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Tyrosinase catalyzed production of 3,4-dihydroxyphenylacetic acid using immobilized mushroom (*Agaricus bisporus*) cells and *in situ* adsorption

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ABSTRACT

3,4-Dihydroxyphenylacetic acid (DHPAA), a catechol derivative with proposed beneficial human health applications, was synthesized in this work from 4-hydroxyphenylacetic acid (HPAA) using an unpurified, tyrosinase-containing, cell preparation from the fruiting body of the edible mushroom *Agaricus bisporus* which were immobilized in silica alginate matrix capsules. The formation of DHPAA was equimolar to the conversion of HPAA, as long as ascorbic acid was present in amounts sufficient for reduction of *o*-quinones generated by oxidation of DHPAA. With a concentration of 5 mM HPAA and 5, 10, or 25 mM ascorbic acid, the maximum yields of DHPAA were 26, 36, or 56%, respectively. When aluminum oxide, pretreated with ammonium acetate, was added for an *in situ* adsorption of DHPAA, the yield obtained with 5 or 10 mM ascorbic acid was increased to 42 or 52%, respectively, with a reaction time reduced by ~25%. In contrast to experiments without *in situ* adsorption, the yield remained almost constant after depletion of ascorbic acid. After desorption, the concentration of DHPAA in the eluent was up to 32 times higher than the concentration of HPAA. The results presented here will be useful for the design of production and purification processes for DHPAA.

1. Introduction

3,4-Dihydroxyphenylacetic acid (DHPAA) is a catechol derivative which has recently received considerable attention since it has been demonstrated to protect against pancreatic β-cell dysfunction induced by high cholesterol. DHPAA has, therefore, been suggested as a promising drug for prevention or delay of the transition from pre-diabetes to diabetes, which is currently a major health issue worldwide [1]. In addition, DHPAA exhibits antiproliferative activity in prostate [2] and colon cancer cells [2–4] and is, therefore, of great interest for human health. DHPAA has been examined as precursor for the synthesis of hydroxytyrosol [5–7], a substance with antioxidative properties [8,9], commonly considered as beneficial for human health [10–12], though DHPAA itself has antioxidant activity too [13–15]. These beneficial medicinal characteristics could increase the demand for DHPAA and economic processes for its production.

The catechol structure of DHPAA contains two adjacent hydroxyl groups which can be generated enzymatically from 4-hydroxyphenylacetic acid (HPAA) using tyrosinase as a catalyst [16,17]. Tyrosinase catalyzes the orthohydroxylation of monophenols (HPAA) to *o*-diphenols (DHPAA) and the oxidation of *o*-diphenols to *o*-quinones using molecular oxygen [18]. The formed *o*-quinones can be reduced to the *o*-diphenol precursor by addition of ascorbic acid (Fig. 1) [16,17,19–21]. This reaction pattern has also been examined for the tyrosinse catalyzed production of other *o*-diphenols [16,17], including hydroxytyrosol [22] as well as 3,4-dihydroxy-L-phenylalanine (L-DOPA) [23–29] which is a prescribed drug for treatment of Parkinson's disease.

However, the economic feasibility of these methods suffers from the high cost of purified enzyme preparation when pure tyrosinase is used. To overcome this limitation, enzyme containing protein mixtures, or whole cell biocatalysts are a promising alternative which can achieve the enzymatic conversion while avoiding costly protein purification steps. An example of this approach is the use of tyrosinase containing cells from the fruiting body of the edible mushroom *Agaricus bisporus*, immobilized in silica alginate matrix capsules for degradation of bisphenol A [30,31].

To date, this catalyst system has not been examined for production of *o*-diphenols such as DHPAA. In particular, so far no information about side reactions catalyzed by other enzymes in the cells has been published. Formation of byproducts or secondary

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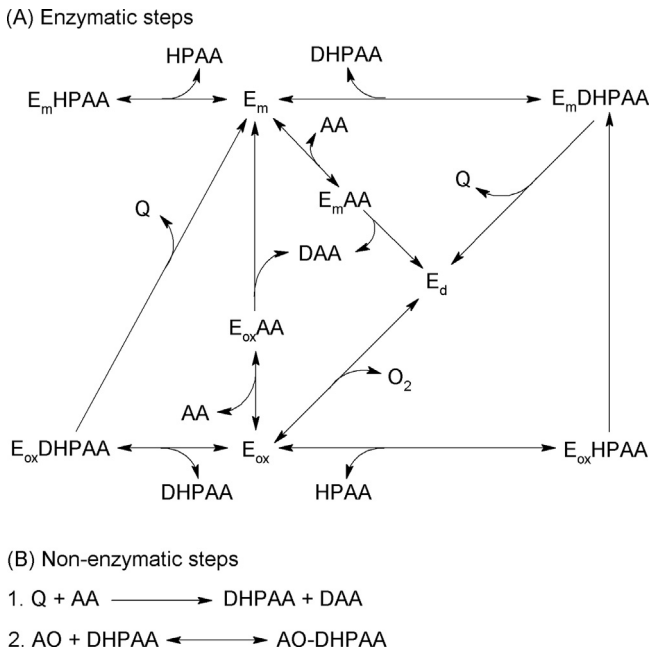

Fig. 1. Hypothetical reaction scheme of the tyrosinase catalyzed production of DHPAA with *in situ* adsorption and possible role of ascorbic acid. (A) Enzymatic steps (transfer of H^+ and formation of H_2O not shown; adapted from Ref. [33]). (B) Non-enzymatic steps: 1. Regeneration of DHPAA [16,17,19–21]. 2. Adsorption of DHPAA (representative equation simplified from Ref. [45,46]). E_m, met-tyrosinase; E_d, deoxy-tyrosinase; E_{ox}, oxy-tyrosinase; AA, ascorbic acid; DAA, dehydroascorbic acid; Q, *o*-quinone; E_mHPAA, complex of E_m with HPAA; E_mDHPAA, complex of E_m with DHPAA; E_mAA, complex of E_m with AA; E_{ox}AA, complex of E_{ox} with AA; E_{ox}HPAA, complex of E_{ox} with HPAA; E_{ox}DHPAA, complex of E_{ox} with DHPAA; AO, aluminum oxide; AO-DHPAA, DHPAA adsorbed on AO.

products is undesirable, as it would reduce the yield of DHPAA and pollute the desired product. This would require extensive purification processes and consequently increase the overall process costs. Therefore, the selectivity of the catalyst is of great importance and deserves further investigation.

The catalytic cycle of tyrosinase includes three intermediate states of the active site (Fig. 1): deoxy-tyrosinase (E_d), oxy-tyrosinase (E_{ox}) and met-tyrosinase (E_m). E_d is able to bind reversibly with molecular oxygen, producing the oxygenated form E_{ox}. E_{ox} can act on monophenols to form *o*-diphenols or *o*-quinones, while being converted to E_m or E_d, respectively. E_{ox} can also oxidize *o*-diphenols with release of *o*-quinones and E_m. E_m can react with *o*-diphenols as well, producing *o*-quinones and E_d, while E_m and monophenols form an inactive complex [32–35]. That means after hydroxylation of a HPAA molecule, a formed DHPAA molecule is oxidized to the *o*-quinone, before another HPAA molecule is hydroxylated, unless the transformation of E_m to E_d is taken over by external reductants [16,33,36]. DHPAA can be regenerated from the *o*-quinone by reduction with ascorbic acid [16,17,19–21], however, competes with the HPAA to be re-oxidized by E_{ox} [32–35], suggesting that high yields of DHPAA only can be attained at high concentrations of ascorbic acid. The effect of ascorbic acid on tyrosinase is currently debated in scientific literature, as several studies observe no effect on tyrosinase activity [29,37–39], other reports suggest that tyrosinase is inactivated by ascorbic acid [40,41], or may even oxidize it [33,42] with reduction of E_{ox} to E_m or E_m to E_d (Fig. 1) [33]. In any case, a high concentration of DHPAA in the reaction medium leads to an inhibition of DHPAA formation (accumulation) and to an acceleration of DHPAA oxidation, thus limiting the overall productivity in batch conversion reactions [27,28].

These limitations could hinder economic DHPAA production processes, therefore, separation of generated DHPAA from the immobilized tyrosinase may be a promising process alternative. Separation of DHPAA and tyrosinase may be accomplished by a selective adsorption of DHPAA during the reaction (Fig. 1). Adsorption and desorption of DHPAA has been examined using aluminum oxide as an adsorbent [43,44]. Both catechol derivatives [45] and carboxyl groups [46], which are present in the molecular structure of DHPAA as well as HPAA, can adsorb onto aluminum oxide. Therefore, it is desirable to limit the adsorption of HPAA while promoting adsorption of DHPAA to enable conversion of HPAA by the immobilized enzyme. The adsorption of catechol derivatives or carboxylic acids onto aluminum oxide has been demonstrated to be affected by ammonium acetate [43]. However, these issues have not yet been extensively characterized with respect to a targeted reduction of the adsorption of HPAA and the applicability for an *in situ* adsorption in the tyrosinase catalyzed production of DHPAA.

In this report, fundamental insights gained on the tyrosinase catalyzed production of DHPAA with mushroom cells immobilized in silica alginate matrix capsules are presented. Selectivity studies and the effect of the amount of cells, ascorbic acid, as well as the size of the matrix capsules are presented. In addition, the adsorption of DHPAA and HPAA onto aluminum oxide is examined, wherein, the extent of adsorption of HPAA depending on modification of the adsorption system was investigated. The results of these analyses were used to conduct an *in situ* adsorption of DHPAA during tyrosinase catalyzed production from HPAA and presented as an alternative approach to economic generation of this potentially valuable compound.

2. Materials and methods

2.1. Materials

Mushrooms (*A. bisporus*) at developmental stages 2–3 [47], were acquired from a local supermarket. Alginic acid sodium salt from brown algae (suitable for immobilization of microorganisms), ammonium acetate (≥98% purity), formic acid (98% purity), HPAA (98% purity) and Ludox® HS-30 colloidal silica 30% (w/w) were purchased from Sigma–Aldrich GmbH (Steinheim, Germany). Acetonitrile (≥99.9% purity), aluminum oxide (neutral, for column chromatography), L-(+)-ascorbic acid (≥99% purity), $CaCl_2 \cdot 2H_2O$ (≥99% purity), ethanol (≥99.8% purity), HCl (37%) and NaOH (≥99% purity) were purchased from Carl Roth GmbH & Co KG (Karlsruhe, Germany). DHPAA (98+% purity) and L-DOPA (98+% purity) were purchased from Alfa Aesar GmbH & Co KG (Karlsruhe, Germany), deuterated dimethyl sulfoxide (DMSO-d_6, 99.8atom%D) from Armar Chemicals AG (Döttingen, Switzerland) and 2-morpholinoethanesulfonic acid (MES, molecular biology grade) from AppliChem GmbH (Darmstadt, Germany). All solutions were prepared with double distilled deionized water (ddH_2O).

2.2. Adsorption experiments

In order to examine the adsorption of DHPAA and HPAA, different amounts of aluminum oxide (0.3–0.6 g) were prepared by addition of 2 ml ddH_2O or ammonium acetate solution (0.2–6 M) and gentle shaking for 1 min. After this pretreatment the adsorbent was allowed to settle (5 min) and the supernatant was removed pipetting. Since phosphate buffer has been demonstrated to prevent adsorption of substances with a similar molecular structure like L-DOPA and dihydroxyphenylserine on aluminum oxide [43], test solutions with either DHPAA (5 mM), HPAA (5 mM), or both (each 2.5 mM) were prepared with 0.1 M MES (pH 5–8 (adjusted to with NaOH)). The concentrations of DHPAA and HPAA were chosen in accordance with the expected concentrations during the synthesis experiments (Section 2.6). The test solution (3 ml) was

added to the wet adsorbent and stirred for 30 min with a magnetic stirrer (300 rpm) at 20 °C. Then the adsorbent was allowed to settle (5 min) and the supernatant was removed. Samples of the supernatant were centrifuged (11,500 × *g*, 10 min) and the residual concentrations of DHPAA and HPAA were quantified by HPLC.

2.3. Desorption experiments

After adsorption, the loaded adsorbent was washed with ddH_2O (10 ml/(g adsorbent)) under gentle shaking for 2 min. Then the adsorbent was allowed to settle (5 min) and the supernatant was removed. Desorption experiments were carried out by incubating the washed adsorbent with different volumes (3.3–10 ml/(g adsorbent)) of ethanol, HCl (0.1–0.8 M), or mixtures of the two for 30–90 min under stirring (300 rpm) at 20 °C. Then the adsorbent was allowed to settle again (5 min) and samples of the supernatant were withdrawn and centrifuged (11,500 × *g*, 10 min). Depending on the solvent used for desorption, the supernatant obtained after centrifugation was diluted with a certain amount of NaOH solution prior to HPLC analysis to avoid a damage of the HPLC column due to acidic pH. After removal of the solvent from the adsorbent, these steps were repeated for a second desorption cycle. The recovery of DHPAA was defined as the percentage mass ratio of desorbed and adsorbed DHPAA.

2.4. Preparation and immobilization of mushroom cells

Preparation and immobilization of mushroom cells were conducted as previously described [30]. After lyophilization and grinding, the mushroom cell preparation exhibited a tyrosinase activity of 80 ± 3 U/(g cell dry weight (cdw)) using L-DOPA as substrate following previously described protocols [30]. Mushroom powder (0.1 or 0.5 g) was added to 0.2 g sodium alginate and 10 ml ddH_2O containing 2.5% (w/w) Ludox® HS-30 (pH 6.8) and stirred with an agitator. After the alginate was dissolved, the polymer solution was dropped into 2% (w/w) $CaCl_2$ solution for gelation using a diameter-controllable droplet generator [30]. The matrix capsules were stored in ddH_2O until use to avoid drying and shrinkage from exposure to air. All capsule masses reported below refer to their wet weight immediately after removal of external water by filtration.

2.5. Characterization of matrix capsules

To estimate the cell content of the matrix capsules, it was assumed that both the volume of the polymer solution and the density of the matrix capsules remained unchanged when the cells were suspended or immobilized. As demonstrated in the previous study [30], the cells and tyrosinase from fractured cells were completely immobilized. The cell content of the matrix capsules was calculated by dividing the cell concentration in the polymer solution by the yield of the matrix capsules (0.6 g/(ml polymer solution)) [30].

The diameter of the matrix capsules was determined utilizing a Traveler SU 1071 USB microscope with Ulead Video Studio 7 SE VCD software (Supra Foto-Elektronik-Vertriebs-GmbH, Kaiserslautern, Germany) and graph paper as a reference. Photographs of matrix capsules were processed by image analysis software, ImageJ 1.46p. Reported diameters (d) are the arithmetic mean of 50 analyzed matrix capsules, taking into account their smallest diameter (d_{min}) and largest diameter (d_{max}) orthogonal to it. The aspect ratio $A_R = d_{min}/d_{max}$ was at least 0.94.

2.6. Production of DHPAA

2.6.1. Reactor configuration

Experiments for production of DHPAA were carried out in a glass vessel equipped with a cannula positioned at the bottom of the vessel for aeration and a magnetic stirrer operating at 300 rpm. Preliminary experiments demonstrated that a suspension of aluminum oxide in combination with the stirrer bar had an abrasive effect on the matrix capsules when they were added directly to the vessel. To ensure the integrity of the matrix capsules, a stainless steel mesh (mesh size 0.5 mm) was positioned above the stirrer bar. The matrix capsules were placed on the mesh so that they were separated from the stirrer bar, but liquid, air bubbles and aluminum oxide could pass.

Since the solubility of oxygen in water in an ambient atmosphere (approximately 0.23 mM at 30 °C) [48], is much lower than the used HPAA concentration (5 mM), the experiments were carried out using an air flow rate of 150 ml/min to avoid an oxygen deficiency and to support agitation of the liquid. This configuration was used for both experiments with and without aluminum oxide (with and without *in situ* adsorption, respectively) to ensure similar reaction conditions.

2.6.2. Production of DHPAA *without* in situ *adsorption*

To examine the tyrosinase catalyzed production of DHPAA, different amounts (0.1–0.9 g) of various matrix capsules (17–83 mg cdw/g, d = 0.9–1.3 mm) were incubated in 9 ml substrate solution (5 mM HPAA, 0–25 mM ascorbic acid, 0.1 M MES with pH 6 adjusted to with NaOH) under stirring and aeration at 30 °C. At certain time intervals samples of the solution were withdrawn and the concentrations of HPAA and DHPAA were quantified by HPLC. The yield of DHPAA in these experiments was determined as the percentage ratio of the amount of DHPAA in the reaction medium and the amount of HPAA initially used.

2.6.3. Production of DHPAA *with* in situ *adsorption*

For production of DHPAA with *in situ* adsorption, 9 ml substrate solution (adopted from Section 2.6.2) was added to 1.8 g aluminum oxide prepared according to procedures in Section 2.2. The reactions were started by addition of 0.9 g matrix capsules (17 mg cdw/g, d = 1.3 mm) and carried out under stirring and aeration at 30 °C. At designated time intervals the reactions were stopped by removal of the matrix capsules. The adsorbent was allowed to settle (5 min) and samples of the reaction medium were centrifuged (11,500 × *g*, 10 min) before they were analyzed by HPLC. After removal of the supernatant, the adsorbent was washed and subjected to the desorption procedure described in Section 2.3. The yield of DHPAA in these experiments was determined as the percentage ratio of the amount of DHPAA in the eluent after desorption and the amount of HPAA initially used.

2.7. HPLC measurements

Prior to HPLC analysis the samples were filtered through a 0.2 μm PTFE filter. HPLC was conducted with a Knauer Smartline series with detection at 227 nm, Eurospher II 100-3C18 A (150 × 3 mm) column (Knauer GmbH, Berlin, Germany), mobile phase water/acetonitrile/formic acid (volumetric ratio 87.3/12.5/0.2), flow rate 0.7 ml/min at 40 °C. The concentrations of HPAA and DHPAA were quantified by comparing the retention time and area of the peaks in the chromatograms with those of authentic samples.

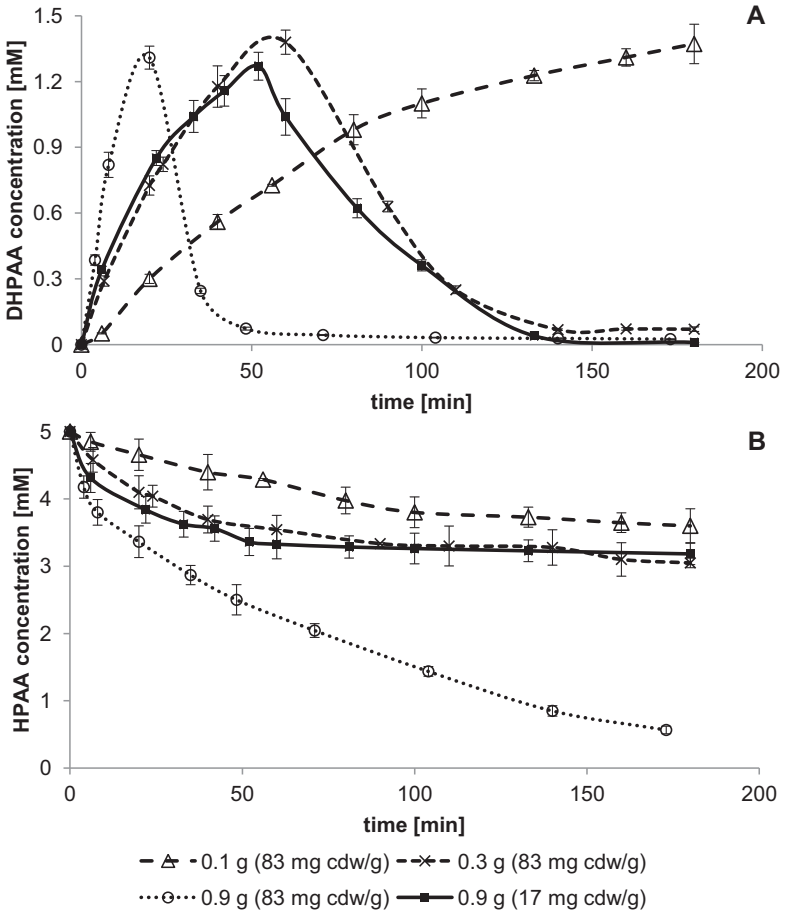

Fig. 2. Formation of DHPAA (A) and decrease in the concentration of HPAA (B) as a function of time, as well as the amount and the cell content of the matrix capsules (d = 1.3 mm) in 9 ml substrate solution (5 mM HPAA, 5 mM ascorbic acid, pH 6) at 30 °C. Dashed and solid lines were interpolated to illustrate the reaction progress in a qualitative manner.

2.8. Identification of DHPAA

Formation of DHPAA in the synthesis experiments (Section 2.6) was confirmed by LC–MS and ^{1}H NMR. For LC–MS, samples of the reaction medium were analyzed with a TSQ Quantum Ultra™ AM Triple Quadrupole mass spectrometer (Thermo Finnigan GmbH, Bremen, Germany) with a Synergi™ Polar-RP (4 μm, 80 Å) column (Phenomenex Ltd., Aschaffenburg, Germany). The mobile phase consisted of solvent A (ddH_2O + 0.2% formic acid) and solvent B (acetonitrile) and the flow rate was 0.4 ml/min. The initial chromatographic conditions were 90% A and 10% B. After 1 min, B was increased to 80% with a gradient time of 8 min and kept constant

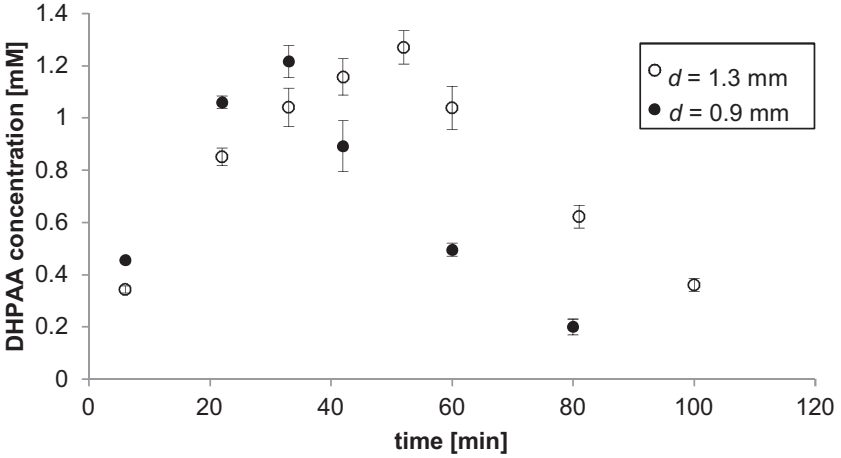

Fig. 3. Production of DHPAA as a function of time and the size of the matrix capsules (0.9 g, 17 mg cdw/g) in 9 ml substrate solution (5 mM HPAA, 5 mM ascorbic acid, pH 6) at 30 °C.

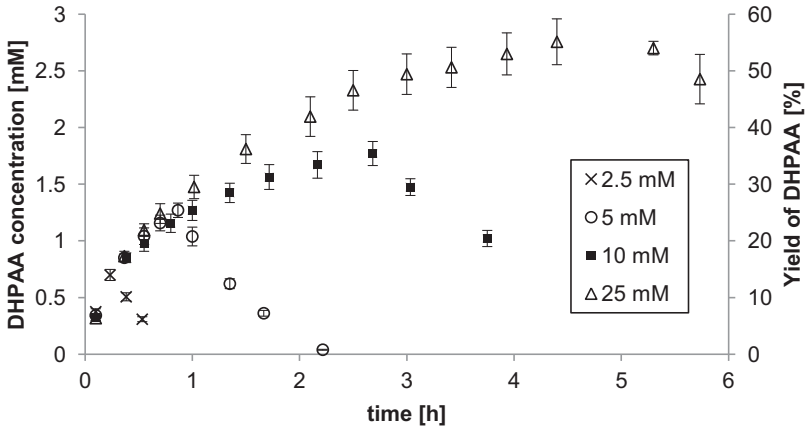

Fig. 4. Effect of ascorbic acid on the production of DHPAA using 0.9 g matrix capsules (d = 1.3 mm, 17 mg cdw/g) in 9 ml substrate solution (5 mM HPAA, pH 6) at 30 °C.

for 2 min. After 11 min, B was returned to 10% and this composition was maintained for 4 min before the next injection. Detection was carried out using an electrospray interface in negative or positive ionization mode. A negative fragment ion was observed at m/z 167.07 which suggested the chemical structure $[DHPAA\text{-}H]^-$.

^{1}H NMR was performed after lyophilization of the product that has been isolated from the reaction medium using the adsorption and desorption procedure (Section 2.3). ^{1}H NMR spectra were recorded in DMSO-d_6 using a Bruker DRX-400 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany).

^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 3.33 (s, 2H, —CH_2—), 6.46–6.49 (dd, J = 7.91, 1.88 Hz, 1H, Ph—H), 6.63–6.64 (d, J = 5.52 Hz, 1H, Ph—H), 6.65 (s, 1H, Ph—H), 8.80 (br s, 2H, Ph—OH), 12.12 (br s, 1H, —COOH).

3. Results and discussion

3.1. Production of DHPAA

3.1.1. Selectivity of the catalyst

Using 0.1 g matrix capsules (d = 1.3 ± 0.08 mm) with a cell content of 83 mg cdw/g and a substrate solution with an equimolar ratio of HPAA and ascorbic acid, almost 2.1 mg DHPAA was produced (Fig. 2A). Although the catalyst is contained within an unpurified cell preparation, peaks of other substances were not detected in the HPLC chromatograms of this experiment. Additionally, the increase in the concentration of DHPAA correlates with the decrease in the concentration of HPAA (Fig. 2B). After 3 h of reaction, the concentration of DHPAA increased to almost 1.4 mM, while the concentration of HPAA decreased from 5 mM to approximately 3.6 mM, indicating near equimolar conversion of HPAA to DHPAA. Therefore, the selectivity of the catalyst of this reaction system may be formally described as almost 100% under these conditions.

After removal of the immobilized cells from the reaction medium, the concentrations of either HPAA or DHPAA did not change, suggesting that the tyrosinase was completely retained in the matrix capsules. Therefore, the use of the immobilized mushroom cells instead of purified tyrosinase could be beneficial to reduce the cost of the tyrosinase catalyzed production of DHPAA.

3.1.2. Effect of the catalyst concentration

For production of DHPAA, HPAA must diffuse into the matrix capsules before it can be converted by tyrosinase in the immobilized cells. Conversion of HPAA is dependent upon the diffusion rate, enzymatic activity, and the cell content of the matrix capsules. The three dimensional shape of the capsules may cause a concentration gradient of HPAA penetration, and therefore, have an influence on its conversion efficiency to DHPAA. For this reason the effect of the amount of cells was examined in two ways: by variation of the amount of matrix capsules and by variation of the immobilized cell content. Fig. 2A depicts that with greater amounts of matrix capsules (83 mg cdw/g) the production of DHPAA was considerably accelerated.

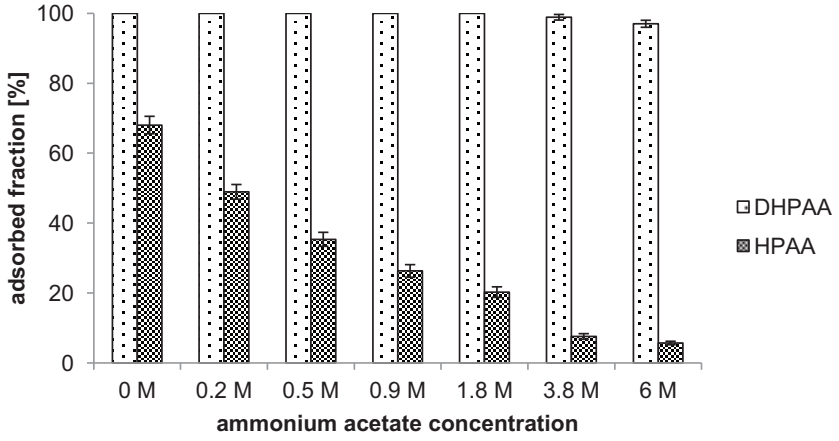

Fig. 5. Effect of the pretreatment of aluminum oxide (0.6 g) with different concentrations of ammonium acetate on the subsequent adsorption of DHPAA or HPAA (5 mM in 3 ml 0.1 M MES (pH 6)).

However, in experiments with 0.3 g and 0.9 g matrix capsules, an apparent maximum concentration of DHPAA was obtained. The time elapsed to reach comparable conversion amounts (60 min or 20 min) was proportional to the amount of matrix capsules, which suggests a proportional conversion rate and that air flow rate and, thereby oxygen consumption, was not limiting for the amount of catalyst used. After the apparent maximum was reached, the concentration of DHPAA decreased and remained below 0.1 mM up to 3 h, although substrate was still available in considerable concentrations (Fig. 2B). It was also observed that the initially colorless solution turned yellowish or brownish. When these samples were analyzed by HPLC, additional peaks, which were not previously observed, were detected in the chromatograms indicating the presence of other substances.

Tyrosinase catalyzes the orthohydroxylation of HPAA to DHPAA and the oxidation of DHPAA to an *o*-quinone, which is reduced by ascorbic acid to form DHPAA again (Fig. 1). However, since the ascorbic acid is consumed in this reaction, the reaction rate of the *o*-quinone reduction becomes lower after a certain number of reduction cycles. This finally results in a lack of *o*-quinone reduction and subsequent loss of DHPAA. In LC–MS analysis, a positive fragment ion was observed at m/z 167.06 which likely originated from $[o\text{-quinone} + H]^+$. The *o*-quinones can undergo polymerization reactions resulting in further secondary products, this likely accounts for the additional peaks observed in the chromatograms. It was also observed that when the last samples were centrifuged, a brown precipitate was obtained, suggesting the formation of insoluble polymeric products.

Using 0.9 g matrix capsules, approximately 0.39 mM DHPAA was produced within the first 4 min of reaction. Simultaneously, a rapid decrease in the initial concentration of HPAA from 5 mM to approximately 4.2 mM was observed (Fig. 2B). The difference in concentration change observed in this short time span is a consequence of the high water content of the matrix capsules (approximately 0.8 ml/g) [31], which results in a dilution of the reaction medium depending on the amount of matrix capsules applied. When 0.9 g matrix capsules without immobilized cells were added to 9 ml substrate solution, the concentration of HPAA decreased from 5 mM to 4.6 mM (data not shown). Therefore, the HPAA conversion in the first 4 min was only 0.4 mM which is consistent with the observed DHPAA formation (0.39 mM). The minor differences in the apparent maximum DHPAA concentrations observed with 0.3 g (1.38 mM) and 0.9 g matrix capsules (1.3 mM) can be attributed to dilution effects as well in accordance with previous observations of high selectivity of the catalyst.

In experiments with 0.3 g matrix capsules (83 mg cdw/g), the apparent maximum DHPAA concentration was obtained after 60 min, however, in experiments with 0.9 g matrix capsules with a cell content of 17 mg cdw/g, the DHPAA concentration was beginning to decrease after the same reaction time (Fig. 2A), despite the lower total mass of cells (15 mg cdw compared to 25 mg cdw). Due to the large DHPAA concentration difference (1.38 mM compared to 1 mM), this observation cannot be explained by dilution effects alone, suggesting that the reaction rate was higher in the latter experiment.

It is likely that the production rate was limited by the diffusion of reactants within the matrix capsules, suggesting that relatively high production rates can be achieved with relatively low amounts of immobilized mushroom cells. This was further examined by variation of the size of the matrix capsules as discussed below.

3.1.3. Effect of the size of the matrix capsules

Fig. 3 depicts that the reaction rate was significantly increased when the diameter of the matrix capsules (17 mg cdw/g) was reduced from 1.3 ± 0.08 mm to 0.9 ± 0.06 mm. After 6 min and 22 min the concentration of DHPAA reached 0.46 mM and 1.06 mM, respectively, when the smaller matrix capsules were used compared to 0.33 mM and 0.85 mM obtained with the larger ones.

The smaller capsules have a higher surface area to volume ratio, therefore, the diffusion distance to reach the immobilized tyrosinase is shorter in the smaller matrix capsules resulting in the more rapid production of DHPAA observed here. It is likely that some degree of investigation is required to optimize the cell content or the size of the matrix capsules to optimally balance cell concentration and capsule size in order to adapt this method to industrial application. However, the obtained results may serve as a basis for reaction kinetic studies.

3.1.4. Effect of ascorbic acid

Since the decrease in concentration of DHPAA after a certain reaction time was correlated with the depletion of ascorbic acid, the molar ratio of ascorbic acid to HPAA was varied to examine the effect on the formation of DHPAA.

In reactions without ascorbic acid, only low concentrations of DHPAA (< 0.02 mM) were detected in the reaction medium over the whole examined reaction time of 3 h. Given that a loss of DHPAA was observed over long reaction times, Fig. 2, it was presumed that DHPAA, which may have been formed from HPAA even without ascorbic acid, was mainly oxidized to the *o*-quinone derivative so that only traces of DHPAA were found in the solution. This would confirm the need for a reducing agent to allow stable formation of DHPAA.

Using 2.5 mM ascorbic acid the concentration of DHPAA increased to approximately 0.7 mM within 14 min (Fig. 4), indicating the positive effect of a certain amount of ascorbic acid in this reaction system. The higher the concentration of ascorbic acid used, the longer and higher the concentration of DHPAA increased (Fig. 4). When 5 mM ascorbic acid was used, a concentration of approximately 1.3 mM DHPAA was obtained after 52 min, which is a yield of 26%. Using 10 mM or 25 mM ascorbic acid, the concentration of DHPAA was increased to approximately 1.8 mM (36% yield) after 2.7 h or 2.8 mM (56% yield) after 4.4 h, respectively, likely resulting from the higher capacity for reduction of *o*-quinones due to ascorbic acid in solution.

Using ascorbic acid at concentrations from 2.5 up to 25 mM, the initial increase in the concentration of DHPAA was $0.33\ \text{mM} \pm 8\%$ within the first 6 min in all experiments. Therefore, the reaction is comparably effected by the concentration of ascorbic acid within the examined range. Even the use of 50 mM ascorbic acid did not change the initial reaction rate (data not shown). Although long-term inactivation of tyrosinase by ascorbic acid [40,41] cannot be excluded, no inhibitory effects were observed here, which is consistent with other studies [29,37–39]. It is indeed possible that no inhibition of tyrosinase activity occurred, although here, a slight inhibition may not have been detected due to the diffusion limitation of the reaction.

Although relatively high yields of DHPAA can be attained with the immobilized mushroom cells, relatively large amounts of ascorbic acid are required to sustain DHPAA in solution after its formation (Fig. 4). After a certain reaction time, DHPAA formed in this reaction is lost, likely due to its competitive oxidation, therefore long reaction times are required to obtain high yields. When the ascorbic acid is consumed the concentration of DHPAA decreases rapidly.

3.2. Adsorption of DHPAA and HPAA

In order to circumvent these disadvantages, a new production concept with *in situ* adsorption of DHPAA was examined. To find a suitable adsorption system, several adsorption experiments were first carried out separately.

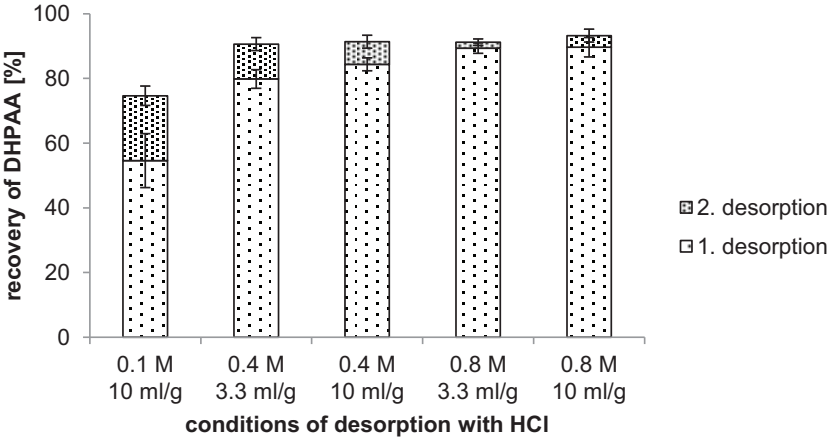

Fig. 6. Recovery of adsorbed DHPAA (load 4.2 mg/(g aluminum oxide)) after desorption with different concentrations and volumes of HCl. The volumes are given in relation to the mass of the aluminum oxide.

First, 0.1 g aluminum oxide/ml at pH 6 resulted in adsorption of the dissolved DHPAA (5 mM) up to 98%, whereas 0.2 g aluminum oxide/ml resulted in adsorption of 100%, at 4.2 mg/(g adsorbent), suggesting that aluminum oxide could be a suitable adsorbent for the proposed reaction system. To maintain a quantitative adsorption of DHPAA, the concentration of aluminum oxide was kept at 0.2 g/ml.

However, as can be seen from Fig. 5 also a considerable adsorption of HPAA occurred with this system. Although the adsorbed fraction, 68%, was lower than that of DHPAA (100%), HPAA adsorption was considered as undesirable, as this would mean loss of substrate for catalysis. Since it has been reported that the adsorption of L-DOPA, dihydroxyphenylserine, and other related compounds on aluminum oxide was decreased in presence of ammonium acetate [43], the adsorbent was treated with this substance prior to adsorption experiments.

As shown in Fig. 5 the extent of the adsorption of HPAA decreased with higher ammonium acetate concentrations. By use of 3.8 M ammonium acetate, only 7% of the HPAA was removed from the solution, while the adsorbed fraction of DHPAA remained almost unaffected (99%). In comparison, pretreatment of the adsorbent with 6 M ammonium acetate had only a slight effect on the

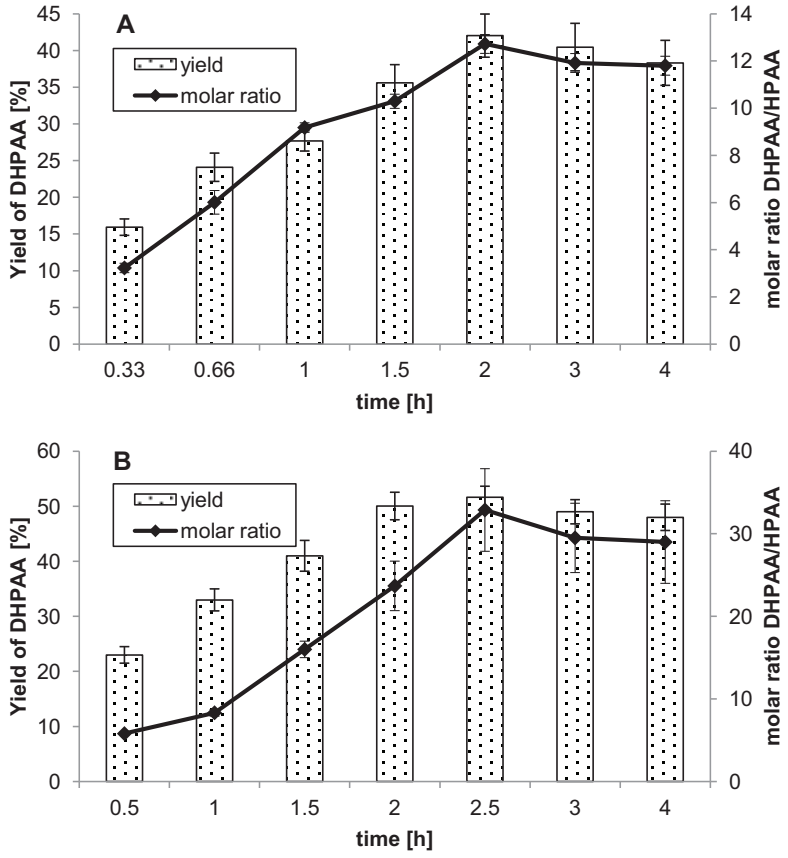

Fig. 7. Production of DHPAA with *in situ* adsorption using 0.9 g matrix capsules (d = 1.3 mm, 17 mg cdw/g), 9 ml substrate solution (5 mM HPAA, 5 mM (A) or 10 mM (B) ascorbic acid, pH 6), 1.8 g aluminum oxide at 30 °C. The diagrams show the yield of DHPAA and the molar ratio of DHPAA and HPAA in the eluent after different reaction times.

reduction of the adsorption of HPAA (5%), but resulted in a slight decrease in the adsorption of DHPAA (97%).

The effect of ammonium acetate was confirmed with equimolar mixtures (2.5 mM) of HPAA and DHPAA (data not shown) suggesting a higher selectivity of the adsorbent prepared with ammonium acetate for DHPAA. These results could be a consequence of previously described differences in the adsorption mechanisms of carboxylic acids [46] and catechols [45]. Due to the favorable ratio of adsorbed DHPAA and HPAA the aluminum oxide was consequently prepared with 3.8 M ammonium acetate for all subsequent experiments.

The solution pH was also considered as an important parameter for reaction optimization. Contradictory results have been reported for the adsorption of catechol derivatives on aluminum oxide at different pH values [43,45,49,50]. However, in this study no significant effect of the pH on the adsorption of these substances was observed between pH 5 and pH 8 (data not shown). It is worth noting that the adsorption can be accompanied by a shift of the reaction medium pH [45]. Therefore, the use of higher concentrations of reactants could require further investigations to determine the effect of pH changes, or ion concentrations, however, this is beyond the scope of this study. In accordance with the pH optimum of the catalyst preparation [31] and the effective pH range of MES buffer, the pH of the substrate solution was, therefore, maintained at pH 6 in all subsequent analyses.

3.3. Desorption of DHPAA

To achieve a high recovery of DHPAA after adsorption (load 4.2 mg/(g aluminum oxide)) different solvents were tested for their desorption capacity.

It was found that ethanol was not able to elute DHPAA from aluminum oxide. Mixtures of ethanol and 0.4 M HCl could recover DHPAA in different quantities (data not shown), however, were less effective than pure 0.4 M HCl. No difference in desorption was observed for either 30 min or 90 min reaction time (data not shown), suggesting that 30 min was sufficient for the highest possible elution under the examined conditions. Therefore, the desorption step was set to 30 min and further experiments were carried out with different concentrations and volumes of HCl (Fig. 6).

When 0.1 M HCl was used in amounts of 10 ml/(g adsorbent) 55% of the adsorbed DHPAA was eluted. Although 0.1 M HCl has been used for elution in a previous study [43], the recovery was considerably lower than the recovery obtained with 0.4 M (84%) or 0.8 M HCl (90%) in the same proportion, suggesting a strong adsorption of DHPAA onto aluminum oxide.

When the adsorbent was subjected to another desorption cycle, the recovery obtained with 0.1 M HCl was increased by 20% to 75% in total. Using 0.4 M HCl or 0.8 M HCl the recovery was increased to 91% or 93%, respectively, demonstrating the feasibility of a high recovery. The loss of approximately 7–9% could be explained by irreversible binding of DHPAA or loss of loaded adsorbent during the separation of liquid and adsorbent after the adsorption or subsequent washing step. Similar total recoveries were also attained when the volume of the 0.4 M or 0.8 M HCl was reduced to 3.3 ml/(g adsorbent). Therefore, this proportion of 0.4 M HCl was adopted for desorption after the DHPAA production with *in situ* adsorption.

3.4. Production of DHPAA with in situ adsorption

The findings from Sections 3.1–3.3 were applied to investigate the tyrosinase catalyzed production of DHPAA with *in situ* product separation. During the experiments, only low concentrations of DHPAA (<0.1 mM) were detected in the reaction medium. However, after the desorption step, considerable amounts of DHPAA were obtained (Fig. 7).

Using 5 mM ascorbic acid in the substrate solution, the maximum yield of DHPAA in the eluent was 42% (Fig. 7A), significantly higher than the maximum yield obtained without *in situ* adsorption, 26% (Fig. 4), an increase of 61%. The yield was even higher than the highest yield of DHPAA without *in situ* adsorption using 10 mM ascorbic acid (36%, Fig. 4). This strategy used considerably less ascorbic acid, at least 50%, compared to strategies without *in situ* adsorption.

The maximum yield with *in situ* adsorption was achieved faster than in the experiments with 10 mM ascorbic acid without adsorption. Here, maximum yield was observed at 2 h compared to 2.7 h, representing a time saving of 25% provided that only the reaction time is considered.

Using *in situ* adsorption, the produced DHPAA was not lost as was observed in longer non *in situ* reactions. Here, 40% yield was observed after 3 h and 38% after 4 h, in contrast to experiments without *in situ* adsorption where a rapid decrease of the yield was observed after the depletion of ascorbic acid (Fig. 4).

The positive effect of the *in situ* adsorption was confirmed using 10 mM ascorbic acid. Here, a yield of 52% was attained after 2.5 h (Fig. 7B). In comparison to experiments without *in situ* adsorption, where the yield was 36% (Fig. 4), this represents an increase of 44%. To achieve this yield without *in situ* adsorption, an ascorbic acid concentration of almost 25 mM was required (Fig. 4). Also in this setup, the observed product yield with *in situ* adsorption was achieved faster than without (approximately 3.4 h) and the product remained stable at 48% yield after 4 h.

It is likely that the separation of the produced DHPAA from the immobilized tyrosinase restricts the oxidation of DHPAA and consequent formation of *o*-quinones. Therefore, the number of reduction cycles and the amount of ascorbic acid required to produce and to maintain a certain amount of DHPAA is reduced. This results in higher yields of DHPAA which are maintained for a longer time, since the adsorbed DHPAA is less prone to oxidation by the immobilized enzyme. It may be the case that sequestration of the DHPAA prevents competition of the enzyme for HPAA conversion, however, this will require kinetic analysis of a purified enzyme.

Although it is uncertain if the adsorbent could wrest the DHPAA from the tyrosinase or promote the release of DHPAA from the enzyme before DHPAA is oxidized, it should be noted that in absence of DHPAA or other reductants the catalytic cycle of tyrosinase (Fig. 1) cannot be closed [16,32–36], leading to a standstill of the reaction. However, as presented here, the *in situ* adsorption concept may offer great potential for process intensification.

Taking into account the produced amounts of DHPAA and the residual concentrations of HPAA in the reaction medium (data not shown), the adsorbed fraction of the initial amount of HPAA was determined to be less than 6%, which is in accordance with the HPAA adsorption studies (Fig. 5). Therefore, only low amounts of HPAA were found in the solvent after desorption (Fig. 7). The concentration of DHPAA in the eluent was up to 12 times (Fig. 7A) or even 32 times (Fig. 7B) higher than the concentration of HPAA, whereas in experiments without *in situ* adsorption, the concentration of DHPAA was always lower than the concentration of HPAA until a yield of 50–56% was attained, when the molar ratio of DHPAA and HPAA was 1–1.5. Although it is still unclear how much ammonium acetate might have entered this system, the results reveal, at least in part, a sequestering effect on the produced DHPAA. Therefore, the results presented here may be also useful to facilitate the separation of DHPAA and HPAA in subsequent downstream processes.

4. Conclusion

It was demonstrated that tyrosinase containing mushroom cells from *A. bisporus*, immobilized in silica alginate matrix capsules, were able to produce DHPAA from HPAA without recognizable enzymatic side reactions of HPAA. The formation of DHPAA was equimolar to the conversion of HPAA, as long as ascorbic acid was present in amounts sufficient for reduction of *o*-quinones generated by oxidation of DHPAA. Therefore, the use of immobilized mushroom cells instead of purified tyrosinase may contribute to reduce the costs of tyrosinase catalyzed production of DHPAA.

The adsorption of HPAA onto aluminum oxide was considerably reduced by pretreatment of the adsorbent with ammonium acetate. Despite this procedure, the adsorption capacity for DHPAA was maintained and adsorbed DHPAA could be recovered in large quantities by use of HCl.

When the prepared aluminum oxide was used for an *in situ* adsorption of DHPAA during tyrosinase catalyzed production, the yield was significantly increased and achieved more rapidly. In contrast to experiments without *in situ* adsorption, the yield remained almost constant after depletion of ascorbic acid, demonstrating a successful introduction of the proposed *in situ* adsorption concept to this reaction system. Since only low amounts of HPAA were found in the eluent, the results presented here could be useful not only for production, but also for purification of DHPAA or other *o*-diphenols.

Acknowledgments

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