

In vitro enzymatic generation of Mn(III)-malonate as a chemical oxidant of Orange II

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The aim of this work was the study of the continuous enzymatic generation of Mn^{3+} -malonate by a fungal versatile peroxidase and its use in the degradation of the industrial azo dye Orange II (OII). The experimental set-up consisted of an enzymatic reactor, providing continuous generation of Mn^{3+} -malonate coupled with a second reactor to perform the degradation of the dye. The enzymatic reactor provided the Mn^{3+} -malonate in steady state conditions and this oxidative species was able to degrade the OII up to 60%.

Introduction

The production of synthetic compounds is associated with the discharge of hardly biodegradable wastes such as industrial wastes. Ligninolytic peroxidases have been used *in vitro* at laboratory scale for the degradation of different contaminants including industrial dyes (Lopez et al., 2004), polycyclic aromatic hydrocarbons (PAHs) (Eibes et al., 2006), pharmaceutical and personal care products (PPCPs) (Tamagawa et al., 2006), etc, and other biotechnological applications, such as bleaching of Kraft pulp (Moreira et al., 1997). These enzymes are extracellular peroxidases produced by white-rot fungi and the onset of their production is associated to secondary metabolism conditions in response to nutrient depletion (Bumpus and Aust, 1985). Two ligninolytic peroxidases, lignin peroxidase (LiP) (Tien and Kirk, 1988) and manganese peroxidase (MnP) (Kawahara et al., 1984), were described in *Phanerochaete chrysosporium* and reported in other white-rot fungi from the group of basidiomycetes (Hatakka, 1994). In addition, some fungi contain another lignin/degrading enzyme, versatile peroxidase (VP), also known as hybrid Mn-peroxidase, which combines properties of LiP and MnP (Martinez, 2002), i.e. they are able to oxidize Mn^{2+} to Mn^{3+} , as well as non-phenolic aromatic compounds. However, its catalytic cycle in presence of Mn^{2+} is much higher than in presence of other aromatic compounds (Heinfling et al., 1998). Mn^{3+} generated by VP is a strong oxidizer (1.54 V) (Cui and Dolphin, 1990) but it is quite unstable in aqueous media. To overcome this drawback, white-rot fungi secrete oxalic and other organic acids that form Mn^{3+} chelates acting as stable diffusing oxidizers of phenolic compounds and dyes (Kuan and Tien, 1993). Taking advantage of this feature of the VP cycle, in the present work we propose the separation of the versatile peroxidase (VP) enzymatic system in two stages to overcome the drawbacks related to the *in vitro* use of this enzyme: consumption and deactivation.

Materials and Methods

Two-stages process for the continuous production of the Mn^{3+} -complex and the degradation of the azo dye Orange II

The enzymatic system used for the continuous production of Mn^{3+} -malonate is presented in **Figure 1**. It is composed by a stirred tank reactor (200 mL working volume) operated in continuous mode coupled to a 10 kDa cut-off ultrafiltration membrane (Prep/Scale-TFF Millipore), which permits the recycling of the enzyme to the reaction vessel. The additional volume of the ultrafiltration unit and the interconnecting tubing was 65 mL, so the total volume of the reaction was 265 mL. Substrates and cofactors were added from two stock solutions, one of H_2O_2 (13 μM) and the other containing Mn^{2+} and sodium malonate (1 and 20 mM respectively) at pH 4.5. The operational parameters were: VP activity, 100 U/L; H_2O_2 feed rate, 5 $\mu\text{M}/\text{min}$; Na-malonate feed rate, 330 $\mu\text{M}/\text{min}$; Mn^{2+} feed rate, 16 $\mu\text{M}/\text{min}$; room temperature. The enzyme was recycled in a recycling:feed flow ration of 12:1. The enzyme solution was added as a single addition of VP at the beginning of the experiment. VP activity was measured by monitoring the amount of the Mn^{3+} -malonate complex at 268 nm at the outlet of the enzymatic reactor. The Mn^{3+} -malonate generated in the enzymatic reactor was used in a second stage for the degradation of the dye Orange II. The decoloration reactor (200 mL of working volume), was fed with 2.5 mL/min of a stock solution of the dye, 50 mg/L.

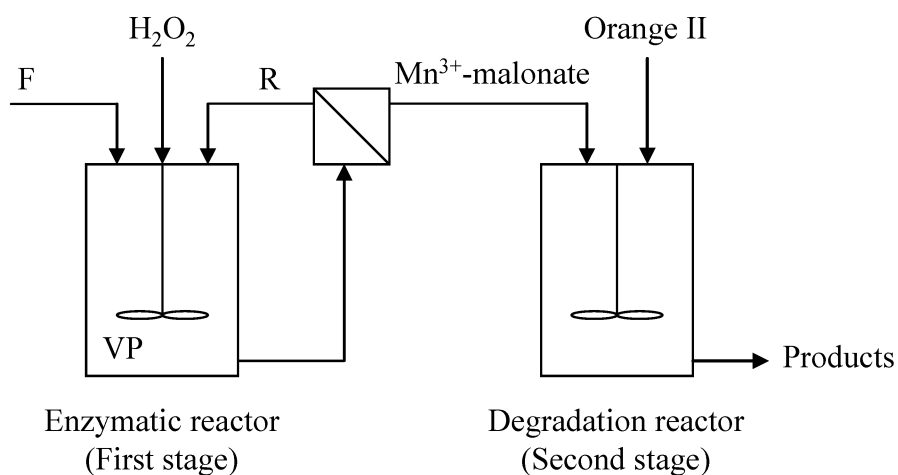


Figure 1. Schematic diagram of the two-stage system. F, feed: Na-malonate + MnSO_4 ; R, recycling.

Determination of the Mn-dependent activity of the versatile peroxidase

Manganese-dependent activity of the versatile peroxidase (VP) was measured by monitoring the oxidation of 2,6-dimethoxyphenol (DMP) spectrophotometrically at 468 nm and 30°C (Cecil CE 7200, UK). The reaction mixture contained 50 mM sodium malonate (pH 4.5), 1 mM DMP, 1 mM MnSO_4 , and up to 600 μL of supernatant in a total volume of 1 mL. The reaction was initiated by adding 0.4 mM H_2O_2 . One MnP

activity unit was defined as the amount of enzyme transforming 1 μmol DMP per minute (Mester et al., 1995).

Measurement of Orange II concentration

During the operation of the two-stage system, Orange II concentration was measured by spectrophotometry at 480 nm (Lopez et al., 2004).

Measurement of Mn^{3+} -malonate concentration

Mn^{3+} -malonate concentration was measured spectrophotometrically at 268 nm as described by (Wariishi et al., 1992). For converting 268 nm absorbance to concentration, a molar extinction coefficient of $11.59 \text{ mM}^{-1}\text{cm}^{-1}$ was used.

Results

The effect of the concentration of the complex was evaluated during the degradation of the dye Orange II. The concentration of the dye was 25 mg/L. The results are shown in **Figure 2**. It was observed that the higher the initial concentration of Mn^{3+} -malonate, the higher the percent of degradation, thus when the concentration of the oxidizing agent was 200 μM , the percent of degradation was 90 %, whereas when the initial concentration was 70 μM , the percent was 42.5 %.

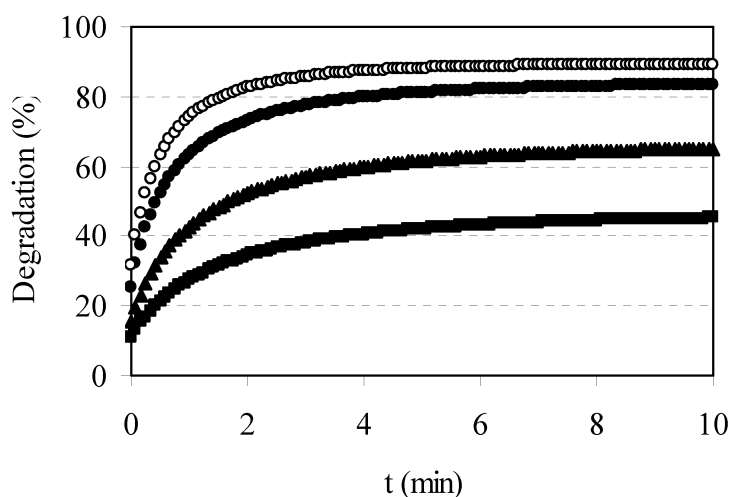


Figure 2. Kinetics of degradation of Orange II with different initial concentrations of Mn^{3+} -malonate: (■) 70 μM , (▲) 110 μM , (●) 165 μM , (○) 200 μM .

The continuous production of Mn^{3+} -malonate in an enzymatic reactor was coupled with a degradation system, operating with the azo dye Orange II (25 mg/L). From previous results, it is known that this compound presented an intermediate degradation rate and its spectrophotometric monitorization is simple and straightforward (Mielgo et al., 2003). In the present work, the amount of Mn^{3+} -malonate produced increased during the first seven hours and then, during five following hours remained stable, reaching a maximal level of approximately 400 μM (**Figure 3**).

At the beginning of the operation the degradation was small but later it increased up to 60% as the concentration of the complex reached its maximal value. However, with the continuous system it was not able to degrade the same amount of Orange II, even when the concentration of the complex was two-fold higher and the residence time was five-fold higher than the time of the batch reaction.

The activity of the enzyme was maintained constant throughout the reaction since the concentration of the complex never dropped during all the operation time.

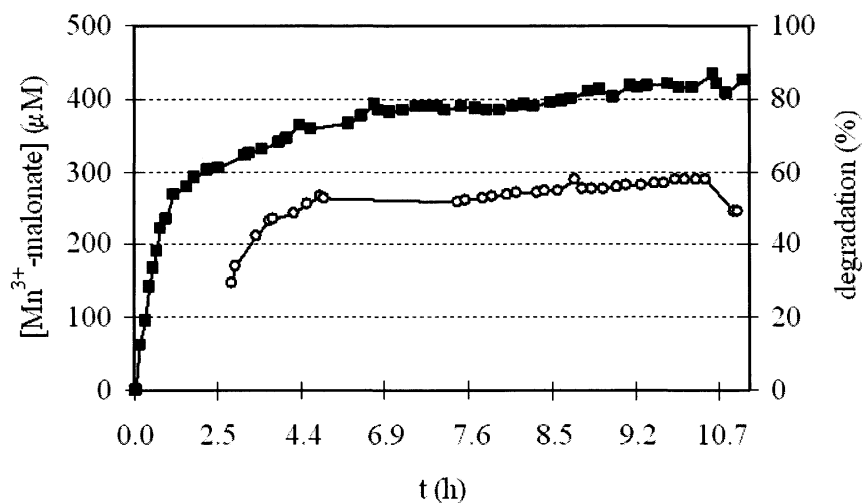


Figure 3. Profiles of production of Mn^{3+} -malonate and degradation of Orange II. (■) Mn^{3+} -malonate produced in the first stage, (○) degradation of Orange II in the second stage.

Other systems for the generation and use of Mn^{3+} -chelates in a two-stage reactor system have been previously reported (Grabski et al., 1998; Sasaki et al., 2001). Both of them proposed enzyme immobilization on two different supports, the NH_2 -Emphaze polymer (Pierce Chemical Co., Rockford, IL) and the FSM-16 (mesoporous material), respectively. Emphaze-MnP column system requires NaCl to prevent the adsorption of Mn^{3+} -chelate to the support matrix, and the salt conditions must be controlled carefully. Hereby, the continuous production of Mn^{3+} -malonate in reactions catalyzed by VP was performed in a stirred reactor coupled with an external ultrafiltration membrane, hence this system does not require enzyme immobilization nor any additive, such as NaCl.

The broad degradation capability of this system points out the use of Mn^{3+} -malonate for the degradation of other xenobiotics. In addition, Mn^{3+} -chelates, being nonproteinaceous, can solve problems related to the use of organic solvents to increase the solubility of certain compounds. Moreover, extreme operational conditions such as acid or basic pH, high temperatures or agitation rates to improve the contaminant degradation rate, could also be investigated.

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