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## A chemo-enzymatic tandem reaction in a mixture of deep eutectic solvent and water in continuous flow†

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The combination of metal- and biocatalysis is a challenging but forward-looking topic in synthetic chemistry. The unique selectivity of enzymes paired with the broad range of applications of chemical catalysts enables an undreamed-of number of novel processes. Herein, we describe the application of immobilized phenolic acid decarboxylase (PAD) for the decarboxylation of *para*-coumaric acid and subsequent Pd-catalyzed Heck cross-coupling with an aryl halide in a fully integrated two-step continuous flow process to synthesize (*E*)-4-hydroxy-stilbene. The application of a choline chloride-based deep eutectic solvent (DES) proved to be crucial to overcome the problem of solvent compatibility and enabled an increase in substrate concentration (from 5 mM in buffer to 20 mM in DES) as well as a process with a homogeneous starting solution. The two-step process was successfully operated for more than 16 h in continuous flow and full conversion was achieved. The results underline the usefulness of DES to overcome compatibility problems in tandem-catalytic processes. The system benefits from its simplicity due to increased substrate solubility, the possibility to conduct both reactions at their optimal temperatures and the elimination of isolating the reaction intermediate, which is prone to polymerization.

### Introduction

Chemo-enzymatic one-pot syntheses have been attracting increasing attention of researchers in the past decade.<sup>1–7</sup> These tandem reactions offer the best of both worlds, the versatility of traditional chemical catalysis is replenished with the unbeatable selectivity and mild reaction conditions of enzymes. Nevertheless, besides numerous advantages these promising

systems also pose several challenges. One obstacle is the compatibility of the considerable differences of ideal reaction conditions for the individual process steps.<sup>4,8</sup> Especially, the choice of an appropriate solvent is crucial. Most enzymes show optimal activity in aqueous systems. However, low substrate solubility and the hydrolysis sensitivity of many chemo-catalysts are crucial arguments against the use of neat buffer systems. Alternative solvents, such as deep eutectic solvents (DESs) can help to overcome this obstacle of solvent compatibility.<sup>9–13</sup> DESs are mixtures of two or more components which have a lower melting point than the individual compounds at a certain mixing ratio.<sup>14</sup> In recent years these solvents have gained attention due to their simple preparation and tuneable properties. With lower intrinsic toxicity than ionic liquids, DESs have emerged as alternative solutions for issues such as low substrate solubility, enzyme activity and stability. Besides these advantages, DESs also bring along some drawbacks including occasional toxicity and high viscosity, depending on the starting materials. The latter is a challenge in the scale-up of processes, but can be tackled by mixing with water.<sup>15–18</sup> Up to 50% of water the characteristics of DESs is preserved. In mixtures containing a lower fraction of DES (in the second step in the process described in this work, after adding ethanol and water) their properties are comparable with salt solutions.<sup>19</sup> This good miscibility with water becomes an issue, when the environmental compatibility is discussed. Therefore, a well thought-out process and post-process treatment of DESs and its mixtures with water is required in order to get DES into industrial application.<sup>20,21</sup> Nevertheless, an impressive series of chemical<sup>22,23</sup> and enzymatic<sup>24–26</sup> reactions and also flow applications<sup>27–29</sup> were successfully conducted in DESs. We have recently shown the feasibility to combine the enzymatic decarboxylation of hydroxycinnamic acids catalyzed by phenolic acid decarboxylase from *Bacillus subtilis* (BsPAD) in water and Ru-catalyzed homo-coupling in organic solvents in a one-pot reaction for the synthesis of a series of bio-based stilbene derivatives.<sup>30</sup> In order to expand the scope of the decarboxylation reaction towards the synthesis of asymmetric

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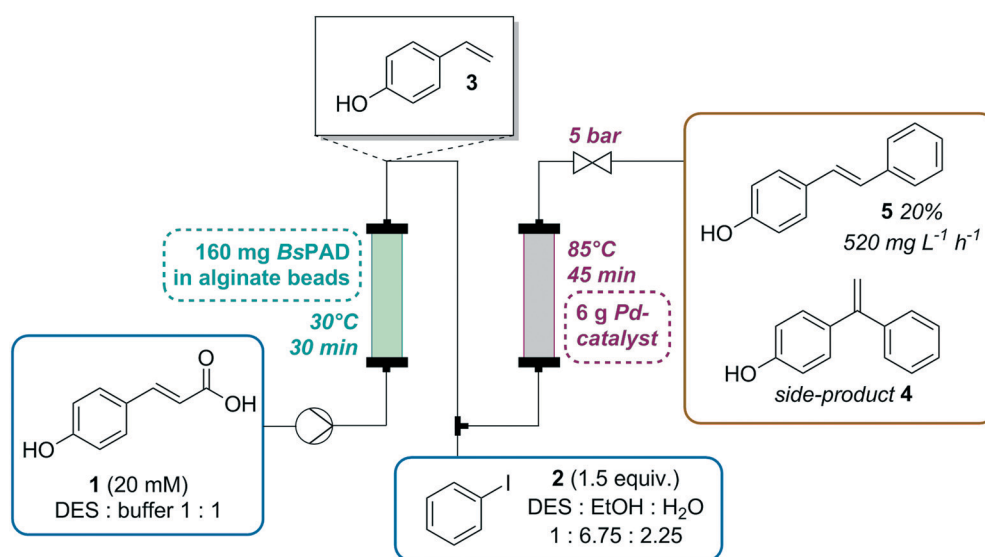
stilbenes, we envisioned a combination of enzymatic decarboxylation with the Heck reaction.<sup>31</sup> The most prominent derivatives of stilbenes are resveratrol and its analogues. Due to their conjugated double bond system, many stilbenes possess anti-oxidant, anti-inflammatory, antidiabetic and antiaging properties<sup>32,33</sup> and are under investigation as active pharmaceutical ingredients (APIs) for cancer preventive drugs.<sup>34</sup> For the synthesis of these important compounds, encapsulation proved to be a practical approach to achieve spatial and temporal separation of the enzymatic in aqueous environment and the metal-catalyzed step in organic solvent.<sup>30</sup> Yet, biphasic systems are complex and difficult to scale. Therefore, the goal of this work is a continuous flow process using a solvent compatible for both catalysts and allowing high substrate loadings. Continuous flow synthesis yields a number of benefits over batch operation. Besides consistent product quality and reduced reaction time, the elimination of separation of the catalyst from the reaction solution is a major advantage.<sup>35</sup> Furthermore, splitting the two reaction steps into separate continuous flow reactors in a row made it possible to conduct the reaction at optimal reaction temperature for both catalysts (30 °C for enzymatic reaction, 85 °C for Heck reaction). Yet, the limited solubility of hydroxycinnamic acids in buffer of a few mM and the need to add additional 30% ethanol for the Heck reaction would require to operate with very diluted substrate solutions in order to prevent reactor clogging. Recent findings showed that PAD is highly active in DES.<sup>36</sup> Experiments on interactions of the used DES and the biocatalyst were not conducted so far and are not scope of this work. A series of recently investigated DES-protein systems can be found in literature.<sup>37</sup> Encouraged by the fact that PAD is highly active in DES, we envisioned that this would be the appropriate solution for the transformation of the one-pot reactions to an integrated multi-step continuous flow process. Furthermore, the

combination of both reactions as cascade in flow lower the risks associated to the spontaneous polymerization of the intermediate hydroxystyrene. While conventional flow chemistry,<sup>38–43</sup> also including C–C coupling reactions,<sup>39,44–47</sup> and continuous biocatalysis<sup>48–50</sup> are currently in the focus of many researchers, examples for their successful combination are rare, which can also be attributed by the difficulties to find suitable reaction media.<sup>51</sup> In this work we present a fully integrated two-step flow setup consisting of an enzymatic decarboxylation with a phenolic acid decarboxylase from *Bacillus subtilis* (BsPAD), followed by a Heck reaction catalyzed by a Pd-substituted Ce–Sn-oxide as shown in Scheme 1. For the proof of concept of the synthesis of asymmetric stilbenes, we chose the synthesis of (*E*)-4-hydroxy stilbene **5** from *para*-coumaric acid **1** and iodobenzene **2** because of the low price and good availability of these substrates.

## Results and discussion

### Decarboxylation in batch

We first started with batch experiments to investigate the compatibility of the systems. We recently showed the excellent compatibility of BsPAD with DESs.<sup>36</sup> Choline chloride (ChCl)-based eutectic mixtures were tested neat and in dilution with potassium phosphate buffer (50 mM, pH 6.0) for the decarboxylation of a variety of phenolic acid derivatives. Our results proved that not only the free enzyme, but also immobilized PAD shows excellent activity in deep eutectic solvents. The 1 : 1 (v/v) dilution of ChCl/glycerol 1 : 2 (mol/mol) with buffer gave the best results for PAD. Mixing DES with buffer has not only the advantage of increased enzyme activity and enhanced CO<sub>2</sub> release from the system, but also decreases the viscosity of the solvent system enabling its application in continuous flow.<sup>29</sup> With up to 50% water the



**Scheme 1** General reaction scheme for enzymatic decarboxylation with a phenolic acid decarboxylase from *Bacillus subtilis* (BsPAD) yielding 4-ethenylphenol **3**, which serves as substrate for a Pd-catalyzed cross-coupling reaction with aryl halide **2**, resulting in (*E*)-4-hydroxy-stilbene **5** and the side product (*para*-hydroxy-1,1-diphenylethylene **4**).



properties of the DES are predominant and substrate solubility is significantly increased.<sup>19</sup> Therefore, this solvent system was chosen for the first step in our two-step synthesis. As the final process requires a heterogeneous biocatalyst, PAD cell-free extract (CFE) was immobilized in 2% alginate beads and tested in the DES:buffer mixture. The formation of the decarboxylation product **3** proceeded linearly (Fig. 1) and full conversion of **1** was reached after 6 h (70 mM) and a yield of **3** of 90% was determined by HPLC. In order to test the heterogeneity of the biocatalyst, a “hot”-filtration test was conducted.<sup>52</sup> Fig. 2 shows the formation of **3** in the reaction mixture until the beads were filtered off after 45 min reaction time, which stopped the reaction, thus indicating that no active enzyme leaches from the alginate beads under these reaction conditions (details in ESI†). Since the hot filtration test does not prove heterogeneity of a catalyst as a standalone method, further investigations regarding enzyme leaching were required in the flow experiment.

### Heck coupling in batch

For the second step, the Heck coupling of **3** with **2**, an in-house developed heterogeneous Pd-catalyst<sup>45,53†</sup> (Pd-substituted cerium-tin-oxide with the molecular formula  $\text{Sn}_{0.79}\text{Ce}_{0.20}\text{Pd}_{0.01}\text{O}_{2-\delta}$ ) was successfully tested for its activity in the novel solvent system. Full conversion of **3** was reached within 30 min (Fig. 3) with a mixture of DES:buffer 1:1 (v/v) (from the first step in the continuous application) plus ethanol:water 1:1 (v/v) containing substrate **2** and the required base,  $\text{K}_2\text{CO}_3$ , (both in 1.5 mol-eq. with respect to **3**).

Due to the similar reactivity of both  $\text{sp}^2$ -atoms in the terminal olefin group, formation of two isomers (Scheme 1) is expected during the Heck reaction, that can either be separated or applied as antioxidant mixture. Unfortunately, there is a number of potential side-reactions, such as homo-coupling of **2** or **3**, subsequent Heck reaction on the double-bond of the product or polymerization of **3**. These reactions led to a low isolated yield of the desired product **5** of 35% in the batch reaction (see ESI†).

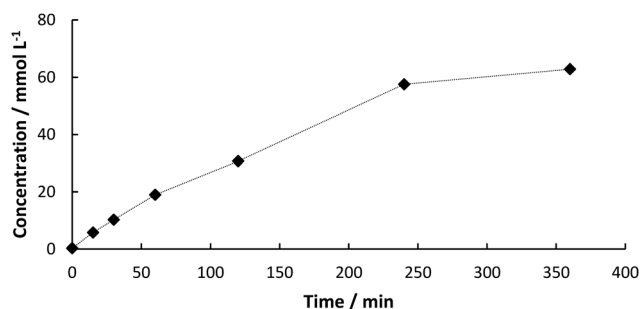


Fig. 1 Concentration of **3** from the decarboxylation of **1** (0.7 mmol) using *Bs*PAD immobilized in alginate beads (2% (w/v) Na-alginate and 38.3 mg *Bs*PAD (CFE) in potassium phosphate buffer (1 mL, 50 mM, pH 6.0), cross-linking was induced by 2% (w/v)  $\text{BaCl}_2$  solution), 10 mL solvent (DES:buffer 1:1 (v/v)), 30 °C.

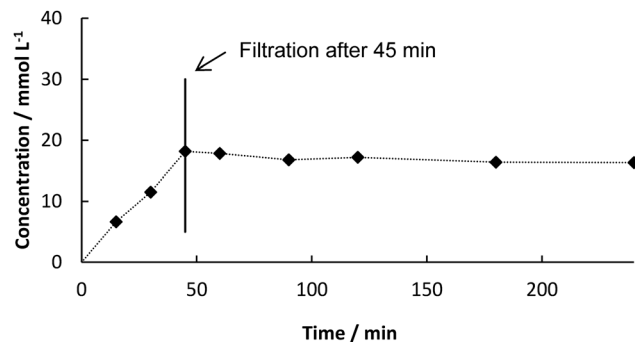


Fig. 2 Concentration of **3** from decarboxylation of **1** using *Bs*PAD immobilized in alginate beads (2% (w/v) Na-alginate and 38.3 mg *Bs*PAD (CFE) in 1 mL potassium phosphate buffer (50 mM, pH 6.0)), 100 mM substrate in 10 mL solvent (DES:buffer 1:1 (v/v)), 30 °C, catalyst beads filtered off after 45 min.

### Combined decarboxylation and Heck coupling in batch

A one-pot reaction was conducted to investigate potential negative impact of the first step on the second one (Fig. 4). A suspension of **1** (70 mM) was stirred together with 80 mg immobilized PAD alginate beads in DES:buffer 1:1 (v/v). After 180 min, the enzyme was filtered off and solvent, substrates and catalyst for the second step were added. Within 2 h all previously formed **3** was converted. Therefore, we can conclude that remaining **1**, which was not converted in the first step, does not negatively affect the performance of the catalyst in the second step and does also not serve as substrate leading to unwanted side-products. It was a very important finding that components from the immobilized CFE do not interfere with the Heck-reaction, which is a crucial requirement for a continuous approach.

### Individual steps in flow

Before both reactions were combined in a two-step flow process, each reaction was tested in continuous mode. Immobilization of PAD in alginate beads with an average diameter of 2 mm proved to be a straightforward method using cell-free extract, thus avoiding any steps for enzyme



Fig. 3 Concentration of **3** (0.7 mmol) and **5** in a batch Heck coupling with **2** (1.5 mol-eq.) in the presence of a base ( $\text{K}_2\text{CO}_3$ , 1.5 mol-eq.) using  $\text{Sn}_{0.79}\text{Ce}_{0.20}\text{Pd}_{0.01}\text{O}_{2-\delta}$  as heterogeneous catalyst (0.05 mol%) in 20 mL solvent (DES:buffer:ethanol:water 1:1:1 (v/v/v/v)), 85 °C.



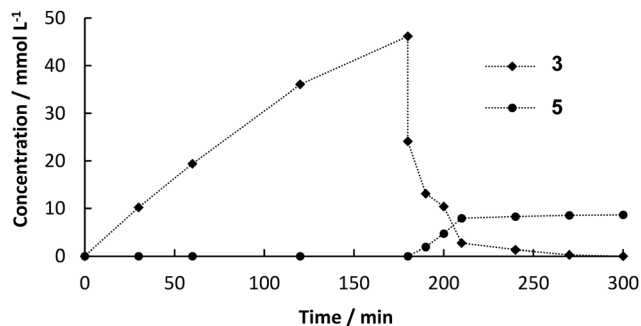


Fig. 4 Product formation over time in one-pot cascade reaction of **1** (0.7 mmol, 70 mM) in 10 mL DES:buffer 1:1 (v/v), 30 °C, 80 mg BsPAD immobilized in 2% (w/v) alginate beads. Beads were filtered off after 180 min and add 10 mL ethanol:water 1:1 (v/v) containing **2** and  $K_2CO_3$  (1.05 mmol, 1.5 mol-eq. to **1**), 25.2 mg (0.05 mol% Pd loading) of Pd-catalyst, 85 °C.

purification. The beads were packed into two stainless steel columns (40 mm × 8 mm i.d.) connected in a series with a total volume of 4 mL. This “packed bed reactor” was heated to 30 °C in a water bath. The reactor was filled and flushed with solvent (DES:buffer 1:1 (v/v)) for 1 h before switching to the feed solution containing **1** (20 mM). This lower concentration was chosen to guarantee complete solubility of the substrate (substrate crystals need to be avoided in order to prevent clogging of the capillaries in the continuous setup). The feed stock was pumped with a syringe pump equipped with a 20 mL stainless steel syringe at a flow rate of 45.5  $\mu\text{L min}^{-1}$  resulting in a residence time of 75 min in the whole set-up, of which 30 min was the residence time within the reactor. Product formation was followed by taking samples of the product stream in 15 min intervals (Fig. 5). The progress of this step could be followed visually as  $CO_2$  bubbles left the reactor as soon a full conversion of the substrate was achieved and no back pressure regulator was used. Details on the flow set-up are available in ESI.† In order to investigate the heterogeneity of the immobilized biocatalyst, samples were taken and not quenched, but additional substrate was added. No further product formation was observed, indicating that there is no active enzyme leaching into to product stream.

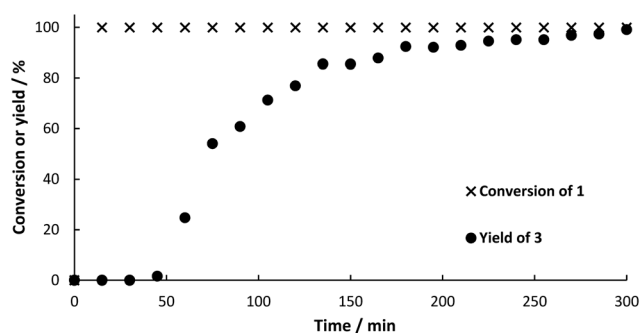


Fig. 5 Conversion of **1** and yield (determined by HPLC) of **3** for the continuous flow synthesis. 160 mg BsPAD immobilized in alginate beads (4 mL beads volume), 20 mM substrate in DES:buffer 1:1 (v/v), 30 °C, flow rate: 45.5  $\mu\text{L min}^{-1}$ .

Continuous Heck coupling was conducted by packing the catalyst powder (2 g,  $Sn_{0.79}Ce_{0.20}Pd_{0.01}O_{2-\delta}$ ) in a stainless steel column (40 mm × 8 mm i.d.).§ This packed bed reactor was heated to 85 °C in a water bath. The reactor was flushed with solvent (aqueous solution of DES:buffer:ethanol:water 1:1:1:1 (v/v/v/v)) for 1 h before switching to the feed stock, which contained **3** (10 mM, simulating the product stream from the first step) as well as 1.5 mol-eq. of **2** and  $K_2CO_3$ , respectively, dissolved in DES:buffer:ethanol:water 1:1:1:1 (v/v/v/v). The feed solution was pumped with a syringe pump equipped with a 20 mL stainless steel syringe at a flow rate of 91  $\mu\text{L min}^{-1}$ . Samples were taken from the product stream in 15 min intervals and analysed with HPLC. After a start-up time of 200 min a approximate steady-state with an average conversion of 90% of **3** was reached. The residence time within the reactor was determined to be 45 min. More information can be found in the ESI.†

### Combined decarboxylation and Heck coupling in flow

Since the outcome of both continuous processes was satisfactory for us, the combination of them was the next goal. For the first step, all the preparations were similar to the single step continuous flow. To improve the performance of the second step, some changes needed to be done. In order to achieve complete dissolution of the substrates, the solvent composition of the feed stock for the second step was adapted to DES:ethanol:H<sub>2</sub>O 1:6.75:2.25. Therefore, the final solvent composition for the Heck reaction was 30 vol% DES, 25 vol% buffer, 34 vol% ethanol and 11 vol% water. Furthermore, the amount of catalyst was increased. Instead of a column of 40 mm in length a column with the dimensions 120 mm × 8 mm i.d. was used (6 g of catalyst). The flow rate was set to 45.5  $\mu\text{L min}^{-1}$  for both pumps (90  $\mu\text{L min}^{-1}$  in sum). Samples of 100  $\mu\text{L}$  were collected every 15 min at the outlet of the reactor. As soon as decarboxylation product was detected at the outlet of the a column packed with PAD, both steps were connected.  $CO_2$  initially leaving the decarboxylation in bubbles, were not observed after connecting the reactors, because the pressure increased to 5 bar and  $CO_2$  dissolved in the reaction solvent. In a first attempt, some leaching of the enzyme led to channel clogging in the pre-heating tube before the Pd-packed reactor after 11 h (see Fig. S3 in ESI†). Blocking of the capillary reduced the supply of intermediate **3** for the second column, which led to increased formation of biphenyl by homo-coupling of the excess **2**. Leaching could easily be overcome by a thorough overnight flushing of loosely bound enzyme from the surface prior to the continuous experiment. In addition to that, a filter (HPLC pre-column filter) was installed after the pre-heating channel, catching the denaturated enzyme. The filter could easily be changed and hence the continuous process could proceed for 16 h in a steady-state with full conversion of both **1** in the first step and **3** in the second step yielding an average of 20% of desired product **5** (Fig. 6). The yield in the batch process was higher than in the continuous setup, because after full conversion we



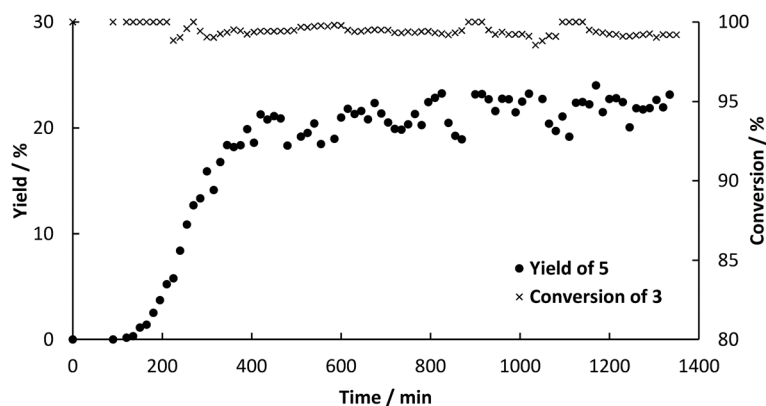


Fig. 6 Yield $\ddagger$  (determined by HPLC) of 5 and conversion $\ddagger$  of 3 over time for the continuous synthesis by enzymatic decarboxylation by *BsPAD* and subsequent Pd-catalyzed Heck reaction using  $\text{Sn}_{0.79}\text{Ce}_{0.20}\text{Pd}_{0.01}\text{O}_{2-\delta}$  to couple 2 and 3.

observed isomerization of 4 to the more stable and desired product 5. The ratio of both products was determined to be 3:1 (mol/mol) and shift towards 5 with increasing reaction time. However, the rate of isomerization is too small to be considered as a way to synthesize 5. The product could easily be isolated by evaporation of ethanol followed by extraction in MTBE. The residence time in the reactors was determined to be 30 min for the decarboxylation and 45 min for the Heck coupling resulting in a space-time yield of  $4.8 \text{ g L}^{-1} \text{ h}^{-1}$  for decarboxylation and  $0.52 \text{ g L}^{-1} \text{ h}^{-1}$  for Heck coupling. Since constant feed of 3 could be achieved this time, side-product formation due to homo coupling of 2 could be limited to a maximum of 0.8 mM (see Fig. S4 ESI $\dagger$ ). Other potential side reactions are polymerization of 3 resulting in high conversion, but not leading to any detectable product.

## Conclusion

In conclusion, we were able to develop a fully integrated flow process combining an enzymatic decarboxylation by encapsulation of the enzyme and subsequent Heck coupling *via* a heterogeneous Pd-catalyst. To our knowledge, this is the first example of a chemo-enzymatic cascade in flow using a non-conventional solvent system of DES with water. The outstanding potential of DES to overcome obstacles like substrate solubility and solvent compatibility is a major step towards broad application of continuous flow chemo-enzymatic reactions. In comparison to the one-pot setup, continuous flow requires a reduction in substrate concentration. However, long-term usage of catalysts in continuous flow is economically and ecologically more favourable. Admittedly, the complex chemistry of the chosen reaction leads to a number of side reactions and thus a low yield of the desired product. Nevertheless, due to the elimination of the isolation of the highly reactive intermediate, the use of bio-based, cheap substrates and the reuse of the expensive Pd-catalyst, this proof-of-concept can be the first step on the path to establish flow reactions in aqueous DES mixtures.

In near future further substrates will be tested for their potential to serve as bio-based substrates for the decarboxylation in order to extend the scope of this setup. Furthermore, experiments regarding catalyst and enzyme activity, stability and heterogeneity are planned. Additionally, optimization of process parameters and product ratio are currently under investigation.

## Conflicts of interest

There are no conflicts to declare.

## Notes and references

$\ddagger A_{(\text{limiting})} + B \rightarrow P$ ; yield  $Y [\%] = (c_{P,t}/c_{A,0}) \times 100$ ; conversion  $X [\%] = (c_{A,0} - c_{A,t})/c_{A,0} \times 100$ , yield and conversion determined by HPLC after calibration.

$\S$  Leaching of the inorganic catalyst was not investigated in this work, but will be topic of future investigations. Leaching data of the catalyst under similar conditions can be found in ref. 45 and 47.

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