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Chemo-Enzymatic Cascade for the Generation of Fragrance Aldehydes

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Abstract: In this study, we present the synthesis of chiral fragrance aldehydes, which was tackled by a combination of chemo-catalysis and a multi-enzymatic in vivo cascade reaction and the development of a highly versatile high-throughput assay for the enzymatic reduction of carboxylic acids. We investigated a biocompatible metal-catalyzed synthesis for the preparation of α or β substituted cinnamic acid derivatives which were fed directly into the biocatalytic system. Subsequently, the target molecules were synthesized by an enzymatic cascade consisting of a carboxylate reduction, followed by the selective C-C double bond reduction catalyzed by appropriate enoate reductases. We investigated a biocompatible oxidative Heck protocol and combined it with cells expressing a carboxylic acid reductase from *Neurospora crassa* (*Nc*CAR) and an ene reductase from *Saccharomyces pastorianus* for the production fragrance aldehydes.

Keywords: chemo-enzymatic cascade; carboxylic acid reductase; ene reductase; Heck-coupling; fragrance aldehydes

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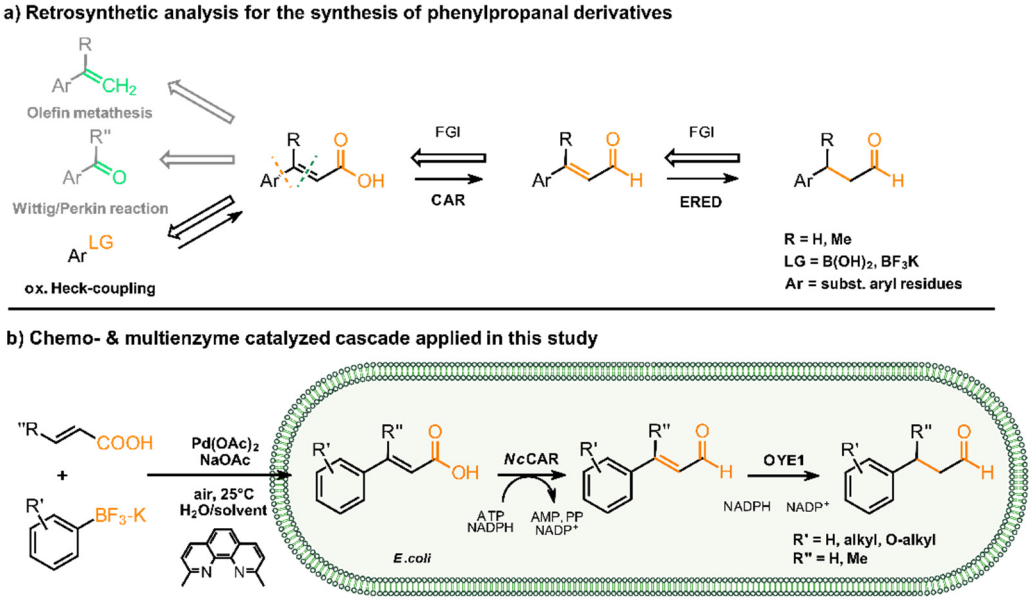
1. Introduction

Biocatalysis keeps adding new and versatile synthetic routes to organic synthesis and has proved to be a valuable part of today's chemistry [1]. The combination of organic chemistry with enzyme catalysis is attracting more and more attention, with high yields, less toxic byproducts and in general more environmentally friendly processes [2]. The first approaches for combining the advantages of the two fields started decades ago and in the 1090s, racemic resolution started to be broadly applied [3]. Since then, numerous applications for combinations of chemo-and biocatalytic steps were explored and employed in single- and multistep reactions. Cascade reactions are defined as consisting of at least two consecutive transformations, where the product of the first step serves as substrate for the subsequent step [2]. The advantages of both fields can be combined if the protocols for both the organic and the biocatalytic step are compatible. Reactions with biocatalysts typically require atmospheric pressure, ambient temperatures, and a nearly neutral pH. Enzymes are adapted to aqueous environments and therefore frequently applied in or around a cellular environment. The key advantage of enzymes is their naturally evolved selectivity (chemo-, regio-, stereoselectivity).

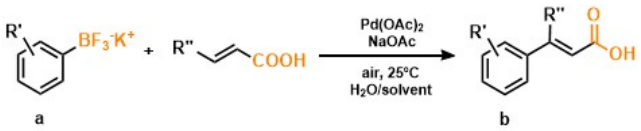
Our aim was to exploit the unique chemoselectivities of reductive enzymes and to explore routes for the generation of aromatic, chiral aldehydes via a chemoenzymatic reaction sequence. Starting with a retrosynthetic analysis (Scheme 1a), the first step in this cascade is the generation of cinnamic acid derivatives. Several synthetic routes are well described, e.g., Wittig, metathesis reactions and others [4]. The perhaps most commonly applied Perkin reaction relies on benzaldehyde derivatives, acetic acid anhydride, an

organic solvent, the addition of a base, and high temperatures for the formation of cinnamic acid derivatives [5]. One equivalent carboxylic acid is released, and the overall atom efficiency of the reaction is low. These reaction conditions are not biocompatible at all. In contrast, palladium-catalyzed cross-coupling reactions (e.g., Heck coupling) [6] are more compatible with enzymatic reactions, because protocols in aqueous systems were developed. These coupling reactions can directly be used and combined in biological applications [7–12] without further purification steps.



Scheme 1. Routes to aryl-aliphatic aldehydes (**a**) Retrosynthetic analysis for the preparation of cinnamic acid derivatives (**b**) Biocatalytic cascade reaction, Reaction scheme for Heck coupling and subsequent reductions steps; a: Trifluorborate, b: cinnamic acid, c: cinnamaldehyde, d: Phenylpropionaldehyde, e: cinnamyl alcohol (unsaturated alcohol of c); for generated compound, please see Table 1.

Table 1. Heck coupling efficiency in water with 10% DMSO.



Entry	Product	Yield [%]
1	**1b** R″ = H, R′ = H	76.0 ± 1.0
2	**2b** R″ = CH_3, R′ = H	61.0 ± 4.0
3	**3b** R″ = H, R′ = 3,4-O-CH_2-O	73.0 ± 1.0
4	**4b** R″ = CH_3, R′ = 3,4-O-CH_2-O	49.0 ± 2.0
5	**5b** R″ = H, R′= 4-*t*Bu	37.0 ± 3.0
6	**6b** R″ = CH_3, R′ = 4-*t*Bu	8.0 ± 0.4
7	**7b** R″ = H, R′ = 4-*iso*Bu	30.0 ± 1.0
8	**8b** R″ = CH_3, R′ = 4-*iso*Bu	11.0 ± 1.0

In the second step, the carboxylic acid is reduced in an enzymatic one-step reaction to the corresponding aldehyde. This functional group transformation is still challenging for chemical as well as enzymatic routes. Carboxylic acids show little reactivity and need a high level of activation energy to participate in reactions. The aldehyde product is more susceptible to reduction than the acid starting material and is easily over-reduced to the respective alcohol. Nature provides a way of selectively reducing carboxylic acids to the corresponding aldehydes: CArboxylate Reductases (CARs). The carboxylate reduction is

dependent on ATP and NADPH, and ATP is used to provide energy for substrate activation and NADPH for the actual reduction step [13].

The reduction of the acid to the aldehyde is the prerequisite step for the reduction of the double bond in the generated cinnamaldehyde (see Scheme 1), because the α,β unsaturated double bond in the cinnamic acid itself is not sufficiently activated to undergo reduction by Ene reductases (EREDs), which represents the third step in the reaction sequence. When the double bond of substituted cinnamaldehydes are reduced, a chiral center is generated [14,15].

We present herein the combination of a Heck coupling reaction in neat water and the subsequent enzymatic reduction of the generated unsaturated acid via a CAR and ERED mediated in vivo cascade (see Scheme 1). The biocatalytic steps of this cascade are predominantly used embedded in whole cells owing to the demand for ATP and NADPH. However, aldehydes are very reactive and tend to interfere with the cellular metabolism and cell components. The most common detoxification mechanism of cells is the elimination of the aldehyde functionality by reduction to the corresponding less reactive alcohol. To circumvent this obstacle, effort went into the development of distinct strains that lack this aldehyde reduction ability [16,17]. A second strategy to increase aldehyde yields is the utilization of in situ product removal (ISPR), e.g., by application of a non-miscible phase [18,19]. Combined strategies have also been reported [20].

2. Results and Discussion

In our chemoenzymatic cascade, the first step was the generation of cinnamic acids. The major aim was to investigate a biocompatible method; hence, we tested oxidative Heck coupling efficiency in the first approach. We adapted and optimized a literature protocol and used $Pd(OAc)_2$, sodium acetate, and neocuproine at room temperature. Yields were between 8% for 3-methyl *tert*-butyl cinnamic acid (**6b**) and 76% for the unsubstituted parent compound cinnamic acid (**1b**) and 10 mM final substrate concentration (Table 1). In general, it seems that solubility has a huge influence on the coupling efficiency. For example, coupling phenyltrifluoroborate with acrylic acid—which generates **1b**—yields 76%, whereas the coupling to *iso-butyl* phenyltrifluoroborate generates only 11% of **8b** (see Table 1). The catalyst concentration was tested from 1 to 10 mol% and seems to reach an optimum at 6 mol%. Coupling phenyltrifluoroborate derivatives with crotonic acid to yield 3-substituted cinnamic acids results in a lower yield than compared to reactions with acrylic acids in all tested cases with a maximum of 61% yield for 3-methyl cinnamic acid (**2b**). Overall, our developed method led to the synthesis of several different cinnamic acid derivatives in up to 76% (Table 1). In addition, we tested the coupling with acrolein and crotonaldehyde but did not achieve *E*-selectivity. Regardless of the conditions, mixtures of *E* and *Z* isomers were observed in our hands.

With the aim to identify a suitable CAR for the subsequent conversion of different cinnamic acid derivatives, a recently developed photometric aldehyde assay was used [21]. This assay is based on the formation of a dihydroquinazoline product in a very fast cyclization-reaction of 2-amino benzamidoxime (ABAO) derivatives and an aldehyde in acidic aqueous sodium acetate buffer. We applied the "ABAO-assay" for the detection of improved CAR mutants in a random mutagenesis library [22]. The formed product has a high absorption coefficient and can be detected at different wavelengths from 380 to 405 nm. This assay was applied to screen for CARs with activity for **1b**, **2b** and **4b** reduction. The results showed that *E. coli* cells expressing *Nc*CAR [23] had the highest activity for all tested substrates.

As reported previously, 1c tends to be reduced to the alcohol (**1e**), however, to a lesser extent compared to non-engineered strains [23] *E. coli* RARE [pETDuet1_EcPPTase_HTNcCAR] showed significant amounts of cinnamyl alcohol, but using hexane for in situ product removal (ISPR) by a two-phase system significantly decreased the alcohol formation [23].

Hexane was rated "hazardous" [24], so alternative solvents were tested in view of whole-cell biocatalysis. A previously described organic solvent that was used for ISPR is

dioctylphthalate (DOP, CAS 117-81-7) [18]. The chemically similar diisononylphthalate (DINP, CAS 28553-12-0), isooctane (IO, CAS 540-84-1), and cyclopentyl methyl ether (CPME, CAS 5614-37-9) were tested as second phases with resting cells and the efficiency of 1a reduction as well as alcohol formation determined. According to Henderson et al. [25], isooctane and CPME show less environmental impact and much lower toxicity as compared to hexane. Solvent selection guides of Pfizer as well as GSK rated these solvents as "usable" for reactions and no substitution is needed [26]. CPME was also described previously by Maugeri et al. [27] as a solvent for the application in a Micro Aqueous Reaction System (MARS) for substrates and products that are not water-soluble, which would be favorable for hydrophobic cinnamic acid derivatives and the corresponding aldehydes. The reaction without any co-solvent generated only 5.5% of **1c** but 7.5% of cinnamyl alcohol (**1e**), respectively, which shows the necessity for a second phase. In our hands, IO was giving the best result (see Figure 1).

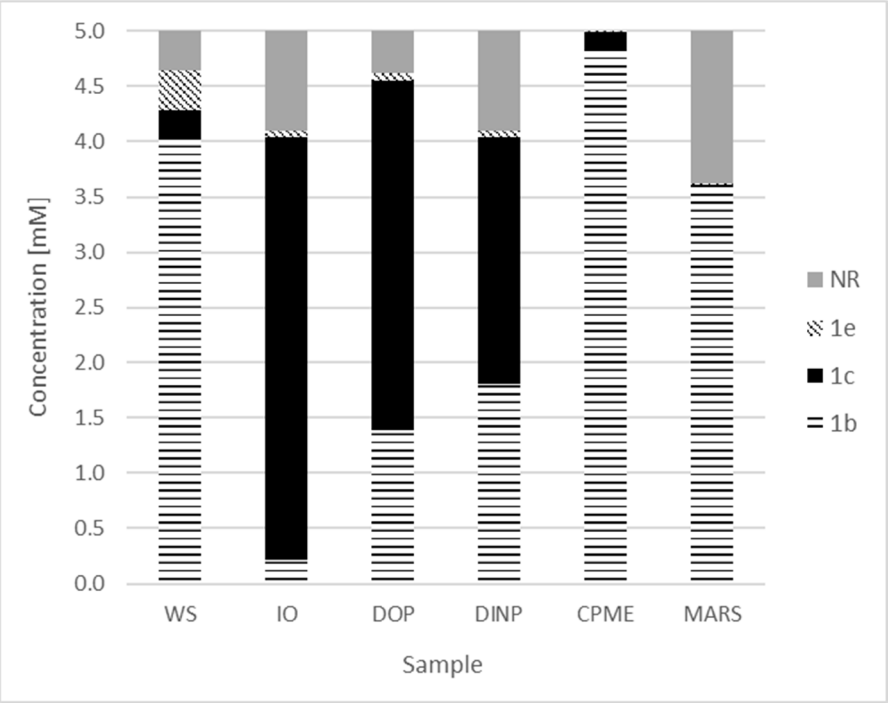


Figure 1. Different co-solvents for in situ product removal in resting cell biotransformations. A total of 5 mM of **1a** was dissolved in KOH, WS: without co-solvent, IO: Isooctane; DOP: Dioctylphthalate; DINP: Diisononylphthalate: CPME: Cyclopentyl methyl ether, 50% *v/v* solvent, 90% *v/v* CPME for MARS. Standard reaction conditions were 100 mM HEPES, 10 mM $MgSO_4$, 50 mM glucose, 40 mg wet cell weight, and total volume of 200 µL in glass vials; 30 min reaction time. Striped **1b**, black: **1c**, diagonal squared: **1e** (cinnamyl alcohol), grey: not recovered.

After 30 min, **1b** was nearly completely converted to **1c**, whereas the reactions with DOP and DINP gave only a conversion rate of 68 and 55%, respectively. CPME at 50% *v/v* concentration, as well as MARS with 90% *v/v*, is not suitable for this whole cell conversion. One reason may be that MARS has been described for lyophilized cells, whereas here we require metabolically active cells as biocatalysts for ATP and NADPH supply. The reactions with CPME did not run to completion even after 24 h while in the presence of IO, full conversion of DOP and DINP was observed within 1 h.

With this two-phase system we were able to reduce 5 mM of **1b** with *E. coli* RARE [pET-Duet1_*Ec*PPTase_HT*Nc*CAR] to the corresponding aldehyde without noteworthy amounts of **1e** (see Figure 1). With the established two-phase system, the activity of *E. coli* RARE [pET-Duet1_*Ec*PPTase_HT*Nc*CAR] and *E. coli* RARE [pETDuet1_*Ec*PPTase_HT*Nc*CAR:OYE1] were used for the reduction of **1b**. Conversion of **1b** to **1c** reached completion (see

Figure 2A). Using the strain that produced the *Nc*CAR:OYE1 fusion protein, *E. coli* RARE [pETDuet1_*Ec*PPTase_HT*Nc*CAR:OYE1], conversion of **1b** to **1d** was slightly slower and gave a yield of 80% after 1 h which is attributable to the additional NADPH consumption through the additional enzyme and the added metabolic burden for the cell (see Figure 2B).

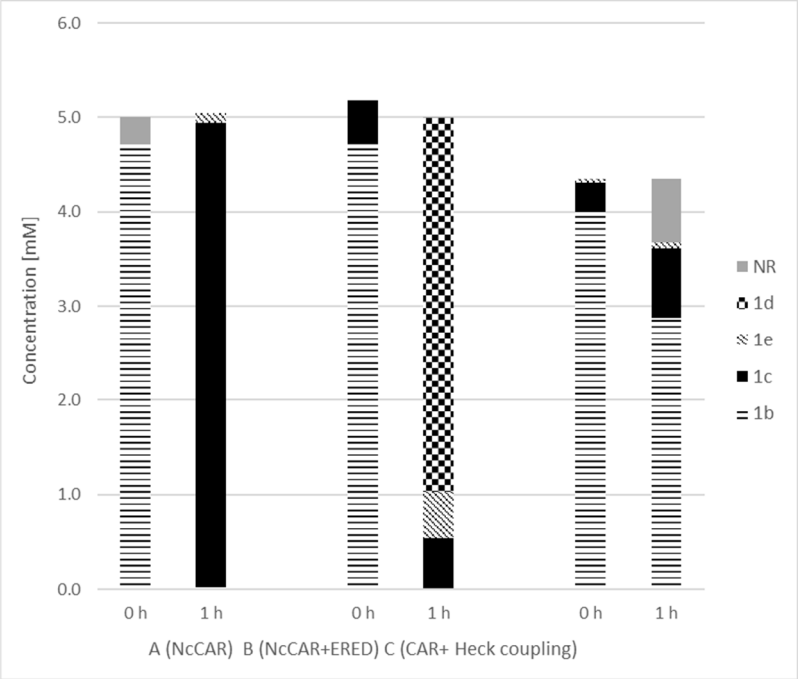


Figure 2. Whole cell biotransformation of 5 mM **1b** or Heck coupling resulting **1b** to **1c** or **1d**. A: *E. coli* RARE [pETDuet1_*Ec*PPTase_HT*Nc*CAR] with 5 mM isolated **1b**, B: *E. coli* RARE [pETDuet1_*Ec*PPTase_HT*Nc*CAR:*OYE*1], C: *E. coli* RARE [pETDuet1_*Ec*PPTase_HT*Nc*CAR] with **1b** forming Heck coupling, 50% *v/v* IO, standard reaction conditions 100 mM HEPES 10 mM $MgSO_4$, 50 mM glucose, 40 mg wet cell weight, total volume of 200 µL in glass vial; Striped: **1b**, black: **1c**, black squared: **1d**, diagonal striped: **1e** (cinnamyl alcohol), grey: not recovered.

Finally, we coupled the in vivo cascade to the oxidative Heck reaction in an aqueous system in a one pot fashion. In comparison to reactions with isolated and purified cinnamic acid derivatives as the substrates, the one-pot reaction showed less analytical yield of 0.74 mM for **1** (see Figure 2C). The overall concentration is lower due to incomplete Heck coupling reaction. Unfortunately, the Heck coupling reactions for the substrates **2b**–**8b** did not give any conversion to the desired saturated aldehyde **2d**–**8d**. The reaction stopped already at the CAR mediated reduction step. We attributed this outcome to the excess of acrylic-, and crotonic acid from the Heck reaction. These acids compete with the cinnamic acid substrates for CAR and the required cofactors. The specific activity of *Nc*CAR for acrylic acid is higher than for substrates (e.g., see Table 2 entry # 3–4) and acrolein is likely formed, which strongly reacts with proteins [28].

Table 2. In vitro activity of *Nc*CAR measured by NADPH depletion. The assay was not conducted with all compounds due to absorption (**3b**, **4b**) and solubility (**5b**–**8b**) reasons.

Entry	Substrate	Activity [U/mg]
1	**1b** Cinnamic acid	0.92 ± 0.07
2	**2b** 3-Methyl cinnamic acid	0.31 ± 0.03
3	Acrylic acid	0.35 ± 0.03
4	Crotonic acid	0.47 ± 0.01

The conversion rate is significantly lower with residual acrylic acid above a concentration of 5 mM (see Table 3). After 1 h, the reaction runs to completion without any acrylic acid (see Table 3, entry # 1), with 5 mM residual acid the reaction only reaches 46% with the best substrate, **1b** (Table 3, entry # 2). With 10 mM acrylic acid only 4% conversion is reached (Table 3, entry # 3). The catalyst, Pd (II) acetate and neocuproine at 6 mol% as used in Heck coupling reactions seemed to have little effect on acid reduction capability of the whole cell catalyst (Table 3, entry # 4).

Table 3. Conversion of isolated **1b** to **1c** with increasing acrylic acid concentration.

Entry	Cinnamic Acid [mM]	Acrylic Acid [mM]	Pd(II)Acetate/Necocuproine	Conversion [%]
1	5	0	0	99
2	5	5	0	46
3	5	10	0	4
4	5	0	6 mol%	93

In conclusion, excess acrylic acid must be avoided. Reduction of **1b** with *E. coli* RARE [pETDuet1_*Ec*PPTase_HT*Nc*CAR] formed in the Heck reaction with equimolar acrylic acid, shows complete conversion. The Heck coupling gives a yield of 68% of **1b**, compared to a yield of 76% with a surplus of acrylic acid (see Table 1). The overreduction to **1e** is slightly increased when compared to a reduction performed with pure **1b**. The concentration of **1e** reaches about 0.4 mM, which is 11.5% of the total conversion and 2% when isolated **1c** is used.

3. Materials and Methods

Catalyst generation: The Palladium catalyst for Heck couplings was generated in a 30 mL glass vial. The dry powder of Neocuproine (CAS 484-11-7, TCI Chemicals, Eschborn, Germany) and Pd (II) acetate (TCI Chemicals, Germany) was weighed in the vial and 4 mL of H_2Odd was added. The concentration was 7 mM for both compounds. The reaction was stirred for 1 h at room temperature and stored at room temperature for up to one day. Aliquots were used as the catalyst for Heck coupling reactions.

Heck Coupling: Heck reactions were set up in water with or without DMSO. The total volume was 4 mL in 30 mL glass vials with a magnetic stirrer. The final concentrations of trifluoroborates and acrylic (TCI Chemicals, Germany) acid or crotonic acid (TCI Chemicals, Germany) (were 10 mM and 20 mM, respectively, if not stated otherwise. The catalyst was added to a concentration of 6 mol%. The synthesis and the spectral data for all cinnamic acid derivatives are given in the Supplementary Materials.

Cells were grown according the protocol of Studier et al. [29,30] and harvested by centrifugation. Standard reaction conditions: 40 mg wet cell weight was dissolved in 100 mM HEPES (Carl Roth, Australia) 10 mM $MgSO_4$ (TCI Chemicals, Germany), 50 mM glucose (Sigma Aldrich, Darmstadt, Germany), total volume of 200 µL in glass vial with 50% *v/v* IO. Vials were incubated at 20 °C and 700 rpm on a Thermomixer comfort (Eppendorf). Reactions were stopped by diluting with 80 vol% EtOH. The mixture was centrifuged at 13,000 rcf for 10 min and the supernatant was subjected to analysis via HPLC. The following gradient was used for **1**: 0–5.00 min/25–55% ACN; 5.00–7.20 min/55–70% ACN; 7.20–7.50 min/70–90% ACN; 7.50–9.00 min/90% ACN; 9.01–15.00 min/25% ACN. Compounds were detected at 254 nm using a Diode Array Detector (Agilent, Santa Clara, CA, USA) (DAD).

4. Conclusions

These results outline the proof of concept of the presented chemoenzymatic cascade reaction. We could investigate a biocompatible oxidative Heck protocol that runs at room temperature in water. Nevertheless, a current limitation of its one-pot implementation as combination of the chemical and the two enzymatic steps is the utilization of acrylic acid

and crotonic acid by the CAR and the generated products thereof. To increase the overall yield, Heck coupling reactions with low or no residual acrylic and crotonic acid need to be developed or a CAR identified or engineered with negligible activity for short chain carboxylates. Furthermore, the coupling could be envisioned with the corresponding esters that would not interfere with the following CAR mediated reduction. This would extend the cascade by an additional esterase to cleave the corresponding cinnamic esters.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/catal11080932/s1, Figure S1: Expression vector pETDuet1_EcPPTaseHTNcCAR, Figure S2: Illustration of the expression vector pETDuet1_EcPPTaseHTNcCAR_Oye1, Figure S3: Expression of NcCAR and NcCAR:OYE1 fusion protein in E. coli RARE, Figures S4 and S5: HPLC chromatograms of the biotransformation with cinnamic acid, Table S1: Primers used in this study.

Author Contributions: Conceptualization, M.W. & F.R.; methodology, D.S., A.K.R., M.E., V.S.; validation, D.S., A.K.R., M.E. and V.S.; investigation, D.S., A.K.R., M.E., V.S.; writing—original draft preparation, D.S.; writing—review and editing, D.S., M.W. and F.R.; visualization, D.S. and F.R.; supervision, M.W. and F.R.; project administration, M.W. and F.R.; funding acquisition, M.W. and F.R. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All data are provided by the manuscript and the Supporting Information.

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