Multi-Enzymatic Cascade Reactions for the Synthesis of cis,cis-Muconic Acid

RESEARCH ARTICLE

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Multi-enzymatic cascade reactions for the synthesis of *cis,cis*-muconic acid

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Abstract. Lignin valorization allows the generation of a number of value-added products such as *cis,cis*-muconic acid (ccMA), which is widely used for the synthesis of chemicals for the production of biodegradable plastic materials. In the present work, we reported the first multi-enzymatic, one-pot bioconversion process of vanillin into ccMA. In details, we used four sequential reactions catalyzed by xanthine oxidase, *O*-demethylase LigM (and the tetrahydrofolate-regeneration enzyme methyl transferase MetE), decarboxylase AroY (based on the use of *E. coli* transformed cells) and catechol 1,2-dioxygenase CatA. The optimized lab-scale procedure allowed to reach, for the first time, the conversion of 5 mM vanillin into ccMA in ~ 30 h with a 90% yield: this achievement represents an improvement in terms of yields and time when compared to the use of a whole-cell system. This multi-enzymatic system represents a sustainable alternative for the production of a high value added product from a renewable resource.

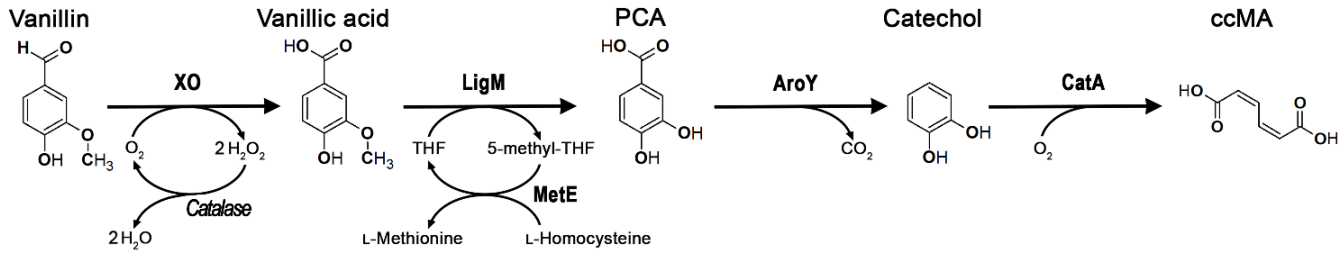
Keywords: Bioconversion; Cascade reaction; System biocatalysis; Lignin valorization; ~~Green chemistry~~; ~~Recombinant enzymes;~~ Enzyme catalysis

Introduction

Lignin is an aromatic three-dimensional amorphous polymer obtained by the radical polymerization of phenylpropane units (*p*-coumaryl, syringyl and guayacyl alcohols). It constitutes with cellulose and hemicellulose the so-called lignocellulosic biomass, a promising second generation renewable feedstock.[1] Although lignin accounts for approximate 15-40% of lignocellulose, representing the first renewable source of aromatics on Earth, it is almost underutilized.[2-4] In fact, as its calorific power is comparable to that of some fossil carbons (26-28 MJ/ton dry lignin), approximately 140 million tons per year of technical lignin is burned for the in loco generation of heat and power.[1,5] Nevertheless, lignin valorisation is feasible by performing its thermochemical or biological depolymerisation into monomeric aromatic compounds.[1,6-9] These compounds represent building blocks for the sustainable synthesis of chemicals, such as adipic acid, *cis,cis*-muconic acid ((2Z,4Z)-hexa-2,4-dienedioic acid, ccMA) and terephthalic acid.[1,10,8] Because of the relevance of these compounds as precursors of commercial plastics, biological routes for their production have been already implemented and scaled up to the pilot scale, representing the masterpiece of lignin valorization.[34,11]

ccMA is one of the three isomers of the linear dicarboxylic muconic acid; ~~which~~ the global market for ccMA and its derivatives is greater than $22 billion[10] due to its relevance for the synthesis of polyamides, unsaturated polyesters, adipic acid and terephthalic acid, which are all chemicals involved in the production of a large number of plastic materials.[3,4,10] Currently, the industrial production of ccMA depends mainly on chemical synthesis using petroleum-based feedstocks (e.g., catechol and phenols) in the presence of acids or heavy metal catalysts.[4,12] In order to limit the use of fossil-sources, the development of green alternatives for the synthesis of ccMA is a crucial objective for biotechnology.[1,4,8]

Recently, the feasibility of a biotechnological process for the ccMA production from an alkali pre-



Scheme 1. Reactions involved in the multi-enzymatic bioconversion of vanillin into ccMA.

treated lignin was demonstrated.[10] The DH1 strain of *Escherichia coli* was engineered with a new anabolic pathway for the bioconversion of vanillin (obtained from the thermochemical pre-treatment of lignin) into ccMA (314 mg ccMA/L culture; 0.69 g ccMA/g vanillin).[10] Nonetheless, the limits of this approach consist both in the difficulty of the microbial pathway regulation (i.e. strain engineering) and in the set up of the optimal microbial growth conditions.[7,13] To overcome these limitations, multi-enzymatic biocatalytic approaches could represent a suitable strategy:[14-16] system biocatalysis allows the combination of different enzymes, also from different source organisms, in a single reactor resulting in the generation of new routes for the product synthesis.[8,15,17,18] Cascade reactions are advantageous over classical step-by-step synthesis since these allow the elimination of the steps for isolation and purification of reaction intermediates, the handling of unstable intermediates, higher yields (by shifting of unfavourable reaction equilibria), and improved atom economy.[19]

In the present work, we optimized a multi-enzymatic one-pot process for the production of ccMA through the bioconversion of vanillin, an aromatic lignin degradation product that can be obtained by a simple and rapid thermo-chemical pretreatment from Kraft lignin.[20] In details, the oxidation of vanillin into vanillic acid is catalyzed by the commercial enzyme xanthine oxidase (XO, UniProtKB ID: P80457). Then, the demethylation of vanillic acid into protocatechuic acid (PCA) is catalyzed by the recombinant *O*-demethylase LigM (UniProtKB ID: G2IQS7) in the presence of tetrahydrofolate (THF) and of the cofactor-regeneration enzyme methyl transferase MetE (UniProtKB ID: Q42699).[17] PCA is converted into catechol (1,2-dihydroxybenzene) by using *E. coli* cells expressing the decarboxylase AroY (UniProtKB ID: B9A9M6). The final reaction, catalyzed by the recombinant catechol 1,2-dioxygenase (CatA, UniProtKB ID: P81422), consists in the production of ccMA through the intradiolic opening of the aromatic ring of catechol (Scheme 1).

Results and Discussion

Set up of single catalytic steps

The bioconversion process of vanillin into ccMA involves four different steps to give the final product which also requires the THF cofactor (see Scheme 1). The feasibility of each step (in 1 mL final volume and starting from 1 mM of each substrate, a concentration at which all reaction components are fully soluble at the conditions tested) was evaluated as formation of the corresponding product by HPLC analyses. The optimal operational conditions for the one-pot bioconversion into ccMA were selected as 100 mM Tris-HCl, pH 8.0 as buffer and an incubation temperature of 30 °C, ~~respectively,~~ on the basis of the optimized reaction conditions reported for the LigM/MetE bi-enzymatic system (the rate-limiting step in the cascade process, see below), that is essential for THF regeneration and to make economically sustainable the overall biocatalytic process.[17]

Concerning the first step, the oxidation of vanillin to vanillic acid was investigated by using the commercial enzyme XO (165 ± 15 U/mg on vanillin as substrate, see Experimental Section), which catalyses the oxidation of a wide range of *N*-heterocycles and aldehydes.[21] The oxidation of vanillin by XO was evaluated at two different pH values, i.e. 7.4 and 8.0 (the optimal condition for XO vs the selected condition for the multi-step process).[21] The reaction performed in Krebs-Henseleit buffer, pH 7.4, showed the complete conversion of 1 mM vanillin into vanillic acid in 1 h at 30 °C using 220 µg of XO (data not shown). On the other hand, the complete conversion of 1 mM vanillin was observed after 2 h of incubation at 30 °C using 90 µg of XO (15 U) in 100 mM Tris-HCl, pH 8.0 (1 mL final volume), (Figure 1A). This represented the minimum amount of enzyme which allows the complete bioconversion of 1 mM vanillin and corresponded to a ~2.4-fold reduction in enzyme consumption compared with previous studies.[21]

The demethylation of 1 mM vanillic acid into PCA was catalysed by the purified LigM/MetE bi-enzymatic system (0.3 and 1 mg/mL, respectively) in the presence of 0.1 mM THF, 2 mM L-homocysteine (MetE can use both L- and D,L-mixtures)[17], and adding 0.5 mg of MetE every 2 h, in 100 mM Tris-HCl, pH 8.0 (1 mL final volume): after 8 h of reaction, 1 mM of PCA was produced (Figure 1B). The specific activity of the LigM enzyme on vanillic acid as substrate was 150 mU/mg.[17] The need for sequential additions of MetE is related to its partial inactivation over time at 30 °C. [17]

Concerning AroY, all the available purification protocols consist in the isolation of the inactive apoprotein followed by a time-consuming reconstitution step with the cofactor FMN.[22-25] In order to facilitate the bioconversion, we opted for the use of transformed *E. coli* cells (grown reaching a volumetric yield of 24 g cells per litre of culture). An apparent specific activity of 0.27 ± 0.03 U/mg on PCA as substrate was determined (see Experimental Section). When ~~300 mg of~~ wet pET24::AroY transformed *E. coli* cells (300 mg/mL) or the corresponding amount of lyophilised cells (60 mg/mL) were incubated with 1 mM PCA in 100 mM Tris-HCl, pH 8.0, in a 1 mL final volume, a peak corresponding to 1 mM catechol was detected after 4 h of incubation at 30 °C (Figure 1C).

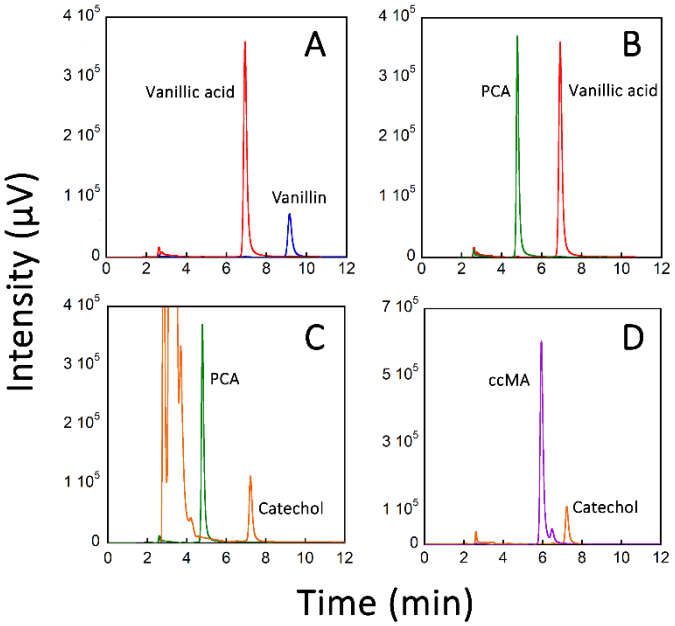


Figure 1. HPLC chromatograms of single catalytic steps in the bioconversion of (A) vanillin to vanillic acid, (B) vanillic acid to PCA, (C) PCA to catechol, and (D) catechol to ccMA. All the reactions were carried out on 1 mM substrate in 100 mM Tris-HCl (pH 8.0, 30 °C, 1 mL final volume). The chromatograms showing the reaction mixtures at the beginning and at the end of the incubation are shown in different colors in each panel. For sake of clarity, the peak corresponding to catechol was reported at 10-fold magnification.

Noteworthy, a similar bioconversion process was achieved by using the lyophilised transformed cells. Taking into account their stability (up to 6 months) and the easy storage, the lyophilised cells were selected for the subsequent studies.

The enzyme CatA showed a similar specific activity (2900 ± 150 U/mg) at 30 °C both using its optimal buffer (i.e. 50 mM HEPES, pH 8.0)[26] and 100 mM Tris-HCl, pH 8.0. The bioconversion of 1 mM catechol by CatA (0.02 mg/mL, 58 U) was ~~thus~~ performed in 100 mM Tris-HCl, pH 8.0 (1 mL final volume), and resulted in the full conversion to 1 mM ccMA after 30 min of incubation (Figure 1D).

Noteworthy, for each bioconversion step, the incubation of the relative control without the corresponding enzyme (or using untransformed *E. coli* cells for the AroY step), did not generate any detectable reaction product.

Multi-step sequential bioconversion of vanillin into ccMA

In order to demonstrate the feasibility of the process, the multi-step bioconversion of vanillin into ccMA was investigated in a sequential way, using the reaction conditions optimised for each single reaction (at pH 8.0, 30 °C, 1 mL final volume): the enzyme catalysing the next step was added when the previous reaction was completed. Firstly, 1 mM vanillin was oxidised in the presence of 90 μg/mL (15 U) XO and 0.1 mg/mL catalase (>200 U; an excess of ~~the latter enzyme~~ catalase was added ~~in order~~ to ~~avoid~~ eliminate ~~that~~ the hydrogen peroxide formed by XO ~~could affect the following enzymatic steps~~): after 2 h of incubation, 1 mM vanillin was completely converted into vanillic acid, as expected (see the Supporting Information, Figure S1A). Then, 0.1 mM THF, 2 mM ~~L-~~ homocysteine and the LigM/MetE (0.3 ~~mg~~ and 1 mg/mL, respectively) bi-enzymatic system were added. After 10 h of incubation, 1 mM vanillic acid was completely converted into 1 mM PCA (see the Supporting Information, Figure S1B). Thirdly, ~~60 mg of~~ the lyophilised pET24::AroY transformed BL21(DE3) *E. coli* cells (60 mg/mL) were added to the reaction mixture: after 4 h of incubation, 1 mM PCA was fully converted into catechol (see the Supporting Information, Figure S1C). Finally, the full conversion of catechol into ccMA was obtained in 2 h by adding 20 μg/mL CatA (see the Supporting Information, Figure S1D). On the whole, ≈~~1 mM~~100% of vanillin was converted into ccMA ~~was thus formed~~ in a total time 18 h at pH 8.0 and 30 °C.

Bioconversion of vanillin into ccMA: one-pot vs tandem process

Because of the apparent difference in reaction kinetics, the optimal incubation conditions for the one-pot production of ccMA from vanillin were identified by first combining in two separate one-pot mixtures the components involved in the bioconversion of vanillin into PCA and those for the bioconversion of PCA into ccMA (see Scheme 1).

The multi-enzymatic system composed by XO, LigM and MetE was applied in the one-pot oxidation of vanillin into vanillic acid, the THF-dependent demethylation of vanillic acid into PCA and the regeneration of the THF cofactor. At first, 1 mL solution containing 1 mM vanillin, 0.1 mM THF, 2 mM ~~L-~~homocysteine, 90 µg/mL XO, 0.3 mg/mL LigM, 1 mg/mL MetE and 0.1 mg/mL catalase was incubated at 30 °C and pH 8.0: after 14 h of incubation, ~55% of PCA was produced and a 35% of vanillic acid, the intermediate reaction product, was still present (Figure 2A).

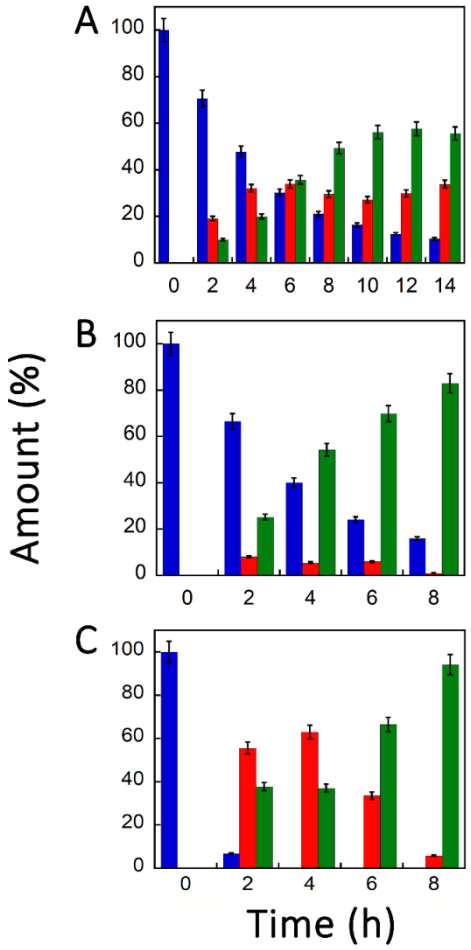


Figure 2. Time course of the ~~1 mL~~ one-pot bioconversion of 1 mM vanillin into PCA (pH 8.0, 30 °C) in the presence of (A) standard amount of XO (90 µg) and LigM (0.3 mg), (B) 10-fold increased LigM (3 mg) and (C) 5- and 10-fold increased XO (0.45 mg) and LigM (3 mg), respectively (1 mL final reaction volume). The column bars of the relative amount of vanillin, vanillic acid and PCA are depicted in blue, red and green, respectively.

In order to speed up the bioconversion of vanillin, the rate of the second step was increased by using a 10-fold excess of LigM (3 mg/mL final concentration). After 8 h of incubation, ~80% of PCA was produced and a 20% of vanillin was still present (Figure 2B). The almost complete conversion of 1 mM vanillin into PCA was achieved in 8 h when the 10-fold increased amount of LigM was used in combination with a 5-fold increase amount of XO (0.45 mg/mL final concentration) (Figure 2C and Figure 3A).

Then, the ccMA production from PCA was carried out in a one-pot reaction (1 mL final volume) containing 1 mM PCA, 60 mg/mL lyophilised pET24::AroY transformed BL21(DE3) *E. coli* cells and 20 µg/mL CatA in 100 mM Tris-HCl, pH 8.0: after 2 h of incubation, the peak corresponding to PCA disappeared and the complete bioconversion of 1 mM PCA into ccMA was achieved (Figure 3B).

The set up of the one-pot bioconversion of vanillin into ccMA by using the complete enzymatic system is hindered by the conversion of vanillin to vanillyl alcohol due to the use of *E. coli* cells in the third step of bioconversion (~~data not shown~~ see the Supporting Information, Figure S2). Previous evidence reported the presence of endogenous aromatic aldehyde reductases responsible of the rapid conversion of aldehydes into undesired alcohols.[27,28] Actually, a 10-fold higher XO amount (0.9 mg/mL final concentration) was required to fully convert vanillin avoiding its reduction to vanillyl alcohol when the one-pot process was set-up with only 10 mg/mL of the lyophilised BL21(DE3) *E. coli* cells. After 7 h, a further addition of lyophilised pET24::AroY transformed BL21(DE3) *E. coli* cells (50 mg) pushed the ccMA production: starting from 1 mM vanillin, ~0.75 mM ccMA was produced in 10 h (~75% yield, not shown). An intriguing option could be the use of the engineered *E. coli* K-12 MG1655 strain with reduced aromatic aldehyde reduction (RARE) for the AroY step: the rationally deletion of six genes, resulted in the RARE strain successfully used for the accumulation of vanillin from vanillate by preventing formation of the byproduct vanillyl alcohol.[28]

An alternative strategy for the one-pot bioconversion of vanillin into ccMA consists in a two-step one-pot procedure (tandem process) which first leads to the production of the PCA intermediate from vanillin, followed by its conversion into ccMA in the same pot.[~~25,26~~] First, the one-pot bioconversion of 1 mM vanillin into the intermediate PCA was performed using the optimized amounts of XO, LigM/MetE, THF, L-homocysteine, and catalase previously identified (i.e., 0.45 mg/mL and 3 mg/mL of XO and LigM, respectively), in 1 mL final reaction volume. After ~8 h of incubation at 30 °C, ~0.95 mM PCA was produced; hence, both the lyophilized pET24::AroY transformed *E. coli* cells and CatA were added to the

reaction mixture (60 mg and 20 µg, respectively; 1.05 mL final volume) and, after further 3 h of incubation at 30 °C, a peak corresponding to ~0.95 mM of the final product ccMA was observed, without any accumulation of intermediates. Under these operational conditions, the bioconversion of 1 mM vanillin into ccMA was achieved in 11 h with a ~95% yield (see Figure 3C).

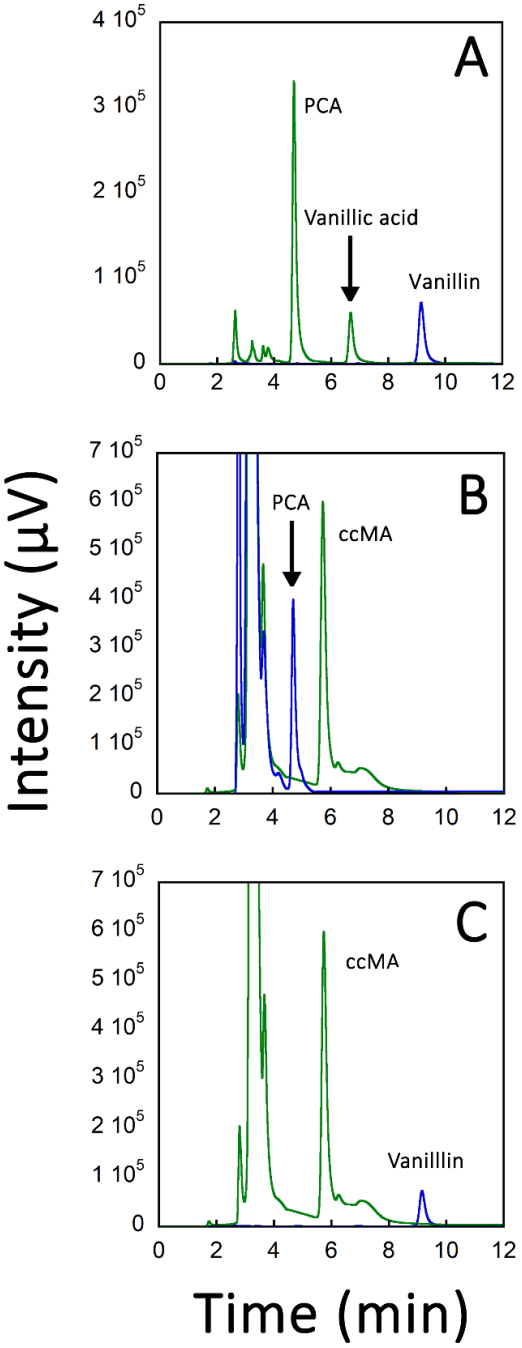


Figure 3. HPLC chromatograms of the mixture composition corresponding to the multi-enzymatic one-pot bioconversion of (A) 1 mM vanillin into PCA, (B) 1 mM PCA into ccMA and (C) the tandem bioconversion of 1 mM vanillin into ccMA, in 100 mM Tris-HCl, pH 8.0, 30 °C, at t0 (blue line) and at the end of incubation (green line) (1.05 mL final reaction volume).

Scaled up production of ccMA

In view of a practical application, the tandem system was performed at 5 mM of vanillin (this concentration represents the solubility limit for vanillic acid)[17] in a 1 mL reaction mixture containing XO (0.45 mg/mL), LigM (3 mg/mL), MetE (1 mg/mL), catalase (0.1 mg/mL), 0.5 mM THF and 10 mM homocysteine, in 100 mM Tris-HCl buffer, pH 8.0. A 0.45 mg/mL of XO and 3 mg/mL of LigM were further added after 10 h incubation to push the vanillin consumption: the peak corresponding to vanillin disappeared after 4 h, this allowing the addition of both the lyophilized pET24::AroY transformed *E. coli* cells (60 mg) and CatA (20 µg) (see Figure ~~4B~~). Following 23 h from the starting of the reaction, ~3 mM ccMA was produced (60% yield, Figure 4B). Adding an additional amount of lyophilized pET24::AroY transformed *E. coli* cells (60 mg; 1.3 mL final volume), residual PCA was almost completely~~fully~~ transformed into the final product ccMA in a total of 30 h, with a ~90% yield (a ~10% PCA was still present, see Figure 4).

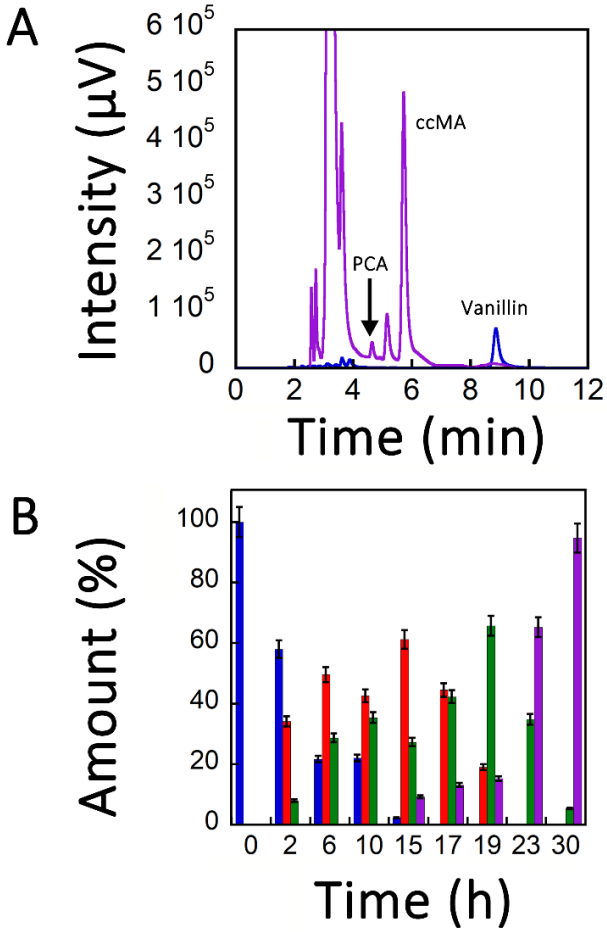


Figure 4. Bioconversion of 5 mM vanillin into ccMA, in 100 mM Tris-HCl, pH 8.0, 30 °C (1.3 mL final reaction volume). (A) HPLC chromatograms of vanillin (t0, blue line) and of ccMA produced after 30 h (purple line). (B) Time course of the reaction. The column bars of the relative amount of vanillin, vanillic acid, PCA and ccMA are depicted in blue, red, green and purple, respectively.

Product was recovered from the reaction mixture by methanol precipitation: a ccMA titer of 1 g L^{-1} in 30 h was apparent. The liquid fraction contains the muconic acid as *cis,cis*-enantiomer only (the peak corresponding to the *trans,trans*-muconic acid was absent in the HPLC chromatogram, see Figure 4A and Supporting Information, Figure S3A). Its identity was demonstrated by ESI-MS (see Supporting Information, Figure S3B): the full scan mass spectrum shows an (M-1)-m/z 141 (100, relative intensity), and an intense product ion at m/z 97 that resulted from a decarboxylation process (Supporting Information, Figure 3B, top). An identical fragmentation pattern was observed when the commercially available *cis,cis*-muconic acid was dissolved in methanol and analysed in the same conditions (Supporting Information, Figure 3B, bottom).

Previously, a biotechnological route for ccMA production from vanillin was reported based on the engineering of the *E. coli* DH1 strain containing four genes encoding for vanillin dehydrogenase, *O*-demethylase, protocatechuate decarboxylase and catechol dioxygenase:[10] 0.69 g ccMA/g vanillin was produced after 48 h of incubation at 30 °C. Our work well compared in terms of yield (0.89 g ccMA/g vanillin) and time with the whole-cell system. In particular, our enzymatic process overcomes the limiting step of the cell-based bioconversion, represented by the protecatechuate decarboxylase.[10] Furthermore, the optimized operational conditions used in our work are compatible with the stability of all enzymes and avoid drawbacks due to both reaction equilibria and side-reactions. In particular, the constant removing of the PCA intermediate by the decarboxylase enzyme allows to push the reaction catalysed by the LigM/MetE by-enzymatic system towards PCA, otherwise reaching at equilibrium a ~50% yield.[17]

In these last years, for the biobased ccMA production, much attention has been given to metabolic engineering of bacteria naturally able to utilize a variety of lignin-derived aromatics as a carbon source for growth and energy production. Interestingly, the engineered *Pseudomonas putida* KT2440 based strain produced ccMA with a ~20% yield while growing on a mixture of vanillic acid and 4-hydroxybenzoic acid, without supplement of glucose. Analogously, the *Sphingobium sp.* SYK-6 based strain showed a sugar-free ccMA production when grown on a mixture of vanillic acid and syringic acid, as sole carbon source, with a ~45% yield.[29]

The economic aspects of ~~this~~ our multi-enzymatic bioconversion were taken into great consideration during the design of the synthetic pathway. The use of recombinant His-tagged enzymes (i.e. LigM, MetE and CatA), which are easily purified with high yields in a low-cost single chromatographic step, offers some obvious benefit when compared to the directly use of crude extract cells (i.e., absence of competing reactions and enzyme inhibitors, simplest product recovery, facilitated reaction control, easy storage, possible immobilization). Noteworthy, the use of an efficient system for the regeneration of the LigM cofactor (which allows a reduction of the lab-scale costs from 20 to 0.07 € per mg of converted vanillic acid)[17] accompanied by the use of racemic D,L-homocysteine instead of the more expensive single enantiomer (approx. 50-fold cheaper),[17] represent main advantages of the proposed biocatalytic system. Remarkably, the choice to adopt lyophilized *E. coli* cells expressing the enzyme protocatechuate decarboxylase AroY drastically reduce both the costs and the times required for the reconstitution of the purified inactive apoprotein AroY with its FMN cofactor,[22] this combined with a high stability and easy storage of the lyophilized cells.

Conclusion

In this work, a high-yield biocatalytic system for production of ccMA starting from vanillin was presented. In comparison with cell-based systems, the multi-enzymatic reactions allow a rapid and easy modification of the reaction parameters in order to improve both the yield and the timespan of the process. The immobilization of this multi-enzymatic system, ~~aimed at increasing the enzyme stability,~~ could represent a further improvement by pushing its use while reducing the costs, once the intrinsic problems of the process have been solved. Over the last few years, the merging of continuous flow synthesis and biocatalysis has been considered one of the most promising technologies for industrial continuous flow applications. The enzyme immobilization onto the reactor wall (wall-coated reactor) or encapsulated into a tube (packed-bed reactor) could increase the enzyme stability, simplify the product work-up, and reduce the required amount of biocatalyst.[30] Moreover, the incorporation of both enzymes and whole cells in biodegradable bacterial nanocellulose discs characterized by a high porosity and water absorption capacity, diffusion properties, and mechanical and chemical stability, could represent a suitable composite hydrogel material for spatially confined biocatalysts, thus reducing the mass transfer problem.[31,32]

Moreover, our study provides the operational basis for the design of novel bio-catalysed synthetic pathways: the availability of a toolbox of enzymatic activities catalysing the conversion of different lignin-derived compounds will accelerate the development of innovative multi-enzymatic cascades, thus providing a green opportunity to transform lignin into high value

added compounds, especially biodegradable plastic materials.

Experimental Section

Reagents and enzymes

Xanthine oxidase from bovine serum milk (Grade I, ammonium sulfate suspension), ~~catalase from bovine liver,~~ THF, analytical grade standards of vanillin (4-hydroxy-3-methoxybenzaldehyde), vanillic acid (4-hydroxy-3-methoxybenzoic acid), protocatechuic acid (3,4-dimethoxybenzoic acid~~, PCA~~), catechol (1,2-dihydroxybenzene), *cis,cis*-muconic acid ~~((2Z,4Z) hexa-2,4-dienedioic acid, ccMA)~~, *trans,trans*-muconic acid, acetonitrile (ACS Grade, ≥99%) and formic acid (ACS Grade, ≥ 98%) were purchased by Merck/Carlo Erba (Merck KGaA, Darmstadt, Germany). Catalase from bovine liver (2000-5000 U/mg) was from Boheringer Mannheim. Methanol of ACS Grade (≥ 99%) was purchased by Carlo Erba. The enzyme catechol 1,2-dioxygenase (CatA) from *Acinetobacter radioresistens* S13, cloned between *NdeI* site and *EcoRI* site in pET30(+) (Novagen)[33], was produced in *E. coli* as previously described;[26] recombinant *O*-demethylase from *Sphingobium* sp. SYK-6 ~~(LigM)~~ and methionine synthase from *Catharanthus roseus* ~~(MetE)~~ were expressed in *E. coli* and purified as previously described.[17]

AroY recombinant expression in *E. coli* cells

The synthetic gene encoding the protocatechuate decarboxylase AroY from *Klebsiella pneumoniae* ssp. *pneumoniae* A170-40 was previously optimized for the recombinant expression in *E. coli* (Accession no. KX774258.1).[10] The *AroY* cDNA was synthesized by TWIST Bioscience (San Francisco, USA) and cloned in the pET24(b) vector using the *BamHI* (GGATCC) and *XhoI* (CTCGAG) restriction sites. During the subcloning procedure, six codons (encoding for six additional histidines, His-tag) were added at the C-terminus of the *AroY* gene.

The pET24::AroY plasmid was transferred to the BL21(DE3) *E. coli* host strain. Cells were grown at 37 °C in Terrific broth and protein expression was induced at an OD_{600nm} ≈ 0.6 by adding 0.2 mM IPTG; cells were further grown at 18 °C for 18 h under shaking.[23] After the harvesting of the cells by centrifugation (8000 g, 10 min, 4 °C) and their washing twice in 100 mM Tris-HCl, pH 8.0, cells were resuspended at the final concentration of 0.8 g cells/mL in the same buffer. Cells have been lyophilized and stored as a dried power at 4 °C. A rehydration step (i.e. 15 minutes of incubation at room temperature on a rotatory wheel in the presence of an amount of deionized water corresponding to the one lost during lyophilisation) was performed before their use.[23,34] Notably, the same growth and expression protocol, followed by the harvesting, washing and lyophilisation steps, were performed also for not transformed BL21(DE3) *E. coli* cells, used as control during the bioconversion reactions.

Protein quantification

The commercial ammonium sulfate suspension of XO from bovine serum milk was dialysed against 100 mM Tris-HCl, pH 8.0 and the amount of XO (≈150 kDa) was determined by measuring the absorbance intensity at 450 nm and using the molar extinction coefficient of 36000 M^{-1} cm^{-1}.[23] The amount of purified LigM (≈55 kDa), MetE (≈85 kDa) and CatA (≈38 kDa) were estimated by measuring the absorbance intensity at 280 nm and using the following molar extinction coefficients: 107950 M^{-1} cm^{-1},[17] 126290 M^{-1} cm^{-1},[17] and 53560 M^{-1} cm^{-1},[26] respectively.

Single-step biocatalytic process

Oxidation of vanillin into vanillic acid

XO was equilibrated in 100 mM Tris-HCl, pH 8.0 buffer by dialysis. The standard oxidase activity of XO was determined on 100 μM xanthine as substrate in Krebs-Henseleit buffer, pH 7.4,[21] or in 100 mM Tris-HCl, pH 8.0, at 25 °C by following for 5 min the change in absorbance at 295 nm on a Jasco V-580 spectrophotometer (Cremella, Italy) and using a molar absorption coefficient of 9600 M^{-1} cm^{-1}.[35] One enzymatic unit was defined as the amount of enzyme which converts 1 μmol of substrate in one minute, at 25 °C.

The activity of XO on 1 mM vanillin was determined by HPLC: the mixture was assayed under the conditions used in the bioconversion reaction (i.e. 90 μg XO and 30 °C) and the initial rate was determined within the linear range, i.e. in the 0-20 min of conversion (see "HPLC analyses" paragraph). A specific activity on vanillin of 165 ± 15 U/mg was determined. For bioconversion studies, the oxidation of vanillin was assayed in a 1 mL reaction mixture containing 1 mM vanillin and 90 μg (15 U) XO in 100 mM Tris-HCl, pH 8.0: the reaction mixture was incubated for 2 h at 30 °C on a rotatory wheel. A control reaction in the absence of enzyme was also assayed. Reaction mixtures withdrawn at different times were analysed using the HPLC method (see below). Catalase (>200 U) is added to remove H_2O_2 generated by XO activity.

Demethylation of vanillic acid into PCA

The vanillic acid demethylation into PCA was carried out using the recombinant enzymes *O*-demethylase THF-dependent LigM and methionine synthase MetE, as previously described.[17] The 1 mL reaction mixture contained 1 mM vanillic acid, 0.1 mM THF and 2 mM L-homocysteine (the substrate of the MetE enzyme, see Scheme 1),[17] in 100 mM Tris-HCl, pH 8.0. LigM specific activity is 0.15 U/mg. Reaction started by adding 0.3 mg (45 mU) LigM and 1 mg MetE.[17] The reaction control was assayed in the absence of enzymes. Every 2 h, 0.5 mg MetE or an equal volume of buffer were added to the mixture and in the control reactions, respectively. Reaction mixtures were incubated at 30 °C for 8 h on a rotatory wheel and 25 μL of sample were withdrawn at different times and analysed by HPLC (see below).

Whole-cell decarboxylation of PCA into catechol

The activity of pET24::AroY *E. coli* transformed cells was determined on 1 mM PCA in 100 mM Tris-HCl, pH 8.0, in the presence of 60 mg of lyophilized cells (corresponding to 0.3 g of wet cells). The initial rate of PCA consumption was determined within the linear range, i.e. in the 0-25 min of conversion (analyses were performed by using the HPLC method, see below). One enzymatic unit is defined as the

amount of lyophilized cells that converts 1 µmol of substrate in one minute, at 30 °C: an apparent specific activity of 0.265 ± 0.03 U/mg of lyophilized cells was determined.
The bioconversion of 1 mM PCA into catechol was performed in a 1 mL reaction mixture containing 60 mg (16 U) of lyophilized pET24::AroY transformed *E. coli* cells in 100 mM Tris-HCl, pH 8.0. The control was carried out using 60 mg of untransformed *E. coli* BL21(DE3) lyophilized cells, as well as omitting the substrate. Reaction mixtures were incubated at 30 °C on a rotatory wheel for 4 h. At different times, 50 µL of each sample were withdrawn and centrifuged for 10 min at 13000 rpm, 4 °C and analyzed by HPLC (see below).

Conversion of catechol into ccMA
One enzymatic unit was defined as the amount of CatA that converts 1 µmol of substrate in one minute, at 30 °C. The activity of CatA was routinely assayed on 2 mM catechol by following for 5 min the ccMA formation at 260 nm (extinction coefficient of 17600 M^{-1} cm^{-1} at 260 nm) in a thermostated spectrophotometer at 30 °C (1 mL): no difference in activity value was observed using 50 mM HEPES, pH 8.0,[26] or 100 mM Tris-HCl, pH 8.0 as buffer. For bioconversion studies, the activity on 1 mM catechol in 100 mM Tris-HCl buffer, pH 8.0, was determined by following the initial rate (15 min) of a reaction ~~assayed in the presence~~added of 3.6 µg CatA (final volume 1 mL, 30 °C) and by monitoring the ccMA formation as reported in the "HPLC analyses" paragraph. A specific activity on 1 mM catechol of 2900 ± 150 U/mg was determined.
The 1 mL bioconversion of 1 mM catechol was carried out using 20 µg (58 U) CatA in 100 mM Tris-HCl, pH 8.0, for 30 min at 30 °C on a rotatory wheel: at different times 25 µL of sample were withdrawn for HPLC analyses (see below).

Combination of biocatalytic steps
Multi-step sequential bioconversion of vanillin into ccMA
The sequential bioconversion of vanillin into ccMA was performed at 30 °C using the reaction conditions optimised for each single reaction in four steps: a) 1 mM vanillin was incubated in 100 mM Tris-HCl, pH 8.0 (1 mL final volume) with 90 µg (15 U) XO and 0.1 mg (>200 U) catalase; b) after 2 h of incubation, 0.1 mM THF, 2 mM L-homocysteine, 0.3 mg (45 mU) LigM and 1 mg MetE (or an equal volume of buffer) were added to the reaction mixture (or to the control). In addition, 0.5 mg of MetE were supplemented after 2, 4, 6 and 8 h of incubation; c) after 10 h, 60 mg (16 U) of lyophilized pET24::AroY transformed BL21(DE3) *E. coli* cells were added to the reaction mixture and incubated for 4 h; d) finally, 20 µg (58 U) CatA were added and the reaction was monitored for further 2 h. At different times, samples were analysed by HPLC to monitor the time course of each reaction step (see below).

One-pot bioconversion of vanillin into PCA
The bioconversion of vanillin into PCA was performed in a one-pot multi-enzymatic reaction. The ~~1 mL~~ reaction mixture containing 1 mM vanillin, 0.1 mM THF and 2 mM ~~L-~~homocysteine in 100 mM Tris-HCl, pH 8.0, was added of XO (90 µg, 15 U), LigM (0.3 mg, 45 mU), MetE (1 mg) and catalase (0.1 mg, >200 U) (1 mL final volume). The control reaction did not contain the enzymes. The reaction mixture was incubated for 14 h at 30 °C on a rotatory wheel. The enzyme MetE (0.5 mg) was added after 2, 4, 6, and 8 h of reaction. At different times, 25 µL of sample were withdrawn for HPLC analyses (see below).

One-pot bioconversion of PCA into ccMA
The mixture for the one-pot bioconversion of PCA into ccMA contained 1 mM PCA, 60 mg (16 U) of lyophilized pET24::AroY transformed BL21(DE3) *E. coli* cells and 20 µg (58 U) CatA in 100 mM Tris-HCl, pH 8.0 (final volume 1 mL). As controls, reaction mixtures containing not transformed lyophilised BL21(DE3) *E. coli* cells or lacking the substrate PCA were used. Reaction mixtures were incubated for 4 h at 30 °C, on a rotatory wheel: at different times, 50 µL of the reaction mixture were withdrawn and centrifuged for 10 min at 13000 rpm, 4 °C. The supernatant was subsequently treated and analysed as described in the "HPLC analyses" paragraph.

One-pot bioconversion of vanillin into ccMA
The one-pot bioconversion of vanillin into ccMA was performed at 30 °C under optimised conditions. The 1 mL reaction mixture contained 0.1 mg (1.4 U) catalase, 3 mg (0.45 U) LigM, 1 mg MetE, 10 mg (2.6 U) of lyophilized pET24::AroY transformed BL21(DE3) *E. coli* cells (let to re-hydrate for 15 minutes at room temperature on a rotary wheel) and 20 µg (58 U) CatA. The reaction was started by adding 0.9 mg (150 U) XO. The 1 mL control mixture contained 1 mM vanillin, 0.1 mM THF and 2 mM ~~L-~~homocysteine in 100 mM Tris-HCl, pH 8.0 buffer. In order to push the bioconversion, 0.5 mg MetE were added every 2 h; after 7 h of incubation, both 50 mg (13 U) of lyophilized pET24::AroY transformed BL21(DE3) *E. coli* cells and 20 µg (58 U) CatA were further added (1 mL final volume). In order to maintain the same final volume, an equal volume of 100 mM Tris-HCl buffer, pH 8.0, was added in the control mixture.

Two-step one-pot bioconversion of vanillin into ccMA and scale-up
The bioconversion of vanillin into ccMA was also carried out in a two-step one-pot reaction. Firstly, 1 mM vanillin, 0.1 mM THF and 2 mM L-homocysteine were incubated in the presence of XO (0.45 mg, 75 U), LigM (3 mg, 0.45 U), MetE (1 mg) and catalase (0.1 mg, 1.4 U) in 100 mM Tris-HCl, pH 8.0 (1 mL final volume). After 2, 4, 6 and 8 h, 0.5 mg MetE were supplemented. After 8 h of incubation at 30 °C on a rotary wheel, 60 mg (16 U) of lyophilized pET24::AroY transformed *E. coli* cells and 20 µg (58 U) of CatA were added in the same pot reaction (1.05 mL final volume). Analyses on samples withdrawn at different times were performed by HPLC (see below).
The scale-up of the bioconversion was performed using 5 mM vanillin in a 1 mL reaction mixture containing 0.5 mM THF, 10 mM ~~L-~~homocysteine and the enzymes XO (0.45 mg, 75 U), LigM (3 mg, 0.45 U), MetE (1 mg) and catalase (0.1 mg, 1.4 U) in 100 mM Tris-HCl buffer, pH 8.0. After 10 h of incubation at 30 °C on a rotary wheel, the reaction mixture was added of 0.45 mg (75 U) XO and 3 mg (0.45 U) LigM. Furthermore, after 14 h, 60 mg (16 U) of lyophilized pET24::AroY transformed BL21(DE3) *E. coli*

cells and 20 µg (58 U) CatA were supplemented. Finally, after 28 h, 60 mg (16 U) of lyophilized pET24::AroY transformed BL21(DE3) *E. coli* cells were added again. Notably, for the first 20 h of incubation, 0.5 mg MetE were added every 2 h. Final reaction volume is 1.3 mL. At different times, samples were analysed by HPLC (see below). A reaction not containing enzymes/cells was used as control.

HPLC analyses

All the HPLC analyses were performed on a Jasco apparatus equipped with a Symmetry C8 column 100 Å, 5 µL, 4.6 x 250 mm (Waters, Milan, Italy) and with an UV detector set at 254 nm. The mobile phase consisted in 34.5% (v/v) methanol, 5.4% (v/v) acetonitrile and 0.1% (v/v) formic acid; the flow rate was 0.8 mL/min and the column oven was set at 30 °C.[17]

Calibration curves were obtained solubilising standards of vanillin, vanillic acid, PCA, catechol and ccMA in 100 mM Tris-HCl, pH 8.0 at the final concentration of 40 mM. Subsequently, after dilution in the 0.04 – 2 mM range, 80 µL of each sample were added with 160 µL of the mobile phase and centrifuged for 2 min at 13000 rpm, 4 °C: 20 µL of the supernatant were injected for HPLC analysis. Each value was reported as the mean of three measurements ± standard deviation. Retention times for standards of PCA, ccMA, vanillic acid, catechol and vanillin were: 4.8, 5.9, 7.2, 7.7 and 9.7 min, respectively. The calibration curves are reported in the Supporting Information, Figure S4.

The biocatalytic processes were monitored by withdrawing, at different times, 25 µL of reaction mixture that were added with 50 µL (350 µL for the scaled-up bioconversion of 5 mM vanillin) of mobile phase. After 5 min of centrifugation at 13000 rpm at 4 °C, 20 µL of supernatant were analysed by HPLC.

Product recovery and characterization

The reaction mixture from the scaled up bioconversion of 5 mM vanillin (1.3 mL) was treated with 130 µL of methanol, centrifuged to eliminate enzyme and *E. coli* cells, evaporated, and then dissolved in 150 µL of methanol. The product ccMA was characterized by mass spectrometry analysis using Waters ZQ-2000 instrument single quadruple in ESI mode.

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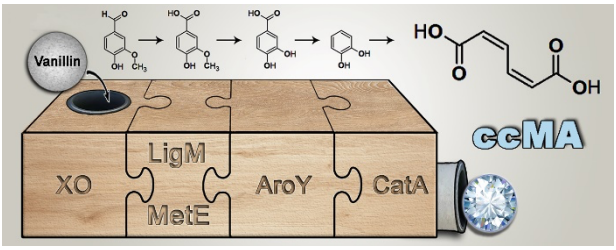
RESEARCH ARTICLE

Multi-enzymatic cascade reactions for the synthesis of *cis,cis*-muconic acid



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