Laccase-catalyzed azodye synthesis

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There is an increasing demand of the chemical industry to develop eco-friendly processes. In numerous cases, biocatalysis represents an attractive route towards one-step safe synthesis. In this context, the finding that laccases are able to create azo bonds represents a good potential for the dye industry. This work investigates the properties and biosynthesis of an azodye named LAR1, which stands for Laccase Acid Red 1. LAR1 is made up of two anthraquinonic moieties coupled through an azo bond and demonstrated good dyeing properties on nylon, polyamide and particularly on leather. LAR1 has been shown not to be cytotoxic, mutagenic and ecotoxic. Further investigations aimed at optimizing the process of LAR1 production. Several laccases from bacterial and fungal (ascomycetes and basidiomycetes) origin proved to be useful to produce LAR1 in short reaction times. Whatever the laccase tested, optimal pH for LAR1 synthesis was 4 while the highest rates of oxidation for ABTS were obtained between pH 3 and 4. In our conditions, two isoenzymes from *Trametes versicolor* have been shown to be the most efficient to transform ABu62. It was also found that oxidation of the anthraquinonic precursor by these laccases did not follow a classical Michaelis-Menten kinetic.

1. From traditional dye industry to biosynthesis

Since the discovery of the first synthetic dye mauvein by a young English chemist William Perkin in 1856, traditional dyes from animal (*Hexaplex trunculus, Kerria lacca, Kermes vermilio* …) or plant (*Isatis indigota, Rubia cordifolia, Rhamnus lycioides*…) origin have been progressively replaced by synthetic dyes (Cardon, 2003). The colour chemical industry was an important activity in Europe until the end of 20th century. It suffers now displacement to the developing world due to the high labour costs in Europe as well as to both worker and environment non-friendly character of dyes synthesis (Integrated Project SOPHIED, 2005). Since several groups (Shaw and Freeman, 2004; Bruyneel *et al.*, 2008) described the ability and the efficiency of laccases to catalyze the formation of coloured compounds, these enzymes now represent an attractive route towards dye synthesis. Laccases (EC 1.10.3.2) are benzenediol:oxygen oxidoreductases coupling the one-electron oxidation of an organic reducing substrate (mainly a phenol or an aromatic amine) to the concomittant reduction

of dioxygen to water (Solomon *et al.*, 1996). By contrast to peroxidases which share with laccases a low specificity for the reducing substrate but require hydrogen peroxide as co-substrate, laccases have a strong preference for dioxygen. As this compound is easily renewed in the reaction medium, this represents an advantage for industrial applications.
Our group (Vanhulle 2007a; Vanhulle *et al.*, 2008) reported the production of a red azo-compound LAR1 (Laccase Acid Red 1) during fungal laccases biotransformation of an anthraquinonic acid compound (Acid Blue 62-ABu62) (Figure 1).

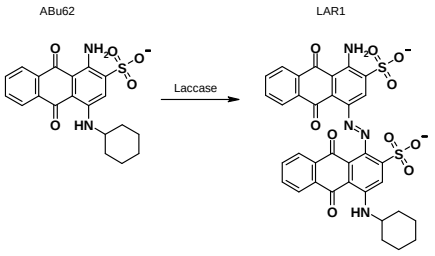

Figure 1: Laccases catalyzed the synthesis of Laccase Acid Red 1 (LAR1) from Acid Blue 62 (ABu62) (from Vanhulle et al., 2008).

LAR1 was tested for its ability to dye textile. In particular, the fastness properties of the dyeings on nylon, polyamide and leather were measured in accordance with ISO standards. All results demonstrated that the dye has a good behaviour, except for light fastness, which was only medium. Besides demonstrating good dyeing properties, LAR1 has also prove its safety through several adequate toxicity studies. Nowadays, 60% to 70% of the dyes used are azodyes and many of them have demonstrated toxicity in the past (Vanhulle *et al.*, 2007b). NRU test with Caco-2 cells was used to evaluate LAR1 cytotoxicity. Mini-Ames and Standard Ames tests allowed to assess its mutagenic potential whereas mortality studies using zebra fish egg, *Vibrio fisherii*, *Daphnia magna* and green alga *Desmodesmus subspicatus* were used to evaluate its ecotoxicity. The new azodye was neither mutagenic nor ecotoxic (Enaud *et al.*, 2008).

2. Laccases in azodye synthesis

Interest in the use of laccase-catalyzed LAR1 synthesis for a future industrial production being established, robust and efficient biocatalysts must be selected. Accordingly, the catalytic properties of several laccases from both bacterial and fungal origins were compared for ABu62 biotransformation: CotA is a recombinant enzyme from *Bacillus subtilis* (Martins *et al.*, 2002); Bioscreen, a commercial laccase from *Trametes versicolor***,** purchased from BIOSCREEN, Germany and delivered under the commercial trademark Oxizym LA - batch n° LA 2000/001; PS7, a laccase from *Pycnoporus sanguineus* MUCL 41582 (Trovaslet *et al.*, 2007); PS344, a laccase from the aquatic fungus *Phoma sp.* strain UHH 5-1-03 (Junghanns and Schlosser, personal communication) and TVA and TVB are two isoenzymes purified from *Trametes versicolor* ATCC 32745 (Bertrand *et al.*, 2002a and b).

These enzymes differ for their kinetic parameters using ABTS as substrate (Table 1). In our conditions, TVA and PS344 laccases showed the highest affinities towards ABTS (K_m < 10 µM while all other K_m values were between 30 and 50 µM) and the k_{cat}/K_m ratios showed that ABTS substrate was more efficiently converted by TVA laccase (54 400 $mM.s^{-1}$) than by all other enzymes, suggesting that this enzyme could be an efficient catalyst for biotransformation applications. Very similar K_m were obtained for TVB and Bioscreen, both *Trametes versicolor* laccases, suggesting that the commercial enzyme Bioscreen could be an inducible form of laccase as TVB.

For all tested laccases, oxidation rate of ABTS decreased when increasing pH from 3-4 to 7-8 and no transformation of ABTS could be detected anymore at pH 7-8, except for PS344 and CotA laccases which retain activity until pH 8-9. Moreover, all fungal laccases displayed a maximal activity between 50 and 70°C and have close activation energy (10-15 $KJ.mol^{-1}$). The activity of the bacterial laccase CotA raised in a linear manner with temperature up to the highest temperature tested (80°C) and its activation energy was two fold higher than the one of other tested laccases.

Table 1: Kinetic characterization of laccase catalyzed ABTS biotransformation.

Laccases	k_{cat} (s^{-1}) *	K_m (µM) *	k_{cat}/K_m (x 10^3 $mM^{-1}.s^{-1}$) *	Optimum pH **	Optimum temperature ***
Cot A	17	39.5	0.4	4	> 80°C
Bioscreen [a]	ND	30.8	ND	3	55°C
PS7	21.4	47.4	0.4	3	65-70°C
PS344 [b]	24.7	9.7	2.5	4	65°C
TVA	386	7.1	54.4	ND	ND
TVB	442	30.5	14.5	ND	ND

[a]: Neither purity nor specific activity of the enzyme preparation were known.

[b]: PS344 laccase is a dimeric enzyme consisting of 75 KDa monomers (Junghanns and Schlosser, personal communication).

*: Reactions were carried out in acetate 0.1 M pH 4.5. The apparent k_{cat} and K_m values were evaluated using six ABTS concentrations and Lineweaver-Burk linearisation.

**: The optimum pH was determined by oxidation of 4 mM ABTS, over a range of pH 2 to 10 in phosphate 0.1 M adjusted to desired pH using HCl or NaOH.

***: The optimum temperature was determined by oxidation of 4 mM ABTS, between 20 and 75°C in phosphate 0.1 M pH 4 solution.

ND: not determined.

2.1. Optimum pH conditions for LAR1 production

For all laccases, the ABu62 transformation rate as a function of pH exhibited a bell-shaped activity profile with an optimum pH around 4 (Figure 2A). At pHs higher than pH 7-8, ABu62 was not transformed, excepted with CotA and PS344 laccases. As previously evidenced for phenylureas derivatives (Jolivalt *et al.*, 2006) and as suggested

from the colour changes of the reaction solution during the biotransformation course, pH influenced the nature of the ABu62 biotransformation products: indeed, a decolourisation was observed at neutral to alkaline pH while the reaction medium turned from blue, the ABu62 colour, to red in acidic conditions. At the optimal pH for ABu62 consumption, the main biotransformed product was identified as LAR1.

Laccases stability studies as a function of the pH showed that for all laccases, the stability increased with pH, up to pH 7 to 9. Acidic pH is required to produce the targeted product, LAR1, but in such conditions, laccases are likely to lose their activity, thus limiting the transformation yield. However, in the presence of ABu62, laccase stability was improved between pH 3 and 8 (Figure 2B). It is noteworthy that bacterial laccase CotA has an unusual stability behaviour, both in the presence or in the absence of ABu62 (data not shown). At pH 2, a rapid loss of activity occurred while, at other pHs, an activity increase (between 2 and 30 folds) was observed when increasing the storage duration. Contrary to fungal laccases, deactivation mechanism of this biocatalyst seems to involve intermediate(s) that has (have) more activity than the initial form of the enzyme. Work is currently under progress to better characterize this (these) intermediate(s).

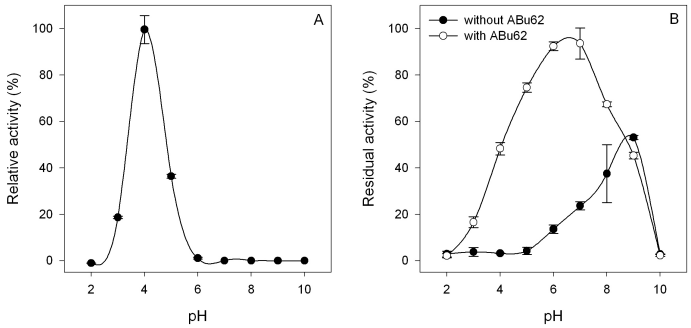

Figure 2: A. Effects of pH on Bioscreen catalyzed ABu62 biotransformation. B. Bioscreen stability, after a one week storage, as a function of pH, in the presence or in the absence of 70 µM ABu62. All reaction media contained 10 U.l^{-1} laccase and phosphate 0.1 M adjusted to desired pH using HCl or NaOH. All experiments were triplicated.

2.2. Kinetic parameter evaluation

Although not really favourable in terms of laccase stability, pH 4.5 was chosen for the kinetic studies for two reasons: (i) pH 4.5 is close to the optimum pH for ABu62 transformation; (ii) at this pH, LAR1 is the main biotransformed product.

To estimate the kinetic parameters of each laccase for ABu62, apparent K_m and k_{cat} values were first evaluated from Lineweaver-Burk linearisations, assuming a Michaelis-Menten behaviour of the enzymes (upper part of Table 2). In these conditions, k_{cat} values were in the same range of those calculated for ABTS substrate. Deducted K_m values varied from 165 µM for CotA laccase to 472 µM for PS7 enzyme, suggesting

that the affinity of the enzymes for ABu62 is significantly lower than the one observed for ABTS.

Table 2: Kinetic parameters for laccases catalyzed ABu62 biotransformation. Excepted for TVA and TVB laccases, reactions were carried out in acetate 0.1 M pH 4.5. The apparent k_{cat} and K_m values were evaluated using height substrate concentrations and Lineweaver-Burk linearisation.

Laccases	k_{cat} (s^{-1})	K_m or $S_{0.5}$ (µM)	k_{cat}/K_m (x 10^3 $mM^{-1}.s^{-1}$)	K_i (mM)	h
Cot A [1]	3.1	165	0.02	-	-
Bioscreen [a] [1]	ND	305	ND	-	-
PS7 [1]	35.7	472	0.07	-	-
PS344 [1]	34.1	194	0.17	-	-
TVA [1]	1 376	302	4.55	-	-
TVB [1]	898	204	4.41	-	-
TVA [2]	1 200	189	6.34	230	-
TVB [3]	477	63	7.57	-	1.6

[a]: Neither purity nor specific activity of the enzyme preparation were known.
[1] Apparent kinetic parameters were evaluated using the classical Michaelis-Menten equation.
[2] Reactions were carried out in 100 mM tartaric buffer pH 4.5. The ABu62 concentrations ranged from 0 to 450 µM. Apparent kinetic parameters were calculated using a modified Michaelis-Menten equation which includes an inhibition constant Ki (*Eqn. 1*).
[3] Reactions were carried out in 100 mM tartaric buffer pH 4.5. The ABu62 concentrations ranged from 0 to 450 µM. Apparent kinetic parameters were calculated using Hill equation (*Eqn. 2*).

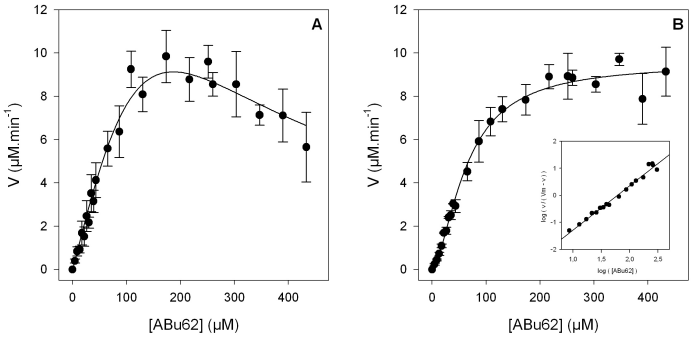

Figure 3: TVA (A) and TVB (B) laccases activity vs. ABu62 concentration. Insert: Hill plot. Reactions were carried out in 100 mM tartaric buffer pH 4.5 as previously described in Trovaslet et al. (2007).

However, observation of Figure 3 shows that the kinetic behaviour of these two enzymes differs somewhat from an ideal Michaelis-Menten mechanism. On the one hand, a substrate-inhibition phenomenon occurred at ABu62 concentrations higher than 150-200 µM for TVA laccase (Figure 3A). Therefore, apparent kinetic parameters were calculated using the modified Michaelis-Menten equation (*Eqn.* 1) which includes an inhibition constant K_i. On the other hand, a sigmoïdal curve was observed when plotting the reaction rate *vs.* substrate concentration for TVB laccase (Figure 3B). To account this behaviour, kinetic data were fitted according to a Hill equation (*Eqn.* 2) including three parameters: $S_{0.5}$, the half-saturating substrate concentration; *h*, the Hill coefficient and k_{cat}. Based on our results (second part of Table 2), TVB laccase presents the highest affinity for ABu62 (K_m value about 63 µM *vs.* 189 µM for TVA) whereas TVA has the highest turnover (1 200 s^{-1} *vs.* 477 s^{-1} for TVB). Overall efficiency of both laccases are therefore very similar. The kinetic behaviour of TVB suggests a positive cooperativity with respect to ABu62 substrate with a Hill coefficient of about 1.6, which is very similar to what was previously obtained for a laccase from *Pycnoporus sanguineus* (Trovaslet *et al.*, 2007).

$$v = \frac{V_m[S]}{K_m + [S] + ([S]^2 / K_i)} \quad (1)$$

$$v = \frac{V_m[S]^h}{S_{0.5}{}^h + [S]^h} \quad (2)$$

An enzymatic kinetic following a Hill equation can be the result of the presence of two different binding sites of the substrate on the enzyme. As ABu62 is substrate (it is transformed by laccase into LAR1), it obviously binds into the oxidation active site of the enzyme. It can thus be assumed that a second binding site for ABu62 exists. Crystallisation experiments of TVB in the presence of ABu62 are currently in progress to address this issue and localise the second hypothetical binding site for ABu62.

2.3. *Oxidation of ABTS in the presence of ABu62*

Based on the hypothesis of two-binding sites for ABu62, this dye may be considered as both a substrate and an activator of TVB laccase. To determine if ABu62 could also increase the transformation efficiency of laccase towards another substrate, ABTS oxidation was carried out with TVA and TVB laccases, in the presence of increasing ABu62 concentrations (Figure 4).

Transformation of ABTS was followed by measuring the absorbance changes at 414 nm. At this wavelength, concomitant oxidation of ABu62 also contributed to absorbance increase. However, it has been verified that the transformation of ABu62 accounts at most for 8% of the total absorbance changes.

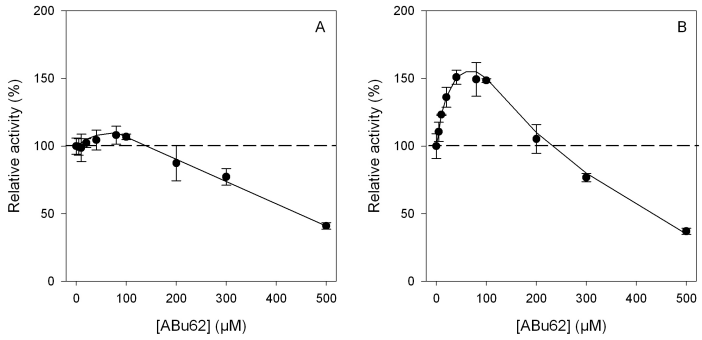

Figure 4: Biotransformation of 20 µM ABTS in the presence of increasing ABu62 concentration (between 0 and 500 µM), in tartaric buffer 100 mM pH 4.5. Activities were expressed as a percentage of activity measured without dye. Reactions were carried out with 10 U.l^{-1} TVA (A) or TVB (B) laccase activity.

From 0 to 100 µM ABu62, the transformation rate of ABTS by TVA laccase is not significantly different if compared to the transformation rate of ABTS alone (Figure 4A). For ABu62 concentrations higher than 100 µM, a decrease of the transformation rate is observed, as expected in presence of two competitive substrates. For TVB laccase (Figure 4B), the kinetic behaviour was different: for ABu62 concentration lower than 200 µM, a significant increase of the transformation rate of ABTS is observed, with a maximum for 100 µM ABu62 concentration. At this ABu62 concentration, ABTS is oxidized more than 1.5 times faster than without ABu62, suggesting an activation effect of this dye. For higher ABu62 concentrations, the transformation rate of ABTS decreased, as a consequence of the competition of both ABTS and ABu62 at the active site of laccase.

3. Conclusion

Laccases are cheap biocatalysts useful in azodye synthesis. Our study focused on the laccase biotransformation of a blue anthraquinonic dye (ABu62) oxidized into a red, non toxic and industrially interesting azodye (LAR1). Based on kinetic parameters evaluation of bacterial and fungal laccases, two enzymes from *Trametes versicolor* (TVA and TVB) have been shown to be the most efficient to transform ABu62. Furthermore, it was evidenced that one of the *Trametes versicolor* laccase (TVB) is activated by anthraquinonic substrate. Work is currently under progress to confirm the occurrence of this activation effect with other laccase substrates and different experimental conditions. These findings represent not only the synthesis of a new compound with good dyeing properties (LAR1), but also open the way to new safe and environmental friendly routes to azodye synthesis.

4. Acknowledgement

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