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Adsorbent-based downstream-processing of the decarboxylase-based synthesis of 2,6-dihydroxy-4-methylbenzoic acid

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KEYWORDS

Downstream-processing (DSP); *ortho*-benzoic acid decarboxylases; biocatalysis; carboxylation; ion-exchange resin

ABSTRACT

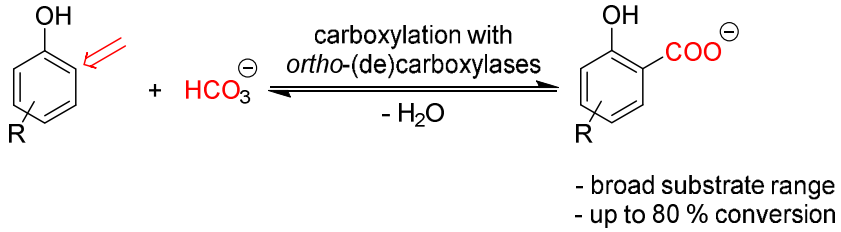
In this case study the regioselective enzymatic carboxylation of 3,5-dihydroxytoluene (orcinol) using the nonoxidative 2,3-dihydroxybenzoic acid decarboxylase from *Aspergillus*

oryzae (2,3-DHBD_Ao), followed by an adsorbent-based downstream approach, has been investigated. The product 2,6-dihydroxy-4-methylbenzoic acid (DHMBA) was herein purified by an adsorption-desorption-cycle and subsequently obtained with purities >99% without a full elimination of the excess bicarbonate from its reaction solution. Ten adsorbent resins were studied in respect of their ability to recover the product from the reaction solution, whereas the strong anion exchange resin Dowex 1x2 in its chloride-form showed affinities >99%, even at bicarbonate concentrations of >3 $mol \cdot L^{-1}$. Desorption from loaded resin was carried out by a 2 $mol \cdot L^{-1}$ HCl/acetone mixture, followed by product crystallization during acetone evaporation. This presented concept does not require a final column preparation step and improves overall atom efficiency of the biocatalytic reaction system.

1. INTRODUCTION

The synthesis of hydroxybenzoic acids was originally introduced into industrial scale by Hermann Kolbe and Rudolf Schmitt at the end of the 19th century.[1,2] The Kolbe-Schmitt process enables access to important aromatic carboxylic acids such as pharmaceuticals,[3] agrochemicals and building blocks for organic synthesis.[4] Unfortunately, this procedure frequently suffers from unsatisfactory regioselectivity and requires harsh reaction conditions such as high temperatures and CO_2 pressures (120-300 °C, 20-100 atm).[2] Further disadvantages may include the preparation and isolation of completely dry phenoxide from the corresponding phenol, depending on the phenol-substrate.[5] Despite these challenges, the Kolbe-Schmitt reaction is still widely used in industry, especially for the syntheses of salicylic acid, which is a precursor of acetylsalicylic acid and more well known as Aspirin® by Bayer AG.

However, in recent years multiple investigations were carried out to circumvent the disadvantages of the Kolbe-Schmitt-reaction, e.g. by using (transition) metal catalysis,[6] microwave-assisted reactions[7] and ionic liquids.[8] A further promising biocatalytic alternative is the use of nonoxidative (de)carboxylases, which catalyze the reversible introduction/removal of a carboxylic group from organic compounds.[9] This can be synthetically applied for the regioselective *ortho*- and *para*-carboxylation of small or medium-sized phenol derivatives using bicarbonate as CO_2-source.[10] Recently also more complex (poly)phenolic substrates were carboxylated using *ortho*-(de)carboxylases 2,3-dihydroxybenzoic acid (de)carboxylase from *Aspergillus oryzae* (2,3-DHBD_Ao), 2,6-dihydroxybenzoic acid (de)carboxylase from *Rhizobium* sp. (2,6-DHBD_Rs) and salicylic acid decarboxylase from *Trichosporon moniliiforme* (SAD_Tm) (Scheme 1).[14]



Scheme 1. Regioselective enzymatic carboxylation of phenols using bicarbonate as a CO_2-source leading to a wide range of products.

In strong contrast to the original Kolbe-Schmitt reaction, significantly less harsh reaction conditions are required for such a biocatalytic carboxylation reaction with a reaction temperature of only 30 °C at or near atmospheric pressure (using bicarbonate as CO_2-source). Unfortunately, the reaction equilibrium of the biocatalytic alternative is similarly positioned towards the phenol-compounds.[11] Hence, strategies to push the equilibrium of (de)carboxylase-catalyzed reactions toward the formation of carboxylation products are required.[12] Here, bicarbonate or CO_2 is usually applied in excess and recent studies also suggest the use of amines/quaternary ammonium salts for a (partial) equilibrium displacement towards the carboxylic acid.[13] In addition, downstream-processing of (de)carboxylase reactions is still a challenge and, to the best of our knowledge, a total elimination of the excess of bicarbonate (see above) via acid addition is still the preferred route. A column chromatography is still subsequently required to remove remaining substrate and further impurities from the biocatalyst preparation, e.g. from the cell preparation method in LB medium.[14]

In this work, we report the development of an alternative adsorbent-based downstream approach for a (de)carboxylase-catalyzed reaction to facilitate selective product isolation from the reaction mixture without removing the excess of bicarbonate (Fig. 1). This approach was investigated for the highly stereoselective 2,3-DHBD_Ao-catalyzed (2,3-dihydroxybenzoate decarboxylase from *Aspergillus oryzae,*[15] NCBI Reference GI: 94730373) *ortho*-carboxylation of 3,5-dihydroxytoluene (orcinol) to 2,6-dihydroxy-4-methylbenzoic acid

(DHMBA) (Scheme 2).[16] DHMBA is a valuable precursor for the synthesis of intermediates for novel inhibitors to target acetohydroxy acid synthase (AHAS; a major target for herbicide discovery),[17] the synthesis of Debromohamigeran E,[18] and further anti-rheumatic agents and immunosuppressants (Scheme 2).

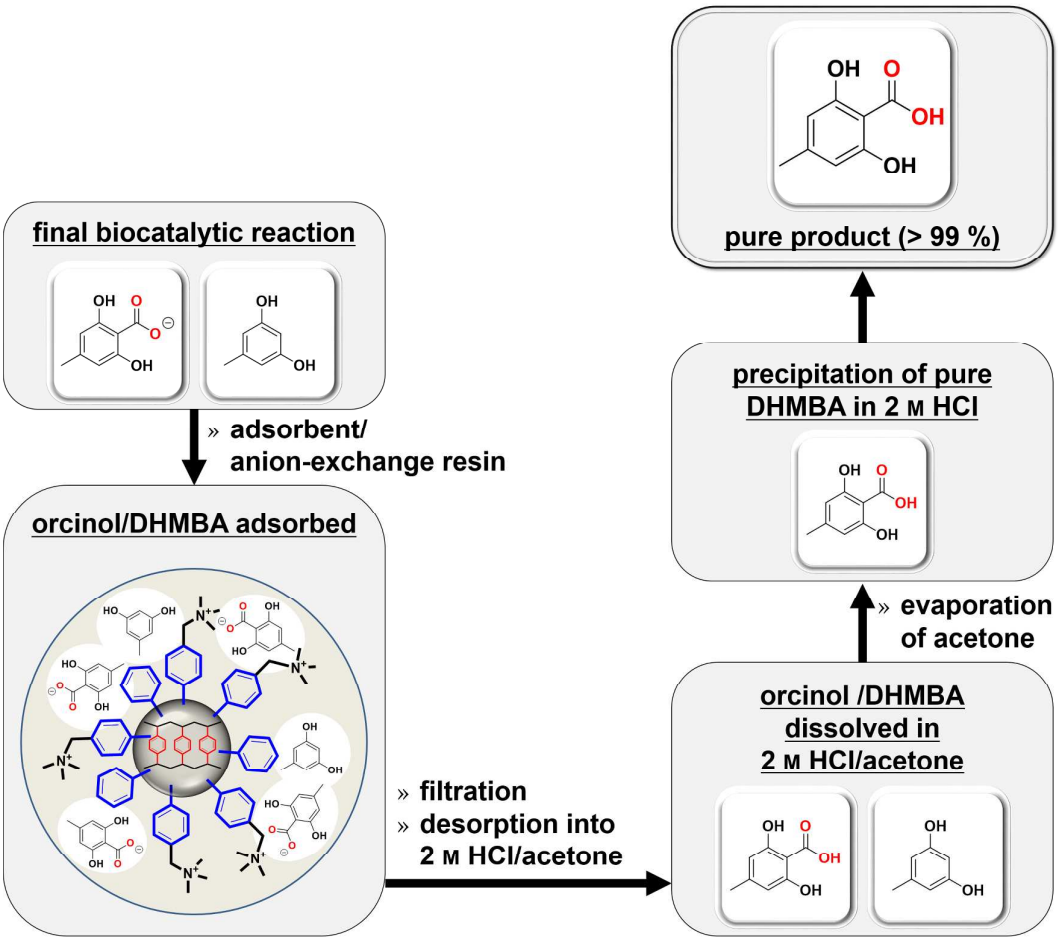
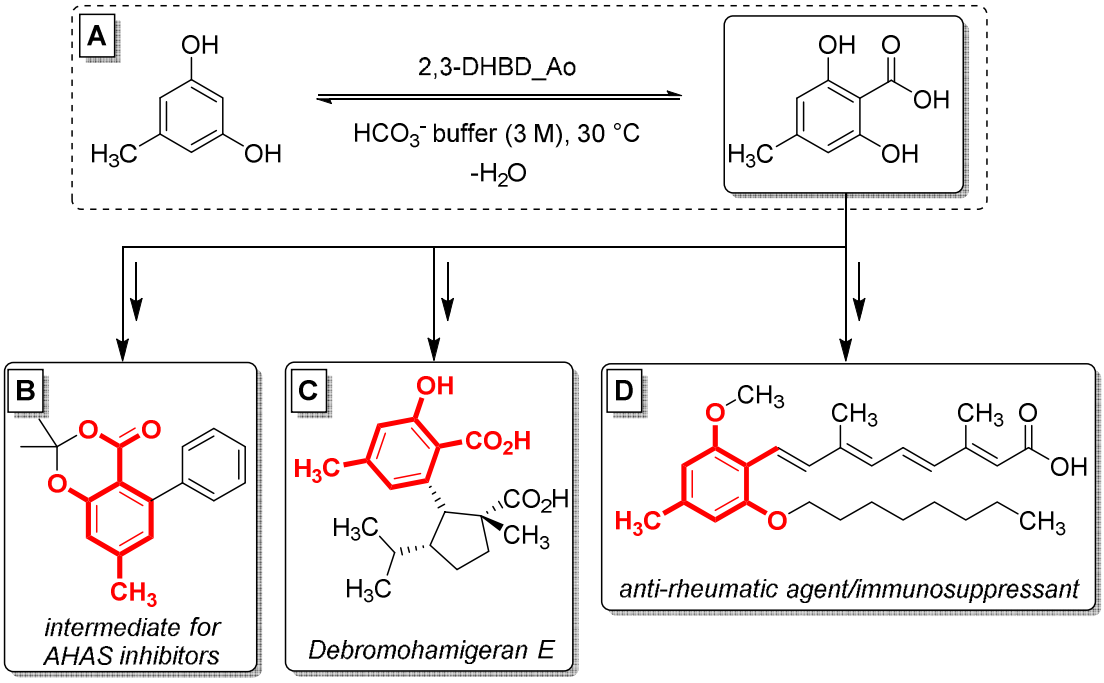


Fig. 1. Schematic representation of adsorbent-based scale-up of the decarboxylase-based synthesis of 2,6-dihydroxy-4-methylbenzoic acid (DHMBA) leading to pure product without further purification steps.



Scheme 2. Enzymatic *ortho*-carboxylation of orcinol catalyzed by 2,3-DHBD_Ao using bicarbonate as CO_2-source (A) and subsequent products [(B) Qu *et al.*[17], (C) Blaisdell *et al.*[18], (D) Hoffmann *et al.*[19]].

2. MATERIALS AND METHODS

General

All compounds were received from commercial suppliers in highest available purity (Sigma-Aldrich, Alfa Aesar, TCI, Fluka) and used as received. Ultrapure water was produced with an 'Ultra Clear Reinstwassersystem' by SG Water (now Evoqua, Guenzburg, Germany) and used throughout this study. 2,3-Dihydroxybenzoic acid decarboxylase from *Aspergillus oryzae* (2,3-DHBD_Ao) was cloned and overexpressed in *E. coli* BL21 (DE3) as previously described.[20]

Resin beads

Resins Diaion PA312(Cl) (LOT: S01C015), Diaion SA10A(Cl) (LOT: T30A023), Amberlite XAD-4 (LOT: 10195020) and Diaion HPA25(Cl) (LOT: U23D040) were purchased from Alfa Aesar and Dowex 1x2(Cl) (LOT: 427259956) was purchased from Carl Roth. Amberlite XAD-1180N (LOT: BCBD7925V) and LEWATIT VP OC 1064 (LOT: BCBB2767V) were purchased from Fluka. Amberlite XAD-7HP (LOT: 041M0001V) and Dowex Optipore L-493 (LOT not given) was purchased from Sigma. Diaion HP-2MG (LOT: 184408F) was purchased from Supelco (see SI).

General Procedure for Biotransformations (small scale)

Lyophilized *E. coli* cells (60 mg whole cells, containing the corresponding overexpressed enzyme 2,3-DHBD_Ao), were rehydrated in 900 µL 50 mmol·L^{-1} acetate buffer pH 5.2 and horizontally shaken at 30 °C and 180 rpm for 30 min. A 500 mmol·L^{-1} stock solution of the substrate was prepared in ultrapure water. 100 µL of the substrate stock solution and 300 mg $KHCO_3$ were added successively to the cell suspension (1 mL final, 50 mmol·L^{-1} final concentration of substrate, 3 mol·L^{-1} final concentration of $KHCO_3$) to give a final pH of 8.5. A blank sample was prepared as described without cells to ensure the absence of a carboxylation reaction without the catalyst. The vials were tightly sealed and horizontally shaken at 30 °C and 180 rpm for 24 h.

After 24 h the reaction was stopped by transferring the whole reaction mixture into 3 mL of a solution containing 2 mol·L^{-1} HCl:MeOH (66:33 v/v). The reaction vessel was additionally rinsed two times with each 500 µL 2 mol·L^{-1} HCl:MeOH (66:33 v/v). The resulting mixture was centrifuged (5 min, 4000 rpm) and the supernatant was filtered through a 10 kDa Centrifugal Filter (VWR Modified PES 10K, 500 µl centrifugal filter) to remove residual proteins (5 min, 14000 rpm). The permeate was diluted in ultrapure water (1:50 total dilution) and the resulting mixture was analyzed by HPLC (see below).

For investigations of the reaction system (conversion vs. time), samples for HPLC were taken after definite time intervals. Therefore 100 µL of the reaction solution was diluted into 400 µL of a solution containing 2 mol·L^{-1} HCl:MeOH (66:33 v/v). The mixture was vortexed to remove remaining bicarbonate and processed as described above (centrifugation, filtration, 1:50 total dilution and analysis by HPLC).

For the evaluation of the biocatalyst concentration the desired mass of whole cell catalyst (0.5, 1, 2, 5, 10, 20, 30 and 60 mg) was weighed in 1 mL HPLC glass vials and the reaction conditions were chosen as previously described.

Characteristics and preparation of the anion–exchange and adsorbents beads

The specific characteristics of the resins are shown in Table S1 and Table S2 (see SI). The anion–exchange resins were used in their native ionic form. Prior to use, all resins were washed by ultrasonification for 5 min successively with 0.1 mol·L^{-1} HCl, ultrapure water and acetone. Afterwards the resin beads were dried under vacuum (rotary evaporator, 40 °C, 20 mbar).

Four anion exchange resins with strong basicities, according to the supplier's specifications, were included in this study. The data in parentheses indicate the type of counterion bound to the resin in its native form. The total exchange capacity of the resin in mmol equivalents per gram (meq g^{-1}) is defined as the total number of counterions available for exchange per unit weight of resin.

Adsorption experiments

To evaluate the capability for a downstream-processing of the case biocatalytic reaction, the capacity of the resins with respect to substrate and product were determined. For these adsorption experiments a 25 mmol·L^{-1} test solution of product and substrate in 2.5 mol·L^{-1} bicarbonate was prepared. 5 mL of this test solution was then added to 0.1 g, 0.5 g and 1.0 g of each resin, respectively. The shaking-flask assays were performed at 30 °C and 180 rpm for 30 min. In earlier experiments (data not shown) adsorption equilibrium was determined to be reached within 30 min. The adsorption of substrate and product was determined by analysis of the residual concentration in solution before and after adding the adsorbent resins via HPLC. Therefore, 100 µL of the supernatant was diluted into 900 µL of a solution containing 2 mol·L^{-1} HCl:MeOH (66:33 v/v) to remove remaining bicarbonate and dilution (2:3 v/v) in ultrapure water. For calculations, the measured concentration of the prepared test solution was set as 100%.

Desorption studies experiments

Because of the promising results from the adsorption experiments, desorption experiments for the anion-exchange resin Dowex 1x2 (Cl) were investigated. Therefore, 0.5 g Dowex 1x2 (Cl) was loaded with substrate and product as mentioned above. Different mixtures of 2 mol·L^{-1} HCl:acetone were then added to the resin beads for the elution experiments at 30 °C and 180 rpm for 30 min. Afterwards the concentrations of substrate and product in solution were determined via HPLC. The supernatant was removed by decantation, and two more elution steps were performed as described.

Biocatalytic carboxylation starting with 2.5 g orcinol, including downstream-processing using anion-exchange resin Dowex 1x2 (Cl)

For a preparative-scale enzymatic reaction, 12 g lyophilized *E. coli* cells containing the corresponding overexpressed enzyme (30 mg cells/mL reaction solution) were rehydrated in 400 mL (50 mmol·L^{-1}) acetate buffer pH 5.2 and stirred in a tempered and closed 1 L flask at 30 °C and 600 rpm for 30 min. 2.5 g of substrate orcinol and 120 g $KHCO_3$ were added successively to the cell suspension (50 mmol·L^{-1} final concentration of substrate, 3 mol·L^{-1} final concentration of $KHCO_3$) to give a final pH of 8.5.

After 24 h the reaction was checked for maximal conversion by adding a 100 µL sample to 400 µL of a solution containing 2 mol·L^{-1} HCl:MeOH (66:33 v/v). The mixture was centrifuged (5 min, 4000 rpm) and the supernatant filtered through a 10 kDa centrifugal filter (VWR Modified PES 10K, 500 µl centrifugal filter) to remove residual proteins (5 min, 14000 rpm). The permeate was diluted in ultrapure water (1:50 total dilution) and the resulting mixture was analyzed by HPLC (see below).

For the removal of DHMBA and orcinol from reaction solution, the cells were first separated from reaction solution by centrifugation (20 min, 4000 rpm). Then, 40 g of washed anion-exchange resin Dowex 1x2 (Cl) (0.1 g of resin/mL) was added to the supernatant and stirred for 2 h at 30 °C and 600 rpm. Afterwards, the supernatant was checked for full adsorption as previously described. The loaded adsorbent beads were separated from the solution and washed with 20 mL water. The loaded resins were then treated with 400 mL of 2 mol·L^{-1} HCl:acetone (20:80 v/v) for 30 min at 30 °C and 600 rpm to recover product and substrate. Afterwards, the resin beads were filtered off and the permeate evaporated in a rotary evaporator to remove acetone (40 °C, from 400 mbar at the beginning to 0 mbar in the end) until white crystals appeared in the remaining aqueous 2 mol·L^{-1} HCl solution. After vacuum filtration, the crystals of DHMBA were dried for 2 h by lyophilisation. This desorption procedure was performed two more times.

After carrying out detailed analytics (^{1}H-NMR, ^{13}C-NMR, HSQC, methanolic solution of received crystals for HPLC and LC-MS) no further purification steps were necessary to obtain pure product in high purity (>99%).

Analytics

HPLC analysis

Analyses were performed on a HPLC system from Knauer (pump: WellChrom K-1001, degasser: Smartline Online Degasser, autosampler: Spark Marathon Basic, software: Clarity Chrome) equipped with a 4-channel UV detector (Knauer UV Detector S 2550) and a reversed phase Phenomenex Kinetex C18 column (100 Å, 150 × 3.00 mm, 2.6 µm, item number 00F-4462-Y0, column temperature 35 °C). Conversions were determined by using calibration curves for substrate and product prepared with commercial reference materials. All compounds were spectrophotometrically detected at 273 nm (orcinol) and 248 nm (DHMBA). The method was run over 40 min (flow rate 0.2 mL min^{-1}) with water (+0.1% formic acid, solvent A) and MeOH (+0.1% formic acid, solvent B) as the mobile phase with the following gradient: 0-6 min 30% B, 6-10 min 30-80% B, 10-18 min 80% B, 18-28 min 80-30% B, 28-40 min 30% B). Retention times: 8.6 min (orcinol) and 13.2 min (DHMBA).

NMR analysis

^{1}H NMR and ^{13}C NMR-spectra were recorded with a Bruker AVANCE (300 MHz) in DMSO-d_6. Chemical shifts are reported in parts per million (ppm) relative to the solvent peak, coupling constants *J* are given in Hz. Splitting patterns are indicated as follows: s, singlet; d, doublet; t, triplet; m, multiplet; quint, quintet, dt, double triplet; dd, double doublet.

LC-MS analysis

The purity of the target compound (DHMBA) was determined to be >99% by LC-MS using a Thermo Finnigan LTQ Velos Pro LC-MS (software: Xcalibur) equipped with an ion trap detector and reversed phase column (Phenomenex Kinetex Phenyl Hexyl column (100 Å,

150 × 3.00 mm, 2.6 µm, column temperature 35 °C). The method was run as mentioned earlier (see "HPLC analysis").

3. RESULTS AND DISCUSSION

The biocatalytic carboxylation of orcinol to DHMBA was initially reported by Wuensch *et al.*[16] A substrate screening showed that 2,3-DHBD_Ao selectively converts a broad substrate scope of substrates to the corresponding *ortho*-carboxylated derivatives. Moderate conversions were found with a 300-fold excess of bicarbonate, whereas fortunately only *ortho*-regioisomeric carboxylation products were formed.[16,20] However, major limitations are low substrate loading and the requirement to remove the entire excess bicarbonate via acidification during downstream-processing, followed by column chromatography to purify the desired product. This study targets to (partly) overcome these limitations.

3.1 Optimization of the enzymatic carboxylation reaction

Initial experiment targeted the increase of the substrate concentration to facilitate higher yields within the biocatalytic process. Since the equilibrium conversion is directly linked to the excess of bicarbonate over orcinol, higher substrate concentrations will inevitably result in lower equilibrium conversions at a constant bicarbonate concentration of 3 $mol \cdot L^{-1}$ (K_{eq} is approximately $5 \cdot 10^{-4}$) (Fig. 2, A).

While a substrate concentration of 5 $mmol \cdot L^{-1}$ will lead to >80% conversion, higher substrate concentrations of 10 $mmol \cdot L^{-1}$ and 50 $mmol \cdot L^{-1}$ result in only 80 and 68% conversion, respectively. A further increase of orcinol concentration unfortunately leads to a full inhibition of the catalytic reaction system. Consequently, a substrate concentration of 50 mmol L^{-1} was used throughout this study. Catalyst loading was optimized here to facilitate optimal catalyst usage within the desired process with recombinantly expressed 2,3-DHBD_Ao in *E. coli* BL21 (DE3) whole cells. As previously reported, a lower substrate loading of only 5 mmol L^{-1} is easily converted by ca. 10 mg whole cell biocatalyst/mL, but for 50 $mmol \cdot L^{-1}$ a significant higher catalyst loading of 60 mg whole cell biocatalyst/mL is required to reach equilibrium conversion (Fig. 2-B and 2-C). This clearly indicates that further improvements of the catalytic robustness towards higher substrate loadings and higher

specific activities are required to improve overall process competitiveness. The final optimized reaction conditions carried out at 30 °C, a substrate concentration of 50 $mmol \cdot L^{-1}$ and 3 $mol \cdot L^{-1}$ $KHCO_3$ resulted in a conversion of 60% (Fig. 2-D, highlighted with star). It is important to note that the reaction time course of the up-scaled application (see below) differs from these results since the required sample drawing procedure causes multiple losses of gaseous carbon dioxide from the reaction mixture. This results in a reduced excess of bicarbonate/CO_2, which induces lower equilibrium conversions. Hence, a closed vessel reaction set-up was preferred throughout this study, especially at a larger scale.

A

c (orcinol)/mmol·L^{-1}	conversion/%
5	80
10	80
50	68
100	2

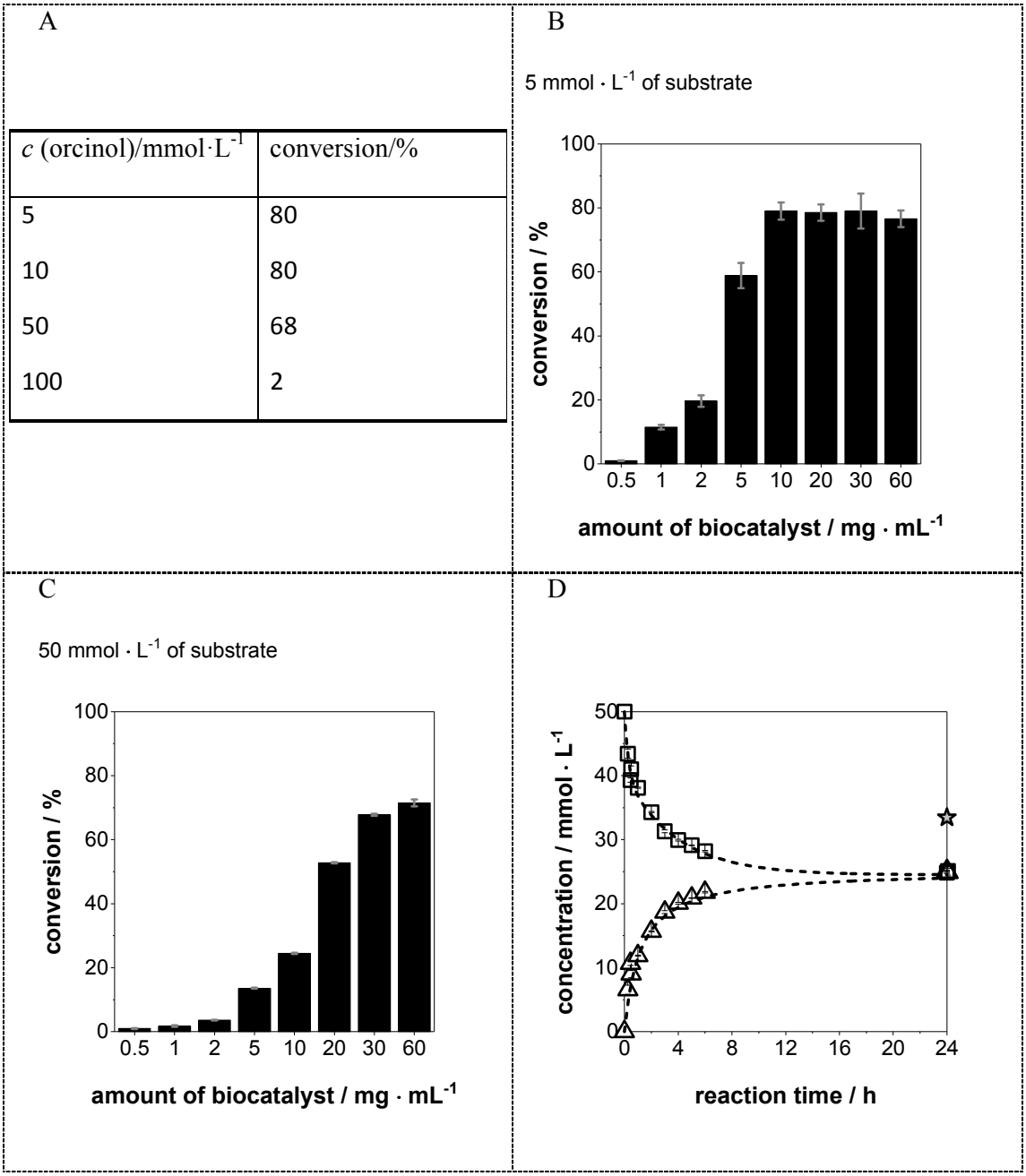


Fig. 2. A) conversion of different orcinol concentrations with a fixed bicarbonate concentration of 3 mol·L^{-1} with 30 mg·L^{-1} biocatalyst at 30 °C; B) required catalyst loading to reach equilibrium conversion at 5 mmol·L^{-1} orcinol and 3 mol·L^{-1} bicarbonate; C) required catalyst loading to reach equilibrium conversion at 50 mmol·L^{-1} substrate and 3 mol·L^{-1} bicarbonate; D) time course and final reactant concentration at optimized conditions; reaction conditions: 50 mmol·L^{-1} NaOAc buffer solution, 3 mol·L^{-1} $KHCO_3$, T = 30 °C, U = 180 rpm,

$\sum t = 24$ h, final pH 8.5. All experiments were carried out in triplicate and the calculated standard deviations are shown.

3.2 Selection of adsorbents resin

Classical downstream concepts for carboxylic acids, e.g. from fermentation processes, include mainly the use of adsorption,[21] extraction[22] or crystallization.[23] Unfortunately, to date, no effective recovery method of aromatic hydroxy acids for an enzymatic carboxylation reaction have been reported in scientific literature. This mainly involves the significant excess of 3 $mol \cdot L^{-1}$ bicarbonate, which is required to push the reaction equilibrium to favor the product side. This excess is afterwards removed via acidification, resulting in a full loss of bicarbonate and thus lowering atom efficiency of the overall reaction system.

The main objective of the first downstream-processing step is to remove both reactants from the bicarbonate solution, which might thereafter be recycled. Different commercially available anion–exchange and non-ionic adsorbents resins were screened for their ability to remove both product (DHMBA) and remaining substrate (orcinol) from a decarboxylase reaction solution in the presence of excess bicarbonate, as mentioned above. Therefore, test solutions containing 25 $mmol \cdot L^{-1}$ of product and substrate in 2.5 $mol \cdot L^{-1}$ $KHCO_3$ were prepared to simulate the reaction system at a conversion of 50 %. Furthermore, three different loading capacities (0.1, 0.5 and 1.0 g per 5 mL test solution, respectively) were evaluated to estimate the required loading of the adsorber materials. The residual concentration in solution was measured via HPLC and the results are shown in Table 1.

Table 1. Adsorption of DHMBA and orcinol onto ten different adsorbers (anionic and non-inonic form) at 30 °C and 180 rpm after 30 min from test solution containing 25 mmol·L^{-1} either of product and substrate in 2.5 mol·L^{-1} $KHCO_3$.

applied adsorber	DHMBA adsorption/%			orcinol adsorption/%		
	0.02 g·mL^{-1}	0.1 g·mL^{-1}	0.2 g·mL^{-1}	0.02 g·mL^{-1}	0.1 g·mL^{-1}	0.2 g·mL^{-1}
a) anion exchanger resins						
Dowex 1x2 (Cl)	93	**>99**	**>99**	74	**>99**	**>99**
Diaion PA312 (Cl)	77	99	**>99**	51	96	**>99**
Diaion HPA-25 (Cl)	83	98	**>99**	52	98	**>99**
Diaion SA10A (Cl)	74	99	**>99**	46	96	**>99**
b) non-ionic adsorbent						
Dowex Optipore L493	48	97	**>99**	57	98	**>99**
Lewatit VP OC	20	44	61	21	55	72
Diaion HP-2MG	33	90	97	44	93	**>99**
Amberlite XAD-7HP	42	93	98	47	94	**>99**
Amberlite XAD-4	1	7	16	3	19	29
Amberlite XAD-1180N	2	4	13	1	8	14

In general, ion exchange resins exhibit a much stronger affinity towards orcinol and DHMBA in comparison to non-ionic adsorber materials. The affinity of the ion exchange resins ordered in decreasing order of affinity was: Dowex 1x2 (Cl) > Diaion HPA-25 (Cl) > Diaion PA312 (Cl) > Diaion SA10A (Cl), while the non-ionic adsorbers were ordered as Dowex Optipore L493 > Amberlite XAD-7HP > Diaion HP-2MG > Lewatit VP OC > Amberlite XAD-4 > Amberlite XAD-1180N. The highest adsorption affinity towards orcinol and DHMBA was found for Dowex 1x2 (Cl), which requires only 0.1 g adsorber per mL reaction solution to fully remove both main reactants. Thus, Dowex 1x2 (Cl) was subsequently used throughout the study.

In addition, all anion–exchange resins show a slightly higher adsorption affinity towards the product DHMBA in comparison to the substrate orcinol. The highest difference between the

adsorption of DHMBA and orcinol can be achieved with the Diaion HPA-25 (Cl) resin (31%), which might be used in future application within a (partial) *in situ* product removal-strategy (ISPR). Here the shown difference in adsorption is based on the presence of the respective microspecies of both reactants. At pH 8.4, which originates from 3 mol·L^{-1} $KHCO_3$ (highlighted as dashed line in Fig. 3), the carboxylic group of DHMBA is fully deprotonated forming the corresponding monoanion of DHMBA. In contrast, the phenolic hydroxyl group of orcinol is only partly deprotonated and thus the majority of the substrate is present in its neutral form.

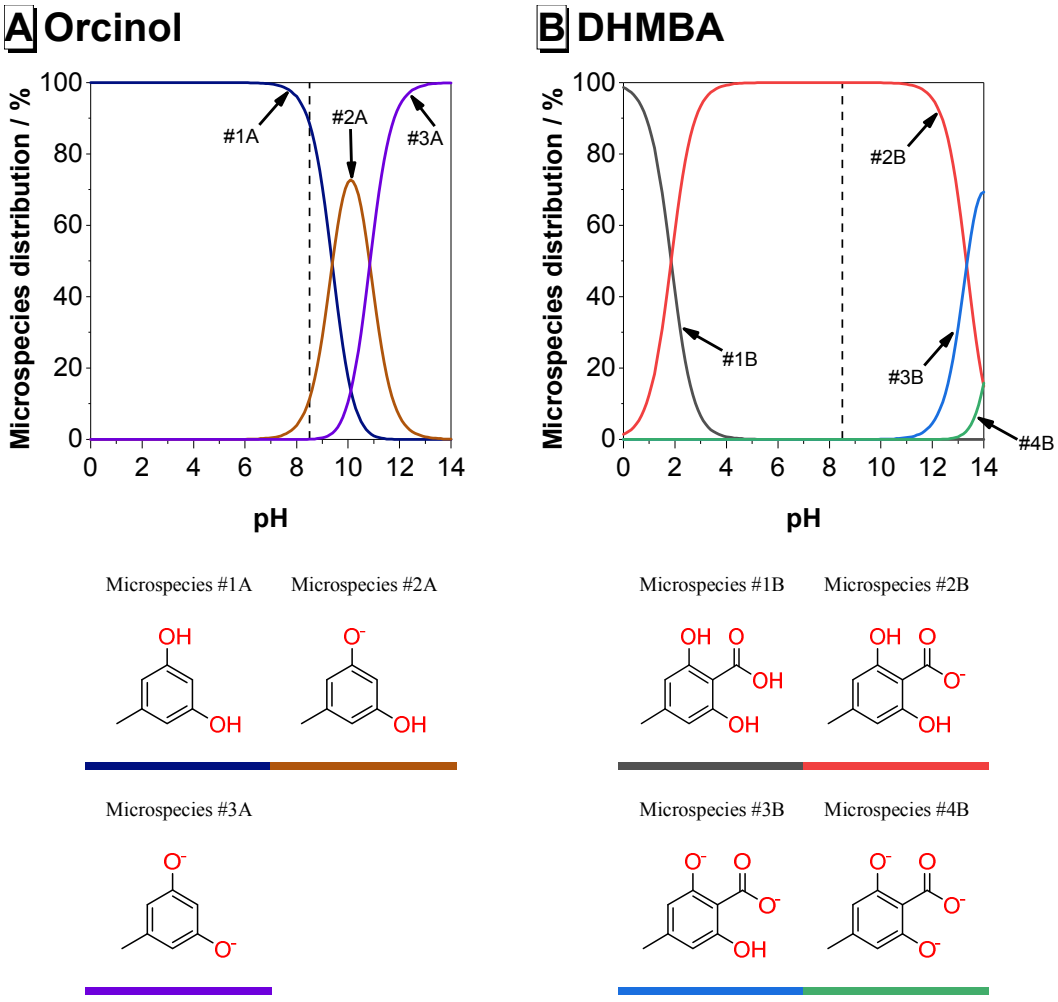


Fig. 3. Undissociated fraction (1A, 1B), monodissociated fractions (2A, 2B), double dissociated fraction (3A, 3B) and triple dissociated fraction (4B) of the total of orcinol [A]

and DHMBA [B] species. Diagrams and pK_s values calculated with "chemicalize" (www.chemicalize.com).

Consequently, DHMBA adsorbs more strongly via ionic interactions towards ion exchange resins than its counterpart orcinol. These ionic interactions overlap with non-specific hydrophobic adsorption of both reactants towards the polystyrene (ST)/*p*-divinylbenzene (DVB)-polymer backbone of the ion exchange resin, which in combination result in the observed affinities (Fig. 4).

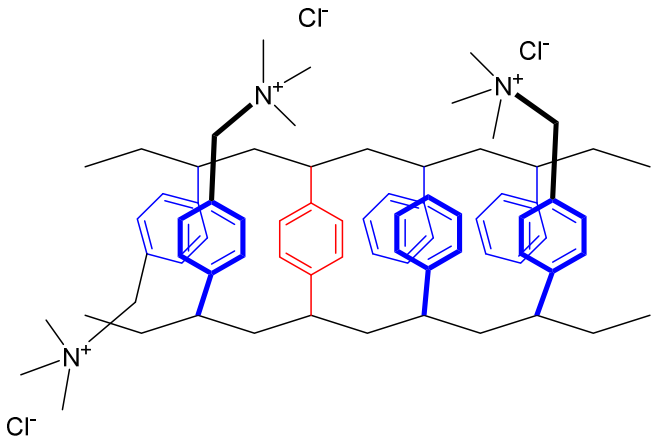


Fig. 4. General presentation of ST (blue)/DVB (red) based anion exchange resin structure with trimethylbenzylammonium functional groups.

In comparison, the investigated non-ionic adsorber materials facilitate only hydrophobic interactions and thus show no significant differences in adsorption between orcinol and DHMBA.

3.3 Desorption from Dowex 1x2 (Cl) and Final Precipitation of DHMBA

After loading, the adsorbent including both reactants were removed from the reaction mixture to facilitate its separation from the remaining concentrated bicarbonate solution.

After loading experiments, elution experiments were performed with different organic solvents and aqueous solutions. Optimal desorption from the loaded adsorbent was found with a solvent mixture consisting of 2 mol·L^{-1} HCl and acetone, which allows a direct combination with the final precipitation step (see below). Different mixtures were investigated, and the results are presented in Fig. 5.

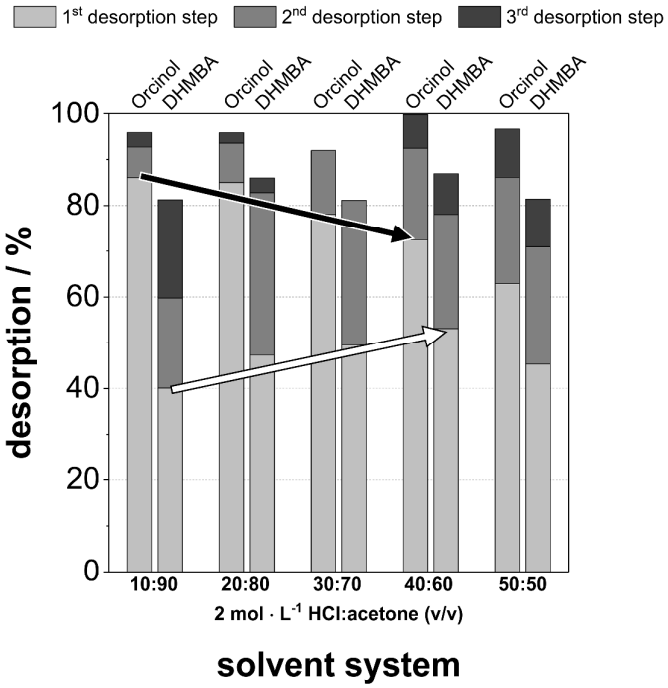


Fig. 5. Desorption of orcinol and DHMBA from loaded anion-exchange resin Dowex 1x2 (Cl) at different mixtures of 2 mol·L^{-1} HCl/acetone at 30 °C after 30 min for each desorption step. Arrows are shown only as a guide (filled arrow: decrease of orcinol desorption with decreasing acetone content; open arrow: increase of DHMBA desorption with decreasing acetone content).

By increasing the ratio of 2 mol·L^{-1} HCl from 10% to 40% (v/v) the initial desorption of DHMBA increases successively from 40% to a maximum of 50% (open arrow) with a simultaneous reduction of orcinol elution from 86% to 72% (filled arrow). Eventually a

solvent mixture of 2 $mol \cdot L^{-1}$ HCl:acetone (20:80 v/v) was sufficient with at least three desorption steps to reach a recovery rate of ≥90% for both reactants.

After filtration of the depleted adsorbent Dowex 1x2 (Cl) and evaporation of acetone, the free acid DHMBA directly precipitates from the remaining aqueous HCl-solution. The depleted adsorbent and the remaining orcinol (in solution) can then be re-used. After a single filtration, pure DHMBA was yielded. A preparative-scale enzymatic carboxylation of orcinol, as a concept study, yielded 878 mg of DHMBA with >99% purity in crystalline form without any further purification steps, e.g. by column chromatography or re-crystallization. In contrast to classical downstream-processing approaches of biocatalytic carboxylation reactions, a total elimination of the excess of bicarbonate is not required.

4 SUMMARY AND CONCLUSION

In this contribution the process development of the decarboxylase-catalyzed synthesis of 2,6-dihydroxy-4-methylbenzoic acid (DHMBA) by an adsorbent-based downstream-processing approach is presented. The biocatalytic reaction was optimized and a variety of adsorbent resins were investigated for the effective removal of both product and substrate from the bicarbonate-containing reaction solution. Here the strongly basic anion exchange resin Dowex 1x2 (Cl) was found to be the most effective adsorbent and was subsequently used within this study. After elution studies, the resin was successfully used for the downstream-processing of a scaled up reaction (from 1 to 400 mL) to produce highly pure (>99%) DHMBA without further purification steps.

The developed process concept herein enables the synthetic use of the biocatalytic carboxylation reaction without the full removal of excess bicarbonate during downstream-processing. Unreacted substrate and the depleted resins can be obtained from the reaction mixture and re-used. This case study highlights the synthetic potential of biocatalytic carboxylation reactions and may serve as a model for other carboxylation biotransformations, especially at a larger scale.

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ABBREVIATIONS

2,3-DHBD_Ao, (de)carboxylase from *Aspergillus oryzae*; DHMBA, 2,6-dihydroxy-4-methylbenzoic acid; DSP, downstream-processing; PS-DVB, Polystyrene/*p*-Divinylbenzene

SUPPORTING INFORMATION

The Supporting Information is available free of charge on the ACS Publications website at DOI: XXX and contains a summary of characteristics of the investigated resins and NMR-data of the obtained 2,6-dihydroxy-4-methylbenzoic acid.

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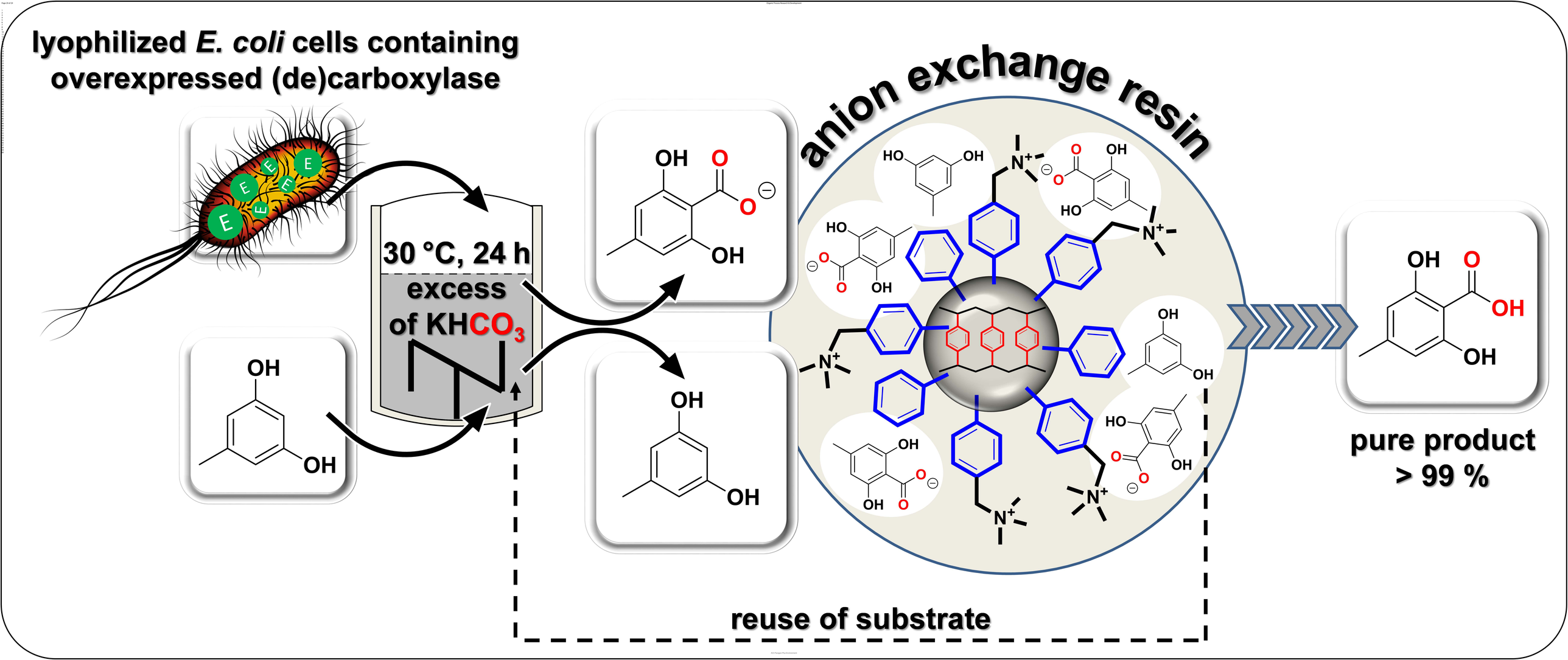
lyophilized E. coli cells containing overexpressed (de)carboxylase
30 °C, 24 h
excess of KHCO3
anion exchange resin
reuse of substrate
pure product
> 99 %