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Synthesis of 2,6-Dihydroxybenzoic Acid by Decarboxylase-Catalyzed Carboxylation Using CO_2 and In Situ Product Removal

Daniel Ohde *, Benjamin Thomas, Paul Bubenheim and Andreas Liese *

Institute of Technical Biocatalysis, Hamburg University of Technology, 21073 Hamburg, Germany; paul.bubenheim@tuhh.de (P.B.)
* Correspondence: daniel.ohde@tuhh.de (D.O.); liese@tuhh.de (A.L.)

Abstract: For the enzymatic carboxylation of resorcinol to 2,6-dihydroxybenzoic acid (2,6-DHBA) using gaseous CO_2 in an aqueous triethanolamine phase, an adsorption-based in situ product removal was demonstrated. The aim is to improve the reaction yield, which is limited by an unfavourable thermodynamic equilibrium. First, a screening for a high-affinity adsorber was carried out. Then, the application of a suitable adsorber was successfully demonstrated. This enabled achieving reaction yields above 80% using the adsorber for in situ product removal. The applied biotransformation was scaled up to 1.5 L at lab-scale. Furthermore, a downstream process based on the elution and purification of the product bound to the adsorber was developed to obtain 2,6-DHBA in high purity. Recycling is one of the key factors in this system, making it possible to recycle the reaction medium, the adsorber and the solvents in additional batches.

Keywords: CO_2; carboxylation; decarboxylase; in situ product removal; process development

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1. Introduction

Carboxylic acids are common raw materials for chemical synthesis and are commercially available in a large structural variety. They are produced primarily from petroleum-based feedstocks by chemocatalysis or from carbohydrates in fermentative processes on a large scale [1,2]. To realize a green enzymatic process, research focuses on alternative routes to produce carboxylic acids, for example, by employing decarboxylases for carboxylation reactions [3,4]. However, these reactions are severely limited by thermodynamics. This makes it a challenge to achieve high reaction yields.

Limited success in improving these reactions was achieved by increasing the substrate concentration [5], either the bicarbonate salt or CO_2 partial pressure [6], by substituting $KHCO_3$ with other metal bicarbonate salts [7], and by using different organic solvents as well as ionic liquids [7]. In particular, the utilization of amines for the capture of gaseous CO_2 and the supply of a high C1 substrate concentration was promising, as no bicarbonate salt was required, and an enzymatic carboxylation was possible [8]. In our previous work, we demonstrated that the medium is efficiently loaded with gaseous CO_2 as triethanolamine (TEA) reduces the CO_2 bubble size, thereby improving the mass transfer rate and efficiency [9]. Moreover, we showed that the equilibrium yield depends on the dissolved inorganic carbon (DIC) content, with HCO_3^- being the major contributor [10].

Other approaches focused on the application of in situ product removal (ISPR) to improve enzymatic carboxylation. For example, liquid-liquid extraction [11] and chromatography-based [12] methods are commonly employed for carboxylic acid removal. So far, for the biocatalytic carboxylation of catechol and resorcinol, liquid–liquid extraction has not been successfully implemented to the best of our knowledge [13]. The main reason is that both the substrate and product have very similar properties, such as solubility in solvents and affinity to adsorber [13]. Having too many similar molecular

properties also poses a challenge in identifying suitable materials for a chromatography-based separation approach. However, a major breakthrough was achieved when certain quaternary ammonium compounds were reported to selectively precipitate some carboxylic acid products in enzymatic carboxylations [14,15]. This led to the investigation of different anion exchangers with similar group functionalities. In this manner, an anion exchanger was found and reported to efficiently remove the product of the biocatalytic carboxylation of orcinol in an aqueous $KHCO_3$ system [16]. For the separation of catechol and 2,3-DHBA, several different anion exchangers were studied, all of which showed competitive adsorption of catechol [13]. In this investigation, the weak anion exchangers Amberlite IRA67® (Dow, Midland, TX, USA) and Dianion WA30® (Mitsubishi Chemical Group, Tokyo, Japan), as well as the strong anion exchangers Amberlite A26® (Dow, Midland, MI, USA) and Amberlite IRA958® (Dow, Midland, MI, USA), were investigated [13]. Both weak anion exchangers have tertiary amines as functional groups but different backbone matrices. Dianion WA30® has a styrene-divinylbenzene backbone, whereas Amberlite IRA67® has a hydrophilic acrylic structure. In contrast to these resins, both strong anion exchangers have quaternary ammonium functional groups. In terms of backbone, Amberlite A26® has a styrene-divinylbenzene backbone, and Amberlite IRA958® has an acrylic structure. Finding an adsorber that exhibits sufficient product selectivity can provide an alternative to the in situ product precipitation reported to be possible when using dissolved quaternary ammonium salts, for example, as shown for resorcinol and 2,6-DHBA [14].

In this work, the range of investigated anion exchangers for resorcinol and 2,6-DHBA separation is expanded with the strong anion exchanger Dowex® 1X8-50, which has trimethyl ammonium functionalized groups on 50 µm-sized carriers. This makes the carrier similar to Amberlite A26®, with only slightly different properties, such as a smaller particle size and a different cross-linking percentage. Dowex® resins have already been investigated for the downstream processing of 2,6-dihydroxy-4-methylbenzoic acid with good selectivity [16]. Therefore, and also because it was not yet investigated for the resorcinol/2,6-DHBA system, this resin is examined in the aqueous TEA system. Based on these results, a first lab-scale synthesis was realized using the well-studied 2,6-dihydroxybenzoic acid decarboxylase (2,6-DHBD) from *Rhizobium* sp. strain MTP-10005 [17–20].

2. Results and Discussion

2.1. Adsorber Affinity Investigation for Resorcinol and 2,6-DHBA

For an initial assessment of the binding selectivity, 50 g/L Dowex® 1X8-50 was mixed with an 80 mM equimolar mixture of resorcinol and 2,6-DHBA in 1 M aqueous TEA saturated with CO_2. Such conditions are similar to reaction conditions in previous investigations of this reaction system [10]. In the supernatant, 5% of the product and 40% of the substrate remained, meaning that 38.1 mM (95%) product and 23.9 mM (60%) substrate were bound to the anion exchanger. Consequently, this is a milestone, as it is now the first reported adsorber, to the best of our knowledge, that has a higher selectivity towards 2,6-DHBA compared to resorcinol. Due to its selectivity, it is possible to use the adsorber for in situ product removal and shift the reaction equilibrium to higher reaction yields. To assess the feasibility of such an adsorption-based ISPR, a comparative investigation of the binding capacity and selectivity under different conditions was performed. Therefore, the mole fraction of 2,6-DHBA and resorcinol, as well as the adsorbent concentration, were varied (Figure 1). Furthermore, a fitting of the obtained results was performed with different competitive isotherms for binary mixtures and the single-solute Langmuir isotherm. Especially, the application of the extended Fritz and Schlünder isotherm resulted in the best fit of the experimental data based on its high R^2 of 0.989. Compared to the other applied isotherms, this one has the highest number of fitting parameters and, therefore, is the most flexible tested isotherm. Still, all tested isotherms show high R^2 values and, therefore, are suitable to predict the distribution of resorcinol and 2,6-DHBA on the adsorber and in the liquid medium. The fitting parameters and R^2 values are listed in Table 1.

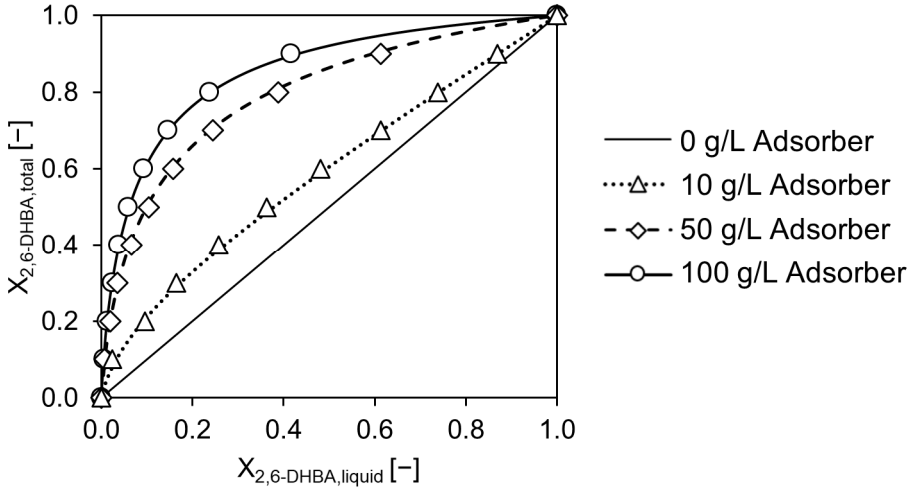

Figure 1. Adsorption isotherm of 80 mM mixtures of 2,6-DHBA and resorcinol on the adsorber Dowex® 1X8-50 at different concentrations in 1 M aqueous TEA saturated with CO_2 at 30 °C.

Table 1. Isotherm parameters of 2,6-DHBA (subscript 1) and resorcinol (subscript 2) adsorbed on Dowex® 1X8-50 at 100 kPa and 30 °C.

Extended Langmuir isotherms								
Jain and Snoeyink (1973) [21]			Butler and Ockrent (1930) [22]			Single Solute		
$q_{m,1}$	mol g^{-1}	1.857	$q_{m,1}$	mol g^{-1}	1.838	$q_{m,1}$	mol g^{-1}	1.807
$q_{m,2}$	mol g^{-1}	1.806	$q_{m,2}$	mol g^{-1}	2.028	$q_{m,2}$	mol g^{-1}	1.946
b_1	mM^{-1}	0.597	b_1	mM^{-1}	0.589	b_1	mM^{-1}	0.560
b_2	mM^{-1}	0.038	b_2	mM^{-1}	0.032	b_2	mM^{-1}	0.026
R^2		0.989	R^2		0.989	R^2		0.959
Extended Freundlich isotherms								
Sheindorf et al. (1981) [23]			DiGiano et al. (1978) [24]			Fritz and Schlünder (1974) [25]		
K_1		0.709	K_1		0.742	$b_{1,1}$		1.633
K_2		0.139	K_2		0.280	$b_{1,2}$		0.257
n_1		0.332	n^{-1}		2.959	$b_{2,1}$		1.180
n_2		0.577	R^2		0.978	$b_{2,2}$		0.214
$K_{1,2}$		0.044				$b_{2,2,1}$		1.880
R^2		0.988				$b_{2,1,2}$		0.037
						n_1		0.911
Extended Tóth isotherm						n_2		0.996
Jaroniec and Tóth (1976) [26]						m_1		0.771
q_m	mol g^{-1}	1.910				m_2		0.872
b_1	mM^{-1}	1.685				$m_{1,2}$		0.991
b_2	mM^{-1}	27.02				$m_{2,1}$		0.728
n		0.985				d_1		1.217
$b_{1,2}$		0.062				d_2		3.518
$b_{2,1}$		16.03				R^2		0.989
R^2		0.989						

In a typical biotransformation, the reaction only occurs in the liquid phase, meaning that the bound substrate and product are inaccessible to the enzyme. However, there is an equilibrium between the substances in the solid and liquid phases. In such a system, the liquid mole fraction $x_{2,6\text{-DHBA,liquid}}$ equals the equilibrium yield without adsorber addition, while $x_{2,6\text{-DHBA,total}}$ equals the improved reaction yield when adding adsorber. Therefore, the overall reaction yield can be increased. From Figure 1, it can be seen that increasing the adsorber concentration has a beneficial effect on the mole fraction of 2,6-DHBA in the multiphase system $x_{2,6\text{-DHBA,total}}$, thus increasing the overall possible reaction yield for a given $x_{2,6\text{-DHBA,liquid}}$.

Application of the isotherms can even be used to predict the reaction yield in relation to the adsorber concentration in the investigated system. This is depicted in Figure 2 with the extended Fritz and Schlünder isotherm. There, the experimentally determined yield of 0.3 without adsorber addition was set as a constant for $x_{2,6\text{-DHBA,liquid}}$. This value was determined experimentally for the enzymatic carboxylation of 80 mM resorcinol in 1 M aqueous TEA saturated with CO_2 at 30 °C (see also [10]). Next, for each adsorber content, the determined isotherm (extended Fritz and Schlünder isotherm) was applied with $x_{2,6\text{-DHBA,liquid}} = 0.3$ to calculate the expected mole fraction of 2,6-DHBA in the solid phase. Then, the total fraction of 2,6-DHBA in the multiphase system ($x_{2,6\text{-DHBA,total}}$) was calculated from both values. This value corresponds to the theoretical reaction yield for each adsorber content (shown as a line in Figure 1).

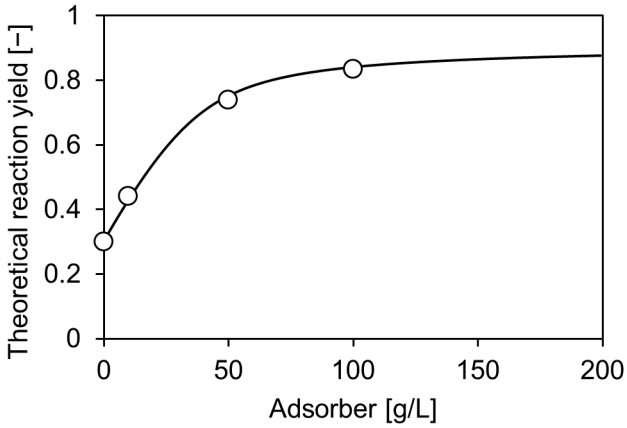

Figure 2. Theoretical reaction yield for the enzymatic carboxylation of 80 mM resorcinol when utilizing Dowex® 1X8-50 in 1 M aqueous TEA saturated with CO_2 at 30 °C. For this approach, the reaction equilibrium is reached when a liquid mole fraction of 2,6-DHBA ($x_{2,6\text{-DHBA,liquid}}$) of 0.3 is reached. This corresponds to the experimentally determined yield of 0.3 without adsorber addition (see also [10]). For each adsorber content, the determined isotherm (extended Fritz and Schlünder isotherm) was applied with $x_{2,6\text{-DHBA,liquid}} = 0.3$ to calculate the expected mole fraction of 2,6-DHBA in the solid phase and in the multiphase system ($x_{2,6\text{-DHBA,total}}$), which corresponds to the theoretical reaction yield (shown as a line).

As the adsorbent concentration is increased, the shape of the predicted reaction yield curve follows a typical saturation profile. Above a reaction yield of about 80%, only minimal improvements are possible by further increasing the adsorbent concentration. Considering this, the utilization of higher adsorber concentrations above 100 g/L becomes unfeasible. It is expected that excessive resorcinol binding slows the reaction speed. Additionally, impairment of the mixing of the suspension through the high solid volume content occurs. Firstly, higher particle volume fractions increase the viscosity, and secondly, the particles get increasingly susceptible to shear stress, as reported in the case of aqueous dispersions of 250 nm poly(styrene-ethylacrylate) particles [27]. In conjunction, the dispersed particles disturb the mixing, leading to extra energy dissipation [28]. Furthermore, the high frequency of contact at high particle volume fractions can damage the particles, causing a decrease in particle size distribution during mixing.

Especially, the undesired binding of substrate to the adsorber is of major concern as it will slow down the reaction and reduce the overall productivity. The extent of the reduction was investigated by performing the enzymatic carboxylation of resorcinol in batches with concentrations of the adsorber, Dowex® 1X8-50, up to 200 g/L. For five days, the reaction yield in the liquid phase was measured (Figure 3). It can be observed that the less adsorber is used, the faster the liquid mole fraction of the product increases and reaches equilibrium at around 0.3. However, the equilibrium liquid mole fraction is not yet reached after 5 days for batches with an adsorber concentration roughly above 100 g/L.

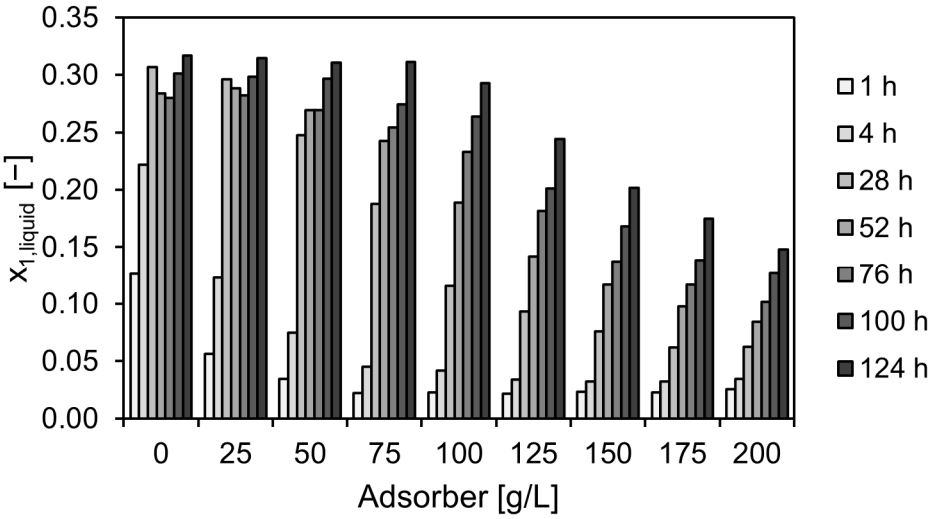

Figure 3. Liquid mole fraction of 2,6-DHBA in the enzymatic carboxylation of resorcinol by 0.139 g/L purified 2,6-DHBD (16.4 U/mg) in aqueous TEA saturated with CO_2 combined with the adsorber Dowex® 1X8-50 at 30 °C.

Converting the liquid mole fraction (Figure 3) into the reaction yield (Figure 4), a maximum within each timeset after the first day can be observed that shifts to a higher adsorber concentration. This demonstrates that even small amounts of adsorber are beneficial, while large amounts reduce productivity. Based on this, a stepwise addition of the adsorber is likely to be beneficial as the overall yield and productivity can be increased simultaneously. In this case, a higher reaction rate can be maintained by minimizing the amount of adsorbed phenolic substrate. Furthermore, high reaction yields above 80% are possible, improving the biotransformation significantly compared to the reaction system without adsorber. It is likely that the styrene-divinylbenzene-based backbone structure of Dowex® is responsible for the undesired substrate binding as it is known to adsorb organic species, such as phenols, due to hydrophobic bonding [29].

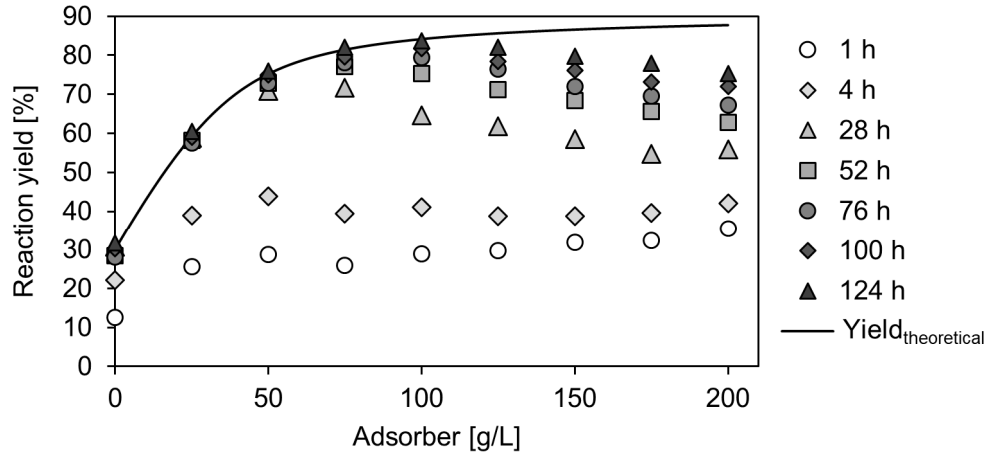

Figure 4. Enhancement of the enzymatic carboxylation of resorcinol by utilization of Dowex® 1X8-50. The enzymatic carboxylation of resorcinol was performed with 0.139 g/L 2,6-DHBD (16.4 U/mg) in aqueous TEA saturated with CO_2 at 30 °C, supplemented with Dowex® 1X8-50. The theoretical reaction yield is shown as a line, as shown in Figure 2. The reactions at high adsorber concentrations have not reached their theoretical reaction equilibrium after 124 h.

2.2. Product Elution and Purification in a Dowex®-Based Downstream Process

After the successful adsorption of 2,6-DHBA and ISPR, the product needs to be efficiently eluted from the adsorber for product isolation. An elution was performed with 1 M HCl in methanol, which removed resorcinol and 2,6-DHBA from the adsorber. In this process, HCl provides chloride ions to compete with the bound 2,6-DHBA, while methanol has a high solubility for both resorcinol and 2,6-DHBA. In contrast, 2,6-DHBA has a low solubility in aqueous HCl. After elution, vacuum distillation was used to remove methanol, causing the precipitation of 2,6-DHBA. Subsequent washing with 1 M aqueous HCl was performed using the product's low solubility in aqueous HCl to remove the remaining substrate and any side products. Side products may have been formed by oxidation reactions [5], which can be minimized by oxygen removal during the downstream processing, i.e., by purging of the used aqueous and organic HCl solutions. After drying at 60 °C and 300 mbar in a vacuum oven, pure 2,6-DHBA was obtained. The downstream process is summarized in Figure 5.

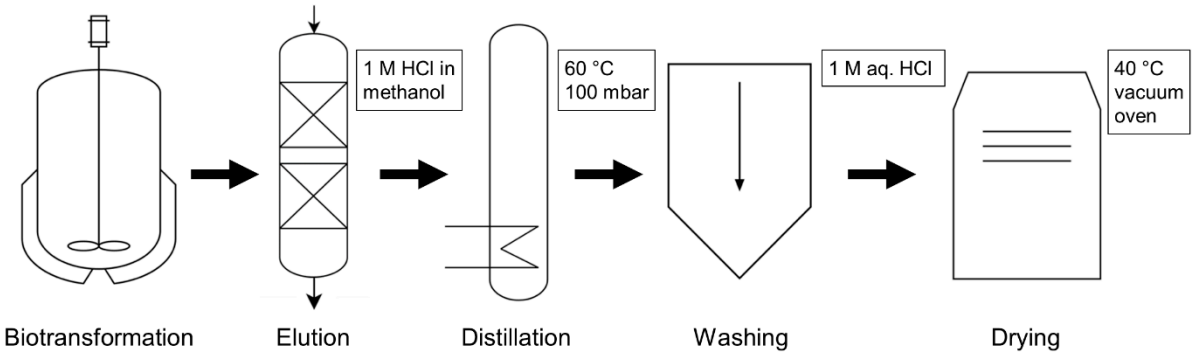

Figure 5. First concept for an adsorber-based ISPR with subsequent downstream processing to obtain 2,6-dihydroxybenzoic acid in high purity.

The described downstream process was applied to test if the product can also be recovered from a representative reaction solution using the adsorber resin, but without applying the resin in the reaction step. For this, a 30 mL carboxylation of 80 mM resorcinol in 1 M aqueous TEA presaturated with CO_2 was performed with 11.6 µg/mL purified 2,6-DHBD (16.4 U/mg). Reaching a product yield of 20.4%, the medium was loaded into a chromatography column containing 0.5 g of Dowex® 1X8-50. At this small scale, gravity column chromatography was performed. After the binding step, product elution and isolation were carried out as described above, and a mass balance analysis was performed. In total, 32.8 mg of product crystals were obtained from the initial 75.5 mg of 2,6-DHBA in the medium. HPLC analysis of the liquid solutions showed that the second largest amount of product remained in the reaction medium at 27.9 mg, while 6.3 mg was detected in the washing fraction of 1 M HCl. Consequently, 8.6 mg of product is not accounted for, which is predicted to be the product that remained bound to the adsorber or was lost due to side-product formation during the downstream processing. The elution with 1 M HCl in methanol proved to be efficient, as 91.7% of resorcinol and 2,6-DHBA were recovered from the adsorber. The high amount of unbound 2,6-DHBA can be reduced by decreasing the flow rate. This is conducted by increasing the contact time, which results in better utilisation of the adsorber. The purity of the product crystals was determined via HPLC analysis. The 2,6-DHBA crystals were 98.9% pure, only containing 1.1% residual resorcinol and no detectable side-products. Optimization of the overall downstream process, especially with respect to the total applied elution volume, flow rate and composition of the solutions, is expected to increase the isolated product yield.

2.3. Enzymatic Carboxylation of Resorcinol on Lab-Scale

A biotransformation in a 3 L bioreactor with 1.5 L reaction medium was performed that improved on the previous small-scale process. Improvements were primarily realized by implementing a stepwise addition of adsorber, increasing the final adsorber loading to 100 g/L and active CO_2 gassing. In Figure 6, the experimental set-up is shown.

Figure 6. Set-up for the enzymatic carboxylation of resorcinol and chromatographic product isolation. (1) CO_2 supply; (2) thermostat; (3) stirred tank reactor with baffles; (4) overhead stirrer performing at approx. 450 rpm (Rushton turbine); (5) pH probe connected to (10), pH meter; (6) adapter connected to a 0.5 µm porous CO_2 sparger; (7) chromatography glass column containing the Dowex® 1X8-50 in the process of washing; (8) piston pump connected to the chromatography column; (9) mass flow meter.

Analogous to the small-scale experiment, the liquid concentrations were measured, which were used to calculate the liquid mole fraction of 2,6-DHBA (Figure 7) and the reaction yield (Figure 8). Based on these results, the initial enzyme activity was calculated to be 18.4 U/mg. The stepwise (20 g/L) addition of adsorber proved to be suitable to maintain a liquid mole fraction of 2,6-DHBA below 10% in order to achieve a first-order kinetics. Furthermore, the third and subsequent adsorber additions were able to reactivate the biocatalyst after the reaction was nearly stopped after 8 days (192 h) (Figure 8). The competitive binding of resorcinol was likely one of the main reasons for the slowing of the reaction. Furthermore, a deactivation of the biocatalyst is expected to have occurred, which reduced the overall productivity.

After two weeks, the adsorber was harvested, as the reaction only progressed very slowly at this point. The material was transferred into a glass column for product elution. In comparison to the small-scale elution process, a lot of foaming and pressure build-up occurred during the elution at this scale. It is expected that the anion exchanger not only bind resorcinol and 2,6-DHBA but also CO_2, which was released by the acidic condition during the elution process. The semi-open chromatography column utilized in the elution process prevented a build-up of pressure inside the glass column. On a large scale and for

future work, the possible build-up of pressure needs to be considered to prevent equipment failure. At the same time, this gives us the opportunity to recycle the desorbed CO_2.

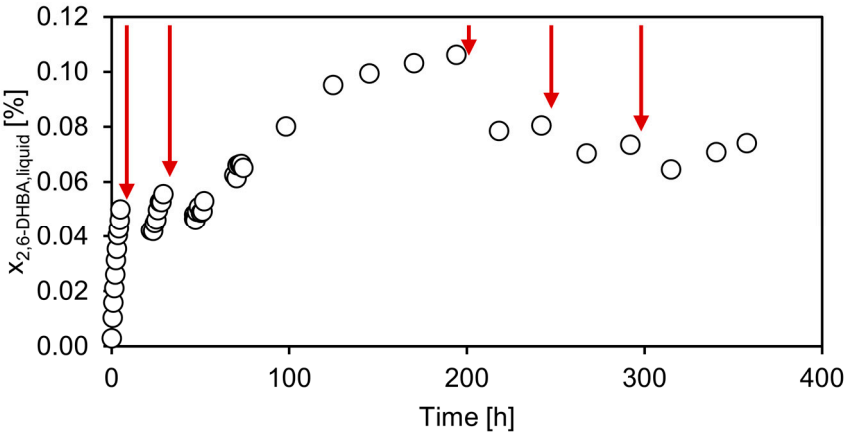

Figure 7. Liquid mole fraction of 2,6-DHBA during the enzymatic carboxylation of 1.5 L of 80 mM resorcinol in 1 M aqueous TEA saturated with CO_2 in a 3 L stirred tank reactor with baffles. In each step (indicated by red arrows), 30 g of Dowex® 1X8-50 were added to the 1.5 L reaction medium containing 64.2 mg/L 2,6-DHBD cell-free extract. The medium was stirred at 450 rpm, heated to 30 °C and continuously gassed with 20 mL/min CO_2 through a 0.5 µm porous sparger.

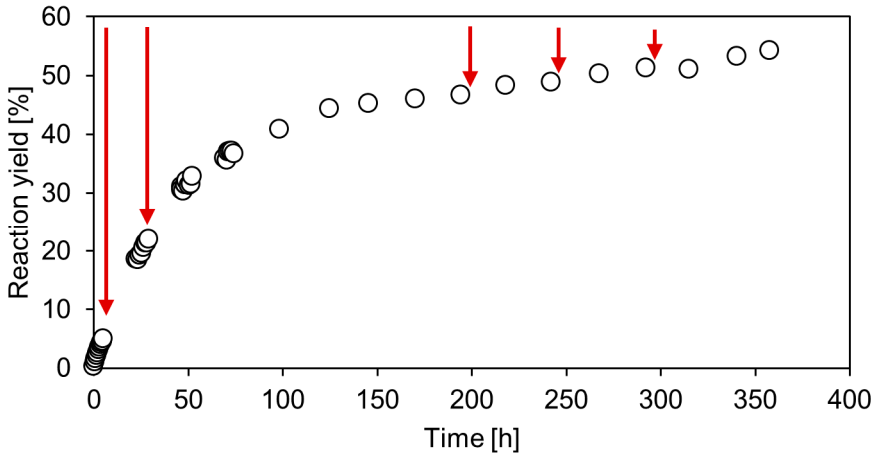

Figure 8. 2,6-DHBA reaction yield for the enzymatic carboxylation of 80 mM resorcinol in 1 M aqueous TEA saturated with CO_2 in a 3 L stirred tank reactor with baffles. In each step (indicated by red arrows), 30 g of Dowex® 1X8-50 were added to the 1.5 l reaction medium containing 64.2 mg/L 2,6-DHBD cell-free extract. The medium was stirred at 450 rpm, heated to 30 °C and continuously gassed with 20 mL/min CO_2 through a 0.5 µm porous sparger.

The obtained product mixture was concentrated by vacuum distillation after elution. Here, the separated methanol was reused for additional product elutions, minimizing the required eluent volume and waste. In total, 1 L of 1 M HCl in methanol was used for the elution step. Figure 9A depicts the precipitate before the first washing step with 1 M HCl. The dark color is likely the result of side-products formed by the oxidation of phenols [5]. These side-products were visible in the HPLC chromatograms as additional peaks with retention times above 9 min. Analysis of the resorcinol peak at 7.1 min and the 2,6-DHBA peak at 8.4 min was not impaired. Repeated washing with aqueous 1 M HCl removed leftover resorcinol and side-products, causing the residual 2,6-DHBA precipitate to gain a slightly pinkish color (Figure 9B). After drying the product at 40 °C, 2,6-DHBA powder was obtained with a greyish color, as shown in Figure 9C. The powder has a purity above

99.8% as determined by HPLC analysis, with minimal residual resorcinol being present. In total, 6.71 g of 2,6-DHBA was isolated.

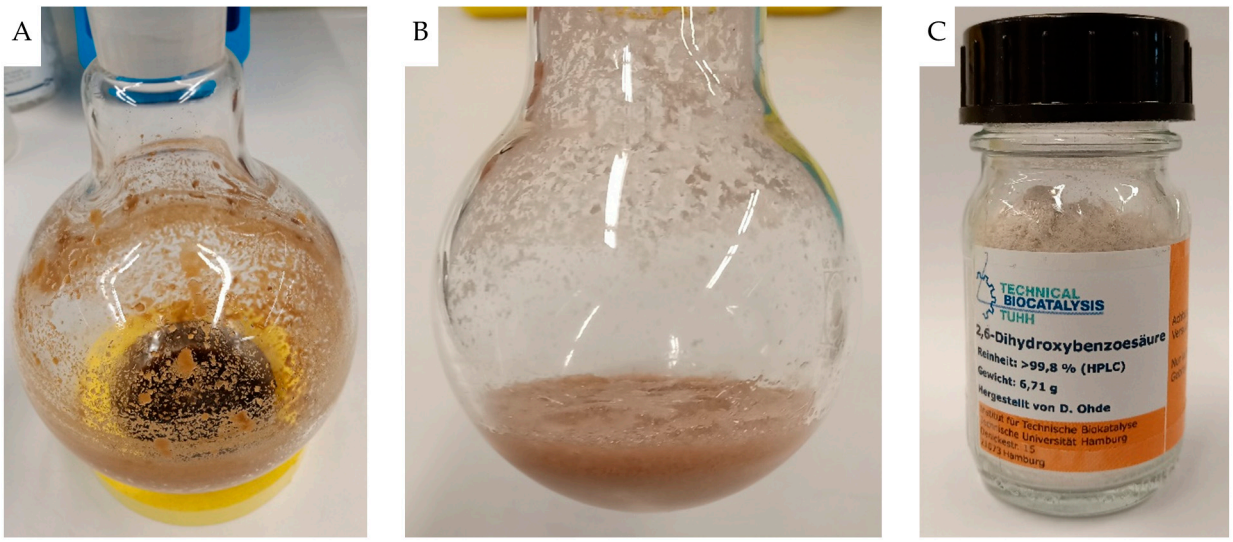

Figure 9. Purification status of 2,6-DHBA eluted from Dowex® 1X8-50. Following the downstream processing, (**A**) shows the precipitate from the elution fraction, (**B**) shows the 2,6-DHBA precipitate after washing with 1 M HCl and (**C**) shows the isolated and dried product in a glass bottle.

The ratio of the isolated product from the reaction medium could be significantly improved, as shown in the following. In the 30 mL scale biotransformation, 43.4% (32.8 mg) of the product was isolated. In comparison, 66.9% (6.71 g) were isolated on the larger scale from 10.03 g and produced 2,6-DHBA. It is expected that the use of 100 g/L instead of 16.7 g/L adsorber is the primary reason for this. For further improvement, the amount of applied adsorber for the adsorption can be increased, and a second chromatographic run of the leftover product in the reaction medium can be performed to maximize the recovery of 2,6-DHBA. Moreover, the application of higher total enzyme activity can enhance productivity and reaction yield. In theory, a reaction yield of 84% is enabled when using 100 g/L adsorber. However, this yield could not be reached in a timely manner as only a total enzyme activity of 1772 U was employed. In turn, a biocatalyst yield [30], also referred to as the 'productivity number' [31], of 69.7 $g_{product}/g_{catalyst}$ was achieved. This value places the process in the fine chemical sector, as provided by Tufvessen et al., a publication on the cost analysis for catalyst production in biocatalytic processes [32]. In this fine chemical sector, an enzyme preparation of free enzyme usually costs between 1000–2500 €/kg enzyme. Therefore, the biocatalyst cost would be in the range of 14.35 to 35.87 € to produce one kilogram of 2,6-DHBA for this process. However, the value of the obtained biocatalyst yield should be used with caution as it was only achieved for long-running biotransformation (>14 days), which makes this process not practical for applications. Finding an adsorber with significantly increased selectivity and capacity would be necessary to increase productivity, as it is expected that a higher enzyme loading would make the process too expensive.

2.4. Repetitive Batch Operation

Performing the enzymatic carboxylation in an aqueous amine solution has the advantage that the reaction medium can be easily reused. Only fresh substrates (CO_2 and resorcinol) and, if necessary, fresh enzymes need to be provided. The recycling of the reaction medium (1 M aqueous TEA) is demonstrated in a second biotransformation, which was performed using the reaction medium of the first batch. In this second batch, only 0.60 U/mg initial enzyme activity remained, corresponding to a 96.8% decrease. In

Figure 10, it can be seen that the liquid mole fraction starts at 0.008, which is due to the leftover substrate and product from the first batch. The addition of adsorber doubled the remaining activity to 1.21 U/mg, highlighting the benefit of using an ISPR, even if it is still not fully practical.

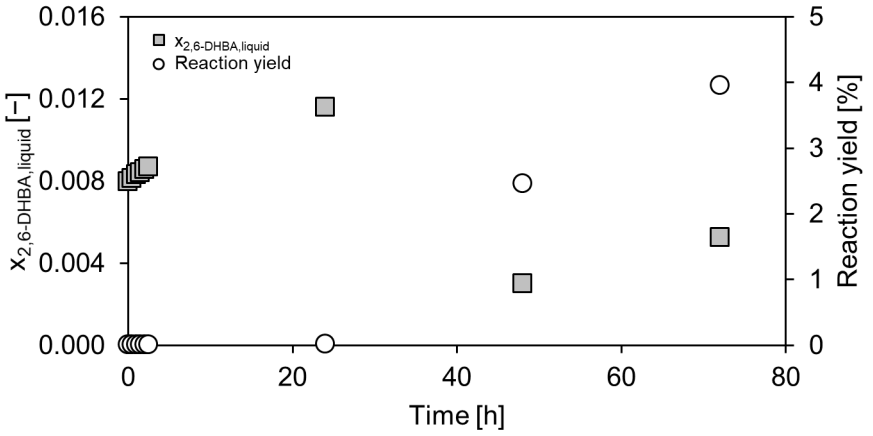

Figure 10. Repetitive batch of the enzymatic carboxylation of 80 mM resorcinol in 1 M aqueous TEA saturated with CO_2 in a 3 L stirred tank reactor with baffles. After 24 h, 30 g (20 g/L) of Dowex® 1X8-50 was added to the 1.5 L reaction medium containing 64.2 mg/L 2,6-DHBD cell-free extract. The medium was stirred at 450 rpm, heated to 30 °C and continuously gassed with 20 mL/min CO_2 through a 0.5 µm porous sparger.

The high enzyme deactivation can be caused by several effects, among others, stirrer-introduced pressure differentials and shear forces, such as when using piston pumps [33], the interaction of the enzyme with the bubble interface [34], temperature [35] and contact with resorcinol [5]. Regarding the temperature-dependent deactivation, 2,6-DHBD is known to be quite stable even above 60 °C [35]. An additional effect was observed that most likely contributed to a large loss of active enzyme, namely the binding of the enzyme to the adsorber. Even though binding to the adsorber might have stabilized or even activated the enzyme, all the bound enzymes will be deactivated after the biotransformation. During the elution step, a protein precipitate appeared on top of the chromatography column after product elution with 1 M HCl in methanol. It is expected that the acidic and organic conditions caused the desorption and denaturation of any present enzyme. In consequence, this enzyme is no longer available for a second (repetitive) batch and, in part, has already been removed from the final product. In this regard, anion exchangers are known to bind proteins, which are generally influenced by the isoelectric point of the protein [36]. Therefore, it is recommended to work with immobilized enzymes on suspended carriers to prevent a direct interaction between the enzyme and adsorber. Alternatively, ultrafiltration can be used for spatial separation not only in the reactor (enzyme membrane reactor) but also as a purification step, preventing the carryover of any enzyme in the final product. Which way to go depends often on the efficacy of enzyme immobilization, which is often low in regard to recovered activity [37]. However, it has the advantage of increasing convenience in handling, separation and reuse, potentially increasing thermal and pH stability, and can also permit the use of various reactor configurations [38]. Using the immobilized enzyme, it would be crucial to spatially separate the adsorber and catalyst, as treating the solids with 1 M HCl in methanol during the elution step would deactivate the bound enzyme. To address the mentioned challenges, a three-part reactor configuration can be envisioned. Such a system would, for example, contain a CO_2 gassing unit, a packed bed with catalyst and a packed bed with adsorber, with the medium being circulated continuously through the three units. In this manner, the elution can be performed selectively on the packed bed with the adsorber.

3. Materials and Methods

3.1. General

All chemicals were obtained with purities of ≥99% from Sigma-Aldrich (Darmstadt, Germany), except triethanolamine, which was purchased from Carl Roth (Karlsruhe, Germany). Carbon dioxide 4.5 (≥99.995%) was obtained from Linde (Pullach, Germany).

3.2. Biocatalyst Preparation

E. coli BL21(DE3) cells containing the plasmid pET21a+ with the gene for 2,6-DHBD from *Rhizobium* sp. strain MTP-10005 were used for decarboxylase preparation. Production of cell-free extract and purified 2,6-DHBD was performed according to previously reported protocols [5].

3.3. Adsorber Characterization without Enzyme Addition

Different 80 mM mixtures of resorcinol and 2,6-DHBA were prepared in 1 M aqueous TEA. Resorcinol and 2,6-DHBA liquid fractions of 0-1 with increments of 0.1 were prepared. The mixtures were saturated with CO_2 by sparging the solutions via cannula at 30 °C. During sparging, the pH was measured. Reaching a constant pH indicated achieving CO_2 saturation, and the sparging was stopped.

Experiments were carried out in closed 1.5 mL HPLC screw-cap vials with Dowex® 1X8-50 as adsorber. First, different amounts of adsorber were added to the vials. Then, the CO_2-saturated mixtures were added. For each prepared resorcinol:2,6-DHBA ratio, the final adsorber contents of 0, 10, 50 and 100 mg/mL were investigated. The suspensions were incubated for several days on a thermoshaker at 30 °C and 1000 rpm. Samples of the supernatant were taken and measured via HPLC.

3.4. Small-Scale Enzymatic Reactions with Adsorber

Small-scale reactions were carried out in closed 1.5 mL HPLC screw cap vials with or without the absorber Dowex® 1X8-50. The procedure was similar to the one described in Section 3.3. A resorcinol solution was prepared in aqueous TEA, saturated with CO_2, and added to the vials already containing the adsorber. The reaction was started by adding 2,6-DHBD to reach final concentrations of 80 mM resorcinol, 1 M TEA and 55.7 µg/mL purified 2,6-DHBD. The stock solution of purified 2,6-DHBD contained 1392 mg/mL 2,6-DHBD and had an activity of 16.4 U/mg, which was determined as previously described [10]. Various adsorber contents were tested. The reactions were incubated on a thermoshaker at 30 °C and 500 rpm. At different time intervals, samples of the supernatant were taken and measured via HPLC. After each sampling (20 µL), the vials were flushed with CO_2 to counteract CO_2 leakage.

3.5. Product Purification of the Reaction Medium

Similar to the small-scale biotransformation with adsorber, a 30 mL reaction was performed at 80 mM resorcinol, 1 M aqueous TEA presaturated with CO_2, and 11.6 µg/mL purified 2,6-DHBD (16.4 U/mg). The reaction was performed at 30 °C with stirring using a magnetic stirrer to provide a representative medium for product purification using Dowex® 1X8-50.

Reaching a product yield of 20.4% after two days, the medium was loaded into a chromatography column containing 0.5 g of Dowex® 1X8-50. The yield corresponds to the sum of 2,6-DHBA in the liquid and solid fractions in relation to the starting resorcinol. Gravity column chromatography was performed with the suspension. After the binding step, product elution was carried out using 1 M HCl in methanol. Using vacuum distillation (60 °C, 100 mbar), methanol and water were removed, and crude product crystals formed. The crude product was washed twice with 1 M aqueous HCl, and the supernatant was discarded. The wet product was dried in a vacuum oven at 40 °C overnight. Samples were taken at various stages and analyzed via HPLC.

3.6. Lab-Scale Carboxylation with Product Purification

The setup of the lab-scale carboxylation and downstream processing is shown in Figure 6. A 3 L bioreactor with baffles containing 1.5 L reaction medium was used. The medium consisted of 80 mM resorcinol, 1 M aqueous TEA saturated with CO_2 and 64.2 mg/L 2,6-DHBD cell-free extract. The reaction mixture without enzyme was pre-saturated with CO_2, using a 0.5 µm porous sparger until the pH was constant. The medium was stirred at 450 rpm, heated to 30 °C and, after the addition of enzyme, continuously gassed with 20 mL/min CO_2 through a 0.5 µm porous sparger. In 5 intervals, 30 g of Dowex® 1X8-50 were added to the reaction medium. In this manner, a final adsorber content of 100 g/L was reached.

After reaching the final yield, the suspension was transferred to a chromatography column. The reaction medium was passed twice over the created packed bed. Elution and product purification were performed as described in Section 3.6, except that the methanol mixture obtained from the distillation step was reused for elution. Furthermore, a piston pump was used to apply the elution and washing solution to the chromatography column. Samples were taken at various stages and analyzed via HPLC.

3.7. Sampling Procedure and RP-HPLC Method

Reaction samples were diluted in trifluoroacetic acid to stop the reaction and remove amine-bound CO_2. Samples were mixed and centrifuged for 5 min at 13.000 rpm. Supernatant was injected into an Agilent (Waldbronn, Germany) LC-1100 HPLC system equipped with a diode array detector and a LichroCART 250-4 Lichrospher 100 RP column (5 µm). The separation was performed according to the previously reported protocol [39]. Typical retention times were 7.0 min for resorcinol and 8.3 min for 2,6-dihydroxybenzoic acid.

3.8. Adsorber Characterization and Adsorption Isotherms

For the characterization of the adsorption characteristics of Dowex® 1X8-50, different adsorption isotherms were applied to mixtures of resorcinol and 2,6-DHBA in 1 M aqueous TEA saturated with CO_2. The adsorbent mass concentration as well as the molar ratio of resorcinol and 2,6-DHBA in 80 mM mixtures were varied. Adsorption isotherms were applied to fit the experimental data. Usually, thermodynamic models such as the ideal adsorbed solution theory are used to predict multicomponent adsorption. This method uses only single-solute isotherm parameters but is not applicable to multisolute systems of unknown composition [40]. In these cases, empirical adsorption isotherms are applied. In the aqueous TEA solution, at least two adsorbates, resorcinol and 2,6-DHBA, are present. Adsorption of dissolved inorganic carbon (DIC) species, such as bicarbonate and enzymes, was not included as additional adsorbates. Interference of DIC species was assumed to be neglectable due to ensuring constant conditions by not varying the TEA concentration and pH. In regard to enzyme adsorption, no enzyme was added to the experiments to determine adsorption isotherm parameters. It is expected that enzyme-binding interference in the biotransformations can be neglected as only a very dilute concentration of enzyme was used.

Different empirical adsorption isotherms for binary mixtures as well as the single-solute Langmuir isotherm as a control were applied (Equations (6)–(10)). The single-solute Langmuir isotherm represents the first and one of the simplest adsorption models to describe complete adsorption on a surface [41]. In Equation (1), the q_i gives the amount adsorbed on the surface, while $q_{m,i}$ represents the maximum capacity of the adsorbant. b_i gives the equilibrium constant and c_i the adsorbate concentration at equilibrium. For the binary mixture of resorcinol and 2,6-DHBA, the single solute Langmuir isotherm was applied for each adsorbate.

$$q_i = \frac{q_{m,i}\, b_i\, c_i}{1 + b_i\, c_i} \tag{1}$$

Various empirical extended adsorption isotherms were created to describe multicomponent systems. These have, similar to the single-solute Langmuir isotherm adsorption

constants, constants for the capacity of different adsorption sites, which can differ for each component depending on the isotherm, and additional isotherm parameters. Butler and Ockrent (1930) created an extended Langmuir isotherm (Equation (2)) for multicomponent systems [22].

$$q_i = \frac{q_{m,i}\, b_i\, c_i}{1 + \sum_{j=1}^{N} b_j\, c_j} \tag{2}$$

Similarly, Jain and Snoeyink (1973) described an extended Langmuir isotherm (Equations (3) and (4)), but only for binary mixtures, with the assumption that a fraction of the adsorption sites that are available for component 1 can also be occupied by component 2 [21].

$$q_1 = \frac{(q_{m,1} - q_{m,2})\, b_1\, c_1}{1 + b_1\, c_1} + \frac{q_{m,2}\, b_1\, c_1}{1 + b_1\, c_1 + b_2\, c_2} \tag{3}$$

$$q_2 = \frac{q_{m,2}\, b_2\, c_2}{1 + b_1\, c_1 + b_2\, c_2} \tag{4}$$

Similarly, several extended Freundlich isotherms exist. The extended Freundlich isotherm from DiGiano et al. (1978) is based on the assumption that all components have the same value of Freundlich exponent n and only differ in their Freundlich coefficients K (Equation (5)) [24].

$$q_i = \frac{K_i^{1/n}\, c_i}{\left(\sum_{j=1}^{N} K_j^{1/n}\, c_j\right)^{1-n}} \tag{5}$$

In contrast, the extended Freundlich isotherm from Sheindorf et al. (1981) only applies for a bisolute adsorbate system (Equations (6) and (7)) [23].

$$q_1 = \frac{K_1\, c_1}{(c_1 + K_{1,2}\, c_2)^{1-n_1}} \tag{6}$$

$$q_2 = \frac{K_2\, c_2}{\left(c_2 + K_{1,2}^{-1}\, c_1\right)^{1-n_2}} \tag{7}$$

In addition, the extended Tóth isotherm from Jaroniec and Tóth (1976) is applied to the experimental data (Equations (8) and (9)) [25].

$$q_i = \frac{q_m\, c_i}{\left[b_i + \left(c_i + b_{i,j}\, c_j\right)^n\right]^{1/n}} \tag{8}$$

$$b_{i,j} = \frac{b_i}{b_j} \tag{9}$$

The Fritz and Schlünder isotherm is one of the most versatile isotherms due to the wide range of isotherm parameters it covers. This isotherm was extended for multicomponent systems in 1974 by Fritz and Schlünder (Equation (10)) [26].

$$q_i = \frac{b_{1,i}\, c_i^{n_i}}{d_i + \sum_{j=1}^{N} b_{2,i,j}\, c_j^{m_{i,j}}} \tag{10}$$

Data fitting was performed in Origin 2021 from OriginLab (Northampton, MA, USA) using the non-linear fitting tool.

4. Conclusions

It is demonstrated that the enzymatic carboxylation of resorcinol in aqueous TEA is improved by the application of the strong anion exchanger Dowex® 1X8-50. Consequently, an ISPR was realized and demonstrated, including an elution and washing step, to obtain

2,6-DHBA in 99.8% purity at a lab scale. On the one hand, reaction yields above 80% are possible, and an incremental addition of adsorber improves productivity. On the other hand, competitive binding of the enzyme and the substrates occurs, which slows the reaction, especially at high adsorber concentrations. Enzyme adsorption may be prevented by switching to an immobilized biocatalyst, making the insufficient selectivity of the adsorber for 2,6-DHBA the main challenge in the investigated process. Therefore, further screening of adsorbers as well as the design of new adsorbers is recommended to make the approach more economical. Here, the showcased reusability of the reaction medium, including the dissolved amine, contributes to making the process more efficient. It can easily be reloaded with CO_2, thereby providing a cheap C1 substrate for biotransformation. Furthermore, simple downstream processing was established for obtaining pure 2,6-DHBA, with recycling of the adsorber and the solvents used for product isolation, highlighting the potential of the reaction system.

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Abbreviations

2,6-DHBD, 2,6-dihydroxybenzoic acid decarboxylase from *Rhizobium* sp. strain MTP-10005; 2,6-DHBA, 2,6-dihydroxybenzoic acid; TEA, triethanolamine; DIC, dissolved inorganic carbon concentration [mol/L]; ISPR, in situ product removal.

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