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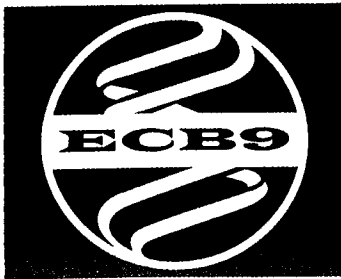
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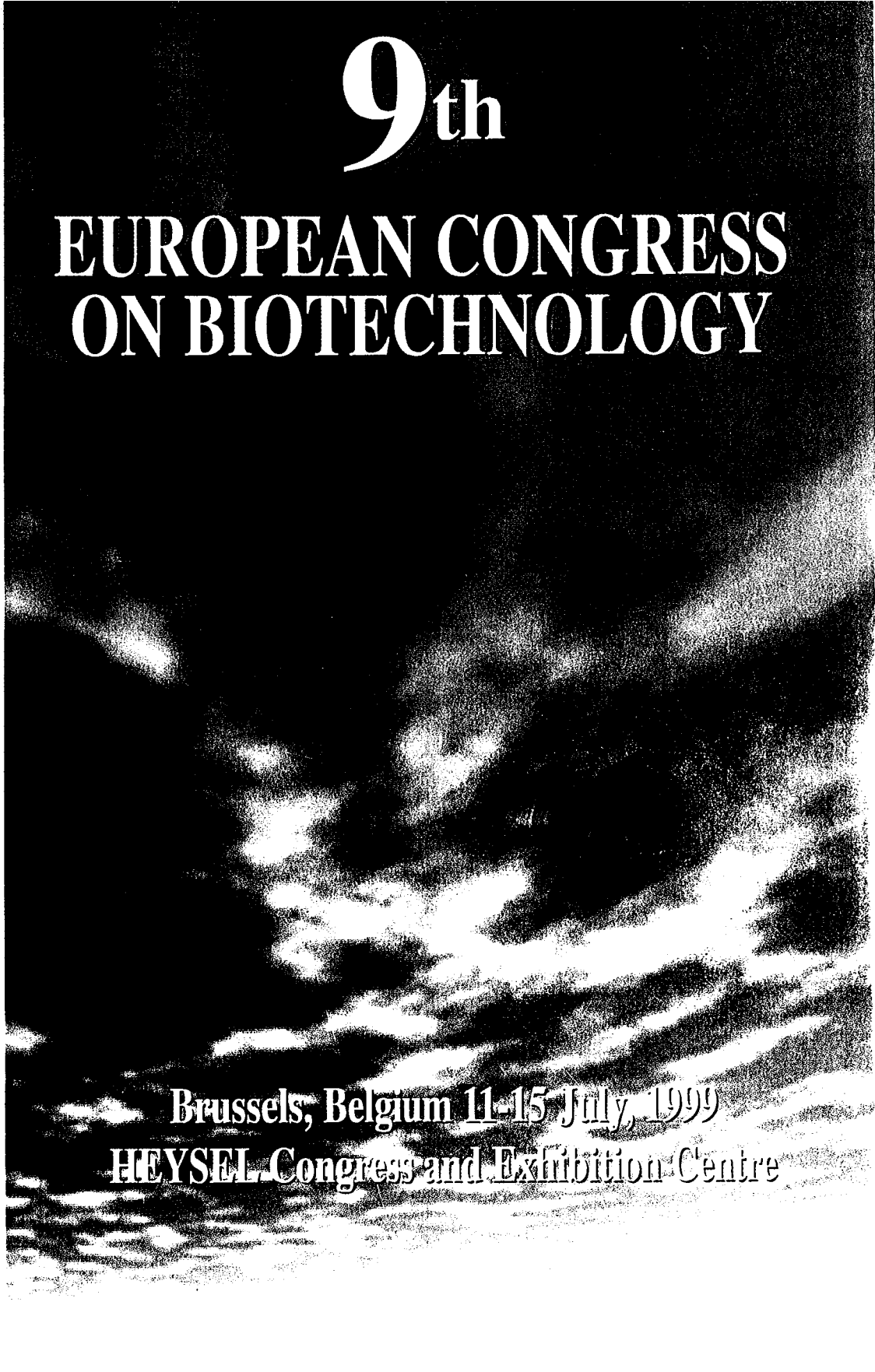
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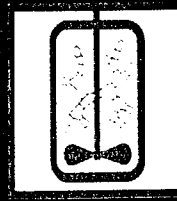
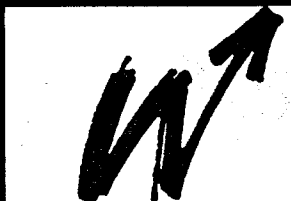
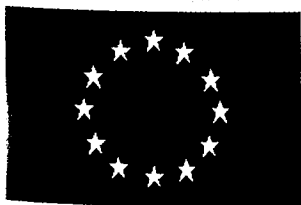
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EFFECT OF THE PH ON THE TRANSFORMATION OF N',N'-DIMETHYL-N-(HYDROXYPHENYL)UREAS BY LACCASE FROM *TRAMETES VERSICOLOR*

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Summary

Phenylureas are widely used herbicides which are likely to undergo aryl hydroxylation in plants and soil and thus to become suitable substrates for laccases, oxidising enzymes from white rot fungi. We studied the transformation of N',N'-dimethyl-N-(hydroxyphenyl)ureas by a laccase from *Trametes versicolor*. The *meta*-hydroxyphenyl derivative remained unchanged in the presence of laccase whereas the *para* and the *ortho*-derivatives were oxidized. The pH has a marked influence on both the rate of the degradation reaction and the nature of the oxidation products.

Materials and methods

Trametes versicolor (from the culture collection of the Unité of Phytopathologie, INRA Versailles, France) was grown on a medium described by Collins *et al.* (1996) The five-liter culture was shaken by continuous air bubbling and grown for seven days. Laccase production was induced with 2,5-xylidine (0.2 mM) at the beginning of the culture. The laccase containing exocellular fluid was filtered through decreasing porosity filters before a concentration step by ultrafiltration on YM10 Amicon membrane. The crude enzyme concentrate was then frozen at -80 °C and laccase activity recovered by purification on a DEAE column.

Laccase activity assays. Laccase activity was measured as the oxidation of 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). Ten microliters of enzyme solution were added to 990 microliters of a 1 mM ABTS solution in 0.1 M phosphate citrate buffer pH 3. The buffer was air saturated by bubbling prior to the experiment. The experiments were performed at 30°C. The oxidation of ABTS was photometrically monitored at $\lambda=420$ nm ($\Delta\epsilon=36\ 000$ l/mol5cm). One unit

of laccase (U) was defined as the enzyme amount that oxidized 1 $\mu\text{mol}/\text{min}$ ABTS.

The oxidation kinetic parameters using hydroxyphenylurea as laccase substrate were also determined spectrophotometrically using a diode array spectrophotometer (HP 8452A). The initial absorbance changes at two wavelengths 280 and 266 nm were used to calculate the initial reaction rate (ν), assuming that the extinction coefficients of 4-HF was 1547 $\text{l}/\text{mol}\cdot\text{cm}$ at 280 nm and 1323 $\text{l}/\text{mol}\cdot\text{cm}$ at 266 nm. For 4, 6- N',N' -dimethyl- N -[(2,5-cyclohexadiene-1-one)-4-ylidene]urea, the ratio $\epsilon_{280}/\epsilon_{266}$ was estimated to be 0.767. The V_{max} and K_M values were then calculated by fitting ν and the substrate concentration according to a linear Lineweaver-Burk representation. k_{cat} was determined from $k_{\text{cat}}=V_{\text{max}}/[\text{laccase}]$. All experiments were carried out in air-saturated solutions, so that the initial O_2 concentration remained constant, and the calculated kinetic parameters were « apparent » values.

N',N'-dimethyl- N -(hydroxyphenyl)ureas (HF) synthesis and transformation by laccase. N',N' -dimethyl- N -(hydroxyphenyl)ureas were synthesized in our laboratory according to methods adapted from the literature and detailed in Jolivalt *et al* (1999).

A typical enzymatic transformation assay of the hydroxyphenylureas was performed at 30°C usually for 3 hours in a 4 ml aerated solution containing 0.89 mM substrate and 0.4 U of partly purified laccase in 0.1 M citrate-phosphate buffer at various pH values. In reaction product identification experiments, about 10 mg of substrate in a 10 ml solution at the suitable pH were incubated with laccase (4 U). When the transformation was completed, the samples were saturated with NaCl, acidified at pH 2 with concentrated HCl and extracted in methylene chloride (3*10 ml). The characterization of the oxidation products was based on electron impact mass spectroscopy analysis (70 eV, quadrupole mass spectrometer) with sample introduction by direct insertion probe. NMR spectra were taken on a 200 MHz instrument in deuteriochloroform.

Analysis. The substrate as well as the soluble reaction products concentrations were followed by HPLC analysis using a C_{18} NovaPak column (150x3.9 mm) from Waters (Saint Quentin Fallavier, France). The elution was performed with a gradient of water/acetonitrile. UV detection was carried out at 244 nm. An extensive description of the methodology used for HF analysis is given in Jolivalt *et al* (1999).

Results and discussion

Laccase was not able to oxidize the *meta*-hydroxyphenyl urea derivative (3-HF) whereas the *para* and the *ortho*-derivatives (respectively 4-HF and 2-HF) were transformed with the following reaction rate at pH 5: *para*<*ortho*. Similar effect of the position of substitution on phenolic compounds has been reported in the literature, notably in case of various chlorophenols (Kadhim *et al*, 1999; Dec and Bollag, 1990). The efficiency of laccase can be significantly improved by appropriate pH conditions: at a given incubation time, the proportion of 4-HF transformed varies as a function of the pH with a maximum at pH 5 (figure 1). At pH 6, only 37 % of the initial 4-HF were transformed after two hours incubation while 93 % were transformed at pH 5. The laccase efficiency decreased very rapidly when increasing the pH above 6: at pH 7, no changes in the initial 4-HF concentration were observed after 6 hours incubation. Such a bell

shape profile of *Trametes versicolor* laccase activity has already been described by Leonowicz *et al* (1984) using 2,6-dimethoxyphenol and syringaldazine or by Dec and Bollag (1990) with chlorophenols. More recently, Xu (1997) showed that the activity profile of non phenolic substrates decreased monotonically as the pH increased whereas three 2,6-dimethoxyphenols exhibited a bell shape profile as a function of the pH. According to Xu analysis, the effect of pH on laccase activity results from two phenomena: first, increasing the pH favors the deprotonation of the phenolic substrate, thus increasing the difference between the redox potential of the type 1 copper laccase and the substrate and consequently the efficiency of the oxidation. On the other hand, the type 2 copper laccase is inhibited by hydroxide ions at higher pH. The bell shape profile of the laccase activity appears to be a consequence of these two opposite effects.

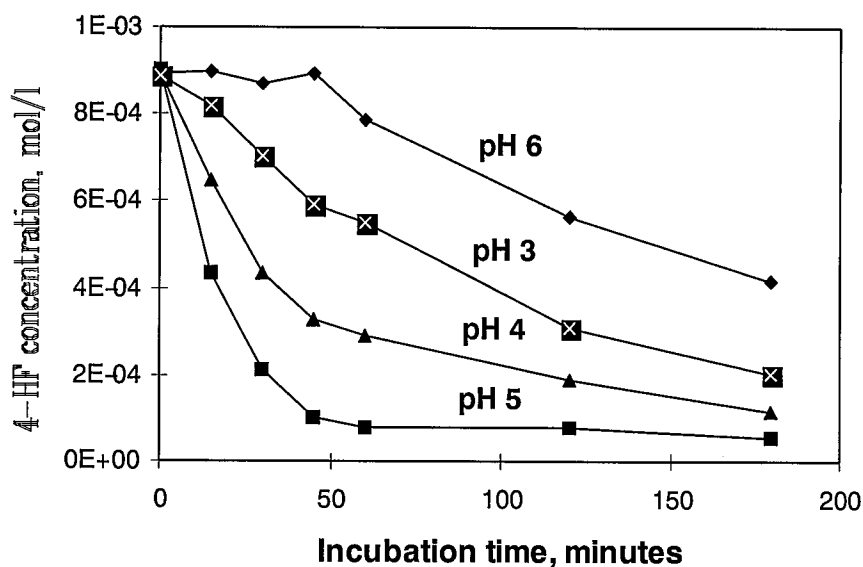


Figure 1: Effect of pH on 4-HF transformation in the presence of laccase

Reaction conditions: 0.1 M citrate-phosphate buffer pH 3 to 6; 4-HF initial concentration 0.89 mM laccase activity 0.4 U per ml medium

Besides its effect on the reaction rate, the pH also influenced the nature of the 4-HF oxidation product formed. At pH 3, the main reaction product of the *para* hydroxylated compound was identified as *p*-benzoquinone, while at pH 6, *N,N'*-dimethyl-*N*-[(2,5-cyclohexadiene-1-one)-4-ylidene]urea was mainly formed, as illustrated in figure 2. For these two pH, the

formation of the product can be directly correlated to the transformation rate of 4-HF. At pH 3, the identification of *p*-benzoquinone as the reaction product allowed to quantify its concentration in the reaction samples, using a calibration curve determined with an authentic control. HPLC analysis of both 4-HF and *p*-benzoquinone concentrations as a function of time showed that for the first 15 minutes, 4-HF was quantitatively transformed into *p*-benzoquinone by laccase. For higher incubation times, *p*-benzoquinone concentration represented only two third of the transformed 4-HF, suggesting that *p*-benzoquinone may undergo further non enzymatic reaction. For intermediate pH values, namely 4 and 5, it was found that the two identified reaction products were formed. At pH 5, the two products are formed simultaneously while at pH 4, 6-*N,N'*-dimethyl-*N*-[(2,5-cyclohexadiene-1-one)-4-ylidene]urea was measured only for incubation times lower than 45 minutes and thus appeared as an intermediary product.

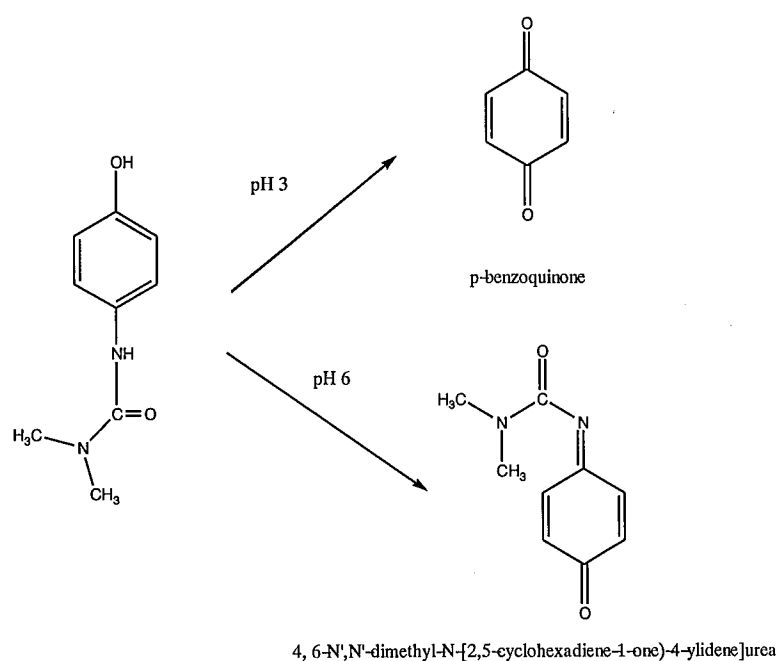


Figure 2: Main degradation products of *N,N'*-dimethyl-*N*-(4-hydroxyphenyl)urea in presence of laccase

Measurements of the kinetic parameters (K_M and k_{cat}) of laccase with 4-HF as a substrate were performed. UV-visible spectroscopy was preferred to HPLC analysis because of its ability to acquire reaction rate values by continuously measuring the absorbance changes as a function of

time. However, this method requires that the substrate and the reaction have significantly different absorbance spectra to monitorate the reaction accurately. That condition was fulfilled at pH 6: 4-HF exhibited a maximum absorbance at 280 nm whereas the only product, namely 4, 6-N',N'-dimethyl-N-[(2,5-cyclohexadiene-1-one)-4-ylidene]urea maximum absorbance was at 266 nm. The simultaneous measurement of the absorbance at these two wavelengths as a function of time allowed to calculate the transformation rate of 4-HF and thus the corresponding kinetic parameters. The Michaelis constants were found to be $K_M=0.140$ mM and $k_{cat}=65$ s⁻¹. Comparison with the values determined in this work for ABTS at pH 3, i.e. for optimum pH conditions ($K_M=0.062$ mM and $k_{cat}=217$ s⁻¹) shows that laccase was slightly less efficient in oxidizing 4-HF than ABTS, generally used as a model substrate for laccase. However, we know that laccase transformed 4-HF more efficiently at pH 5, so k_{cat} is expected to be higher at this pH.

Conclusions :

In conclusion, laccase has been shown to catalyse the oxidation of both *para* and *ortho*-hydroxylated N',N'-dimethyl-N-(hydroxyphenyl)ureas quite efficiently, which first extends the range of application for this enzyme and second, indicates that laccase is likely to be involved in the biodegradation of phenylureas herbicides in plants and soil. The degradation of hydroxylated metabolites of monuron and methoxuron, which are commonly used herbicides, are under progress.

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