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Kinetic characterization of tyrosinase containing mushroom (*Agaricus bisporus*) cells immobilized in silica alginate

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ABSTRACT

A tyrosinase containing cell preparation from the edible mushroom *Agaricus bisporus* was immobilized in silica alginate matrix capsules. This catalyst system was characterized in terms of its reaction kinetics using the orthohydroxylation of bisphenol A (BPA) as a model system. The effect of BPA concentration on the reaction rate could be described with the Michaelis–Menten kinetics with an apparent Michaelis–Menten constant of K_m = 50.4 μmol/l. The reaction rate was proportional to the immobilized cell concentration in the investigated range. The highest observed apparent maximal reaction rate v_{max} related to the wet weight of matrix capsules at 30 °C was 51.5 nmol/(min g)). The diffusion coefficient D of BPA within the matrix capsules was determined to 4.9×10^{-10} m^2/s ±10% at 30 °C. The studies revealed that the observed reaction rate was not limited by the BPA diffusion rate in the matrix material. The immobilized cells exhibited tyrosinase activity over the whole examined pH range (pH 3–11) with a broad maximum between pH 6 and pH 10. The apparent activation energy E_A for the BPA conversion was determined to 37.6 kJ/mol, whereas the apparent activation energy $E_{A,inact}$ for the thermal inactivation of the catalyst was determined to 71.7 kJ/mol. It was demonstrated that the determined parameter values were suitable to predict the change of the BPA concentration with respect to time under different reaction conditions and therefore could be useful for future applications of the catalyst, for example in synthesis of *o*-diphenols or bioremediation processes.

1. Introduction

Tyrosinase (EC 1.14.18.1) is a copper containing enzyme widely distributed in animals, plants, fungi and bacteria [1]. The enzyme is able to utilize molecular oxygen to catalyze the orthohydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones [2] (Fig. 1).

This reaction pattern has been examined for tyrosinase catalyzed production of various *o*-diphenols [3,4], among these the antioxidant hydroxytyrosol [5], 3,4-dihydroxyphenylacetic acid [4], a substance with antiproliferative activity against cancer cells [6], and 3,4-dihydroxy-L-phenylalanine (L-DOPA) [7–9] which is a prescribed drug for treatment of Parkinson's disease. The formation of the *o*-quinones in these reaction systems can be reduced by addition of ascorbic acid as a reducing agent [3–5,7–9].

Apart from these synthesis routes tyrosinase has also been investigated for degradation of phenols like bisphenol A (BPA) [10,11] which is an important bulk chemical with identified endocrine disrupting activity for which reason it has received considerable attention [12–21], as BPA has been found in waste waters [22–25], surface waters [26–28], food [29,30] and mineral water [31], as well as in human blood and urine [32–34].

However, a drawback of these methods is the high cost of the enzyme preparation when pure tyrosinase is used. This could prevent an application of tyrosinase catalyzed reactions on an industrial scale.

The use of tyrosinase containing cells could reduce the costs for tyrosinase based catalytic processes, because expensive enzyme purification is avoided. Tyrosinase is present in the fruiting body of the edible mushroom *Agaricus bisporus* [35,36], which is produced in large amounts for human consumption, inexpensive, and readily available throughout the year. In order to protect the catalyst from deactivation as well as to facilitate its handling, separation, and reutilization the cells can be immobilized, for example in silica alginate matrix capsules. It has recently been demonstrated that such immobilized cells from the fruiting body of *A. bisporus* were able to degrade BPA in environmental water samples [37].

However, so far little is known about the reaction kinetics of this catalyst system. With regard to catalyst design and process development more detailed information is required for an

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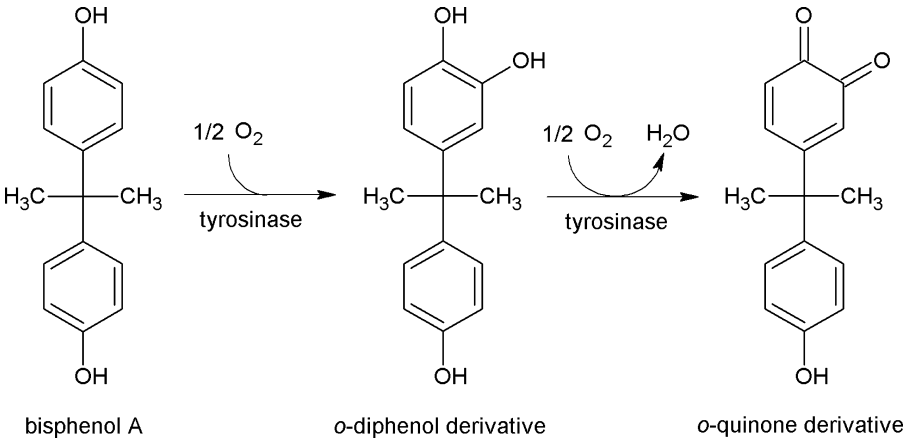

Fig. 1. Hypothesized reaction scheme of the tyrosinase catalyzed oxidation of bisphenol A based on van Gelder et al. [2] and Yoshida et al. [11].

appropriate employment of the catalyst in an industrial application. This report provides a contribution to remedy these deficiencies and presents a kinetic characterization of tyrosinase containing mushroom cells immobilized in silica alginate matrix capsules using the orthohydroxylation of BPA as a model system and considering the effect of BPA concentration, cell concentration, pH, temperature and intra-particle BPA diffusion.

2. Materials and methods

2.1. Materials

Mushrooms (*A. bisporus*) at developmental stages 2–3 [38] were acquired from a local supermarket. Alginic acid sodium salt from brown algae (suitable for immobilization of microorganisms), Ludox® HS-30 colloidal silica 30% (w/w) and BPA (≥99% purity) were obtained from Sigma–Aldrich GmbH, Steinheim, Germany. Acetonitrile (≥99.9% purity), $CaCl_2 \cdot 2H_2O$ (≥99% purity), HCl (37%) and NaOH (≥99% purity) were purchased from Carl Roth GmbH & Co KG, Karlsruhe, Germany, L-DOPA (98%+ purity) from Alfa Aesar GmbH & Co KG, Karlsruhe, Germany and 2-morpholinoethanesulfonic acid (molecular biology grade) from AppliChem GmbH, Darmstadt, Germany. All solutions were prepared with double distilled deionized water (ddH_2O).

2.2. Preparation and immobilization of mushroom cells

The mushrooms were cut into small pieces and lyophilized on the day of purchase. After lyophilization they were ground mechanically with a Retsch S1 planetary ball mill (Retsch GmbH, Haan, Germany) to a fine powder and stored in a flask at 21 °C. The tyrosinase activity of the obtained product was 68 ± 2 U/(g cell dry weight (cdw)) using L-DOPA as substrate according to the assay described previously [37]. The preparation of silica alginate matrix capsules and the immobilization of the mushroom cells were accomplished using a previously published protocol [37]. Briefly, 0–600 mg cdw were added to 0.2 g sodium alginate and 10 ml ddH_2O with 2.5% (w/w) Ludox® HS-30 (pH 6.8) and stirred by use of an agitator. After the alginate was dissolved the polymer solution was dropped into a 2% (w/w) $CaCl_2$ solution for gelation using a self-designed droplet generator. The matrix capsules were stored in ddH_2O until use to avoid drying and shrinkage from exposure to air. Therefore it was assumed that they were completely filled with water at the beginning of the experiments. All capsule masses reported below refer to their wet weight immediately after removal of external water by filtration.

2.3. Characterization of matrix capsules

2.3.1. Determination of size and shape

To determine the diameter of the matrix capsules they were photographed 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. The photographs were processed by image analysis software, ImageJ 1.46p. Reported diameters (d) were determined as the arithmetic mean of 50 analyzed matrix capsules with standard deviation of less than 7%, taking into account their smallest diameter (d_{min}) and largest diameter (d_{max}) orthogonal to it with an aspect ratio $A_R = d_{min}/d_{max}$ of at least 0.94.

2.3.2. Determination of the diffusion coefficient of BPA

To determine the diffusion coefficient of BPA within the matrix capsules, diffusion experiments were carried out by incubating 60 matrix capsules with (5–35 mg cdw/(ml of the polymer solution)) or without immobilized mushroom cells in 3 ml BPA solution (292 μmol/l) under stirring with a magnetic stirrer (300 rpm) at 30 °C for 30 min. The temperature was chosen, because most of the reaction kinetic studies (Section 2.4) were carried out at the same temperature. To avoid tyrosinase catalyzed conversion of BPA during diffusion experiments, matrix capsules with immobilized mushroom cells were pre-incubated at 80 °C for 10 min for thermal inactivation, cooled in ice water and warmed to 30 °C prior to diffusion experiments.

Since the diffusion of BPA from the solution into the matrix capsules was relatively fast, big matrix capsules (d = 3.52 mm) were used to elongate the diffusion process and to decelerate the establishment of the equilibrium in order to monitor the concentration decrease over a longer time period for higher accuracy. The volumetric ratio of matrix capsules to BPA solution was chosen to observe a significant concentration decrease in the BPA solution.

To follow the diffusion progress, samples of 0.8 ml were withdrawn from the solution at intervals of 2–5 min, transferred into a quartz cuvette and BPA concentration was determined measuring the absorbance at 280 nm in a Libra S12 UV/vis spectrophotometer (Biochrom Ltd., Cambridge, United Kingdom). After measurement, the analyzed solution was returned to the matrix capsules immediately to keep the liquid volume as well as the total amount of BPA constant.

The diffusion coefficient (D) was calculated from the decrease in BPA concentration under the following assumptions:

- the BPA concentration in the solution is uniform and initially c_0 = 292 μmol/l