# ISOLATION AND CHARACTERISATION OF A NEW *Bjerkandera* SP. STRAIN B33/3 PEROXIDASE

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## ABSTRACT

The main enzyme responsible for the decolourisation of the anthrone-type dye, Remazol Brilliant Blue R (RBBR) by *Bjerkandera* sp. strain B33/3, was isolated. This enzyme is also able to oxidise manganese, as well as veratryl alcohol and 2,6-dimethoxyphenol in a manganese-independent reaction.
The influence of temperature and pH on the decolourisation of RBBR by this novel enzyme was studied. The optimum conditions for the RBBR decolourising activity of this enzyme were ca. 35°C and pH 4.0, using 100 mM sodium tartrate buffer.
The influence of those parameters on the oxidation of DMP in the presence of $MnSO_4$ and EDTA was also studied.

## INTRODUCTION

Decolourisation of specific dyes has been used since the eighties as a rapid screening method for detection of ligninolytic activity in fungal strains. Dye decolourisation is an enzymatic process involving peroxidases or phenoloxidases produced by lignin degrading fungi, namely lignin peroxidases, manganese peroxidases and laccases. In fact, dyes are usually aromatic or heterocyclic compounds, with molecular structures similar to those of aromatic compounds that are substrates for ligninolytic enzymes.
Recently, a novel class of ligninolytic peroxidases, with high affinity for dyes, has been described; these enzymes can also oxidise manganese and veratryl alcohol and particularly 2,6-dimethoxyphenol (DMP) in a manganese-independent reaction. Those versatile enzymes thus possess a high biotechnological interest because of their broad substrate specificity, and lack of requirement for mediators to efficiently oxidise and decolourise several dyes; until now, they have only been found in *Pleurotus eryngii* and two *Bjerkandera* sp. (1-7).
From 109 fungal strains isolated on rotting ligninocellulosic material, a new *Bjerkandera* sp. strain was selected with high decolourisation activities upon Poly R-478 and Remazol Brilliant Blue R (RBBR) dyes. Analysis of peroxidase activities on its extracellular fluid demonstrated the existence of lignin peroxidase and manganese-dependent and independent peroxidase activities (8). Decolourisation of RBBR is based on an enzyme-mediated process, which is dependent on the presence of hydrogen peroxide in the reaction medium and independent of the presence of manganese (8).
Furthermore, after several purification steps, it was possible to isolate the main enzyme responsible for that decolourisation process (9). This enzyme is also able to oxidise manganese, as well as veratryl alcohol and 2,6-dimethoxyphenol in a manganese-independent reaction, thus exhibiting properties similar to those of the novel class of peroxidases previously referred to (8,9).
In this work, the ability to decolourise RBBR and oxidise DMP by the new enzyme from *Bjerkandera* sp. strain B33/ was studied in reference to certain parameters such as pH, and temperature.

## MATERIALS AND METHODS

### Enzyme

The enzyme from *Bjerkandera* sp. strain B33/3 was purified as previously described (9).

### Chemicals

2,6-dimethoxyphenol (DMP) was purchased from Fluka. Perhydrol [$H_2O_2$, in 30% (v/v)] and Remazol Brilliant Blue R (RBBR) were purchased from Sigma; the RBBR dye content was 50% (w/w). All other chemicals (unless otherwise indicated) where purchased from Sigma and were of analytical grade.

### Decolourisation assay

The RBBR decolourising activity was assayed spectrophotometrically by measuring the decrease in absorbance at 595 nm and 30 ° C. The enzymatic standard reaction mixture consisted of 0.05 mM RBBR and 100 mM sodium tartrate buffer (pH 3.5). The reaction was initiated via addition of 0.1 mM $H_2O_2$. The RBBR decolourising activity was calculated from the time slope of the first linear stage of reaction, using a molar extinction coefficient $\varepsilon_{595} = 11800\ M^{-1}\ cm^{-1}$.

### DMP oxidation

The activity for DMP oxidation was estimated by measuring absorbance at 469 nm, at 30°C, during the oxidation of 1mM of DMP in 50 mM sodium malonate buffer (pH 4.5), in the presence either of 1mM $MnSO_4$ or EDTA (ethylene diamine tetra-acetic acid). The reaction was started via addition of 0.4 mM $H_2O_2$. The molar extinction coefficient used was $\varepsilon_{469}=24800\ M^{-1}\ cm^{-1}$. The activity with $MnSO_4$ was corrected subtracting the activity obtained in the presence of EDTA. The last one was corrected for the laccase activity, by subtracting the activity obtained without $H_2O_2$ and EDTA.

### Activity at different pH

Activities of RBBR decolourisation and DMP oxidation were measured as described above in 100 mM sodium tartrate buffer and 50 mM sodium malonate buffer respectively, with pH 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 and 5.5.

### Activity at different temperatures

Activities of RBBR decolourisation were measured as described above at 15, 20, 25, 30, 35, 40, 50, 60 and 70°C. For each temperature a molar extinction coefficient for the RBBR was determined and used to calaculated the activity.

### Stability at different pHs

Samples of the pure enzyme were incubated overnight at 4°C in 100 mM sodium tartrate buffer with pH 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 and 5.5. The activities were measured as described above.

### Stability at different temperatures

Samples of the pure enzyme in 10 mM cacodylate buffer (pH 6.0) were incubated at 30°C, 50°C, and 70°C for 1, 2, 3, 5 and 10 minutes. The samples were immediately after incubated at 4°C and the activities measured as described above.

## RESULTS AND DISCUSSION

The first kinetic studies for the dye decolourisation reaction with extracellular fluid indicated a first order kinetics from 5 μM (26.44 $IU.L^{-1}$) up to 25 μM (43.51 $IU.L^{-1}$) of RBBR. One unit of enzyme activity (IU) was defined as the amount of enzyme that oxidises 1 μmole of RBBR per min.
Above this concentration inhibition was observed: 105 μM RBBR actually led to an activity that was 32% below the maximum obtained at 25 μM.
The RBBR decolourising activity is strictly $H_2O_2$-dependent: with 0.05 mM RBBR the rate increased with increasing amounts of $H_2O_2$, from 10 μM (12.13 IU $L^{-1}$) up to 75 μM (37.60 IU $L^{-1}$). Higher concentrations of $H_2O_2$ caused a gradual decrease of RBBR decolourising activity, with full inhibition at 1 mM $H_2O_2$ (8).
Using this extracellular fluid, the decolourisation rate is not dependent on the manganese concentration (0.001 - 8 mM) in the reaction medium (8).

A range of pH values (2.0 to 5.5) was used for determine the dependence of RBBR decolourisation, by the pure enzyme, on the pH of the reaction medium. The optimal pH for the RBBR decolourisation was ca. 4.0 (Fig. 1). It could be also determined that the pure enzyme is stable at pH values higher than 3.0, but at pH 2.0 loses all the activity, as shown in Fig. 2.

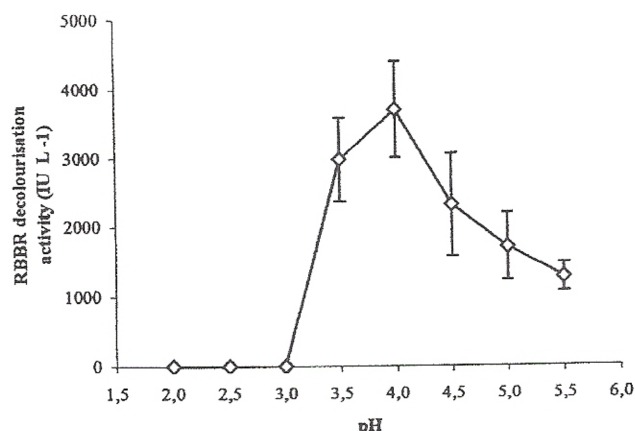


Fig. 1. Influence of pH (mean and standard deviation) upon RBBR decolourisation activity by the pure enzyme, using 100 mM sodium tartrate buffer.

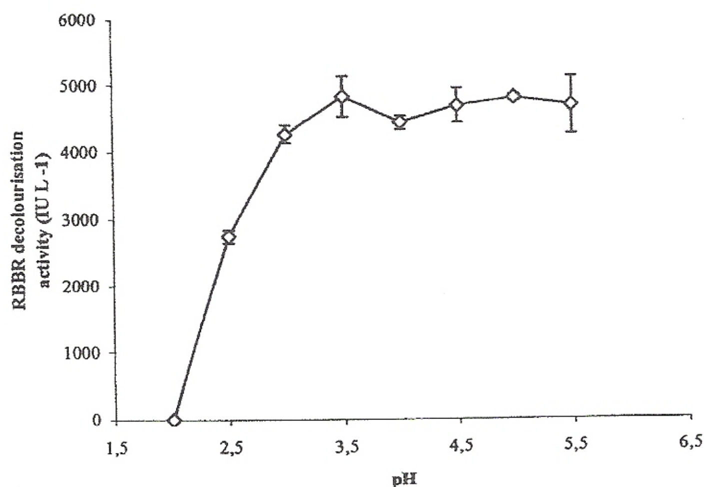


Fig.2. Stability of pure enzyme (mean and standard deviation) upon RBBR decolourisation activity at different pH.

A range of pH values (2.0 to 5.5) was used to determine the dependence of DMP oxidation by the pure enzyme (in the presence of $MnSO_4$ or EDTA), on the pH of the reaction medium. The optimal pH for oxidation of DMP in the presence of $MnSO_4$ was ca. 4.5 (Fig. 3). For the oxidation of DMP in the presence of EDTA, the optimal pH was ca. between 3.0 and 3.5. As already shown for the RBBR decolourising activity, the activity towards DMP is also stable at pH higher than 3.0, but at pH 2.0 loses all the activity.

A range of temperature values (15°C to 70°C) was used to determine the dependence of RBBR decolourisation, on the temperature of the reaction medium. The optimal temperature was ca. 35°C (Fig. 4).

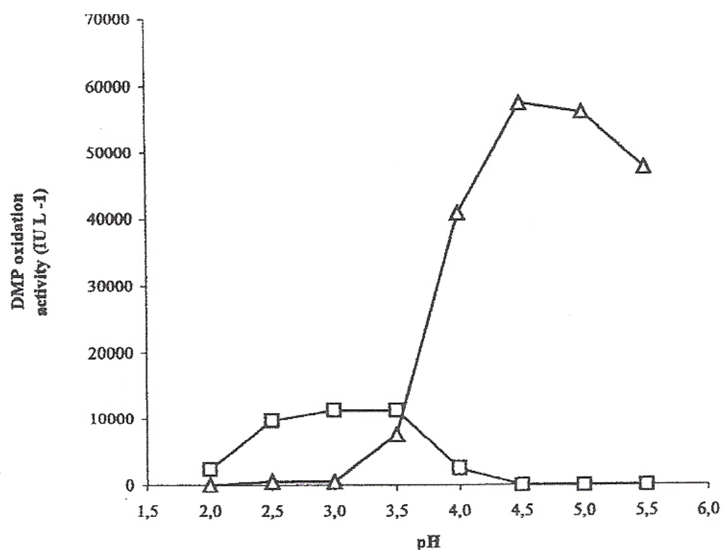


Fig. 3. Influence of pH (mean) upon DMP oxidation by the pure enzyme, in the presence of $MnSO_4$ (△) or in the presence of EDTA in the reaction medium (□).

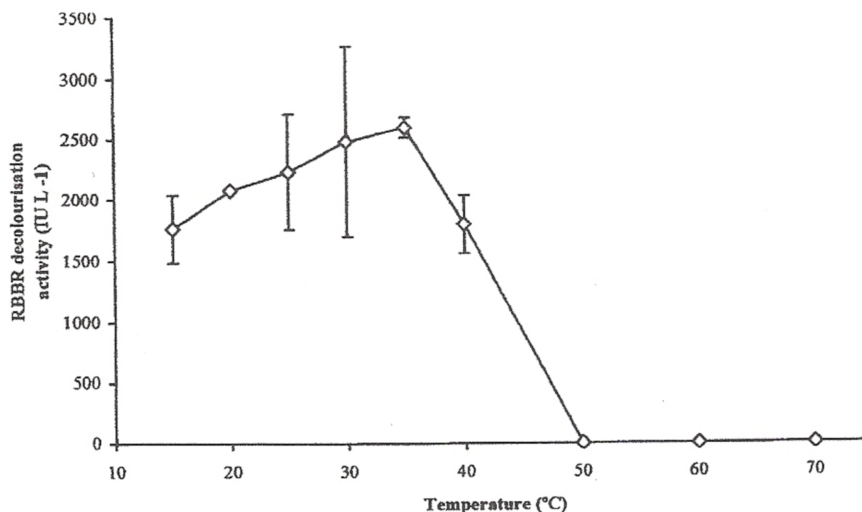


Fig.4. Influence of temperature (mean and standard deviation) upon RBBR decolourisation activity by the pure enzyme.

The enzyme's RBBR decolourising activity is stable at 30°C and 50°C for at least 10 minutes. At 70°C after 3 minutes presents only approximately 25% of initial activity and after 10 minutes loses completely RBBR decolourising activity (Fig. 4).
In a similar way, the enzyme DMP oxidation activity is stable at 30°C and 50°C for at least 10 minutes. At 70°C after 5 minutes the enzyme presents only approximately 50% of initial activity and after 10 minutes only 20% of the initial RBBR decolourising activity (results not shown).

The optimum conditions for the RBBR decolourising activity of this enzyme were ca. 35°C and pH 4.0, using 100 mM sodium tartrate buffer. These values agree with those described for ligninolytic enzymes with dye decolourising activities (10-13).
The optimal pH for the DMP oxidation is higher than in the presence of EDTA. These data are also in agreement with other work done in these versatile peroxidades from other strains. The oxidation of phenolic compounds in the presence of Mn is similar to the classical MnP activity, having an optimal pH close to those of the classical MnP. The DMP oxidation in the presence of EDTA, which is particular to this type of versatile enzymes, has a lower optimal pH, closer to those of the classical LiP, as has been shown in this work.

These results further confirm that the novel peroxidase isolated from *Bjerkandera* sp. strain B33/3 belongs to the novel class of versatile peroxidases presenting LiP, MnP and MIP-like activities.
Kinetic studies on several phenolic and aromatic substrates are now underway, in order to clarify the substrate affinity of this new class of peroxidases, particularly this novel enzyme.

A clearer understanding of the relation between the enzyme ligninolytic and decolourisation activities will certainly allow development of powerful and environment friendly technologies to be applied in textile and pulp and paper industries based on the enzyme ability to efficiently oxidise certain dyes and lignin related compounds with or without mediators (Fig .5).

Fig. 5. Scheme of proposed catalytic cycle for the versatile peroxidases (adapted from reference 7). Cycle include a two electron oxidation of the enzyme by hydrogen peroxide to compound I, followed by two one-electron reductions of compound I to compound II and then to native enzyme, producing concomitantly two oxidations of the electron donor. Electron donors could be either $Mn^{2+}$ or aromatic compounds (Arom. H) both phenolic or non-phenolic.

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