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Enzymatic degradation of azo dyes using three macrophyte species: *Arundo donax*, *Typha angustifolia* and *Phragmites australis*

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ABSTRACT

The aim of this study was to investigate the potential role of three macrophyte species (*Arundo donax*, *Typha angustifolia* and *Phragmites australis*) for degradation of azo dyes. Activities of peroxidases enzymes involved in plant protection against stress were tested for dye decolourization. In order to conduct the enzyme activity assay, the fresh extract was obtained from crude extracts of leaves. The oxidation capability of peroxidase (POD) on direct azo dyes (amaranth and amido black) was investigated and found to be an effective treatment methodology. The performance of peroxidase was evaluated in soluble and free form in the presence of H_2O_2. The oxidation was tested as a function of peroxidase at constant POD activity. Parameters such as temperature (5–70˚C), concentration of H_2O_2 (0–1000 μM/L), pH (4–10), dye concentration (0.002–1 mM/L) and enzyme amount (5–20 μL) were studied. Results show that *P. australis*, leaves contained the highest peroxidase activity. The dye decolourization is about 87% for amido black and 93% for amaranth after 120 h of reaction time. The bio removal efficiency depends on the reaction time, initial dye and enzyme concentrations, pH and temperature.

Keywords: Phragmites australis; *Arundo donax*; *Typha angustifolia*; Peroxidase; Oxidation; Dye

1. Introduction

The treatment of effluents containing dyes, which come mainly from textile industries, is a challenging issue among environmental technologies [1], due of their complex aromatic structure, designed to resist fading on exposure to sweat, soap, water, light and oxidizing agents [2]. Different physical, chemical and biological techniques have been applied to remove dyes from wastewater and each has some technical and economical limitations. Most physico-chemical dye removal methods are expensive, have limited versatility, are greatly inhibited by other wastewater constituents and/or generate waste products that must be handled. As an alternative, biological

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treatments present a relatively inexpensive way to remove dyes from wastewater [3].

The removal of azo dyes in aqueous solution by peroxidase (POD) enzyme and laccase has been widely reported, in last year's, especially by white-rot fungi [4]. The enzymatic decolourization of amido black using white-rot fungus, *P. chrysosporium*, with a maximum decolourization of 98% is achieved on the third day under normal conditions [5]. Theerachat et al. [6] reported that laccase isolated from *Trametes versicolor* has a decolourization of 96% for 100 ppm amaranth, achieved in 8 h with optimized medium containing 2% starch and 0.125% yeast extract.

Phytoremediation has emerged as an inexpensive and less invasive cleanup strategy. The phytoremediation of various dye effluents by plants such as *Phragmites australis, Blumea malcommi, Brassca juncea, Typhonium flagelliforme* has been reported [7–9]. This degradation process relies mostly on peroxidases, enzymes typically activated as an oxidative stress response.

Antioxidative defence system of plant is composed of reactive oxygen species-scavenging enzymes that function to interrupt the cascades of uncontrolled oxidation in each organelle. The first line of antioxidative defence is superoxide dismutase enzyme (SOD) that converts the $O_2^{\cdot-}$ radical into H_2O_2, preventing the generation of a highly toxic oxidant (hydroperoxyl radical, $HO_2^{\cdot}$). POD's function is the scavenging of H_2O_2 produced by SOD dismutation, avoiding its accumulation [10].

The catalytic cycle of peroxidases in presence of hydrogen peroxide is already presented [11]. The heme group of the enzyme first reacts with one molecule of hydrogen peroxide. During this reaction step, the hydrogen peroxide is reduced to water while the enzyme is oxidized, and oxidizes the reduced substrate (RH) to give a substrate radical (R•).

Several studies have shown that plant peroxidases (POD) are capable of degrading some textile and other important dyes [12]. It has recently been demonstrated that extracellular enzymes of white rot fungi such as peroxidases (lignin peroxidase-LiP, horseradish peoroxidase-HRP and manganese peroxidase-MnP) and phenoloxidases (laccases) [13,14] can be used to degrade and detoxify polyaromatic hydrocarbons, polychlorinated biphenyls and certain dyes [15,16]. Davies et al. [7] reported that for AO7 solutions in contact with stem and root crude extract of *P. australis* after 120 h, not only the azo bond was cleaved, leading to the solution decolourization, but also the degradation of the aromatic amines was detected as the typical peaks attributed to aromatic rings disappeared [17].

This strategy appears to be very interesting as enzymes not only allow the pre-treatment of specific recalcitrant compounds by changing their physico-chemical properties and making them more amenable for treatment, but also act in their transformation into innocuous products [18]. POD is involved in scavenging of hydrogen peroxide with oxidizing a wide range of organic and inorganic compounds.

The present work was aimed to assess bio removal potential of amaranth and amido black by *P. australis, Arundo donax* and *Typha angustifolia*. Since amaranth possesses exceptionally good solubility in water, its removal by common chemical treatments or by physical treatments like coagulation, floatation, etc. is not efficient. Generally, biological aerobic wastewater systems are not successful for decolourization of majority of dyes [19]. The study will compare the response of the selected three macrophytes species to oxidative stress by measuring the concentration of POD activity in the leaves collected from polluted sampling site. Also, to investigate their potential role for decolourization of the two dyes. In order to reach the optimal removal of amido black and amaranth dyes, the effect of main variables such as reaction time, peroxidase concentration, initial dye concentration, pH and temperature was investigated.

2. Materials and methods

2.1. Chemicals and reagents

Azo dyes used in this study are, amido black ($C_{22}H_{14}N_6Na_2O_9S_2$), (Sigma–Aldrich) and amaranth ($C_{20}H_{11}N_2Na_3O_{10}S_3$), (Sigma A1016). All the chemicals used were of the highest purity available and of analytical grade.

2.2. Plants

The studied plants were collected from natural polluted ecosystem (a river collecting secondary treated wastewater from urban and food industry). The aim of this study was to compare the response of these three species to oxidative stress and to investigate their potential role for detoxification of azo dyes (Fig. 1).

2.3. Enzymatic assays

In order to conduct enzyme activity assay, the fresh leaves extract was homogenized in phosphate buffer (0.01 M/L, pH 7.0), containing 0.2% polyvinylpyrrolidone. The homogenate was centrifuged at

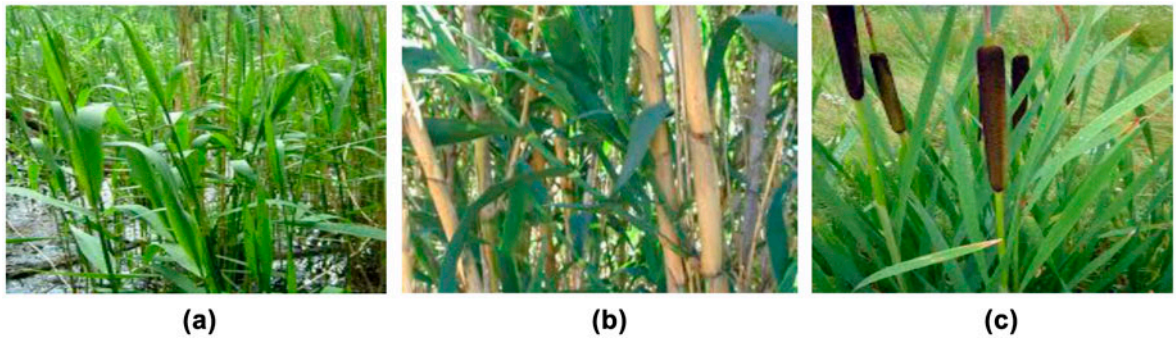

Fig. 1. Photographic image of the three macrophytes species: (a) *P. australis*, (b) *A. donax* and (c) *T. angustifolia*.

2000xg at 4°C for 20 min. The supernatant was used as the crude extract for the following assay:

Protein content was determined according to the method of Bradford with bovine serum albumin as a standard [20]. A BSA standard (0–1 mg/mL) was prepared from dilution of a 2 mg/mL stock solution. Bradford reagent (3 mL) was mixed with 0.1 mL of extract and allowed to develop for 5 min at room temperature before absorbance measurements at 595 nm. A blank was prepared by using 0.1 L of extraction buffer.

Peroxidase (POD EC. 1.11.1.7) activity was determined according to Khan and Robinson [21] by using the reaction medium of: 1.5 mL of guaiacol 1% (v/v) (Vetec, 97%, v/v), 0.4 mL of H_2O_2 0.3% (v/v) (Vetec, PA), 0.1 mL of enzyme (kept in ice bath) and 1.2 mL of 0.05 M/L phosphate buffer pH 6.5. The reaction was carried out for 5 min at 30°C in a spectrophotometer coupled to a thermostatic bath. One unit of peroxidase activity represents the oxidation of 1 μM of guaiacol per minute in the assay conditions and it was calculated by using data relative to the linear portion of the curve.

2.4. Dye decolourization

To evaluate the effects of operational factors on the efficiency of dye decolourization, a reaction medium of: 0.5 mL of dye (0.05 mM/L), 0.4 mL of phosphate buffer, 0.1 mL of H_2O_2 (100 μM/L) and 20 μL enzyme extract was prepared. Experiments were carried out with different initial dye concentrations (0.02–0.1 mM/L), pH values (4–10) and quantities of plant enzyme (5–20 μL). Experimental study of temperature effect on POD activity was performed at the range of 5–70°C. The initial pH of dye solution was adjusted using diluted KOH and H_2SO_4 solutions and was measured by pH metre. The concentration of H_2O_2 was varied to examine its reusability by *P. australis*, *A. donax* and *T. angustifolia* peroxidases to remove amido black and amaranth colours.

2.5. Analytical method

Peroxidase activity extracted from plants leaves was evaluated in soluble form in the presence of H_2O_2. The oxidation was tested as a function of peroxidase at fixed concentration of H_2O_2 (100 μM/l) and at constant POD activity (Table 1).

The degree of decolourization is usually calculated from the decrease of absorbance at a selected wavelength, most conveniently at the maximum absorbance (Fig. 2), $\lambda_{max} = 619$ nm for amido black and 521 nm for amaranth, using a UV–vis spectrophotometer (Thermospectronic UV1). Colour removal percentage was calculated using Eq. (1), [22].

$$\text{Colour removal}\,\% = \frac{(\text{Initial absorbance} - \text{Final absorbance}) \times 100}{\text{Initial absorbance}} \quad (1)$$

The UV–vis spectrum of POD enzyme of different species (*P. australis*, *A. donax* and *T. angustifolia*) dissolved in water was presented in Fig. 3. Two bands are observed at 275 and 330 nm. These values are similar for the three plants. Interference of POD absorbance in the visible region, with the maximum of absorbance of dyes (619 nm for amido black and 521 nm for amaranth) is not expected (Fig. 3). However, it is not the case in the UV region. The decrease of UV absorbance cannot be linked only to the dye degradation as presented in other studies [23].

In order to compare the results between treatments and between species, Student–Newman tests were performed using XLStat pro. In all cases, significance was defined by $p < 0.05$.

Table 1
Peroxidase activity and specific peroxidase activity in *P. australis*, *A. donax* and *T. angustifolia* leaves

Macrophytes species	POD activity (U/g/min)	Specific POD activity (U/mg of protein)	Protein mg/g	[POD] U/mL of extract
P. australis	69.92 ± 0.0 3	5.65 ± 0.03	12.75 ± 0.03	69.92 ± 0.03
A. donax	63.15 ± 0.04	4.95 ± 0.04	15.37 ± 0.04	63.15 ± 0.04
T. angustifolia	43.92 ± 0.0 1	2.65 ± 0.01	12.83 ± 0.01	43.92 ± 0.01

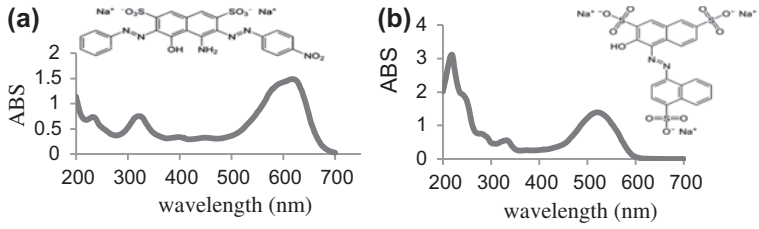

Fig. 2. UV–vis spectrum of amido black (a) and amaranth (b).

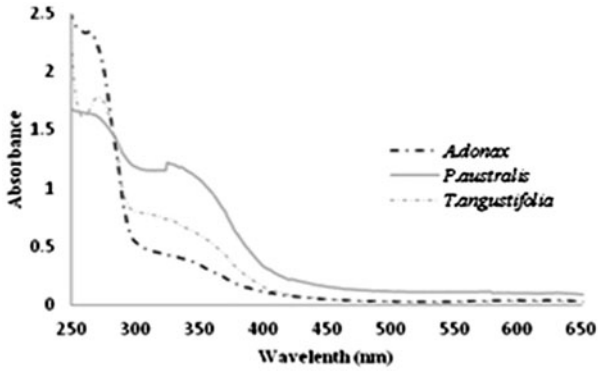

Fig. 3. UV–vis scan of POD enzyme of *P. australis*, *A. donax* and *T. angustifolia*, peroxidases between 250 and 650 nm.

3. Results and discussion

3.1. Enzymatic activity

To compare the response of macrophytes species (*P. australis*, *A. donax* and *T. angustifolia*) to oxidative stress, peroxidase activity was measured in fresh leaves.

As shown in Table 1, peroxidases activities were overall higher for the three plants according to the concentration of organic pollutant. *P. australis* contained the highest specific peroxidase activity (5.65 U/mg proteins). The peroxidase activity in *P. australis* species conducted as cell cultures in laboratory under high-stress condition lead to specific peroxidase activity of 24U/mg protein [24].

3.2. Effect of temperature on POD activity

Temperature's variations have an important effect on biological activity of peroxidase for the three macrophyte species. This parameter was studied at the range of 5–70˚C. As shown in Fig. 3, the efficiency of POD activity increased with rising temperature (5–30˚C) suggesting that the optimal temperature for the activity is about 25–30˚C. After 30˚C, POD activity decreases with increasing of temperature, when reaching an inactivation after 70˚C. *T. angustifolia* appeared less dependent on temperature that increases until 40˚C (Fig. 4, $T > 30$˚C). Peroxidase has been reported to lose activity (~ 91%) at high temperatures such as 50˚C [25,26].

Sciancalepore et al. [27] reported 50% reduction in grape POD activity for 2 min of heating at 65˚C. Also

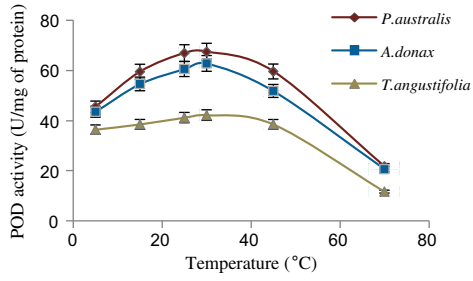

Fig. 4. Effect of temperature on POD activity: peroxidase activity extracted from leaves of plants was evaluated at fixed H_2O_2 concentration and different temperature degree.

in another study [28], for Borbon grape cultivar, POD activity decreased about 58% at 60°C for 6 min of heating. Cano et al. [29] treated whole peeled bananas in boiling water for 11 min and observed 96–100% reduction in the activity of POD enzyme. The energy that is needed to overcome the activation can be supplied by heat, so temperature increases the rate of enzymatic reactions by supplying energy to activate the reaction (Fig. 4, until 30°C). At high temperature (>40°C) the protein denatures which changes the enzymes structure [29].

3.3. Effect of hydrogen peroxide concentration

To evaluate the dye decolourization by the enzymatic extracts, controls were carried out in the absence of H_2O_2. It was observed that for the three species, there was no decolourization of dyes.

The decolourization at different concentrations of H_2O_2 (without extract addition) showed an oxidizing power of peroxide that reached 38% for amaranth and 56% for amido black after 120 h as a reaction time (Fig. 3). This removal can be considered stable at $[H_2O_2] > 100$ μM/L. In the presence of H_2O_2 catalysed by POD (Fig. 5), same behaviour is obtained for the three species and the two dyes. An increase of colour removal related to low H_2O_2 concentration (≤100 μM/L) followed by strong decrease at high concentration of H_2O_2 (≥500 μM/L).

As POD concentration is different for the three species extracts, Fig. 6 shows that the decolourization of the two dyes depend strongly in the ratio $[H_2O_2]$/[POD]. In low ratio (8–10), the percentage of decolourization, obtained after 120 h, was about 20–30%, respectively, for amaranth and amido black by the three plants enzymes. The increase of $[H_2O_2]$/[POD] ratio from 128 to 446, increases the percentage of colour removal. After 120 h, it reaches 93–87% for amaranth and amido black, respectively, when $[H_2O_2]$/[POD] ratio is in the range of 81–128. While, for $[H_2O_2]$/[POD] ratio higher than 600, an inhibitory effect was observed. The H_2O_2 stepwise addition to the reaction medium can avoid the inactivation of the enzyme by H_2O_2 excess [30]. This is due to the denaturation of peroxidase enzyme. Fig. 6 shows the same behaviour for the two dyes with higher colour removal reached for amaranth. Also, same behaviour is observed for the three species with different concentration of H_2O_2. However, the curves of the three species are not confused, (Fig. 6), showing

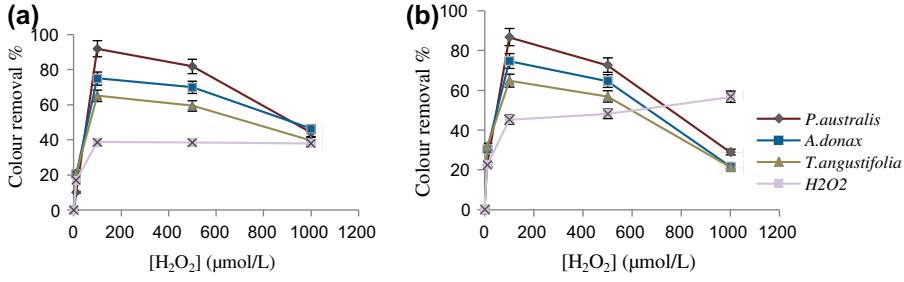

Fig. 5. Influence of H_2O_2 concentration on the decolourization of amaranth (a) and amido black (b). Reaction conditions: pH 7, temperature = 30°C, dye concentration = 0. 05 mM/L; reaction time = 120 h and load enzyme = 20 μL.

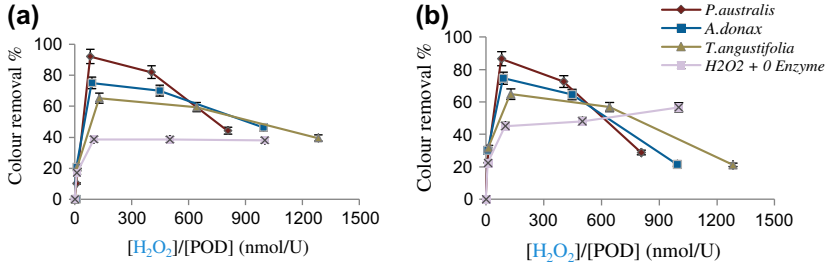

Fig. 6. Influence of $[H_2O_2]$/[POD] ratio on the decolourization of amaranth (a) and amido black (b). Reaction conditions: pH 7, temperature = 30°C, dye concentration = 0.05 mM/L; reaction time = 120 h and load enzyme = 20 μL.

that not only the concentration of peroxidase involved in the decolourization process, but it depends also strongly on the nature of the peroxidase. So H_2O_2 is acting differently with the different component of peroxidase.

3.4. Effect of enzyme concentration

To study the optimum dose of POD for enzymatic treatment of amido black and amaranth solution, experiments were carried out at various POD doses with fixed dye concentration of 0.05 mM/L and H_2O_2 concentration of 100 μM. Enzyme amounts were varied from 5 to 20 μL. The POD concentration is increased from 0 to 24.8U/mL for *P. australis*, from 0 to 22.4 U/mL for *A. donax* and from 0 to 15.6U/mL for *T. angustifolia*. As shown in Fig. 7, the increase of the decolourization of amaranth and amido black is dependent on the amount of catalyst added for fixed contact time. There is thus an optimum relationship between the concentration of enzyme and dye for achieving maximum activity. The enzyme dose was found to have significant influence on colour removal reaction. The increase in the [POD]/[Dye] ratio from 6 to 26 resulted a gradual increase in the colour removal. That was 16–92% for *P. australis* having the highest POD activity, 25–71% for *A. donax* and 21–63% for *T. angustifolia* that has the lowest POD concentration. This increase can be explained on the basis that in the initial stages, the reaction between dye and POD is quite fast and becomes slower when POD concentration increases, as there are not enough dye molecules available for the reaction. Similar trend has also been reported with chloroperoxidase-mediated degradation of Sunset Yellow dye [31]. Khataee et al. [32] reported that an increase in the efficiency is due to an increase in the number of active sites on peroxidase enzyme available for the reaction, which increases the rate of radical formation. The three curves are merged which means that, at the investigated POD/Dye ratios, the POD activity is more important than its origin (plant species).

3.5. Effect of initial dye concentration

A different initial dye concentration of amaranth and amido black (0.05–0.1 mM/L) was added to 20 μL of POD in order to explore a higher ratio of [Dye]/ [POD].

In this study, the maximum colour removal was obtained by *P. australis* peroxidase and the lower colour removal was obtained by *T. angustifolia* peroxidase.

It has been shown experimentally in Fig. 8 that in constant [POD] an increase of amido black and amaranth concentration will increase the enzymatic decolourization process for the three species. The maximum colour removal efficiency was observed in the [Dye]/ [POD] ratio in the range of 40–65. This may be attributed to the initial dye concentration that provided an important driving force to overcome all mass transfer resistances of the dye. Hence, high initial concentration of dye may enhance the process efficiency [33]. Another explanation for above observation is that increasing the initial concentration of the dye increases the probability of contact between dye molecules and enzyme. The finding was in agreement with literature reports where high initial concentration of pollutants would result in high biological decolourization efficiency [34]. After this optimum, the decolourization percentage decreases with further increase in [Dye]/[POD] ratio, due to the limited POD activity. The decrease observed on colour removal at higher dye concentration should be linked to available active sites on POD for decolourization action that is strongly dependent on POD origin.

Comparing both dyes decolourization, observed optimum is similar. However, decolourization rate is different, especially for *T. angustifolia* where higher

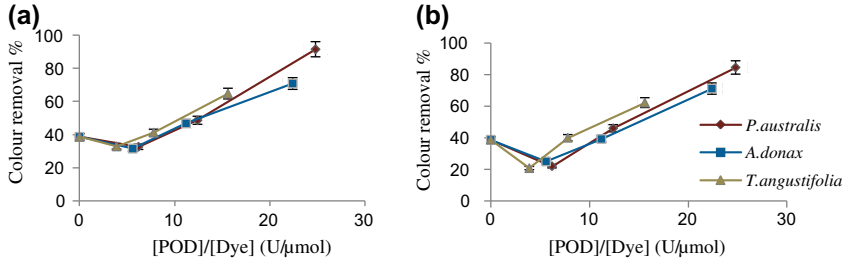

Fig. 7. Effect of POD concentration on the decolourization of amaranth (a) and amido black (b). Reaction conditions: temperature = 30°C, dye concentration = 0.05 mM/L, reaction time 120 h, pH 7, and H_2O_2 concentration = 100 μM/L.

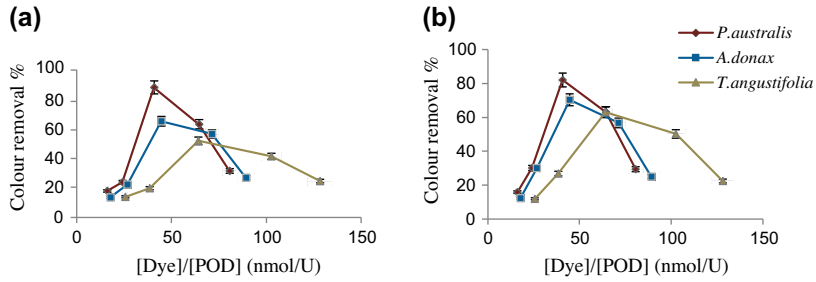

Fig. 8. Effect of dye concentration on the decolourization process. Reaction conditions: temperature = 30°C, pH 7, enzyme = 20 µL, reaction time = 120 h and H_2O_2 concentration = 100 µM/L.

efficiency is obtained for amido black. Interaction between dye functional groups and POD active sites is another important parameter to consider.

3.6. The effect of pH

The effect of initial solution pH on enzymatic decolourization of amido black and amaranth was analysed over a pH range from 4 to 10 (Fig. 9). These experiments show that the decolourization process is dependent on the pH of the solution. Since the decolourization of amaranth and amido black solutions has been identified in presence of hydrogen peroxide and in absence of POD as well, we can affirm that the increase of dye decolourization observed in these experiments is exclusively due to the peroxidase activity of these macrophytes species.

As shown in Fig. 9, in absence of enzyme, initial decolourization (30%) caused by hydrogen peroxide, increased in pH 4 for both amaranth and amido black.

In pH 4 and at fixed dye and enzyme concentration, 50–60% of the colour is removed in 120 h. However, in the pH range of 6–7, approximately 80–92% of the decolourization occurred. At alkaline pH values, decolourization decreases and drops near to 20% (pH 10). As a result, the optimum pH range for *P. australis*, *T. angustifolia* and *A. donax* for colour removal is obtained in the pH range of 6–7 and the optimum pH appears independent on dyes characteristics as pKa. These data are proved that the decolourization appropriate pH which corresponds also to POD activity of macrophytes, is in the range of 6–7 [35,36]. A similar trend of result was previously reported for the removal of Methylene Blue dye using *Lemna minor* peroxidase [32].

3.7. The efficiency removal of amido black and amaranth by P. australis, A. donax and T. angustifolia and kinetic study

In the optimum identified experimental conditions (pH 7, Temperature = 30°C, dye concentration = 0.05 mM/L, reaction time = 120 h, load enzyme = 20 µL, H_2O_2 concentration = 100 µM/L), the amido black decolourization efficiencies by *P. australis*, *A. donax* and *T. angustifolia* were, respectively, 87, 75 and 65%. For the amaranth dye, the highest percentage of decolourization is, respectively, 93, 74 and 66%, achieved after 120 h as a contact time (Fig. 10). These results support that *P. australis* peroxidase has the highest ability to

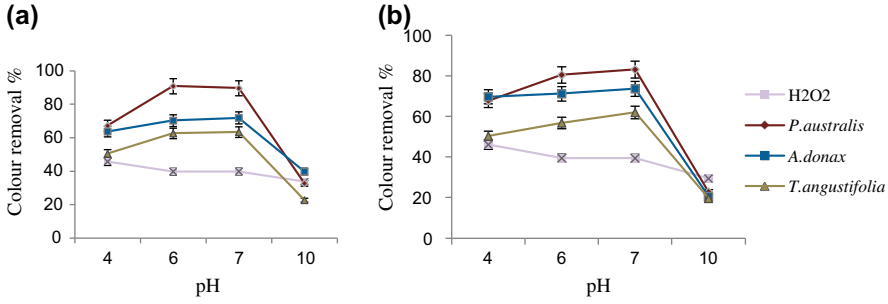

Fig. 9. Effect of pH on colour removal of amaranth (a) and amido black (b) solution. Reaction conditions: temperature = 30°C, dye concentration = 0.05 mM/L; reaction time = 120 h, load enzyme = 20 µL, and H_2O_2 concentration = 100 µM/L.

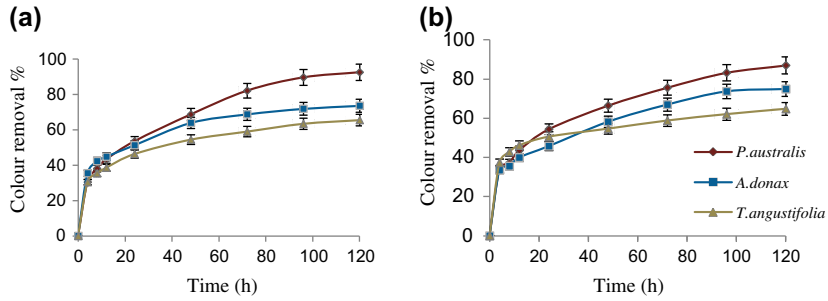

Fig. 10. Reaction progress on the decolourization of amaranth and amido black. Reaction conditions: enzyme 20 µL, dye concentration = 0.05 mM/L, temperature = 30 ℃, pH 7 and H_2O_2 concentration = 100 µM/L.

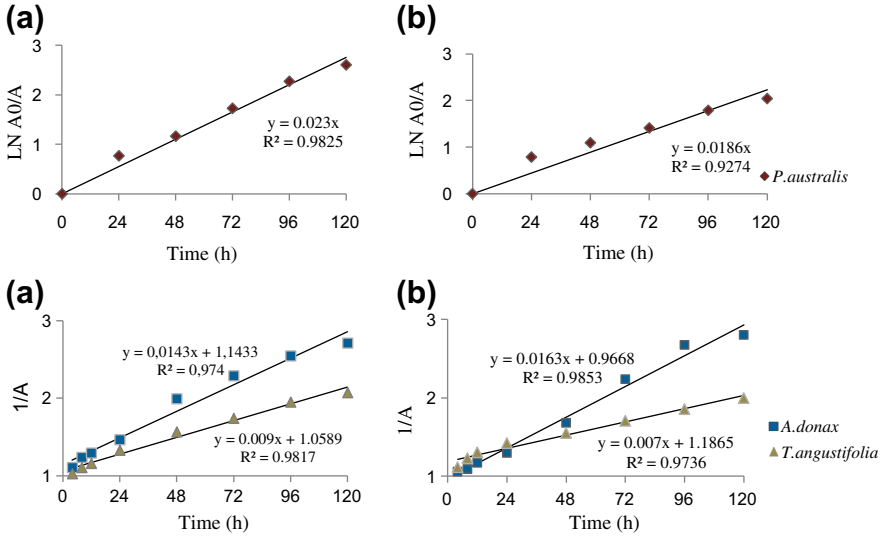

Fig. 11. Kinetic reaction for a first- and second-order in the decolourization process of amaranth and amido black by *P. australis*, *A. donax and T. angustifolia.*

Table 2
Optimum conditions for amaranth and amido black colour removal by *P. australis*, *A. donax* and *T. angustifolia*

	P. australis		*A. donax*		*T. angustifolia*	
Optimum condition for colour removal	Amaranth	Amido black	Amaranth	Amido black	Amaranth	Amido black
Temperature (℃)	30	30	30	30	30	30
PH	7	7	7	7	7	7
K	0.023*	0.019*	0.014**	0.016**	0.009**	0.007**
R^2	0.98	0.93	0.97	0.98	0.98	0.97
[H_2O_2]/[POD] (nM/U)	80.6	80.6	89.3	89.3	128.2	128.2
Colour removal (%)	93	87	75	74	65	66
[POD]/[Dye] (U/µM)	>24.8	>24.8	>22.4	>22.4	>15.6	>15.6
Colour removal (%)	93	87	75	74	65	66
[Dye]/[POD] (nM/U)	40.3	40.3	44.64	44.64	65.78	65.78
Colour removal (%)	93	87	75	74	65	66

*k (h^{-1}).
**k (h/abs).

degrade azo dyes (93% of amaranth and 87% of amido black) with slightly high efficiency with amaranth; where for the two other plants, equivalent dicolourization is obtained for the two dyes.

As seen in Fig. 11 the enzymatic decolourization process is fitting an apparent first-order kinetic reaction for *P. australis*. The kinetic apparent constants of the reaction for both amaranth and amido black is higher (0.023 and 0.019 h^{-1}) using *P. australis* POD. Several authors [37–39] have reported that colour removal obey the first-order kinetic. However, in the case of *A. donax* and *T. angustifolia*, reactions are better fitting the second-order kinetic (Fig. 11).

Halliwell [40] has referred that POD may use a large variety of electron donor substrates, so that the activation of *P. australis* POD could be linked to the ability of this enzyme to use dyes as an electron donor substrate, and thus participate in this dye oxidation and consequent degradation [41]. By analogy with the HRP catalytic cycle, the first reaction step of colour removal uses H_2O_2 as a substrate, and in the following two steps POD are able to use a wide variety of reducing substrates producing its radicals of products [42,43].

Table 2 summarizes the identified experimental conditions.

4. Conclusion

Based on the experimental results presented above, it has been found that the solution pH, the initial H_2O_2 concentration, the initial enzyme concentration, the initial dye concentration and the temperature are the main factors that have strong influences on the degradation of amido black and amaranth, by peroxidase oxidation process. The optimal operation parameters for the POD oxidation of amido black and amaranth were 100 μM/L [H_2O_2], 20 μL enzymes amount and 0.05 mM/L dye concentration in an initial pH of 7 in 30°C. Efficiency of dye decolourization is about 93% of amaranth and 87% of amido black using *P. australis* peroxidase, after 120 h of reaction time, suggesting that an enzymatic breakdown process is occurring. Therefore, *P. australis* has the highest peroxidase activity (69.92 U/g/min) to confirm the participation of these enzymes in the degradation of these azo dyes. Our results indicate that these three macrophytes could be used in bioprocesses to remove colour from effluents. However, only a better understanding of the mechanisms used by these plants will allow the development of technologies to apply these organisms to the cleaning-up of aquatic and terrestrial environments. Optimum identified ratios can be base for process design.

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