Optimization of a Catalytic Chemoenzymatic Tandem Reaction for the Synthesis of Natural Stilbenes in Continuous Flow

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Abstract: In view of the development of efficient processes for the synthesis of high-value compounds, the combination of bio- and chemocatalysis is highly promising. In addition, implementation of immobilized catalysts into continuous setups allows a straightforward separation of the target compound from the reaction mixture and ensures uniform product quality. In this work, we report the optimization of a chemoenzymatic tandem reaction in continuous flow and its extended application for the synthesis of pharmacologically active resveratrol and pterostilbene. The tandem reaction involves enzymatic decarboxylation of coumaric acid employing encapsulated phenolic acid decarboxylase from *B. subtilis* and a Heck coupling of the obtained vinylphenol with an aryl iodide using heterogeneous Pd-Ce-Sn oxides implemented in a packed bed reactor. By optimization of the reaction conditions for the limiting cross-coupling step, the yield of (E)-4-hydroxystilbene using the fully continuous setup could be more than doubled compared to previous work. Furthermore, the improved chemoenzymatic cascade could also be applied to the synthesis of resveratrol and pterostilbene in a continuous fashion. Leaching of the metal catalyst at high temperatures limited the process in many perspectives. Therefore, the feasibility of a reactor setup with reversed flow was experimentally evaluated and approved.

Keywords: catalysis; catalyst leaching; chemoenzymatic tandem; continuous flow chemistry; decarboxylation; Heck reaction; optimization; palladium; stilbenes

1. Introduction

In view of the development of sustainable processes for the production of pharmaceuticals and fine chemical intermediates, chemoenzymatic processes have high potential [1]. Not only do they often rely on the use of cheap and renewable feedstocks [2], they also combine the benefits of traditional chemocatalysts with emerging biocatalysis. While well-established metal catalysts stand out due to their versatility [3], enzymatic transformations can be performed under mild reaction conditions including usually an aqueous reaction medium, moderate temperature, and ambient pressure [4]. Hence, the concept of chemoenzymatic cascade reactions in one-pot or in a sequential fashion is applied for the synthesis of polymers [5], natural products [6], and active pharmaceutical ingredients [7] to name but a few. However, the majority of these processes are performed in batch, although continuous flow chemistry offers a plethora of advantages [8]: on the one hand, the use of micro- and mesosfluidic flow equipment implicates fast mass and energy transfer, which is highly profitable for very fast or exothermal chemical reactions in terms of reaction yield, selectivity, and safety [8,9]. Slow transformations instead can be sped up considerably by applying high temperature and pressure, thus intensifying the
process and opening Novel Process Windows [10]. On the other hand, the implementation of inline analytical techniques ensures real-time process control and a constant quality, whereas process costs are reduced by automation, efficient energy consumption, as well as reduced waste generation [11,12]. Regarding the performance of catalytic processes in flow, the heterogeneous nature of solid-supported catalysts in combination with their recyclability make them the perfect choice for implementation in continuous setups [8]. In particular, heterogeneous palladium catalysts received increasing attention due to their ability to catalyze a variety of important transformations, including hydrogenation [13], oxidation [14], and cross-coupling reactions [15]. The latter in particular became a fundamental synthetic tool for organic chemists [16], extensively being applied for the synthesis of pharmaceutically and biologically active molecules [17]. In this respect, the class of polyphenolic stilbenes is of substantial interest, most importantly resveratrol and pterostilbene [18]. The compounds feature two substituted benzene rings connected by a C-C double bond resulting in a conjugated double bond system, which gives them antioxidant, anti-inflammatory, anti-obesity [19,20], and cancer-preventive properties [21]. Regarding the synthesis of this class of molecules from renewable resources, Baraibar et al. developed a chemoenzymatic approach giving 4-4′-dihydroxystilbene in two steps. They employed an encapsulated, cofactor-free decarboxylase (phenolic acid decarboxylase from Bacillus subtilis, BsPAD) in combination with a ruthenium metathesis catalyst in one-pot, achieving an overall yield of the target compound of 90% (3 g/L substrate solution in t-butyl methyl ether) [22]. Taking up this promising approach and translating it to continuous flow, our group recently reported a chemoenzymatic tandem process combining an enzymatic decarboxylation step with a Pd-catalyzed Heck coupling for the synthesis of (E)-4-hydroxystilbene in a continuous fashion [23]. First, decarboxylation of the substrate para-coumaric was achieved by BsPAD encapsulated in alginate beads and implemented in a packed bed reactor. To improve the poor substrate solubility and avoid solvent compatibility problems, a mixture of choline chloride/glycerol deep eutectic solvent (DES) and phosphate buffer was utilized for the biotransformation. After addition of ethanol, the obtained vinylphenol was then coupled with iodobenzene in a Heck coupling reaction, which was catalyzed by a fixed bed of heterogeneous Pd catalyst Ce0.20Sn0.79Pd0.01O2−δ. The preparation of this type of catalyst is straightforward and can be achieved via a solution-combustion method within several hours and in quantitative yield [24]. Regarding the palladium species, it is incorporated into the crystal lattice of the mixed metal oxide in predominant cationic Pd(II) form and is believed to be reduced in-situ to the catalytically active atomic Pd species [25]. In previous work, various Pd oxides comprising different ratios of cerium and tin were tested for their activity towards the synthesis of (E)-4-hydroxystilbene in continuous flow, proving Ce0.20Sn0.79Pd0.01O2−δ to perform best [26]. Using a fully integrated continuous setup, the target compound (E)-4-hydroxy-stilbene could be successfully synthesized with an average yield of 20% over 16 h. While the enzymatic step worked with full conversion, the yield of the process was limited by the Pd-catalyzed cross-coupling step. In particular, homocoupling of iodobenzene as well as spontaneous polymerization of the styrene intermediate were observed. Addressing these issues, in this article we report the optimization of the chemoenzymatic continuous tandem process by finetuning of reaction conditions for the metal-catalyzed Heck reaction. By adjusting the pH-value and the potassium carbonate concentration relative to the amount of the alkene coupling partner, the two-step cascade for the synthesis of (E)-4-hydroxystilbene could be significantly improved. Furthermore, the process could be extended to the synthesis of the pharmaceutically relevant compounds resveratrol and pterostilbene in continuous flow. Regarding the use of heterogeneous Pd catalysts, leaching of the catalytically active species from the solid matrix is known to be a major issue [27,28]. It is a consequence of the "quasi-homogeneous" nature of immobilized Pd catalysts, a theory that has been widely accepted among the scientific community. During the catalytic cycle, a soluble palladium(II) species is formed, which redeposits onto the support material after the reaction [29]. While in batch mode this "catch-and-release" mechanism does not pose any problems, in continuous flow it will eventually push out the catalytic species from the reaction system [30,31]. From previous studies, we already know that our in-house developed
heterogeneous Pd catalysts of type Ce$_{0.99-x}$Sn$_x$Pd$_{0.01}$O$_{2-\delta}$ are prone to Pd leaching when employed in continuous flow, especially in the start-up phase of the reaction [32]. In order to prevent the loss of catalytically active palladium, we have adopted the concept of a “palladium reversed flow reactor”, which was described by Fan et al. [33]. More precisely, inside a packed bed reactor we implemented sections of inert material before and after the zone of the heterogeneous Pd catalyst Ce$_{0.20}$Sn$_{0.79}$Pd$_{0.01}$O$_{2-\delta}$. In doing so, we hypothesized that palladium species leached from the central region during a continuous experiment would be captured by readsoption onto the subsequent inactive material. Before its capacity is exceeded and Pd elutes from the reactor, the flow direction through the fixed bed is reversed and the process can be continued. Hence, by alternating the mounting direction of the packed bed reactor after certain time intervals, leached palladium can in theory be trapped inside the system. Indeed, we were able to show experimentally that solubilized palladium species can actually be retained by adsorption onto the inactive support material, which in turn was demonstrated to exhibit catalytic activity. Future work will focus on further investigating this concept, in particular the determination of suitable time intervals for reversing the flow direction through the packed bed reactor as well as long-term applicability.

2. Results and Discussion

2.1. Optimization of Process Conditions in Batch

As published previously [23], the investigated chemoenzymatic cascade to produce stilbenes comprises an enzymatic decarboxylation part employing encapsulated phenolic acid decarboxylase from Bacillus subtilis (BsPAD) as well as a subsequent Heck coupling section involving a heterogeneous Pd catalyst implemented in a fixed bed reactor. While the first decarboxylation step gives full conversion and results in quantitative yield, the metal catalyzed cross-coupling reaction was identified to be the bottleneck of the cascade, thus requiring optimization. The two reaction parameters basicity as well as carbonate concentration showed to significantly impact the Heck coupling of vinylphenol 2 with iodobenzene 3 for the synthesis of the model substance (E)-4-hydroxystilbene 4 in batch. Therefore, the influence of these two factors on the product yield was studied in more detail using the Design of Experiments (DoE) approach. DoE considers the fact that the outcome of an experiment is the result of a number of interacting reaction parameters. Utilizing the statistical tool, it is possible to gain the maximum information while minimizing required time and resources as well as reveal relationships between input factors [34].

Regarding the optimization of the Heck coupling step in batch using the DoE approach, we targeted to maximize both yield as well as the desired product 4 to side product 4b ratio (DP/SP). In this respect, we chose to determine the impact of the two factors pH-value and vinylphenol 2 concentration on the reaction outcome. Therefore, we employed a full factorial DoE design without center point replication and determined the respective responses resulting of every possible combination of the two selected reaction parameters, varying the pH-value from 10.5–13 and vinylphenol 2 concentration from 10–40 mM at a temperature of 80 °C. Analysis of the experimental data utilizing the software MODDE Pro (© Sartorius Stedim Data Analytics AB) revealed, that the basicity of the reaction solution, represented by the pH-value, has the most significant influence on the reaction yield (Figure 1a) as well as DP/SP (Figure 1b). Obtained mathematical models suggest a pH optimum of 11.52 for the yield and 11.68 for DP/SP, regardless of the substrate concentration in the investigated range (see Supporting Information for experimental raw data, a detailed description of conducted DoE study as well as statistical model parameters). In view of our chemoenzymatic tandem reaction, we decided to perform the continuous Heck coupling step at a pH-value of about 11.75 (to compensate for the lowering of the pH by dissolution of CO$_2$ formed in the first step of the reaction tandem) using a substrate concentration of 10 mM vinylphenol 2.
Apart from that, we found that not only the pH-value but also the carbonate concentration affects the reaction outcome. Apparently, a high concentration of K$_2$CO$_3$ (100 mM) suppresses the formation of side product by more than half but also decreases the yield by 24%. Instead, low concentrations of K$_2$CO$_3$ (15 mM) proved to give a satisfying DP/SP ratio but still maintain acceptable yield. Performing the Heck coupling step in batch at a pH of 11.5 (according to the pH optimum for yield identified by DoE) and employing 15 mM vinylphenol, hydroxystilbene 4 could be obtained in 23% yield at a temperature of 80 °C. When only 10 mM vinylphenol were used (as envisaged for the continuous tandem reaction) and obtained yield was compared to the outcome of an experiment performed at reaction conditions reported by Grabner et al. (pH = 11.35, 100 mM K$_2$CO$_3$, 80 °C)\cite{23}, optimization of pH and carbonate concentration increased product formation by 38%.

Regarding the other two compounds of interest, resveratrol and pterostilbene, they could not be synthesized at a reaction temperature of 80 °C, which was limited by the boiling point of employed solvent mixture DES:Buffer (50 mM KPi):H$_2$O:EtOH = 6:5:2.25:6.75 (v/v) under ambient pressure.

### 2.2. Heck Coupling in Continuous Flow

Following the identification of the optimum pH-value and the ideal carbonate concentration for the model Heck coupling of vinylphenol 2 with iodo benzene 3 in batch, it was intended to further optimize the process in terms of reaction temperature. In this respect, continuous flow chemistry allows to heat above the atmospheric boiling point of the reaction solvent of 80 °C by applying a constant backpressure to the system. By implementing a backpressure regulator into our continuous setup for the Heck coupling step, reaction temperatures of up to 200 °C could be realized without bubble formation. In this way, individual optimization of the reaction temperatures for the Heck reactions giving stilbenes 4, 6, and 8 (molecular structures see Figure 2) was performed in a continuous fashion employing a packed bed of Ce$_{0.20}$Sn$_{0.79}$Pd$_{0.01}$O$_{2-\delta}$ (1.6 g, 10 mmol, 1 mol% Pd), which has already been characterized and studied in detail\cite{25}. In doing so, a significant increase in yield was observed at higher temperatures. However, unfortunately catalyst degradation as well as the formation of homocoupling product were also found to be more profound (see graphic representation in Supplementary Materials). Regarding the synthesis of (E)-4-hydroxystilbene 4, at a reaction temperature of 160 °C satisfying product formation and selectivity were achieved and the target compound was obtained in 50% average HPLC yield. Whereas in batch only the synthesis of the model substance (E)-4-hydroxystilbene 4 could be achieved, utilizing the flow setup at >140 °C the natural occurring and biologically active substances resveratrol 6 and pterostilbene 8 could be successfully obtained as well. The required iodoaryl substrates 5 and 7 for the coupling reactions were synthesized in batch.

**Figure 1.** Surface plots of the Design of Experiments (DoE) analysis; (a) yield (y-axis), vinyl phenol concentration (z-axis), pH-value (x-axis); (b) desired product to side product ratio (DP/SP) (y-axis), vinylphenol 2 concentration (z-axis), pH-value (x-axis).
Obtained time average HPLC yields (60 min) at the respective optimum reaction temperature were determined to be 22% for resveratrol 6 (150 °C) and 48% for pterostilbene 8 (140 °C) (Table 1). As leaching of catalytically active species is known to be a serious issue when using heterogeneous catalysts in a continuous setup, the amounts of metals in the outlet flow were measured by ICP-MS in previous experiments. At a reaction temperature of 110 °C, Pd concentration in the product solution was determined to be moderate 1.09 ± 0.01 mg/kg, whereas the amounts of soluble cerium and tin species in the reaction solution were negligible. A possible methodology for retaining the catalytically active Pd species within the system is presented in 2.4.

In principle, the substrate scope for the Heck coupling in flow could be further extended by using various other iodoaryl substrates as starting materials as long as they are soluble in the applied solvent mixture DES:EtOH:H2O.
2.3. Chemoenzymatic Cascade in Flow

After optimization of the critical Heck coupling step in batch, the two-step chemoenzymatic synthesis of stilbene derivatives in a continuous fashion was targeted. Concerning the utilized reaction solvent, in previous work [23] deep eutectic solvents were already proved to be ideal for the chemoenzymatic reaction cascade due to their solubilization and miscibility properties as well as their compatibility with enzymes. The first step of the chemoenzymatic cascade, the enzymatic decarboxylation of coumaric acid 1 [in DES: KPi-buff (50 mM) = 1:1], was facilitated by a packed bed of BsPAD encapsulated in alginate beads at 30 °C. In doing so, the intermediate product of the process, vinylphenol 2, was obtained in quantitative yield. For the subsequent continuous cross-coupling step, process conditions as optimized in batch (15 mM K₂CO₃, pH 11.75) were employed. Regarding the pH-value, a slightly higher value than the DoE optimum was chosen to compensate for the expected lowering of pH-value due to the dissolution of CO₂ formed in the enzymatic decarboxylation step. After addition of the respective iodoaryl coupling partner (3, 5, or 7; in DES:EtOH:H₂O 1:6.75:2.25 containing K₂CO₃ and KOH) to the vinylphenol 2 stream, Heck coupling was achieved utilizing an HPLC column packed with Ce₀.20Sn₀.79Pd₀.01O₂−δ (1.6 g, 10 mmol, 1 mol% Pd) at 145 °C (Figure 2). At this temperature, yield and catalyst stability were satisfying, whereas at higher temperatures high catalyst activity but also significant degradation and leaching of employed Pd catalyst were observed.

Utilizing the two-step chemoenzymatic setup, hydroxystilbene 4 could be obtained in a stable process with 54% average HPLC yield (60 min) for over 300 min (Figure 3; Table 1). Compared to Grabner et al. [23], the yield of 4 could be more than doubled using the optimized process parameters of this work. Encouraged by these findings, the syntheses of natural occurring and biologically active compounds resveratrol 6 and pterostilbene 8 were targeted utilizing the same setup. Employing 3,5-dihydroxy-iodobenzene 5 as well as 3,5-dimethoxy-iodobenzene 7 as iodoaryl coupling partners, resveratrol 6 (Figure 3) and pterostilbene 8 were successfully synthesized in continuous flow via the chemoenzymatic tandem with 32% and 50% time average HPLC yield over 60 min, respectively (Table 1). While quantitative conversion of coumaric acid 1 to 2 was observed in all performed continuous experiments, the conversion of the intermediate product vinylphenol 2 varied between 76.7–93.1%, indicating that there is still room for improvement. Also, a steady formation of homocoupling products 4a, 6a, and 8a could be observed throughout the continuous experiments, with molar ratios of desired product to homocoupling product between 3.2–7.4.

![Figure 3](image-url)

**Figure 3.** Results of the tandem experiments in continuous flow; (a) synthesis of 4-hydroxystilbene 4, conversion of vinylphenol 2 (crosses) and yield (circles); (b) synthesis of resveratrol 6, conversion of vinylphenol 2 (crosses) and yield (circles); (c) synthesis of pterostilbene 8, conversion of vinylphenol 2 (crosses), and yield (circles).

Regarding possible future tasks, the substrate scope of the cascade could be further extended to achieve the synthesis of other high-value compounds. In this respect, the employed enzyme BsPAD
was reported to also accept other substrates than coumaric acid [23,37], such as caffeic acid or ferulic acid. By variation of the substrate undergoing enzymatic decarboxylation along with choosing the respective aryl iodide coupling partner, even more valuable APIs and natural substances such as rhapontigenin or isorhapontigenin could be synthesized in the reported continuous setup facilitating a chemoenzymatic reaction cascade.

2.4. Palladium Reverse Flow Reactor

Catalyst degradation and leaching of the catalytically active species are often observed when using heterogeneous Pd catalysts and can limit a flow process in many perspectives. Therefore, we wondered about the feasibility of a reverse flow reactor to increase the lifetime of our catalyst, which functions via a Pd catch-and-release principle, in the employed system. By periodically reversing the flow direction through the column containing the heterogeneous Pd catalyst as well as recovery sections at front and end, leached Pd species shall be retained inside the system, in this way preventing a loss of catalytic activity (Figure 4). A similar methodology was already described by Fan et al. for a heterogeneous palladium catalyst on a carbon support and was adopted to our metal oxide matrix [33]. The practical feasibility of the Pd reverse flow reactor was tested experimentally. In a regular Heck coupling experiment in continuous flow at a temperature of 150 °C, the catalytically active species was pushed towards the end of the catalyst bed. Then, catalyst samples (10 mg) were drawn from different spatial positions (front, middle, end) of the catalyst column. Afterwards, the flow direction was inverted, and the active species was pushed to the other end of the column, the former front. Again, catalyst samples were drawn from the same positions. To evaluate the remaining specific activity of taken catalyst samples, Heck coupling experiments in batch were conducted similarly to the optimization experiments. Compared to other methods for analyzing catalyst activity and leaching in flow [30,38], this method allows a spatial resolution and gives information about the remaining specific activity of the catalyst correlating to the available Pd species. As illustrated in Figure 5, in the performed experiment the active material could indeed be pushed towards the end of the column. Expectedly, after reversing the flow direction the catalytically active species was transported back to the former front. However, in order to identify the optimal time intervals between inverting the flow directions, the kinetics of the catalyst leaching still have to be evaluated to avoid pushing active material out of the system. Apart from that, in another experiment we implemented recovery sections comprising unsubstituted Ce-Sn-oxides before and after the active catalyst bed to minimize the loss of the active species (Figure 4). The experiment proved that the active species can readorb onto the unsubstituted Ce-Sn-oxide support as a catalyst samples taken from the recovery section showed a specific activity of 0.70 mg (4) mg (cat.)⁻¹ h⁻¹ compared to 1.1 mg (4) mg (cat.)⁻¹ h⁻¹ of the Pd catalyst bed. The activity was referred to the dry mass of catalyst as the actual palladium concentrations were unknown due to the leaching. However, the leaching kinetics still need to be evaluated to identify suitable time intervals for inversion of the flow direction and making this method more feasible for large scale applications.

![Figure 4. Methodology of a “Palladium Reverse Flow Reactor”](image-url)
3. Materials and Methods

3.1. General Information

Chemicals and solvents were purchased from commercial suppliers and used as received unless stated otherwise (Sigma Aldrich (Darmstadt, Germany): potassium carbonate (99%), vinylphenol (10 w% in propylene glycol), iodobenzene (98%), chlorogluconol (99%), tin(II) oxalate (98%), palladium(II) chloride (99%), potassium iodide (99%); Carl Roth (Karlsruhe, Germany): ammonia solution (30%), sodium nitrite (99%), potassium hydroxide (50w%), glycerol (99%); TCI (Tokio, Japan): resveratrol (99%), 3,5-dimethoxyaniline (98%), choline hydrochloride (98%); Chempur (Karlsruhe, Germany): ammonium cerium(IV) nitrate (99%); Glentham (Corsham, UK): glycine (99%).

For the DoE analysis, the software MODDE Pro (Version 12.1.0.5491, Copyright © Sartorius Stedim Data Analytics AB, Göttingen, Germany) was utilized. For measurement of the pH-value, a Mettler Toledo InLab® Expert Pro-ISM 0-14 pH meter was utilized. Calibration was done weekly with pH = 4.0, 7.0 and 10.0 buffers from Mettler Toledo LTD (Columbus, OH, USA).

3.2. Flow Equipment

For the flow experiments, LAMBDA VIT-FIT HP (Baar, Switzerland) syringe pumps were used equipped with 20-mL stainless steel syringes. Used capillaries (inner diameter 0.03 inch), fittings, and syringe adapters were standard HPLC equipment (Sigma Aldrich, Darmstadt, Germany). As backpressure regulator, a BPR cartridge 75 psi (5.17 bar) Gold Coat P-762 from IDEX Health & Science (Oak Harbor, WA, USA) was used. Preparative stainless steel HPLC columns (VDS optilab, Berlin, Germany, L × I.D. 120 × 8 mm for decarboxylation reactions, L × I.D. 40 × 8 mm for Heck reactions) filled with heterogeneous catalyst were utilized as packed bed reactors.

3.3. Analytical Methods

For High Performance Liquid Chromatography, an Agilent 1100 series HPLC system (Santa Clara, CA, USA) equipped with online degasser, quaternary pump, autosampler, thermostated column compartment and UV-VIS diode array detector was utilized. Compounds were separated using a ThermoFischer Scientific Accucore™ C18 (Waltham, Massachusetts, United States) reversed phase column (50 × 4.6 mm ID; 2.6 µm) using an injection volume of 2.0 µL. As eluents, Buffer (A, H₂O:H₃PO₄ = 300:1, v/v, Sigma Aldrich, Darmstadt, Germany) and HPLC grade methanol (B; Chemlab, Zedelgem, Belgium) were used in following compositions: Method a) 0–1 min 60/40 (A/B), 1–12 min gradient to 10/90 (A/B), 12–14 min gradient to 60/40 (A/B), Method b) 0–1 min 80/20 (A/B), 1–8 min 30/70...
(A/B) gradient, 8–12 min 60/40 (A/B) gradient, 12–17 min 60/40 (A/B), 17–20 min 30/70 (A/B) gradient. Calibration: 3-point calibration with R² ≥ 0.99; calibration slopes [area/mM]: coumaric acid 1 (a) 1012 (b) 714, vinylphenol 2 (a) 375 (b) 480, 4-hydroxystilbene 4 (a) 1159, resveratrol 6 (b) 2357, pterostilbene 8 (a) 519; measurement wavelengths [nm]: coumaric acid 1 282, vinylphenol 2 237, 4-hydroxystilbene 4 282, resveratrol 6 310, pterostilbene 8 282.

NMR-measurements were recorded using a Bruker Avance III 300 MHz spectrometer using CDCl₃, C₆D₆ and DMSO-d₆ as solvents.

3.4. Synthesis of Palladium Catalyst Ce₀.₂Sn₀.₇₉Pd₀.₀₁O₂−δ Using the Solution Combustion Method (SCM)

In order to prepare the heterogeneous palladium catalyst, the solution combustion method reported by Baidya et al. and modified by Lichtenegger et al. was used. In a mortar, ammonium cerium(IV) nitrate (6.370 g, 11.6 mmol; Chempur, Karlsruhe, Germany), tin(II) oxalate (9.489 g, 45.9 mmol; Sigma Aldrich, Darmstadt, Germany), glycine (10.038 g, 133 mmol; Glentham, Corsham, UK), and palladium(II) chloride (0.102 g, 0.575 mmol; Sigma Aldrich, Darmstadt, Germany) were pestled and suspended in 6 mL deionized water. After ultrasonic treatment, the homogeneous solution was heated in the muffle furnace (AHT, Weitersfeld, Austria) for one hour at 350 °C for combustion. Then, it was pestled again in a mortar and heated to dryness overnight at 350 °C. The catalyst was obtained as a yellow powder in almost quantitative yield (~9 g). Using this method, the material for the Pd free recovery section (applied in 2.4) could be synthesized by exactly following this procedure; however, without adding PdCl₂.

3.5. Enzyme Immobilization for the Preparation of Alginate Beads

The enzyme BsPAD used for the decarboxylation step was obtained as cell-free extract in form of a freeze-dried powder from the Institute of Molecular Biotechnology (TU Graz, Graz, Austria) and needed to be encapsulated prior to use in a flow setup. To prepare the alginate beads, 80.0 mg cell-free extract and 40.0 mg sodium alginate were dissolved in 2 mL KPi-buffer (50 mM). The yellowish viscous solution was added dropwise to a 2 w% BaCl₂-solution under gentle stirring. In this way, beads of 1–3 mm diameter were formed and stirred for 1 h to further solidify. After washing with 0.9 w% NaCl solution and drying under ambient conditions, they were ready for use.

3.6. Heck Coupling Reaction in Batch

Vinylphenol 2 (10–40 mM, 1.00 mol eq.; Sigma Aldrich, Darmstadt, Germany), iodobenzene 3 (15–60 mM, 1.50 mol eq.; Sigma Aldrich, Darmstadt, Germany) and potassium carbonate (0–100 mM; Sigma Aldrich, Darmstadt, Germany) were dissolved in DES:Bu₅ff (50 mM KPi):H₂O:EtOH = 6:4, eluent for 4:8. The pH-value was adjusted to 10.5–13 with 1M KOH in solvent mixture. After heating the substrate solution to 80 °C, 1.9 mg Ce₀.₂Sn₀.₇₉Pd₀.₀₁O₂−δ SCM catalyst (11.9 µmol, 1 mol% Pd) were added. Samples (50 µL) were withdrawn after 20 min, quenched with MeOH: Buffer(H₃PO₄) = 7:3 (v/v), 500 µL and analyzed by HPLC according to 3.3.

3.7. Single Step Heck Coupling Flow Experiments

A Lambda VIT-FIT HP syringe pump (Baar, Switzerland) equipped with a 20-mL stainless steel syringe was used to pump a mixture of vinylphenol 2 (10 mM, 1.0 mol eq.), aryl iodide (15 mM, 1.5 mol eq.), K₂CO₃ (15 mM) and KOH (22 mM) dissolved in DES:Buffer (50 mM KPi):H₂O:EtOH = 6:5:2.25:6.75 (v/v) with a flowrate of 0.2 mL/min through an HPLC column filled with 1.6 g Ce₀.₂Sn₀.₇₉Pd₀.₀₁O₂−δ SCM catalyst (10 mmol, 1 mol% Pd) at a temperature of 140–160 °C. After the column, a backpressure regulator (5.2 bar) was installed. Samples (50 µL) were taken at the outlet every 20 min, quenched with MeOH: Buffer(H₃PO₄) = 7:3 (v/v), 500 µL and analyzed by HPLC according to 3.3. To isolate 4-hydroxystilbene 4, resveratrol 6 and pterostilbene 8, the collected outlet flow was extracted with ethyl acetate (EtOAc). Then, the crude products were purified by column chromatography [SiO₂, eluents for 4 and 6: EtOAc:Cyclohexane (CH) = 6:4, eluent for 8: EtOAc:CH = 2:8].
4-Hydroxystilbene 4: 1H-NMR (300 MHz, CDCl₃) δ [ppm] = 7.49 (d, J = 6 Hz, 2H, Ar-H), 7.42 (d, J = 9 Hz, 2H, Ar-H), 7.35 (t, J = 15 Hz, 2H, Ar-H), 7.24 (t, J = 12 Hz, 1H, Ar-H), 7.02 (dd, J = 45 Hz, 2H, H-C=C-H), 6.84 (d, J = 9 Hz, 2H, Ar-H), 4.77 (s, 1H, O-H). 1H-NMR data were in accordance with literature [39].

Resveratrol 6: 1H-NMR (300 MHz, DMSO-d₆) δ [ppm] = 9.37 (s, 1H, O-H), 9.01 (s, 2H, O-H), 7.22 (d, J = 8.3 Hz, 2H, Ar-H), 6.76 (d, J = 16.4 Hz, 1H, C=C-H), 6.66 (d, J = 16.4 Hz, 1H, C=C-H), 6.58 (d, J = 8.3 Hz, 2H, Ar-H), 6.21 (s, 2H, Ar-H), 5.94 (s, 1H, Ar-H); 13C-NMR (76 MHz, DMSO-d₆) δ [ppm] = 158.5 (C₁₅), 157.2 (C₁₆), 155.4 (C₁₇), 149.2 (C₁₈), 139.2 (C₁₉), 128.1 (C₂₀), 127.8 (C₂₁), 127.8 (C₂₂), 125.6 (CH), 115.5 (C₂₃), 104.3 (C₂₄), 101.7 (C₂₅). 1H- and 13C-NMR data were in accordance with literature [40].

Pterostilbene 8: 1H-NMR (300 MHz, CDCl₃) δ [ppm] = 9.79 (s, 1H, O-H), 7.32 (d, J = 8.5 Hz, 2H, Ar-H), 6.95 (d, J = 16.2 Hz, 1H, C=C-H), 6.81 (d, J = 16.3 Hz, 1H, C=C-H), 6.75 (d, J = 8.5 Hz, 2H, Ar-H), 6.57 (s, 2H, Ar-H), 6.31 (s, 1H, Ar-H), 3.75 (s, 6H, CH₃). 13C-NMR (76 MHz, CDCl₃) δ [ppm] = 161.0 (C₁₅), 155.4 (C₁₆), 139.7 (C₁₇), 130.2 (C₁₈), 128.7 (CH), 128.0 (C₁₉), 126.7 (CH), 115.7 (C₂₀), 104.4 (C₂₁), 99.7 (C₂₂), 55.4 (CH₃). 1H- and 13C-NMR data were in accordance with literature [41].

3.8. Chemoenzymatic Tandem Flow Experiments

A Lambda VIT-FIT HP syringe pump equipped with a 20-mL stainless steel syringe was used to pump coumaric acid I solution [20 mM, 1.00 mol eq., in DES:Buffer (50 mM KPi) 1:1 (v/v)] with a flowrate of 0.1 mL/min through an HPLC column filled with B₅PAD enzyme (160 mg, encapsulated according to 3.5) at a temperature of 30 °C. The outlet stream containing the intermediate product vinylphenol 2 was merged in a T-mixing element with a solution containing aryl iodide [either iodobenzene 3, dihydroxyiodobenzene 5 or dimethoxyiodobenzene 7 (30.0 mM, 1.50 mol eq., in DES:EtOH:H₂O = 1:6.75:2.25), K₂CO₃ (30.0 mM, 1.50 mol eq.) and KOH (43.7 mM, 2.18 mol eq.) delivered by another syringe pump at a flowrate of 0.1 mL/min. The resulting mixture with a total flowrate of 0.2 mL/min was then pumped through an HPLC column filled with 1.6 g Ce₀.₁₂Sn₀.₇₆Pd₀.₀₁O₂·δ (10 mmol, 1 mol% Pd) at a temperature of 145 °C. After the column, a backpressure regulator (5.2 bar) was installed to avoid bubble formation. For the different iodoaryl substrates, a bed of fresh palladium catalyst was utilized. Samples (50 µL) were withdrawn from the outlet flow every 20 min, quenched with MeOH: Buffer (H₃PO₄) = 7:3 (v/v), 500 µL) and analyzed by HPLC according to 3.3.

3.9. Leaching Experiment

A continuous Heck coupling experiment according to chapter 3.7 was performed using 10 mM vinylphenol 2, 15 mM iodobenzene 3, 15 mM K₂CO₃, and 22 mM KOH at 150 °C and a flow rate of 0.2 mL/min. After 120 min reaction time (~9 times the mean residence time), a sample was taken and the experiment was stopped to take samples of the catalyst (~15 mg) from the front, middle, and back of the column after flushing with solvent. The column was screwed again upside down into the setup to reverse the flow direction. After another 120 min of reaction time, the column was flushed with 10 mL solvent and catalysyt samples were withdrawn again from the same positions. With these catalyst samples, batch experiments according to chapter 3.6 were performed using 10 mg of the wet catalyst, 10 mM vinylphenol 2, 15 mM iodobenzene 3, 15 mM K₂CO₃, and 22 mM KOH. Sampling was done after 20 min. To calculate the specific activity the dry mass of the wet catalyst samples was analyzed by drying roughly 100 mg of wet catalyst for 24 h at 300 °C in a muffle oven.

3.10. Synthesis of Iodoaryl Substrates 5, 7

The synthesis of 3,5-dihydroxy-iodobenzene 5 was performed as reported in literature [35], giving the target compound in 25% isolated yield.

3,5-dihydroxyiodobenzene 5: 1H-NMR (300 MHz, CDCl₃) δ [ppm] = 7.16 (s, 2H, O-H), 6.43 (s, 2H, Ar-H), 5.96 (s, Ar-H); 13C-NMR (76 MHz, CDCl₃) δ [ppm] = 157.8 (C₁₅), 117.3 (C₁₆), 102.8 (C₁₇), 94.0 (C₁₈). 1H- and 13C-NMR data were in accordance with literature [35].
The synthesis of 3,5-dimethoxy-iodobenzene 7 was accomplished according to [36] in 23% isolated yield.

3,5-dimethoxyiodobenzene 7: \(^1\)H-NMR (300 MHz, CDCl\(_3\)) \(\delta\) [ppm] = 6.89 (s, 2H, Ar-H), 6.44 (s, 1H, Ar-H), 3.79 (s, 6H, CH\(_3\)); \(^{13}\)C-NMR (76 MHz, CDCl\(_3\)) \(\delta\) [ppm] 161.1 (C\(_{Ar}\)), 115.8 (C\(_{Ar}\)), 100.7 (C\(_{Ar}\)), 94.1 (C\(_{Ar}\)), 55.5 (CH\(_3\)). \(^1\)H- and \(^{13}\)C-NMR data were in accordance with literature [36].

4. Conclusions

In this paper, the potential of chemoenzymatic tandem reactions for the synthesis of pharmacologically relevant compounds in continuous flow is highlighted. By optimization of a previously reported setup comprising two sequential packed bed reactors hosting encapsulated BsPAD facilitating enzymatic decarboxylation as well as a heterogeneous Pd catalyst for Heck coupling, stilbene derivatives could be successfully synthesized in a continuous fashion. Tuning of reaction conditions of the cross-coupling step in terms of pH-value, carbonate concentration and temperature allowed to increase the yield of 4-hydroxystilbene compared to previous work as well as to broaden the product scope to resveratrol and pterostilbene, giving the target compounds with time average yields between 32–54%. The developed chemoenzymatic cascade is promising to be further extended to the synthesis of other high-value stilbenes by varying the substrate undergoing enzymatic decarboxylation and using more complex iodoaryl coupling partners, which will be the subject of future work. To address the issue of metal leaching, the feasibility of a palladium reversed flow reactor was demonstrated for our particular catalyst. In this respect, metal oxide support sections proved to retain the catalytic species inside the packed bed reactor, which might be an option to render the chemoenzymatic tandem more sustainable.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4344/10/12/1404/s1: Experimental procedures for Evaluation of Pd leaching, Leaching experiments, Chemical synthesis of 5 and 7, Preparation of DES, Residence time distribution measurements, Heck coupling in flow, Design of experiments; Figure S1: Heck reaction in continuous flow for formation of hydroxystilbene 4; Figure S2: Heck reaction in flow for synthesis of resveratrol 6; Figure S3: Heck reaction in flow for synthesis of pterostilbene 8; Figure S4: Overview plot of the yield model created with MODDE Pro; Figure S5: Overview plot of the DP/SP model created with MODDE Pro; Table S1: Experimental raw data for DoE analysis of Heck coupling in batch in terms of yield of 4 and DP/SP; Table S2: Model settings and statistical parameters of the DoE-models for yield of 4 and DP/SP.

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