference
Fdc	CAC18719.1	phenolic acid decarboxylase	*Bacillus pumilus*	161	18
Cso2 (A49P-Q390A)	ADG10219.1	9-*cis*-epoxycarotenoid dioxygenase	*Caulobacter segnis*	491	18
Ado	XP_003665585	aromatic dioxygenase	*Thermothelomyces thermophila*	603	19
LigV	BAK65381.1	vanillin dehydrogenase	*Sphingobium* sp. *SYK-6*	480	14
VanA	AAN69332.1	vanillate *O*-demethylase oxygenase	*Pseudomonas putida KT2440*	355	19
VanB	AAN69333.1	NAD(*P*)H oxidoreductase	*P. putida KT2440*	316	19
AroY	BAH20873	protocatechuate decarboxylase	*Klebsiella pneumonia* subsp.	502	19
C12O	AF182166.3	catechol 1,2-dioxygenase	*Acinetobacter radioresistens S13*	294	19

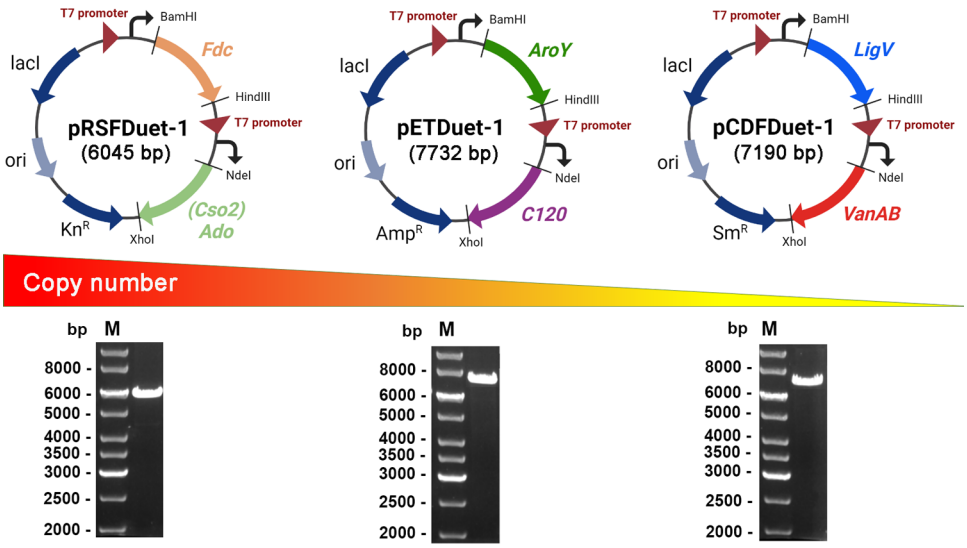

Figure 2. (Top) Physical map of plasmids pRSFDuet-1 (high copy number), pETDuet-1 (medium copy number), and pCDFDuet-1 (low copy number) carrying the genes encoding Fdc (orange) and Cso2/Ado (light green), AroY (dark green) and C12O (purple), and LigV (blue) and VanAB (red) enzymes, respectively. The main functional elements of the plasmids include two multiple cloning sites, both preceded by a T7*lac* promoter and a ribosome binding site as well as a sequence encoding a 6-His tag at the N-terminal end and an S-tag at the C-terminal end. The plasmids also carry the kanamycin (Kn^R), ampicillin (Amp^R), and streptomycin (Sm^R) resistance genes. (Bottom) For each construct, the nucleic acid gel electrophoresis analysis of the DNA plasmid linearized by the *BamHI* restriction enzyme is shown. M = GeneRuler 1 kb DNA ladder (Thermo Fisher Scientific).

hyde), vanillic acid (4-hydroxy-3-methoxybenzoic acid), protocatechuic acid (3,4-dihydroxybenzoic acid), catechol (1,2-dihydroxybenzene), and *cis,cis*-muconic acid were purchased by Merck/Carlo Erba (Merck KGaA, Darmstadt, Germany). The commercial food-grade enzyme UltrafloXL was kindly supplied by Novozymes (Bagsværd, Copenhagen, Denmark), and wheat bran was a generous gift of Molino Dallagiovanna (Gragnano Trebbiense, Piacenza, Italy).

Ferulic Acid Recovery from Wheat Bran. The recovery was carried out using a thermo-enzymatic method similar to the one reported by Bautista-Expósito et al.[7] To improve the release of ferulic acid, wheat bran was pre-treated by milling it to a fine powder (3 min of total time by alternating 30 s of milling to a 30 s pause) before the thermo-enzymatic extraction. Wheat bran powder was suspended at a 1:20 solid to solvent ratio (g/mL) in 100 mM potassium phosphate pH 6.0 and autoclaved at 121 °C, 1 bar, for 30 min. Then, the suspension was cooled to room temperature and 1% UltrafloXL (enzyme to wheat bran powder dry weight ratio, w/w) was added. The enzymatic step was carried out at 37 °C, under shaking (130 rpm), for 24 h. The amount of alkaline-extractable ferulic acid, the reference value used to calculate the thermo-enzymatic recovery yield, was determined as stated by Di Gioia et al.[16] In detail, 0.5 g of wheat bran was suspended in 30 mL of 2 M NaOH and incubated at 30 °C on a rotatory shaker for 2 h; 25 μL of the reaction mixture was added to 50 μL of solvent A (see the HPLC Analytical Method section) and centrifuged for 5 min at 11,000*g*, 4 °C, and 20 μL of the supernatant was withdrawn for HPLC analyses.

The amount of soluble carbohydrates in the supernatant (diluted 1:250) was quantified using the phenol-sulfuric acid method. Briefly, the concentrated sulfuric acid was able to break down all polysaccharides into monosaccharides that react with phenol to form stable yellow-gold compounds that can be measured spectrophotometrically; the absorbance value at 480 nm was measured using a Jasco V-580 spectrophotometer (Cremella, Italy). The calibration curve was built using standard solutions of xylose in the 100−300 μg/mL concentration range.

Ferulic Acid Purification. To selectively recover ferulic acid from the crude extract after the thermo-enzymatic treatment, a method adapted from Di Gioia et al. was used.[16] The solid residue was removed from the crude extract by paper filtration, and then, the strong anion exchange resin Amberlite IRA 401 Cl^- (DuPont, Wilmington, USA) was added (1 g of resin to 40 mL of solution) to the enzymatic hydrolysate. The solution was incubated at room temperature on a rotatory shaker for 3−4 h. The suspension was dried, added to ethanol with 4% (v/v) HCl (15 mL per g of resin), and then incubated at room temperature on a rotatory shaker for 1 h to elute the bound ferulic acid. The alcoholic solution containing ferulic acid was separated from the resin, neutralized by adding 2 M NaOH, and then concentrated by a rotavapor. The resulting powder was solubilized in 100 mM Tris−HCl buffer, pH 8.0 and used for HPLC analyses (to evaluate the recovery yield) and as the substrate for the whole-cell biotransformation. A schematic representation of the recovery and purification of ferulic acid from the wheat bran is depicted in Figure 1.

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Vanillin Recovery from Kraft Lignin. Vanillin was isolated from Kraft lignin following the procedure described by Vignali et al.[13] In detail, a 100 mL mixture containing 0.5 g of Kraft lignin and 2 mM TEMPO was added to the recombinant laccase from *Bacillus licheniformis* (BALL) (100 U, 0.2 U enzyme per mg of lignin).[13,15,17] After 2 h of incubation at 30 °C under shaking (100 rpm) in 0.5 L baffled flasks, 1 N HCl was added until a final pH of 1.0 was reached. The sample was extracted using 3:1 (v/v) ethyl acetate as the organic solvent; the dewatered organic phase was then filtered using Whatman filter paper, dried in a rotary evaporator, and resuspended in 200 mM Tris−HCl buffer, pH 8.0 for HPLC analyses (to evaluate the recovery yield) and as the substrate for the whole-cell biotransformation. Following this procedure, vanillin was obtained with a recovery yield of ≈4.5 mg per gram of lignin, as evaluated by GC−MS analyses.[13]

Construction of Plasmids and Whole-Cell Biocatalysts. The synthetic genes encoding the enzymes involved in the biosynthetic pathway (Scheme 1) were designed by *in silico* back translation of the amino acid sequence reported in the GenBank database (Table 1), and the codon usage was optimized for expression in *E. coli*.

To facilitate subcloning into pRSFDuet-1, pETDuet-1, and pCDFDuet-1 (Novagen, Darmstadt, Germany), the sequences corresponding to *BamHI* (GGATCC) and *HindIII* (AAGCTT) restriction sites were added at the 5′- and 3′-ends of *Fdc*, *AroY*, and *LigV* genes, while the sequences corresponding to *NdeI* (CATATG) and *XhoI* (CTCGAG) restriction sites were added at the 5′- and 3′-ends of *Cso2*, *Ado*, *C12O*, and *VanAB* genes (Figure 2). The synthetic genes were produced by Twist Bioscience HQ (San Francisco, USA). The synthetic genes and the three plasmids were digested with the corresponding restriction enzymes (Fast Digest, Thermo Fisher Scientific, Monza, Italy) and ligated with T4 DNA ligase (Thermo Fisher Scientific), see Figure 2.

The ligation mixtures were used to transform NEB10-β (pRSFDuet-1 and pETDuet-1 plasmids) or JM109 (pCDFDuet-1 plasmid) *E. coli* chemically competent cells (the latter strain was selected since the NEB10-β cells are streptomycin-resistant). The presence of the insert was verified by digestion with the corresponding restriction enzymes. The *Fdc* and *Cso2*/*Ado* genes were inserted into the pRSFDuet-1 plasmid obtaining pRSFD:Fdc-Cso2 and pRSFD:Fdc-Ado, *VanAB* and *LigV* genes were inserted into the pCDFDuet-1 plasmid obtaining pCDFD:VanAB-LigV, and *AroY* and *C12O* genes were inserted into the pETDuet-1 plasmid obtaining pETD:AroY-C12O (Figure 2). Finally, different combinations of plasmids were introduced into the *E. coli* MG1655 RARE[20] strain through chemical transformation to construct the whole-cell biocatalysts. The cell biocatalyst containing pRSFD:Fdc-Cso2 was also co-transformed with the pGro7 (Takara Bio Europe, Saint-Germain-en-Laye, France) plasmid encoding the GroEL and GroES chaperone proteins.[8]

Cultivation of the Whole-Cell Biocatalysts. For the starting culture, the engineered strains used as the biocatalyst were inoculated in the LB medium containing the appropriate antibiotic (30 μg/mL kanamycin, 100 μg/mL ampicillin, or 50 μg/mL streptomycin) and grown at 37 °C, 130 rpm, for 18 h. For the strain containing the pGro7 plasmid, 25 μg/mL chloramphenicol was also added. The next day, 100 mL of Terrific broth containing the appropriate antibiotic was inoculated with an amount of starting culture to have an initial OD_{600nm} = 0.1 and the culture was incubated at 37 °C, 130 rpm, until OD_{600nm} ≈ 0.6. Then, the incubation temperature was lowered to 20 °C and the proteins' expression was induced by adding 0.5 mM IPTG. The cells were further grown at 20 °C, 130 rpm, for 18 h. When required, 1 mM $FeSO_4$ was also added together with IPTG. The cells expressing the Fdc and Cso2 enzymes were prepared following the protocol reported by Furuya et al.:[18] 100 mL of inoculated LB broth containing 100 μg/mL ampicillin (adding 25 μg/mL chloramphenicol and 4 mg/mL arabinose for recombinant cells carrying pGro7) was incubated at 37 °C, 130 rpm, until OD_{600nm} ≈ 0.8−1.0; the cell culture was then added to 0.1 mM IPTG and 1 mM $FeCl_2$ and incubated at 25 °C, 130 rpm, for 18 h. The cells were harvested by centrifugation (8000*g*, 10 min, 4 °C), washed once in reaction buffer (see the Whole-Cell Biotransformation section), and resuspended in the same buffer to have a final concentration of 350 mg/mL. Notably, the same expression protocol, followed by the harvesting and resuspension steps, was performed for untransformed *E. coli* MG1655 RARE cells, used as control during the bioconversion reactions.

Whole-Cell Biotransformation. The whole-cell biotransformation reactions were set up using the analytical grade substrates and the recombinant *E. coli* cells (70 mg cww/mL), in either 100−200 mM Tris−HCl pH 8.0, 100 mM NaOH-glycine pH 9.5, or M9 medium. The reaction was carried out in a 1 mL final volume, into a 4 mL glass vial. To test the effect of different additives on the biotransformation yield, glucose (10 g/L), glycerol (10% v/v), and DMSO (10% v/v) were added to the reaction mixture. The biotransformation reactions using wheat bran-derived ferulic acid or lignin-derived vanillin were set up similarly using wheat bran or lignin extracts instead of the analytical grade standards, adjusting the extract volume based on the substrate concentration. All biotransformation reactions were performed at 30 on a rotatory shaker. The biocatalytic processes were monitored by withdrawing at different times 50 μL of the reaction mixture, centrifuged for 5 min at 16,000*g*, 4 °C, to remove the cells. Then, 5 μL of the supernatant was added to 145 μL of solvent A (see the HPLC Analytical Method section) and centrifuged for 5 min at 16,000*g*, 4 °C, and 20 μL of the supernatant was analyzed by HPLC (see below).

Product Isolation and Characterization. The reaction mixture from the bioconversion of 10 mM ferulic acid (100 mL) was treated with 100 μL of methanol, centrifuged to eliminate *E. coli* cells, evaporated, and then dissolved in 500 μL of methanol. The product ccMA was characterized by mass spectrometry analysis using Waters ZQ-2000 instrument single quadruple in ESI mode,[12] and ^{1}H-NMR spectra were recorded in deuterated DMSO-d_6 (δ = 2.50 ppm) on a spectrometer operating at 400.16 MHz.

HPLC Analytical Method. HPLC analyses were performed on a Jasco apparatus equipped with a Kromaphase C8 column 100 Å, 5 μm, and 4.6 × 250 mm (Scharlab, Barcelona, Spain) and with a UV detector set at 276 nm. The flow rate was 1 mL/min, and the column oven was set at 25 °C. A binary system of solvent A (2.5% v/v formic acid) and solvent B (methanol) was used with the following gradient: 0 min, 90% solvent A + 10% solvent B; 0−20 min, ramping up to 25% solvent A + 75% solvent B; 20−21 min, ramping up to 100% solvent B. Calibration curves were obtained by solubilizing standards of ferulic acid, 4-vinylguaiacol, vanillin, vanillic acid, protocatechuic acid (PCA), catechol, and ccMA in 100 mM Tris−HCl, pH 8.0, at a final concentration of 40 mM. Subsequently, after dilution in the 0.04−2 mM range, 25 μL of each sample was added to 50 μL of solvent A and centrifuged for 2 min at 11,000*g*, 4 °C: 20 μL of the supernatant were injected for HPLC analyses. Retention times for standards of PCA, ccMA, catechol, vanillic acid, vanillin, ferulic acid, and 4-vinylguaiacol were 8.6, 10.4, 11.0, 12.8, 14.0, 15.1, and 21.1 min, respectively. The calibration curves are reported in the Supporting Information, Figure S1.

RESULTS AND DISCUSSION

Ferulic Acid Recovery from Wheat Bran. The wheat bran predominantly contains non-starch polysaccharides (38%), starch (19%), proteins (18%), and lignin (6%), which is mainly a (G-S)-type (i.e., a ratio S:G:H of 0.9:1.0:0.19).[21,22] Noteworthy, ferulic acid is the most abundant phenolic compound in wheat bran lignin followed by *p*-coumaric acid: figures of 9.7 ± 0.4 and 2.6 ± 0.5 mg/g wheat bran have been reported.[22] Ferulic acid is linked to arabinoxylans through ester links:[7] in the context, to develop a sustainable process for its release, the enzymatic hydrolysis of ester bonds is particularly attractive because of its environmentally friendliness and energy efficiency.[23] On the basis of its high efficiency for the release of free ferulic acid from wheat bran,[7] the UltrafloXL (containing cellulase and xylanase activities) was selected. To evaluate different pre-treatments,

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reactions were set up starting from 2 g of wheat bran by (i) autoclaving at 121 °C, 1 bar, for 30 min; (ii) enzymatic treatment alone; (iii) autoclaving followed by the enzymatic treatment; and (iv) milling, autoclaving, and enzymatic treatment. The optimization of the "green" recovery process, representing the best choice to produce nature-identical vanillin compared to a chemical approach,[7,16] was set up based on the ferulic acid recovery yields using the value achieved employing an alkaline extraction treatment as reference. The latter method was used to determine the maximal amount of alkaline-extractable ferulic acid, under experimental conditions widely reported in literature:[24,25] a maximal value of 3.4 mg of ferulic acid per gram of wheat bran was achieved.[7,16] As shown in Table 2, the autoclaving and the enzymatic treatment alone reached an 7 and 22% recovery yield in comparison to the amount of ferulic acid obtained by the alkaline treatment, respectively, while a synergistic cooperative effect was observed for the combination of these two treatments: a 70.1 ± 3.2% yield was achieved. The obtained results suggest that the hydrothermal pretreatment allows easier access of hydrolytic enzymes by opening the cell wall structure, thus enhancing the breakdown of phenolic cross-links, as reported by Merali et al.[22] Noteworthy, compared with other pretreatment strategies, the hydrothermal treatment shows a significantly lower environmental impact, lower capital investment, the lack of chemicals, and a low byproduct generation.[26] The highest recovery yield (≈85% of the alkaline recovered ferulic acid) was obtained by milling the wheat bran to a fine powder before the combined (autoclaving and enzymatic hydrolysis) treatment: starting from 1 g of wheat bran, 3 mg of ferulic acid was recovered. Based on these results, the full treatment (milling and autoclaving pretreatments followed by the enzymatic hydrolysis; Table 2 and Figure 1) was selected for the ferulic acid isolation.

Table 2. Wheat Bran-Derived Ferulic Acid Recovery Yield

treatment	mg ferulic acid/g wheat bran	yield (%)
alkaline hydrolysis[a]	3.4	100
(i) autoclaving	0.3 ± 0.1	7.4 ± 1.5
(ii) enzymatic hydrolysis	0.8 ± 0.1	22 ± 3
(iii) autoclaving + enzymatic hydrolysis	2.3 ± 0.2	70 ± 3
(iv) milling + autoclaving + enzymatic hydrolysis	3.0 ± 0.2	85 ± 6

[a]Treatment used to calculate the maximal amount of achievable ferulic acid, taken as 100% recovery yield.

Ferulic acid was then purified through selective binding to an anion exchange resin: after the incubation with ethanol +4% HCl, more than 70% of the extracted ferulic acid was eluted. The solution was concentrated by a rotavapor obtaining a fine powder that could then be solubilized in the bioconversion buffer to be used as the substrate for the whole-cell biotransformation reactions (Figure 1). Notably, the water phase following the combined treatment contained 0.65 ± 0.06 g of carbohydrates per gram of wheat bran, allowing a valorization of such a component too.

Vanillin Production from Ferulic Acid. To explore the vanillin production from ferulic acid, a cofactor-independent enzymatic reaction system was set up and optimized: ferulic acid is converted to 4-vinylguaiacol by the ferulic acid decarboxylase Fdc from *B. pumilus*, and the carotenoid cleavage oxygenase Cso2 from *C. segnis* converts 4-vinylguaiacol to vanillin (pathway 1; Scheme 1).[18] The synthetic genes encoding Fdc and Cso2 (Table 1) were cloned in the high copy number pRSFDuet-1 plasmid, designed for the co-expression of two target genes (Figure 2). In particular, it was previously reported that the low catalytic efficiency of Cso2 and its thermal instability could affect the vanillin production.[27] In our study, the amino acidic substitutions A49P and Q390A were introduced in Cso2 (Table 1), as suggested by the site-directed mutagenesis study performed by Yao et al.,[28] and the high-copy number plasmid was used for its recombinant production.

The whole-cell biocatalyst was established in the engineered *E. coli* K-12 MG1655 RARE,[20] a strain showing reduced aromatic aldehyde reduction activities and aimed at preventing the conversion of vanillin into the byproduct vanillyl alcohol,[12] see below. Biotransformation reactions using resting cells were carried out using the engineered *E. coli* cells (35, 70, or 140 mg cww/mL), harvested and suspended in an M9 minimal medium or 100−200 mM Tris−HCl buffer, pH 8.0. As shown in Figure 3A, the whole-cell biocatalyst expressing Fdc and Cso2 (70 mg/mL cww incubated at 30 °C in 100 mM TrisHCl buffer, pH 8.0, hereinafter defined as "standard conditions") rapidly decarboxylated 10 mM ferulic acid into 4-vinylguaiacol while a limited amount of the final product vanillin was generated (reaching a 25% bioconversion yield only). The addition of 10 g/L glucose, 10% (v/v) glycerol, or 10% (v/v) DMSO to the reaction mixture significantly inhibited vanillin production: in all cases, the almost complete conversion of ferulic acid into 4-vinylguaiacol was achieved with no vanillin production (see Figure S2A). The co-expression of the GroEL and GroES chaperone proteins increased the vanillin yield up to a 50% value after 20 h of

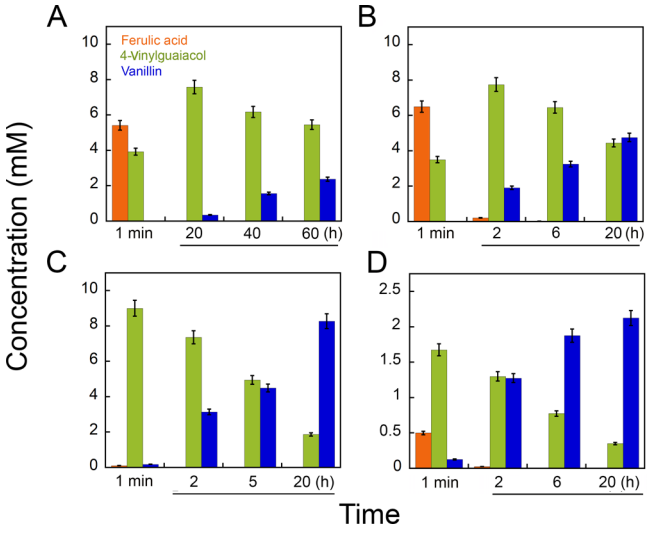

Figure 3. Time course of the one-pot bioconversion of 10 mM ferulic acid into vanillin (pH 8.0, 30 °C) by the *E. coli* whole-cell biocatalyst (100 mg/mL) expressing the enzymes (A) Fdc and Cso2; (B) Fdc, Cso2, and the chaperone proteins GroES and GroEL; and (C) Fdc and Ado. (D) Time course of the one-pot bioconversion of 2.5 mM ferulic acid extracted from wheat bran by the *E. coli* cell system expressing Fdc and Ado (strain *E. coli* II, 1 mL final reaction volume). The low level of ferulic acid at 1 min of reaction highlights the quick import and conversion (by the enzyme Fdc) of this compound. The values are reported as mean ± standard deviation (n = 3).

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incubation under standard conditions (see Figure 3B and Figure S2B), indicating that the efficient folding of Cso2 protein is largely responsible of vanillin production. On the other hand, the addition of compounds able to favor protein folding, such as glycerol (10% v/v) and/or DMSO (10% v/v), as well as the incubation in 100 mM glycine-NaOH buffer, pH 9.5, did not improve the bioconversion yield.

To bypass the rate-limiting step catalyzed by the oxygenase Cso2 in the production of vanillin, the use of the alternative enzyme aromatic dioxygenase Ado from the thermophilic fungus *T. thermophila* (showing a 44% amino acid identity with Cso2) has been evaluated (Scheme 1).[19] The purified Ado enzyme shows a catalytic efficiency 78,500-fold higher than that of wild-type Cso2.[19] The *E. coli* biocatalyst expressing both Fdc and Ado enzymes, and named *E. coli* II, converted 10 mM ferulic acid in one-pot into the final product vanillin in a total of 20 h under standard conditions with a 90% yield (Figure 3C and Figure S2C). The incubation of 10 mM ferulic acid without the whole-cell biocatalyst or using untransformed *E. coli* cells did not generate any detectable reaction products. Taking into account that the Ado enzyme belongs to the carotenoid cleavage oxygenase family containing iron as a prosthetic group,[29] the effect of Fe^{2+} on the oxygenase reaction was investigated: adding 1 mM $FeSO_4$ in the medium (see the Materials and Methods section) did not affect the reaction rate and the bioconversion yield of the overall biotransformation of ferulic acid into vanillin. Moreover, the simultaneous addition of IPTG and 10 mM benzyl alcohol, to induce chaperone co-expression,[30] did not improve the bioconversion yield, and the supplement of 10 g/L glucose in the reaction mixture, to enhance the NAD(*P*)H cofactor regeneration,[31] significantly inhibited the vanillin production: a 30.2 ± 1.4% bioconversion yield was observed after 24 h of incubation.

As shown in Figure 3C, the oxidation of 4-vinylguaiacol to vanillin is the rate-limiting step of pathway 1 (Scheme 1). The bottleneck cannot be ascribed to a difference in the expression levels of Fdc and Ado enzymes (3 mg/g cells and 9.5 mg/g cells, respectively, see Figure S3). Although at present not supported by evidence, a chemical reaction between 4-vinylguaiacol and the formaldehyde produced by the oxygenase enzyme could explain the experimental observation, as reported for the same bioconversion pathway by Saito et al.[32]

The fast bioconversion of ferulic acid into 4-vinylguaiacol suggests that *E. coli* II cells can support high rates of substrate uptake, making this system useful for industrial applications. Moreover, the almost complete transformation of ferulic acid into vanillin demonstrates that the selected *E. coli* strain has no endogenous enzymes metabolizing these compounds. Noteworthy, the *E. coli* II whole-cell biocatalyst converted 85.4 ± 3.2% wheat bran-derived ferulic acid (2.5 mM) into vanillin in 20 h (Figure 3D), highlighting the possibility to obtain a fine specialty chemical such as vanillin at significant yield from an agricultural byproduct, a topic of high interest in wheat-producing countries.[33]

Bioconversion of Vanillin into *cis,cis*-Muconic Acid. Starting from the enzymatic cascade process set up to convert vanillin into ccMA,[12] now, we evaluated the assembling of this pathway in a single-engineered *E. coli* K-12 MG1655 RARE strain to promote the diffusion of intermediates in the confined intracellular space, thus reducing the mass transfer problem.[10] Compared to the cell-free bioconversion system,[12] the commercial enzyme xanthine oxidase catalyzing the oxidation of vanillin into vanillic acid and the THF cofactor-dependent demethylase LigM catalyzing the demethylation of vanillic acid into protocatechuic acid (PCA) have been substituted by the enzymes vanillin dehydrogenase LigV from *Sphingomonas paucimobilis* SYK-6 and vanillic acid *O*-demethylase VanAB from *P. putida* KT2440 (see Scheme 1; this strain encoding five recombinant enzymes was named *E. coli* V).

Recent synthetic biology studies highlighted an enhanced yield and productivity by balancing the expression of genes encoding enzymes involved in whole-cell biotransformation.[34,35] Accordingly, the synthetic genes encoding LigV and VanAB (Table 1) were cloned in the low copy number pCDFDuet-1 plasmid and the genes encoding AroY and C12O were cloned in the medium copy number pETDuet-1 plasmid (see Figure 2). The pETDuet-1 plasmid was selected for AroY expression since it harbors the same replication origin of pET24 previously used to express this decarboxylase.[12]

The cells expressing the vanillin dehydrogenase LigV completely converted 10 mM vanillin into vanillic acid in 20 h (Figure 4A). The co-expression of the demethylase activity consisting of the two-component enzymatic system VanA, an iron-dependent monooxygenase, and its partner electron transfer protein VanB, accepting both NADH and NADPH as the cofactor, pushed the vanillin oxidation and the oxidative demethylation reaction, thus resulting in the PCA production in a total of 2 h of incubation with a 95.2 ± 3.5% yield (Figure

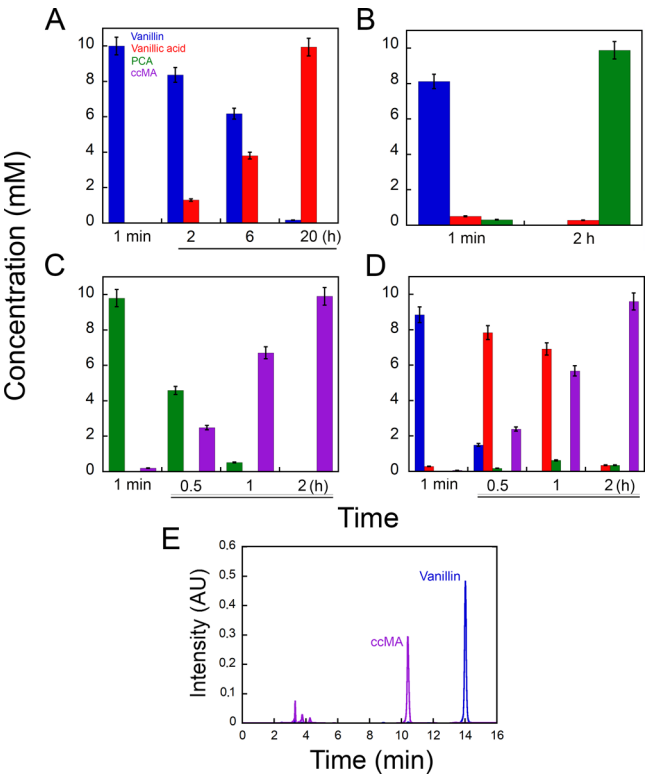

Figure 4. Time course of the one-pot bioconversion of (A) vanillin into vanillic acid, (B) vanillin into PCA, (C) PCA into ccMA, and (D) vanillin into ccMA catalyzed by the *E. coli* whole-cell biocatalyst expressing the enzymes LigV (A), LigV and VanAB (B), AroY and C12O (C), and LigV, VanAB, AroY, and C12O (strain *E. coli* V) (D). All the reactions were carried out on a 10 mM substrate in 200 mM Tris−HCl, pH 8.0, 30 °C (1 mL final volume). The values are reported as mean ± standard deviation (n = 3). (E) HPLC chromatograms of the mixture composition at the end of the biotransformation reaction reported in panel (D).

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4B). Concerning the last two steps of the biocatalytic pathway, the whole-cell biocatalyst expressing AroY and C12O rapidly decarboxylated PCA into catechol and completely converted 10 mM of this substrate into ccMA (Figure 4C).

Noteworthy, the whole-cell biocatalyst was implemented by assembling the overall synthetic pathway (made of five enzymes) in a single strain and completely converted in one-pot 10 mM vanillin into ccMA in a total of 2 h (Figure 4D,E). The co-expression of all enzymes in the same biocatalyst shifted the reaction equilibria toward the production of the final, desired product ccMA and sped up the reaction rate, with no accumulation of intermediates (Figure 4E): a 95.2 ± 3.8% bioconversion yield was obtained in 2 h. Interestingly, the *E. coli* V whole-cell biocatalyst achieved the almost complete conversion of 1 mM lignin-derived vanillin in 30 min of incubation, thus highlighting the possibility to produce ccMA from a renewable and inexpensive biomass: under optimized conditions, 4.2 mg of ccMA has been produced from 1 g of Kraft lignin.

Production of *cis,cis*-Muconic Acid from Ferulic Acid. The whole-cell biocatalysis has been further implemented by assembling both pathways (see Scheme 1) in a single strain, thus offering the potential for production in a single pot: the *E. coli* VII whole-cell biocatalyst harbors three plasmids encoding all enzymes catalyzing the conversion of ferulic acid into ccMA (pathways 1 and 2 in Scheme 1). Under optimized conditions, 10 mM substrate was converted into ccMA with a 40.0 ± 2.1% yield in a total of 22 h of incubation: at such a time, a peak corresponding to ≈6 mM vanillic acid was still present (see Figure S4A). This could be due to the inhibition of the enzymatic activity of VanAB by 4-vinylguaiacol. Actually, an inhibition of the demethylase enzyme has been observed at increasing concentrations of isoeugenol (>5 mM), an aromatic compound structurally resembling 4-vinylguaiacol.[36] In agreement with this hypothesis, when the whole-cell biocatalyst expressing the enzymes LigV, VanAB, AroY, and C12O (*E. coli* V) was incubated with 10 mM vanillic acid in the presence of 5 mM 4-vinylguaiacol, the final product ccMA was obtained with a 30.1 ± 1.7% bioconversion yield after 2 h of incubation: two peaks corresponding to ≈5 mM vanillic acid and ≈3 mM PCA were still present at the end of the incubation (see Figure S4B). Noteworthy, in the absence of 4-vinylguaiacol, the almost complete conversion of vanillic acid into the final product was apparent (Figure S4B). Accordingly, the supplementation of an additional amount of the *E. coli* VII whole-cell biocatalyst (100 mg cww/mL final concentration) after 6 h of incubation (i.e., when the peak corresponding to 4-vinylguaiacol disappeared) allowed the almost complete conversion of ferulic acid into the final product ccMA in a total of 10 h of incubation (see Figure 5A).

Interestingly, the optimized reaction conditions were also used for the bioconversion of wheat bran-derived ferulic acid (2.5 mM): based on the intensity of the peak corresponding to ccMA, a ferulic acid bioconversion yield of 95.1 ± 4.1% after 10 h of incubation was apparent (see Figure 5B). The established bioconversion process produced 2.2 mg of ccMA from 1 g of the wheat bran biomass (see Figure 1).

The scale up of the bioconversion was performed at 10 mM ferulic acid in a 100 mL final reaction volume: the substrate was converted into the final product ccMA with a 96.1 ± 3.8% yield in a total of 10 h of incubation (see Figure 6). The product was recovered from the reaction mixture by methanol precipitation (15 mg) and identified by NMR and ESI-MS (see

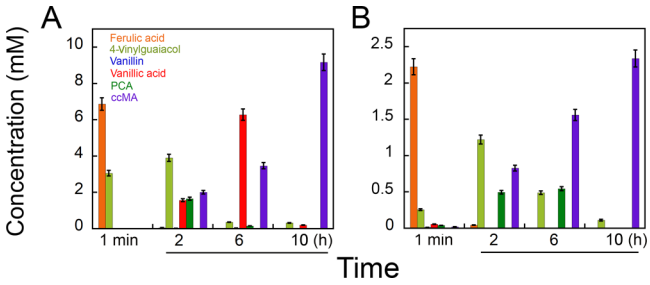

Figure 5. Time course of the one-pot bioconversion of (A) 10 mM standard ferulic acid and (B) 2.5 mM ferulic acid extracted from wheat bran into ccMA by the *E. coli* VII cell system, in 200 mM Tris–HCl pH 8.0, 30 °C (1 mL final volume). In both cases, after 6 h of incubation, an additional amount of cells (100 mg ccw/mL final concentration) was added. The low level of ferulic acid at 1 min of reaction highlights the quick import and conversion (by the enzyme Fdc) of this compound. The values are reported as mean ± standard deviation (n = 3).

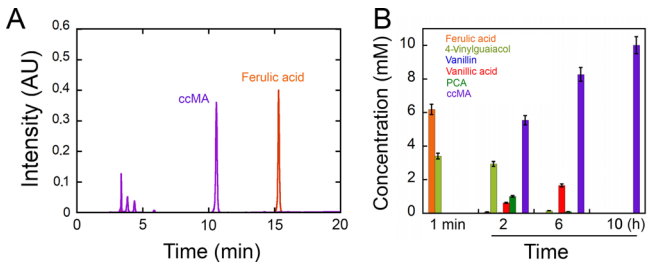

Figure 6. Bioconversion of 10 mM ferulic acid into ccMA, in 200 mM Tris–HCl, pH 8.0, 30 °C (100 mL final reaction volume). (A) HPLC chromatograms of ferulic acid (t0) and of ccMA produced after 10 h. (B) Time course of the reaction: the column bars report the relative amount of ferulic acid, 4-vinylguaiacol, vanillin, vanillic acid, PCA, and ccMA. The low level of ferulic acid at 1 min of reaction highlights the quick import and conversion (by the enzyme Fdc) of this compound. The values are reported as mean ± standard deviation (n = 3).

Figure S5): the ESI-full scan mass spectrum shows an (M-1)- m/z 141.27 and an intense product ion at m/z 97.05 that resulted from a decarboxylation process. An identical fragmentation pattern was observed for the commercial ccMA dissolved in methanol.[12]

CONCLUSIONS

The global market potential of ccMA and its derivatives was estimated to exceed $22 billion per year,[14] compared to a global bio-vanillin market expected to reach $400 million by the end of 2025.[37] Accordingly, the production of ccMA from low-cost, renewable feedstocks using an engineered *E. coli* strain (and through the generation of ferulic acid and vanillin) represents a significant improvement toward the generation of relevant bioproducts from biomasses (such as adipic acid, a useful building block for the production of nylon 6,6-polyamide). Bacterial production of ccMA from lignin or lignin-model compounds has been recently revised.[36,38] The engineered strains such as *P. putida*, *Amycolatopsis* sp., *Rhodococcus opacus*, *E. coli*, and *Corynebacterium glutamicum* have been reported to produce high ccMA yields from lignin-based aromatics. The metabolic engineering of synthetic pathways, combined with the optimization of fermentation conditions in a fed-batch process, achieved high production levels of ccMA starting from different lignin-derived aromatics.

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Table 3. Production of ccMA by Engineered Bacterial Strains

organism	production (g/L)	productivity (g/L·h)	fermentation process	reference
		p-Coumaric acid → ccMA		
P. putida KT2440 CJ103	13.5	0.17	fed-batch	42
P. putida KT2440 CJ238	55.4	0.77	fed-batch	39
P. putida KT2440 CJ242	49.7	0.50	fed-batch	47
P. putida KT2440 CJ781	43.0	0.40	fed-batch	48
		Guaiacol → ccMA		
Amycolatopsis sp. ATCC 39166 MA-2	3.1	0.13	fed-batch	49
		Catechol → ccMA		
C. glutamicum MA-2	85.0	2.4	fed-batch	40
P. putida KT2440 MA-9	36.1	4.4	fed-batch	41
		Pre-treated lignin → ccMA		
P. putida KT2440 CJ103	0.7	0.03	batch	42
P. putida KT2440 TMBHV002	0.3	0.01	batch	43
P. putida KT2440 MA-9	13.0	0.24	fed-batch	41
Amycolatopsis sp. ATCC 39166 MA-2	0.2	0.02	fed-batch	49
C. glutamicum MA-2	1.8	0.07	fed-batch	40
R. opacus PD630-MA6	1.6	0.02	fed-batch	50

Table 4. Comparison of Whole-Cell Production of ccMA from Vanillin and Ferulic Acid

organism	volume (mL)	substrate (mM)	time (h)	production (g/L)	productivity (g/L·h)	g ccMA/g Van or FA	reference
			vanillin (Van) → ccMA				
engineered *E. coli*	5	3.3	24	0.341	0.014	0.690	14
engineered *R. opacus* PD63	50	0.5	24	0.711	0.030	0.934	51
engineered *E. coli*	1	1	3	0.120	0.040	0.805	52
engineered *E. coli* (strain V)	1	10	2	1.421	0.710	0.934	this paper
engineered *E. coli* (strain V) starting from lignin	1	1	0.5	0.142	0.284	0.934	this paper
			ferulic acid (FA) → ccMA				
engineered *P. putida* KT2440	25	20	72	0.796	0.011	0.204	53
engineered *E. coli* (strain VII)	100	10	10	1.421	0.142	0.731	this paper
engineered *E. coli* (strain VII) starting from wheat bran	2.5	2.5	10	0.355	0.035	0.731	this paper

Recent state-of-the-art whole-cell conversion studies are reported in Table 3. The production of ccMA from *p*-coumaric acid reached 55 g/L using the engineered *P. putida* KT2440 CJ238, in which a global regulator of carbon catabolite repression was eliminated.[39] Becker et al.[40] engineered the *C. glutamicum* MA-2 strain with the elimination of muconate cycloisomerase enzyme (CatB) and the overexpression of the catechol-1 and 2-dioxygenase (CatA): final titers of 85 and 1.8 g/L ccMA were produced from catechol and hydrothermal pre-treated softwood lignin, respectively (Table 3). Noteworthy, a titer of 13 g/L ccMA was reached starting from a hydrothermal treated-softwood lignin in supercritical water (mainly containing catechol) using the engineered strain *P. putida* KT2440 MA-9 after 54 h of incubation.[41] Concerning batch processes in a shake flask, a titer of 0.3−0.7 g/L of ccMA was obtained by two different engineered *P. putida* KT2440 strains starting from an alkaline pre-treated liquor stream and the acid-soluble residue was obtained by the corn stover.[42,43]

The success of our proposed process using resting cells in a batch process is due to the optimization of the extraction procedures for selected starting molecules (i.e., vanillin from lignin and ferulic acid from wheat bran, the latter procedure generating a sugar-rich water fraction of further usefulness) and to the genetic engineering of an *E. coli* strain (with low aromatic aldehyde reduction activities) based on the use of plasmid combinations differing in copy numbers for the expression of the various recombinant enzymes. Actually, using the *E. coli* II strain, vanillin was produced at 85.4 ± 3.2% yield from 10 mM ferulic acid (Figure 3C), with a productivity of 1.3 g/L, in agreement with data reported in literature using an engineered *P. putida* KT2440 strain.[44] Noteworthy, the *E. coli* II strain produced nature-identical vanillin from 2.5 mM wheat bran-derived ferulic acid in 20 h (Figure 3D): vanillin produced from renewable resources can be labeled as "natural" by the European and U.S. legislation,[45] thus increasing its value. A similar process based on Fdc and Cso2 enzymes was previously reported:[18] we substituted the rate-limiting Cso2 with the most active aromatic dioxygenase Ado and employed a high-copy number plasmid for expression. Indeed, the analysis of the conversion of 10 mM ferulic acid by the *E. coli* VII strain (expressing all the enzymes of the novel biosynthetic pathway reported in Scheme 1) did not show any intermediate accumulation and highlighted the efficient transport of ferulic acid by the bacterial inner membrane transporters. The process generated 0.73 g of ccMA/g of ferulic acid following the isolation of the final product, corresponding to 1.4 g of ccMA/L in 10 h. Table 4 reports a comparison with previous whole-cell bioconversion processes of vanillin and ferulic acid into ccMA in a batch process: a faster ccMA production and a higher conversion of ferulic acid were obtained using the *E. coli* VII cell system.

Usually, a newly established biosynthetic process does not immediately fulfill the economic requirements for commercial operation. Biological lignin valorization has not yet been

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conducted at an industrial/commercial scale; thus, a technoeconomic analysis (TEA) has not yet been reported.[38] From an economic point of view, the cost of a product = A + B/yield + C/Pv, where A is the capital cost, B is the raw material cost, C is the operating cost, and Pv is the volumetric productivity.[10] The use of a cheap raw material, such as wheat bran and lignin, an efficient bioconversion yield (0.73 g of ccMA/g of ferulic acid), a good volumetric productivity (1.4 g/L), and an easy product recovery, allows us to greatly reduce the production cost and indicate that our proof-of-concept study not only is technically feasible as demonstrated but also possesses significant potential to improve the profitability of renewable biomass valorization. Moreover, compared to a biocatalytic process, the use of whole cells circumvents the need for cell lysis and enzyme purification, thus reducing the catalyst cost. Taking into account the commercial cost of ferulic acid (1 €/g) and the low operational cost (40 €/L cell culture), our optimized whole-cell biocatalyst produces 1 g of ccMA from 1.4 g of ferulic acid starting from 20 mL of fermentation broth at approximately 5% of the commercial cost of the final product ccMA (40 €/g) and the use of ferulic acid recovered from an inexpensive renewable biomass such as wheat bran (at a commercial cost of 0.2 €/kg) leads to a 300-fold increase in economic value.

The ability of an engineered *E. coli* strain to generate ccMA from different natural and renewable feedstocks (instead of petroleum) makes this system well suited to set up a number of innovative processes aimed at generating bioproducts and bioplastic monomers: bacterial lignin valorization seems no more a "field of dreams".[46]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.2c06534.

HPLC calibration curves for ferulic acid, 4-vinylguaiacol, vanillin, vanillic acid, PCA, catechol, and ccMA; HPLC chromatograms of the bioconversion of ferulic acid into vanillin; SDS-PAGE analysis of cell pellets of the strain expressing Fdc and Ado enzymes; HPLC chromatograms of the bioconversion of ferulic acid and vanillic acid into ccMA; and identification of the final product ccMA by ESI-MS and ^{1}H-NMR analyses (PDF)

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Notes

The authors declare no competing financial interest.

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