Available online at www.sciencedirect.com
ScienceDirect
Ultrasonics Sonochemistry 14 (2007) 355–362
Ultrasonics SONOCHEMISTRY
www.elsevier.com/locate/ultsonch

Stability and decolourization ability of *Trametes villosa* laccase in liquid ultrasonic fields

Carlos Basto [a], Carla Joana Silva [a], Georg Gübitz [b], Artur Cavaco-Paulo [a,*]

[a] *Department of Textile Engineering, Minho University, 4800-058 Guimarães, Portugal*
[b] *Department of Environmental Biotechnology, Graz University of Technology, Graz, Austria*

Received 4 February 2006; received in revised form 15 July 2006; accepted 18 July 2006
Available online 20 September 2006

Abstract

We report in this study that the sonication of laccase from *Trametes villosa* and bovine serum albumin promotes the formation of protein aggregates with high molecular weight. The formation of aggregates leads to the deactivation of the enzyme, fact that was confirmed by the analysis of the enzyme stability (half-life time) upon ultrasound treatment. This inactivation was mainly caused by the radicals formed by the cavitation phenomenon. It was verified that the addition of polyvinyl alcohol to laccase had a protecting effect against enzyme inactivation.

The performance of laccase in the decolourization of indigo carmine was studied. It was observed that the best results were attained when the dye solution was treated with ultrasound and enzyme stabilized with polyvinyl alcohol, where more than 65% of decolourization was achieved. This value is remarkably higher than that attained for the enzyme alone, which was only able to decolourize 20% of the dye solution within 1 h of treatment. These results have important implications for the exploitation of sonication in textile industry, where the pollution caused by the release of dyes into effluents is one of the major concerns.

Keywords: Ultrasound; Sonochemistry; Enzyme stability; Protein aggregation; Dyes decolourization

1. Introduction

The application of enzymes in the textile industry has known a vast growth over the last 20 years, being the third most significant segment of the industrial enzymes market [1]. Those textile industrial processes are heterogeneous, where the substrate are insoluble fibres while the enzyme is soluble, resulting in high and unacceptable processing times due to mass transport limitation.

It is well known that ultrasound enhances the mass transference in wet processes and thus power ultrasound appears to be a very promising alternative technique as a means of accelerating mass transfer in textile wet processes such as dyeing or scouring [2–8].

Generally the sonication of a liquid causes two primary effects, namely cavitation and heating. When microscopic cavitation bubbles collapse at the surface of the solid substrate, they generate powerful shock waves that cause effective stirring/mixing of the adjusted layer of liquid, being the effect of cavitation several hundred times greater in heterogeneous systems (e.g. all textile wet processes) than in homogeneous systems [7]. Moreover, the sudden collapse of these bubbles induces high-energy phenomena and produces reactive free radicals that are able to enhance a wide variety of chemical and physical processes with several potential applications in industrial processes [3,9,10].

The enhancement of enzymatic textile processes by the use of ultrasound technology, such as the cotton bio-scouring, was reported by Yachmenev and co-workers [6,7]. However, very little is known about the effect of ultrasound in enzymes, and many contradictory results have been observed when enzymes are treated with high-intensity

* Corresponding author. Tel.: +351 253 510271; fax: +351 253 510293.
E-mail address: artur@det.uminho.pt (A. Cavaco-Paulo).

1350-4177/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ultsonch.2006.07.005

ultrasound. Suslick and collaborators described the effect of ultrasound on some proteins (bovine serum albumin and hemoglobin), where microspheres were formed due to the chemical cross-linking of cysteine residues oxidized by the radicals produced during sonolysis [11,12]. The formation of stable proteinaceous microspheres, based on the sonochemical method, was also reported by Avivi and Gedanken [13,14]. According to a recent work, only some of the enzymes are inactivated by application of high-intensity ultrasound since ultrasonication does not denature all proteins, contrarily to heat denaturation [15]. This would explain the contradictory reports on the influence of sonication in enzyme inactivation. For example, the ultrasound treatment at 20 kHz and several powers (50, 100 and 120 W) did not change the character and selectivity of an alkaline protease from *Bacillus subtilis* in the transesterification process in non-aqueous solvents [16], but contrarily, a significant decrease in the proteolytic activity was verified upon ultrasound treatment at 26.4 kHz and 26 W/cm^2 [17]. Also, trypsin showed decreased activity with increasing ultrasound power from 100 W to 500 W at 20 kHz, where the addition of Tween 80 and mannitol had a protective effect against the inactivation caused by ultrasound [18]. De Gennaro verified that the deactivating efficiency of ultrasound on a horseradish peroxidase becomes very low with increasing power of the ultrasound waves [19]. Inactivation of glucose-6-phosphate dehydrogenase after long exposure to ultrasound was reported, but similarly treated alkaline phosphatase remained fully active [20]. Finally, an activity increase was reported for an invertase in sugar hydrolysis and acid phosphatase in soils with ultrasonication [21,22]. All these reports seem to suggest that differences in response to sonication are the result of differences in energy input, and emphasize the difficulties of comparing ultrasonic work of different researchers due to the estimation of the ultrasonic power applied.

In the present study we have used a combined enzyme-ultrasound treatment to study the potential of this technology in the decolourization of textile effluents. The effect of ultrasound on the stability of a laccase from *Trametes villosa* was also investigated by measuring the residual activity as a function of sonication energy and by analysing the ultrasound-treated enzyme by electrophoresis and size-exclusion chromatography.

2. Materials and methods

2.1. Reagents

Laccase (EC 1.10.3.2) from *T. villosa* (5.3 mg protein/ml, 600 U/ml) was kindly provided by Novozymes (Bagsvaerd, Denmark). Bovine serum albumin (BSA), terephthalic acid (TA), 2.2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS), polyvinyl alcohol (PVA) with average molecular mass of 30,000–70,000 g/mol, polyethylene glycol (PEG) with average molecular mass of 10,000 g/mol and indigo carmine (IC), CI Acid Blue 74, were all purchased from Sigma–Aldrich (St. Louis, USA). All other reagents used were of analytical grade.

2.2. Protein concentration

The total protein concentration was determined by the Bradford method, using BSA as standard [23].

2.3. Enzymatic activity

The assay mixture contained 0.5×10^{-3} M of 2.2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS), 0.1 M sodium acetate buffer pH 5.0, and a suitable amount of enzyme. Laccase activity was assayed spectrophotometrically (Helyos γ, Unicam) at 50 °C by measuring the increase of absorbance at 420 nm ($\varepsilon_{420} = 3.6 \times 10^4$ M^{-1} cm^{-1}) due to the oxidation of ABTS. The interference of PVA in the assay was verified and its contribution was found to be negligible. Enzyme activity (U) was defined as μmol of substrate oxidized per min.

2.4. Hydroxyl radicals produced by acoustic cavitation

The terephthalic acid (TA) in aqueous solution produces terephthalate anions which react with hydroxy radicals to produce highly fluorescent hydroxyterephthalate ions (HTA), whose concentration can be determined by fluorescence measurements [24]. Solutions of 500 μmol/l of TA were prepared in 0.1 M acetate buffer pH 5.0 and treated with ultrasound at the power intensities of 25, 62 and 100 W for the 20 kHz frequency, 26 and 32 W for the 150 kHz frequency and 20 and 35 W for the 500 kHz frequency, at 50 ± 2 °C and varying the time of treatment from 0 min to 30 min.

The 2-hydroxyterephthalic acid (HTA) solution was prepared following the orientations described in the work of Mason and collaborators [24]. The standard solution of HTA was prepared in 0.1 M acetate buffer pH 5.0 with an approximate concentration of 2000 μmol/l. Several dilutions of known concentration were then prepared from the mother solution. The HTA formation was estimated by measuring the fluorescence, using a spectrofluorophotometer (RF-1501, Shimadzu), at an excitation wavelength of 315 nm and an analysing wavelength of 425 nm [24]. It can be assumed that the fluorescence intensity generated by sonication is proportional to the concentration of the HTA in the samples and is thus a measure of the quantity of HO radicals released by cavitation. For that reason, a graph of fluorescence intensity against HTA concentration was plotted (see Fig. 1).

2.5. Sonolysis of protein (BSA and laccase) solutions

The protein solutions (150 ml of total volume) were prepared in 0.1 M acetate buffer pH 5.0 with a total protein content of about 125 mg/l, determined by the Bradford method [23]. The solutions were treated with ultrasound

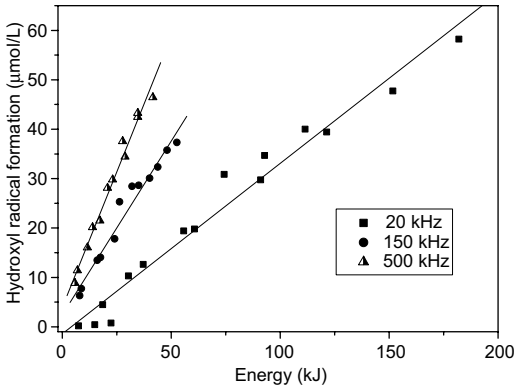

Fig. 1. Formation of OH (μmol/l) in function of the energy introduced in the system for the frequencies of 20 kHz (■), 150 kHz (●) and 500 kHz (▲).

at a power intensity of 50 W for the 20 kHz frequency, 72 W for the 150 kHz frequency and 47 W for the 500 kHz frequency, at a temperature of 50 ± 2 °C. The treated samples were then analysed by SDS–PAGE electrophoresis.

The stability of laccase upon ultrasound treatment was evaluated by determining the half-life time ($t^*_{1/2}$) of the laccase solutions, which is the time it takes for the activity to reduce to a half of the initial value. The enzymatic activity was measured at the beginning and at the end of the incubation period and the activity decay results were fitted to a first order deactivation model by using Origin 6.1 software (OriginLab Corporation, Northampton, USA). Also, the effect of the addition of 10% solutions of polyethylene glycol and polyvinyl alcohol on the stability of the enzyme was studied. A control assay (laccase treated at the same temperature of 50 ± 2 °C and 40 rpm of stirring) was run simultaneously.

2.6. SDS–PAGE electrophoresis

To determine the molecular weights of the proteins, SDS–PAGE was carried out using the Hoefer miniVe system from Amersham Pharmacia Biotech. The resolving gels (10% acrylamide of about 1.5 mm thickness) were run at a constant voltage (120 V) and prepared according to the method originally described by Laemmli [25]. The current was stopped when the bromophenol blue dye marker had reached about 1 cm from the bottom of the gel. Following electrophoresis, to observe the protein-banding pattern on the gel, silver staining was carried out. Phosphorylase b (97 kDa), Albumin (66 kDa), Ovalbumin (45 kDa), Carbonic Anydrase (30 kDa), Trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa) were used for calibration.

2.7. Size-exclusion chromatography

Analytical gel filtration chromatography was performed on a Superdex 200 HR 10/30 column (Amersham Pharmacia, Uppsala, Sweden), sample volume of 200 μl, detection at 280 nm and using a phosphate buffer (0.1 M, pH 7.0) containing 0.1 M NaCl as eluent, with a flow rate of 0.5 ml/min. Apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine albumin (66 kDa) and carbonic anydrase (29 kDa), all obtained from Sigma, were used for calibration.

2.8. Multi-frequencies ultrasound equipment

- *Ultrasound equipment at 150 kHz and 500 kHz:* the experimental set-up used was composed of an electrical generator of multifrequencies of 150 kHz and 500 kHz. The maximum intensity power, determined by the calorimetric method [24], was 72 W for 150 kHz and 47 W for 500 kHz, supplied by a transducer fixed on a reactor module. The transducer consisted of a piezoelectric ceramic provided by STIMIN (Italy), with a diameter of 60 mm that was directly attached to the bottom of a cylindrical glass cell. The diameter of the glass cell was the same as that of the transducer and the total height was 300 mm. The glass cell was not sealed, so that all experiments were conducted under air. Cold water was circulated around the cell for refrigeration.
- *Ultrasound equipment at 20 kHz:* The experimental set-up used was composed of an electrical generator of frequencies of 20 kHz and intensity powers ranging from 7 W to 100 W supplied by a piezoelectric transducer provided by Sonics & Materials (USA), with probe diameter of 13 mm. This intensity power was determined by the calorimetric method [24]. The diameter of the glass cell was 60 mm and the total height was 200 mm. The glass cell was not sealed, so that all experiments were conducted under air and cold water was circulated around the cell for refrigeration.

2.9. Decolourization of indigo carmine with ultrasound and/or enzyme

Solutions of 10 mg/l of indigo carmine (150 ml of total volume) in 0.1 M acetate buffer pH 5.0 were treated with ultrasound and/or native laccase and also with laccase stabilised with 1% of PVA. The protein concentration of the enzymatic solutions was 7 mg/l. The temperature of treatment was set at 50 ± 2 °C and the time of treatment ranged from 0 min to 60 min. The conditions for the ultrasound treatment were set at

(1) an intensity power of 50 W for the 20 kHz frequency;
(2) an intensity power of 72 W for the 150 kHz frequency;
(3) an intensity power of 47 W for the 500 kHz frequency.

The decolourization of indigo carmine was determined spectrophotometrically (Helyos y, Unicam) by measuring

the decrease of the absorbance at 598 nm, which was the maximum absorbance wavelength for this dye.

3. Results and discussion

In this study we first characterized our ultrasound reactors in terms of radicals produced and energy input (from intensity power and sonolysis time), using the methods of Mason [24], as previously described. Those relations are shown in Fig. 1 for the ultrasound frequencies tested in this study (20, 150 and 500 kHz).

Despite the different diameter of the transducers used, our data confirms the results achieved by Pétrier and co-workers [26–29], for which the greatest sonochemical efficiency in producing OH radicals was attained for the highest applied frequency. A linear relationship between the amount of radicals formed and the energy introduced into the system was achieved, whereas to the same frequency a rise in the input energy brings an increase in the radicals' concentration.

3.1. Sonolysis of aqueous laccase solutions

As stated previously, the radicals formed by cavitation are very reactive and can promote several physical and chemical reactions. The reactions promoted by them, together with the heat generated by ultrasound (cavitation phenomena) can seriously interfere with enzyme activity and stability. To evaluate that, the half-life time ($t^*_{1/2}$) for laccase treated with ultrasound was estimated, and the results are presented in Table 1. The estimated half-life time for native laccase at 50 °C was approximately 36 h, decreasing considerably upon ultrasound treatment. The half-life time decreased around 80% for laccase treated with ultrasound at 20 kHz/50 W and 500 kHz/47 W. When changing the ultrasound frequency and power to 150 kHz/72 W, the loss in $t^*_{1/2}$ was similar, namely of 82%.

The use of additives, such as surfactants, low molecular weight polyols or polymers, to increase the stability of enzymes to extreme environmental conditions is a common practice. In this study, the addition of PEG and PVA was investigated. At 50 °C, the half-life time of laccase changed only slightly by the addition of polyethylene glycol (PEG) or polyvinyl alcohol (PVA) to the laccase solution. Nevertheless, when ultrasonic irradiation was applied, the estimated half-life times of the enzymatic solutions showed a considerable increase, when comparing with the enzyme alone, after the addition of these compounds at all the ultrasound conditions tested. This increase was always higher for the solution of laccase stabilized with PVA.

Table 1
Estimated half-life times ($t^*_{1/2}$) for native laccase and laccase stabilised with 10% solutions of polyethylene glycol (PEG) and polyvinyl alcohol (PVA), upon ultrasound treatment

Half-life time $t^*_{1/2}$ (h)	Laccase	Laccase + PEG	Laccase + PVA
Control	36 ± 1	30 ± 1	39 ± 2
20 kHz–50 W	7.0 ± 0.2	14.8 ± 0.2	17.4 ± 0.7
150 kHz–72 W	6.5 ± 0.2	9.8 ± 0.5	14.4 ± 0.7
500 kHz–47 W	7.2 ± 0.1	14.4 ± 0.7	30 ± 2

These two polymers are similar in chemical composition, but their chemical structure is different [30]. PEG has been demonstrated to be particularly effective at suppressing the inactivation in chloroperoxidase and soybean peroxidase [31–33]. In what concerns laccase, the addition of PEG had a significant protective effect on the activity of laccase [34]. PVA is a known enzyme stabilizer, but surprisingly there are not many studies reporting its effects on laccase activity. In a work carried out by Kulys, laccase inactivation was prevented by the addition of PVA, according to the authors due to their globular structure [35]. It is also reported that the addition of PVA caused an increase in the activity of some enzymes [30], whose activity remained unchanged with the addition of PEG. The authors explained this difference by the hydrophobic character of these two polymers in solution: PEG molecules did not aggregate, while PVA molecules aggregated instantaneously when dissolved in water, thus creating a hydrophobic layer around the enzyme. Thus, in this study, the increased stabilizing effect of PVA over PEG might be due to their behaviour in solution, whereas for laccase stabilized with PVA a more protective environment to the ultrasound treatment was formed.

Therefore, it is our assumption that laccase deactivation under ultrasonication was due to the reaction of the protein with the radicals produced during cavitation (hydroxyl radicals, superoxide and peroxide) that may act as potential cross-linking agents promoting protein aggregation [11,12]. The assessment of the formation of laccase aggregates is considered in the following section.

3.2. Effect of ultrasound on aggregates formation

The formation of macromolecular aggregates was observed in the samples after the ultrasound treatment. An insoluble precipitate was formed in the reaction vessels (data not shown) confirming the formation of aggregates. The soluble fraction of the samples was analysed by size-exclusion chromatography and by SDS–PAGE and we have verified that big molecular aggregates were also formed, as reported subsequently. A solution of bovine serum albumin (BSA) was used to compare with the enzyme, being subjected to the same ultrasound treatment as laccase. This protein was used since it is a cheap widespread protein, with a molecular mass very close to that of the enzyme studied. Both laccase from *T. villosa* and BSA have a molecular weight around 66 kDa (see Table 2).

The protein samples were treated with ultrasound at 150 kHz and were then analysed by SDS–PAGE electrophoresis. The resultant gel of laccase and BSA treated with ultrasound is shown in Fig. 2.

The formation of aggregates of high molecular weight in the samples treated with ultrasound is seen in the electrophoresis gel at the interface of the stacking and running gel (Fig. 2). This formation is more evident when increasing

Table 2
Amount of cysteine residues for the proteins studied [37]

Protein	M_w (kDa)	No. of cysteine residues
Laccase from *T. villosa* (*Swiss-prot no. Q99044*)	63	5
Bovine serum albumin (*Swiss-prot no. PO2769*)	66	35

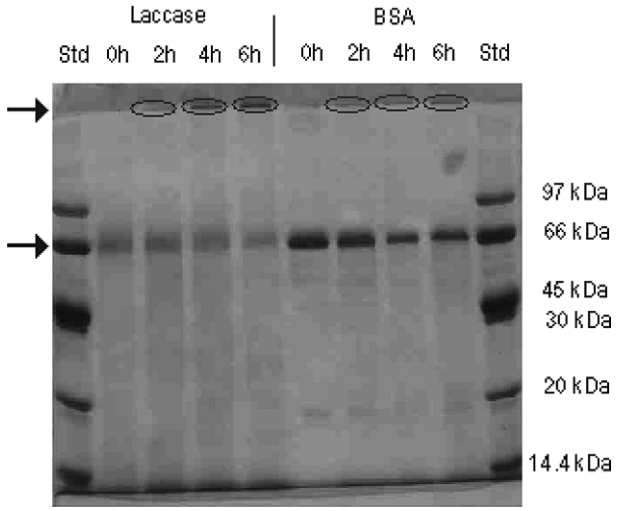

Fig. 2. SDS–PAGE electrophoresis of a solution of laccase and BSA treated with ultrasound at 150 kHz and 72 W. *Lanes*: Std – molecular mass markers, 0–6 h – sample solutions treated with ultrasound respectively from 0 h to 6 h.

the time of treatment to more than four hours. Simultaneously, a progressive decrease of the characteristic band of the native proteins of laccase and BSA is verified, while increasing the time of treatment. This fact is reinforcing the idea that high molecular weight aggregates are being formed from the native protein. These aggregates are being trapped in the interface of the stacking gel and running gel, since that due to their large dimension they cannot penetrate inside the 10% acrylamide running gel.

This result was also confirmed by the HPLC analysis of native laccase and laccase treated with ultrasound for 6 h at the same conditions. Fig. 3 shows the elution pattern of both laccase solutions.

In the above figure the formation of a peak of high molecular weight is observed at the beginning of the elution volume (around 9 ml), while the peak of native laccase is getting smaller. This result corroborates the idea that aggregates are being formed by the ultrasound treatment of the proteins.

The formation of aggregates in proteins upon ultrasound treatment was observed by several authors. According to Suslick, Gedanken and their respective co-workers, these aggregates could be produced by the exposure of the cystine residues of proteins to the reaction with the high reactive generated superoxide radicals formed in the sonication of water under air, thus leading to the formation of disulphide bonds between proteins [11–14].

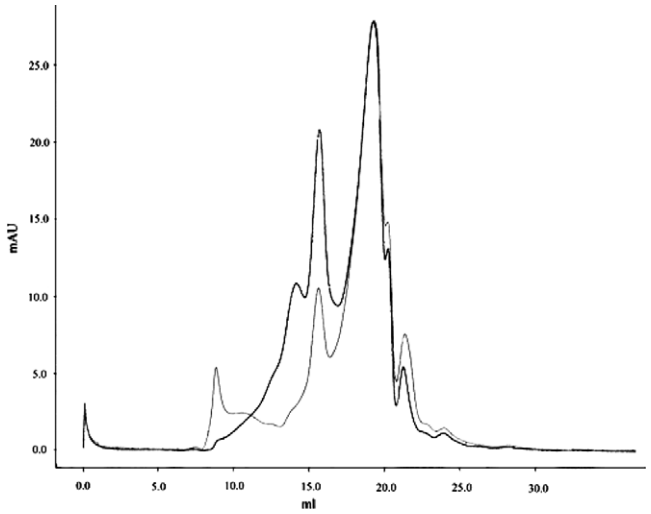

Fig. 3. HPLC profile of native laccase (dark line) and laccase treated with ultrasound at 150 kHz for 6 h (bright line).

The proteins studied in this particular work contain several cysteine residues (Table 2). It is known that during certain conditions, the tertiary structure of the proteins may change in such a way that sulfhydryl and cystine groups are exposed to the solvent and become chemically reactive. The rate of disulphide bond formation of most native proteins is low and it can be increased by unfolding the native structure with application of high pressure, elevated temperature, shear or other conditions. Thus cysteine residues and disulphide bonds that are not accessible in the native conformation can become available and may react to form intermolecular cross-links [36].

The formation of aggregates in enzymes subjected to ultrasonication was also verified by other researchers. The enzyme porcine fumarase was induced to aggregation and had loss of enzymatic activity, by the exposure to hydrogen peroxide and hydroxyl-free radicals produced by irradiation of the aqueous solution with ultrasound [38]. The enzyme inactivation was due to the formation of disulphide aggregates. The main consequence of aggregation, according to the authors, was a rapid decrease in protein solubility, producing a detectable fumarase precipitate. In another work, the inhibition of BSA aggregation was performed by adding a scavenger of OH free radicals [39]. In that particular work, BSA, a completely water soluble protein, was polymerized by the OH free radicals, which resulted in solubility loss. When L-ascorbyl-6-palmitate, an OH free radical scavenger, was added to the system, a reduction of polymerization resulted in a quantifiable decrease of water insoluble protein fraction.

3.3. Decolourization of indigo carmine

Fig. 4 shows the decolourization degree of a dye solution of indigo carmine, when treated with ultrasound and/or laccase, stabilised or not with 1% PVA.

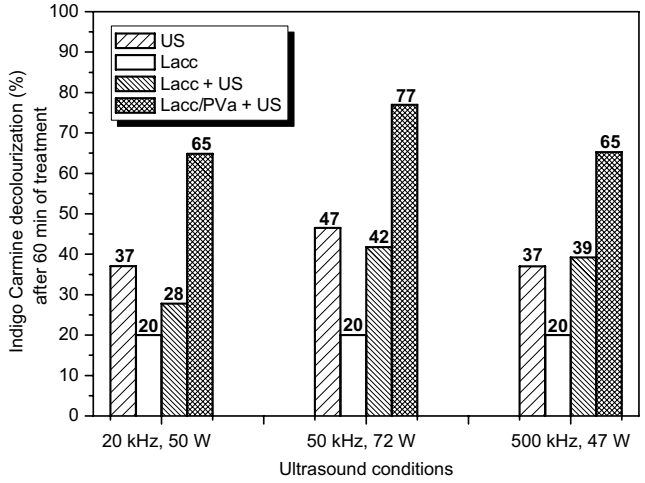

Fig. 4. Indigo carmine decolourization upon ultrasound treatment. US – treatment with ultrasound only; Lacc – treatment with laccase only; Lacc + US – treatment with ultrasound and laccase; Lacc/PVA + US – treatment with ultrasound and laccase stabilized with PVA.

The decolourization of the dye promoted by laccase, without any ultrasound treatment, was approximately 20%. This was the lowest decolourization degree attained, meaning that laccase alone was not able to completely decolourize the indigo carmine solution within 1 h of treatment. This result is in agreement with other reported studies. The complete decolourization of indigo carmine, an indigoid dye, by a laccase from *T. hirsuta* was only achieved when more than 30 h of treatment were used with the enzyme immobilized in alginate beds [40] and it took approximately 3 days when the same laccase was immobilized in stainless steel sponge [41]. It is worth to remind that no mediators were used in this study to improve the dye decolourization reaction. It is reported in a recent study that indigo carmine had a decolourization degree of 50% in 6 h by using laccase from *T. villosa*, increasing to 100% in less than 1 h when mediators were used [42]. The obvious advantage of using PVA to increase the decolourization degree is its low toxicity, comparing with the mediators.

Ultrasound treatment alone had always a higher degree of decolourization than laccase alone although its values were very close to those attained for the decolourization of indigo carmine by laccase stabilized with PVA (data not shown). This data confirms the stabilizing effect of PVA for this enzyme. The mechanism of sono-degradation of dyes involves the cavitation phenomenon and the concentration of the hydroxyl radicals in the aqueous medium [43]. According to Okitsu, the oxidative decomposition of the dyes occurs quickly by the reaction with OH radicals in the interface region of the collapsing bubbles [43]. This assumption of dye degradation by the radicals formed during cavitation is confirmed by the fact that the decolourization degree is accelerated by increasing the power output at a given frequency [44,45].

In this study, the maximum decolourization degree was attained for the 150 kHz frequency, either for the indigo carmine solution treated only with ultrasound, treated with laccase and ultrasound or treated with laccase/PVA and ultrasound. For the other two frequencies studied (20 and 500 kHz), the values attained for the decolourization degree in all these situations were lower and quite similar. The observation of an optimum frequency for the degradation of organic compounds, which is linked with its physical and chemical properties, was previously reported by Pétrier and co-workers [26–29]. According to these authors, the frequency has to be optimized for each particular compound for the reactions induced by OH radicals [26,28,29] in order to achieve the maximum sonochemical reaction rates. This optimum can be explained by considering a two step process. In the first step, H_2O and O_2 are sonolysed inside the cavitation bubble to produce the radicals and in the second step, HO and HOO radicals move to the liquid-bubble interface to react with the organic substrate or recombine with each other to form H_2O_2. The reaction rate hence depends on the number of radicals formed within the bubble and on the extent of radical release to the bulk liquid. As frequency enhances, the pulsation and collapse of the bubble occur more rapidly and more radicals escape from the bubble. However, at high frequency the acoustic period is shorter and the size of cavitation bubbles decreases. As a consequence, the cavitation threshold becomes higher and cavitation intensity decreases; thus reducing the amount of radicals which could be ejected. The optimum find at 150 kHz is a consequence of these two antagonistic phenomena. In other words, to achieve the maximum sonochemical reaction rate, there should be an optimum ultrasonic frequency for the reactions induced by OH radicals [29].

Fig. 5 shows the synergistic effect of PVA in indigo carmine decolourization as a function of ultrasound conditions. As previously stated, the decolourization ability of laccase was 20%, whereas the synergistic effect of PVA was found to be around 9% for all the cases. Thus, the higher decolourization degree found for the 150 kHz

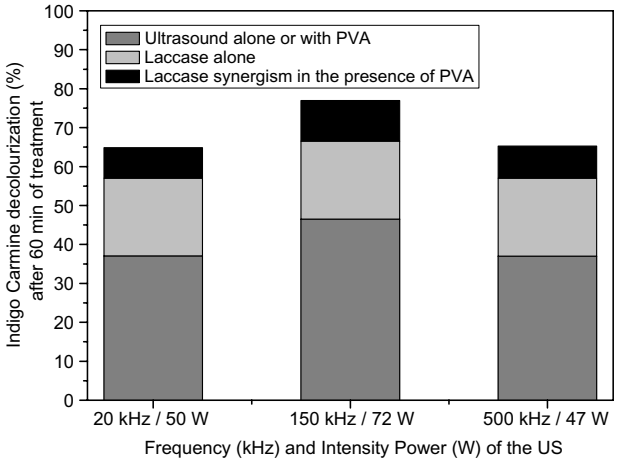

Fig. 5. Diagram showing the synergistic effect of ultrasound and laccase stabilized with PVA.

frequency can only be attributed to the higher number of radicals formed at this frequency and consequently a higher potential for dye decolourization.

These results confirm the potential of applying ultrasound technologies to decolourization systems and that there is no necessity to use mediators to improve the decolourization process of several dyes, such as indigo carmine, by laccase. The performance of laccase in ultrasound treatments can be improved additionally by stabilizing the enzyme with PVA. This may be an indication that the variability of the decolourization efficiency of the enzymatic treatments with ultrasound is also dependent on the enzyme deactivation upon ultrasonic irradiation.

4. Conclusions

The application of enzymes for textile industrial processes is gaining importance rapidly. In the last years the use of the ultrasound as a partner for the enzymatic treatments is being attempted. However, this study shows that the enzymatic activity of laccase from *T. villosa* is strongly affected upon ultrasound treatment. The deactivation of the enzyme can be explained by the exposure of cystine residues and reaction with the highly reactive radicals formed. This leads to an aggregation phenomenon, that may completely hinder the active site, while, at the same time, decreases protein stability.

The addition of polyvinyl alcohol to the medium increases the stability and the catalytic efficiency of the enzyme by minimizing ultrasound effects. Thus, the combined process of ultrasound together with enzymatic treatment can be explored successfully. The decolourization of indigo carmine was considerably higher for the combined treatment of ultrasound and laccase stabilized with PVA, where the decolourization degree ranged from 65% to 77% in only one hour of treatment. It was also verified that indigo carmine decolourization was frequency dependent, and an optimum decolourization degree was attained for the 150 kHz. This optimum can be explained by two antagonistic phenomena: the production and release of radicals to the reaction media.

Acknowledgements

The authors acknowledge financial support from FCT (Portugal) via Ph.D. fellowship, ref. SFRH/BD/8651/2002 and EU funding for ULTRATEC project (NMP2-CT-2003-505892).

References

[1] P. Sørup, C. Tils, O. Wolf, IPTS study, in: P. Sørup, C. Tils, O. Wolf (Eds.), Biocatalysis: state of the art in Europe, Institute for prospective technological studies (IPTS), Sevilla, Spain, 1998, p. 61.
[2] S. Vajnhandl, A.M. Le Marechal, Ultrasound in textile dyeing and the decolourization/mineralization of textile dyes, Dyes and Pigments 65 (2005) 89–101.
[3] V. Sivakumar, P.G. Rao, Studies on the use of power ultrasound in leather dyeing, Ultrasonics Sonochemistry 10 (2003) 85–94.
[4] M.M. Kamel, R.M. El-Shishtawy, B.M. Yussef, H. Mashaly, Ultrasonic assisted dyeing: III. Dyeing of wool with lac as a natural dye, Dyes and Pigments 65 (2005) 103–110.
[5] M.M. Kamel, R.M. El-Shishtawy, H.L. Hanna, N.S.E. Ahmed, Ultrasonic-assisted dyeing: I. Nylon dyeability with reactive dyes, Polymer International 52 (2003) 373–380.
[6] V.G. Yachmenev, N.R. Bertoniere, E.J. Blanchard, Intensification of the bio-processing of cotton textiles by combined enzyme/ultrasound treatment, Journal of Chemical Technology and Biotechnology 77 (2002) 559–567.
[7] V.G. Yachmenev, E.J. Blanchard, A.H. Lambert, Use of ultrasonic energy for intensification of the bio-preparation of greige cotton, Ultrasonics 42 (2004) 87–91.
[8] V.S. Moholkar, V.A. Nierstrasz, M.M.C.G. Warmoeskerken, Intensification of mass transfer in wet textile processes by power ultrasound, AUTEX Research Journal 3 (3) (2003) 129–138.
[9] V.S. Moholkar, M.M.C.G. Warmoeskerken, Mechanism of mass-transfer enhancement in textiles by ultrasound, AIChE Journal 50 (1) (2004) 58–64.
[10] C. Gong, D.P. Hart, Ultrasound induced cavitation and sonochemical yields, Journal of the Acoustical Society of America 104 (1998) 1–16.
[11] K.S. Suslick, M.W. Grinstaff, Protein microencapsulation of non-aqueous liquids, Journal of the American Chemical Society 112 (1990) 7807–7809.
[12] M. Wong, K.S. Suslick, Sonochemically produced hemoglobin microbubbles, Materials Research Society Symposium 372 (1995) 89–94.
[13] S. Avivi, A. Gedanken, S–S bonds are not required for the sonochemical formation of proteinaceous microspheres: the case of streptavidin, Biochemical Journal 366 (2002) 705–707.
[14] S. Avivi, A. Gedanken, The preparation of avidin microspheres using the sonochemical method and the interaction of the microspheres with biotin, Ultrasonics Sonochemistry 12 (2005) 405–409.
[15] D. Güzey, I. Gülseren, B. Bruce, J. Weiss, Interfacial properties and structural conformation of thermosonicated bovine serum albumin, Food Hydrocolloids 20 (2006) 669–677.
[16] Y. Xiao, Q. Wu, Y. Cai, X. Lin, Ultrasound-accelerated enzymatic synthesis of sugar esters in nonaqueous solvents, Carbohydrate Research 340 (2005) 2097–2103.
[17] S.L. Ovsianko, E.A. Chernyavsky, V.T. Minchenya, I.E. Adzerikho, V.M. Shkumatov, Effect of ultrasound on activation of serine protease precursors, Ultrasonics Sonochemistry 12 (2005) 219–223.
[18] Z.M. Tian, M.X. Wan, S.P. Wang, J.Q. Kang, Effects of ultrasound and additives on the function and structure of trypsin, Ultrasonics Sonochemistry 11 (2004) 399–404.
[19] L. De Gennaro, S. Cavella, R. Romano, P. Masi, The use of ultrasound in food technology I: Inactivation of peroxidase by thermosonication, Journal of Food Engineering 39 (1999) 401–407.
[20] B. Özbek, K.Ö. Ülgen, The stability of enzymes after sonication, Process Biochemistry 35 (2000) 1037–1043.
[21] S. Barton, C. Bullock, D. Weir, The effects of ultrasound on the activities of some glycosidase enzymes of industrial importance, Enzyme and Microbial Technology 18 (1996) 190–194.
[22] F. De Cesare, A.M.V. Garzillo, V. Buonocore, L. Badalucco, Use of sonication for measuring acid phosphatase activity in soil, Soil Biology & Biochemistry 32 (2000) 825–832.
[23] M.M. Bradford, A rapid and sensitive method for the quantisation of microgram quantities of protein utilizing the principle of protein-dye binding, Analytical Biochemistry 72 (1976) 248–254.
[24] T.J. Mason, J.P. Lorimer, D.M. Bates, Y. Zhao, Dosimetry in sonochemistry: The use of aqueous terephthalate ion as a fluorescence monitor, Ultrasonics Sonochemistry 1 (2) (1994) 91–95.
[25] U.K. Laemmli, Cleavage of structural proteins during assembly of head of bacteriofage-T4, Nature 227 (1970) 680–685.
[26] A. Francony, C. Pétrier, Sonochemical degradation of carbon tetrachloride in aqueous solution at two frequencies: 20 kHz and 500 kHz, Ultrasonics Sonochemistry 3 (1996) 77–82.

[27] C. Pétrier, A. Francony, Ultrasonic waste-water treatment: Incidence of ultrasonic frequency on the rate of phenol and carbon tetrachloride degradation, Ultrasonics Sonochemistry 4 (1997) 295–300.
[28] C. Pétrier, B. David, S. Laguian, Ultrasonic degradation at 20 kHz and 500 kHz of atrazine and pentachlorophenol in aqueous solution: preliminary results, Chemosphere 32 (9) (1996) 1709–1718.
[29] Y. Jiang, C. Pétrier, T.D. Waite, Sonolysis of 4-chlorophenol in aqueous solution: Effects of substrate concentration, aqueous temperature and ultrasonic frequency, Ultrasonics Sonochemistry 13 (2006) 415–422.
[30] T. Takada, T. Hirai, The PVA solution structure-change effect for α-amylase specific activation, Polymer Bulletin 53 (2004) 63–71.
[31] N. Spreti, R. Germani, A. Incani, G. Savelli, Stabilization of chloroperoxidase by polyethylene glycols in aqueous media: kinetic studies and synthetic applications, Biotechnology Progress 20 (1) (2004) 96–101.
[32] C. Kinsley, J.A. Nicell, Treatment of aqueous phenol with soybean peroxidase in the presence of polyethylene glycol, Bioresource Technology 73 (2000) 139–146.
[33] N. Caza, J.K. Bewtra, N. Biswas, K.E. Taylor, Removal of phenolic compounds from synthetic wastewater using soybean peroxidase, Water Research 33 (13) (1999) 3012–3018.
[34] K. Modaressi, K.E. Taylor, J.K. Bewtra, N. Biswas, Laccase-catalysed removal of bisphenol-A from water: protective effect of PEG on enzyme activity, Water Research 39 (2005) 4309–4316.
[35] J. Kulys, R. Vidziunaite, P. Schneider, Laccase-catalysed oxidation of naphthol in the presence of soluble polymers, Enzyme and Microbial Technology 32 (2003) 455–463.
[36] R.W. Visschers, H.H.J. de Jongh, Disulphide bond formation in food protein aggregation and gelation, Biotechnology Advances 23 (2005) 75–80.
[37] www.expasy.org/sprot.
[38] M. Barteri, M. Diociaiuti, A. Pala, S. Rotella, Low frequency ultrasound induces aggregation of porcine fumarase by free radicals production, Biophysical Chemistry 111 (2004) 35–42.
[39] N. Perricone, K. Nagy, F. Horváth, G. Dajkó, I. Uray, I.Z. Nagy, The hydroxyl free radical reactions of ascorbyl palmitate as measured in various in vitro models, Biochemical and Biophysical Research Communications 262 (1999) 661–665.
[40] A. Dominguez, S.R. Couto, M.A. Sanromán, Dye decolourization by *Trametes hirsuta* immobilized into alginate beads, World Journal of Microbiology & Biotechnology 21 (2005) 405–409.
[41] S.R. Couto, M.A. Sanromán, D. Hofer, G.M. Gübitz, Stainless steel sponge: A novel carrier for the immobilisation of the white-rot fungus *Trametes hirsuta* for decolourization of textile dyes, Bioresource Technology 95 (2004) 67–72.
[42] S. Camarero, D. Ibarra, M.J. Martínez, A.T. Martínez, Lignin-derived compounds as efficient laccase mediators for decolorization of different types of recalcitrant dyes, Applied and Environmental Microbiology 71 (4) (2005) 1775–1784.
[43] K. Okitsu, K. Iwasaki, Y. Yobiko, H. Bandow, R. Nishimura, Y. Maeda, Sonochemical degradation of azo dyes in aqueous solution: A new heterogeneous kinetics model taking into account the local concentration of OH radicals and azo dyes, Ultrasonics Sonochemistry 12 (2005) 255–262.
[44] G. Tezcanli-Güyer, N.H. Ince, Individual combined effects of ultrasound, ozone and UV irradiation: a case study with textile dyes, Ultrasonics 42 (2004) 603–609.
[45] A. Rehorek, M. Tauber, G. Gübitz, Application of power ultrasound for azo dye degradation, Ultrasonics Sonochemistry 11 (2004) 177–182.