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Lignin peroxidase efficiency for methylene blue decolouration: Comparison to reported methods

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Abstract

The oxidative potential and low specificity of peroxidases are distinctive regarding their efficiency for recalcitrant compounds degradation. However, the usefulness of these biocatalysts for environmental biocatalysis needs a stepwise investigation on the reaction conditions that would render these biocatalysts both efficient and cost effective. In a recent work we compared the usefulness of the fungal lignin peroxidase (LiP) to that observed for the plant horseradish peroxidase (HRP) concerning the degradation of methylene blue (MB) and of its demethylated derivatives. We showed that although both enzymes are able to oxidize MB and its derivatives, HRP reactions require higher H_2O_2 concentrations, present a considerably lower reaction rate, and contrary to LiP, HRP is unable to achieve aromatic ring cleavage. The oxidation potential of LiP is roughly double than that of less effective HRP (~0.7 V) and this explains relative efficacy. Thus, lignin peroxidase would be more suitable for phenothyazine dyes degradation and colour removal from waste streams. The present work shows that the use of LiP for the decolouration of MB is competitive in comparison to the majority of the reported methods, regarding reaction time, range of substrate concentration and removal efficiency. In reaction mixtures containing 50 mg/L methylene blue and carried out at 30 °C the dye was degraded within 30 min. Reaction conditions were optimized concerning H_2O_2 addition mode to avoid the inactivation of the enzyme by H_2O_2 excess, the enzyme concentration to minimize cost, and the reaction temperature. Results indicated that the use of an MB:H_2O_2 molar ratio of 1:5 resulted in efficient removal of 90% colour in reactions with MB concentrations up to 50 mg/mL. The enzyme stability was not affected by peroxide concentration up to 990 μM and an LiP:H_2O_2 molar ratio up to 1:900. The stepwise addition of the peroxide extended the possibility of using total peroxide concentrations up to 1980 μM. Lignin peroxidase was stable up to 60 °C.

Keywords: Methylene blue; Lignin peroxidase; Dye oxidation; Dye degradation; Dye removal; Environmental biocatalysis

1. Introduction

Although the use of enzymes in waste treatments was first proposed in the 1930s only as late as in the 1970s the concept of Environmental Biocatalysis, i.e., the applications of enzymes to destroy target pollutants, was established [1]. The remarkable and desirable enzyme characteristics, such as its efficiency, that are responsible for its effectiveness for pollutants degradation, have encouraged the study of different enzymes, from microbial and plant sources, for industrial waste treatment [2,3]. Enzymes may transform pollutants to diminish their toxicity, to increase water solubility allowing their further microbial degradation or to promote insolubility and its subsequent removal from the industrial waste stream. Surely biocatalyst's stability and cost hinder their industrial use. However, the production cost of bulk microbial enzymes can be considerably diminished by the current molecular biology tools. Moreover the use of immobilized enzymes can extend efficiency and lifetime and reduce

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costs [4]. The interest in environmental biocatalysis and the study of new enzymes have grown in the last two decades due to the increasing rate of xenobiotic introduction into the environment, whose degradation to standard levels is a challenge to the majority of the conventional chemical or biological processes. Moreover there is a need to remove target pollutants from industrial wastewater in the range of ppm or ppb due to the increasing growth of industries and urban areas. Table 1, that summarizes industrial sources of pollution, pollutants and enzymes with potential use for waste treatment, indicates that the enzymes, oxidases and peroxidases can degrade four out of the seven listed pollutant categories [2,5]. The obvious interest for environmental biocatalysis would come from the industries that use dyes as around 15% of these compounds are lost in the colouring process and these industries struggle to avoid environmental contamination [6]. Moreover, as the required characteristics for industrial dyes are resistance to light, temperature, wash and microbial attack, these substances are highly recalcitrant. The need to remove dyes is even more imperative as some of them are also mutagenic and carcinogenic [7]. In addition, the discharge of coloured substances in receptor water bodies blocks the sun light absorption harmfully interfering in the ecosystem balance. More than 700,000 tons of dyes and pigments are produced annually worldwide presenting more than 10,000 different chemical structures [8]. As far as Brazil is concerned around 27,000 tons of dyes are industrially used per year [9], whose waste discharge in the environment significantly damages unique and priceless ecosystems. Colour removal from the colouring baths has been studied using adsorption, oxidation, reduction, electrochemistry and tangential filtration [10]. Table 2 lists the advantages and disadvantages of current methods used for dye removal from industrial effluents and includes biocatalysis as an alternative for dyes degradation [11,12]. Many works reported dye degradation by using oxidases and peroxidases or microorganisms that produces oxidative enzymes. Different chromophore structures such as heterocyclic, polymeric, triphenylmethane, phthalocyanin, antraquinones, indigo and azo were degraded by the reported systems [13–32].

Studies related to dye resistance to biodegradation and removal have used methylene blue as a model substance [33]. Previous works, from our laboratories, have studied the reaction mechanism, the kinetics of methylene blue oxidation and the selective effect of the substrate:H_2O_2 stoichiometry on the *N*-demethylation or degradation reactions [12,34]. The mechanistic approach is presented in Fig. 1 [34]. In the present study we have pursued the understanding of methylene blue degradation and decolouration by lignin peroxidase through a stepwise evaluation of reaction conditions and using higher substrate concentrations. Reactions were performed at different temperatures, and reaction mixtures presented different enzyme and H_2O_2 concentration being the peroxide addition mode also evaluated. The results that were obtained

Table 1
Enzymes applicable to industrial pollutants degradation or removal [2,5]

Pollutants	Industrial sources of pollution	Enzymes
Anilines, phenols, dyes, PCBS, PAHS	Chemical industry, oil refining, textile industry	■ Peroxidases ■ Tyrosinase ■ Laccase
Pulp and paper wastes	Pulp and paper industry	■ Peroxidases ■ Laccase ■ Cellulases
Pesticides	Agricultural activities	■ Parathion hydrolase or phosphotriesterase ■ Peroxidases
	Chemical and pharmaceutical industries	■ Cyanidase
Cyanide	Coal processing and metal plating	■ Cyanide hydratase
Food processing wastes	Food processing industry (milk derivatives, meat, poultry and fish processing, starchy material)	■ Proteases ■ Amylases ■ Pectinesterase ■ Lactase ■ Chitinase ■ Pectinase ■ Lipase
Solid waste and sludge	Cellulosic and lignocellulosic processing industries. Municipal solid wastes	■ Ligninases ■ Lipase ■ Lysozima ■ Cellulase
Heavy metal	Industrial activities and mining	■ Phoshatases

Table 2
Current methods used for dye removal from industrial effluents [11,12]

Methods	Advantages	Disadvantages
Fenton's reagent	Effective decolourisation of both soluble and insoluble dyes	Sludge generation
Ozonation	Applied in gaseous state: no alteration of volume	Short half-life (20 min)
Photochemical	No sludge production	Formation of by-products
NaOCl	Initiates and accelerates azo-bond cleavage	Release of aromatic amines
Electrochemical destruction	Breakdown compounds are non-hazardous	High cost of electricity
Activated carbon	Good removal of wide variety of dyes	Very expensive
Silica gel	Effective for basic dye removal	Side reactions prevent commercial application
Membrane filtration	Removes all dye types	Concentrated sludge production
Ion exchange	Regeneration: no adsorbent loss	Not effective for all dyes
Irradiation	Effective oxidation at lab scale	Requires a lot of dissolved O_2
Electrokinetic coagulation	Economically feasible	High sludge production
Biocatalysis	Destruction of contaminant	High cost of biocatalyst

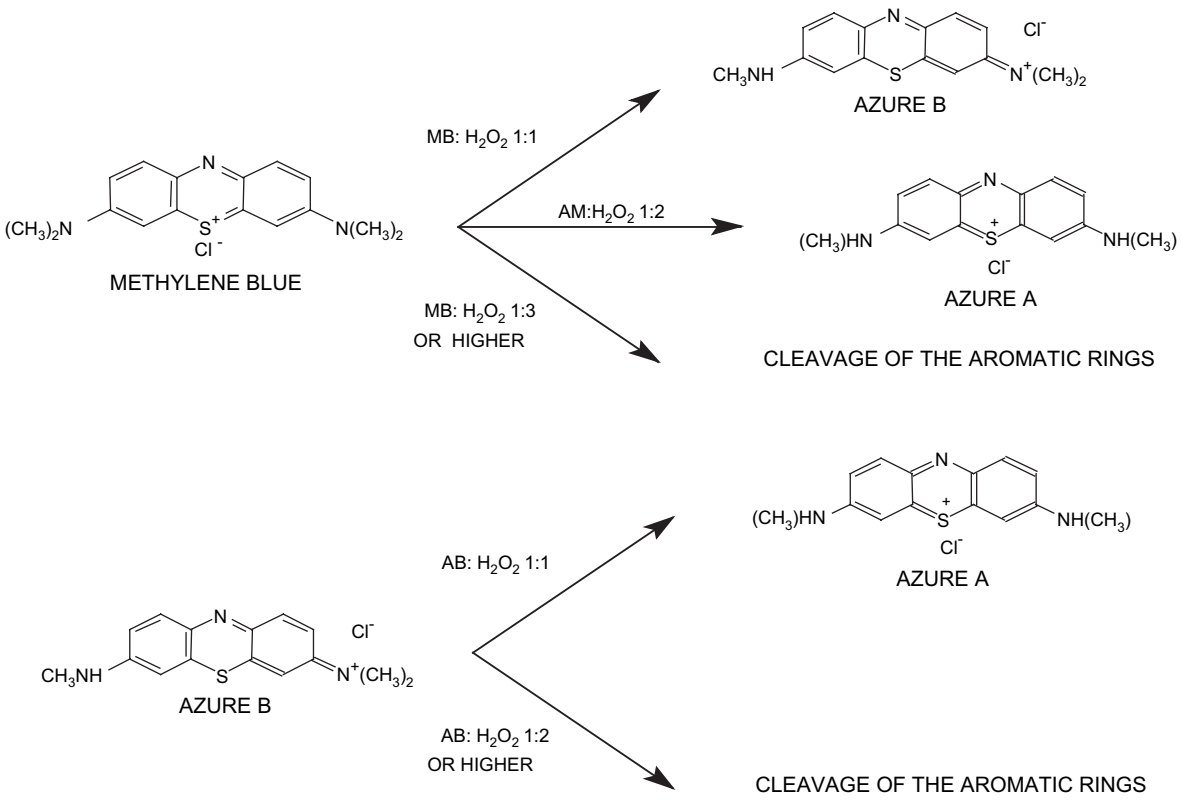

Fig. 1. Dye:H_2O_2 stoichiometry for the *N*-demethylation and aromatic ring cleavage of methylene blue (MB) and azure B by *Phanerochaete chrysosporium* lignin peroxidase [12,34].

were compared to reported methods for methylene blue removal or degradation.

2. Experimental

2.1. Lignin peroxidase source and activity assay

The enzyme was produced by *Phanerochaete chrysosporium* immobilized cells as previously described [35]. The crude lignin peroxidase preparation (culture supernatant) was dialysed for 48 h against deionised water before use. Peroxidase concentration was estimated using $\varepsilon_{403} = 102\ \mathrm{mM^{-1}\,cm^{-1}}$ and lignin peroxidase activity was assayed through the oxidation of veratryl alcohol to form veratraldehyde, which was determined at 310 nm ($\varepsilon_{310} = 9200\ \mathrm{M^{-1}}$) [36]. The crude enzyme activity was of 240 U/L.

2.2. Effect of temperature on methylene blue oxidation by LiP

Reactions were carried out in sodium tartarate buffer 0.2 M at pH 4.0 containing 8 μM MB (2.4 mg/L), 80 μM H_2O_2 (molar ratio MB:H_2O_2 1:10) and 1.1 μM of LiP (0.176 U/mL). Reaction mixtures were incubated at 30 °C, 37 °C, 45 °C and 60 °C and started by H_2O_2 addition. MB consumption was followed at 670 nm (MB λ_{max}) using a spectrophotometer Multispec 1501-Shimadzu.

2.3. Effect of MB concentration, MB:H_2O_2 molar ratios, H_2O_2 addition mode and LiP concentration

Reaction mixtures containing LiP 1.1 μM (0.176 U/mL) and methylene blue 10 mg/L, 30 mg/L or 50 mg/L were performed using different substrate:H_2O_2 molar ratios, 1:5, 1:10 and 1:20 and were incubated for 30 min at 30 °C. Reactions were also carried out by multiple H_2O_2 additions within 5 min intervals, amounting the same final substrate:peroxide molar ratios. MB consumption was followed as already described. Control experiments were performed using reaction media without LiP or H_2O_2 or containing thermally inactivated enzyme. Enzyme load was evaluated in reaction media presenting LiP concentrations ranging from 0.176 to 0.0176 U/mL and methylene blue at 10 mg/L, 30 mg/L and 50 mg/L (final molar ratio MB:H_2O_2 1:5), using multiple H_2O_2 additions.

2.4. HPLC analyses

The formation of MB *N*-demethylated coloured derivatives was monitored by HPLC. Reaction media aliquots of 1 mL were dried using a Speed Vac Plus 110C-Savant. Analysis of the reaction products was performed using a reverse phase C-18 column (7.8 × 300 mm, μbondpack), Waters pump and controller model 600, Waters detector UV–VIS 486 and Waters integrator 746. Elution was performed using a linear gradient of trifluoroacetic acid 0.1% and aqueous 0.07% trifluoroacetic acid in 80% acetonitrile. Solvent flow rate

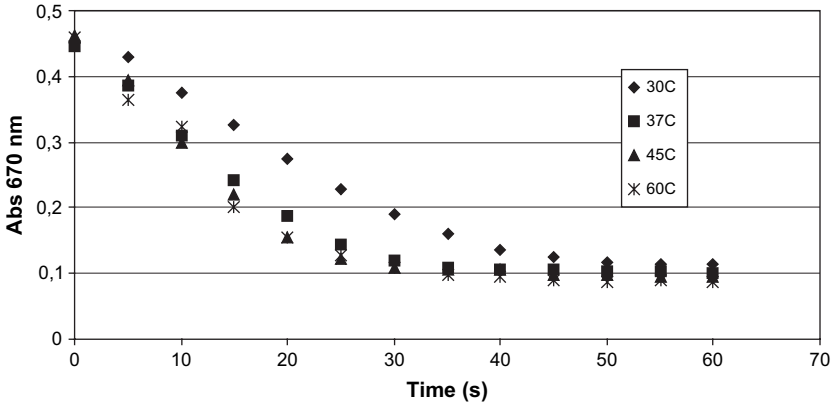

Fig. 2. Progress curves of methylene blue oxidation by lignin peroxidase from *Phanerochaete chrysosporium* at different temperatures using MB:H_2O_2 molar ratio of 1:10.

was set to 3.0 mL/min and elution was monitored at 600 nm. Methylene blue and *N*-demethylated derivatives were used as standards (Sigma Chemical Co).

3. Results and discussion

3.1. Effect of temperature on methylene blue oxidation by LiP

According to Fig. 2, that shows MB oxidation kinetics, reaction takes place within 50 s at 30 °C and within 40 s at 37 °C, 45 °C or 60 °C. Equivalent substrate consumption levels around 80%, as monitored by absorbance decrease at 670 nm, were observed in all cases showing that the enzyme was stable up to 60 °C, within the reaction time interval. As absorbance decrease at 30 °C was similar to that observed at higher temperatures, and as the reaction time difference was negligible, further work was performed at 30 °C. Lower temperatures would favour the energy consumption parameter in large-scale applications. No MB oxidation was observed in control experiments performed in the absence of either LiP or H_2O_2 or using a thermal inactivated LiP, indicating that MB degradation resulted solely from LiP biocatalysis.

3.2. Effect of MB concentration, MB:H_2O_2 molar ratios, H_2O_2 addition mode and LiP concentration on MB degradation

Fig. 3A shows the percentage for colour removal after single addition of H_2O_2 in reactions with LiP 0.176 U/mL (1.1 μM), MB 10 mg/L (33 μM), 30 mg/L (99 μM) and 50 mg/L (165 μM) and presenting MB:H_2O_2 molar ratios of 1:5, 1:10 and 1:20 for each MB concentration. As such MB concentrations ranged fivefold, peroxide concentrations ranged 20-fold, from 165 μM (in reaction medium presenting MB 10 mg/L and MB:H_2O_2 molar ratios of 1:5) to 3300 μM (in reaction medium presenting MB 50 mg/L and MB:H_2O_2 molar ratios of 1:20), and by extension the LiP:H_2O_2 molar ratios also ranged 20-fold, from 1:150 to 1:3000 (Fig. 4). The use of an MB:H_2O_2 molar ratio of 1:5 was efficient to remove 90% colour in reactions with MB concentrations up to 50 mg/mL (165 μM) and peroxide 825 μM. The same colour removal was obtained for the MB:H_2O_2 molar ratio of 1:10 for the MB concentration 10 mg/mL (33 μM), 330 μM peroxide and for the ratio 1:20 for the concentration 10 mg/mL (33 μM), 660 μM peroxide. Reaction media containing peroxide concentrations of 1650 μM (LiP:H_2O_2 molar ratio of 1:1500), 1980 μM (LiP:H_2O_2 molar ratio of 1800) and 3300 μM (LiP:H_2O_2 molar ratio of 3000) resulted in enzyme inactivation as colour removal was severely affected. These data collectively indicate that the threshold peroxide concentration and LiP:H_2O_2 molar ratio, to LiP stability, would be higher than 825 μM and 1:750, and lower than 1650 μM and 1:500, respectively. The media composition presenting MB 30 mg/L (99 μM) and MB:H_2O_2 1:10 (peroxide concentration of 990 μM) and that slightly affect LiP stability (colour

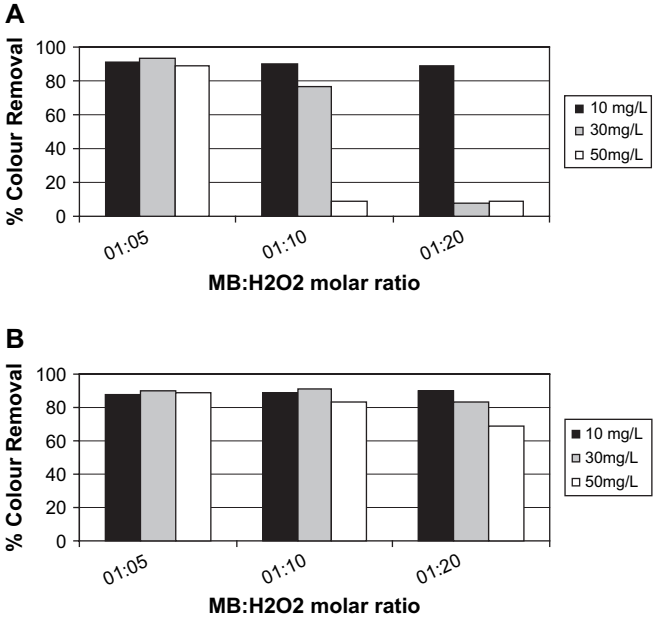

Fig. 3. Percentage of methylene blue colour removal by lignin peroxidase from *Phanerochaete chrysosporium*. (A) H_2O_2 single addition, (B) H_2O_2 stepwise addition. Reaction conditions according to text.

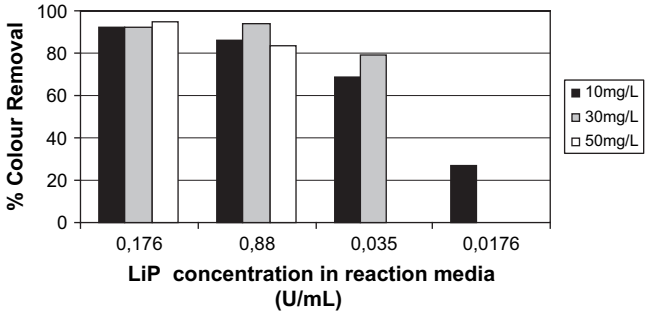

Fig. 4. Percentage of methylene blue colour removal by lignin peroxidase from *Phanerochaete chrysosporium*. The figure shows results from reaction mixtures containing MB:H_2O_2 molar ratio of 1:5 and decreasing lignin peroxidase concentrations.

removal around 70%) narrowed this twofold gap to a likely threshold peroxide concentration around 990 μM and an LiP:H_2O_2 molar ratio of 1:900. Our results regarding peroxidases' susceptibility to higher H_2O_2 concentrations are corroborated by previous findings [34,37–39].

Studies related to H_2O_2 stepwise addition (Fig. 3B) extended the enzyme tolerance to the total peroxide addition to 1980 μM and the LiP:H_2O_2 molar ratio to 1:800 (Fig. 3B). This procedure is quite useful when high H_2O_2 concentrations are required for the degradation of higher dye concentrations or when a higher stoichiometry, substrate:peroxide is needed for chemical bond cleavage [39]. Noticeably, LiP was still able to perform in reaction medium with a final peroxide concentration of 3300 μM.

The study of reactions presenting LiP concentrations lower than 0.176 U/mL was performed using pulse peroxide addition and an MB:H_2O_2 molar ratio of 1:5 that, according to previous results, was effective for dye degradation (Fig. 3A). In reaction media with 0.088 U/L (0.55 μM) it was observed around 90% colour removal, for all three MB concentrations, in reaction media presenting up to 825 μM peroxide and LiP:H_2O_2 molar ratios up of 1:500, in accordance to results from previous experiments. Further enzyme dilution (0.0352 U/L, 0.22 μM) resulted in decrease in the degradation of 33 μM MB (LiP:H_2O_2 molar ratio 1:750) and 99 μM (LiP:H_2O_2 molar ratio 1:2250) or a complete arrest of colour removal for MB 165 μM (LiP:H_2O_2 molar ratio 1:3750). Very poor or lack of the dye degradation was observed in reaction media presenting LiP 0.0176 U/mL.

4. Discussion and conclusions

Table 3 reports methodologies for methylene blue removal or degradation. Removal using adsorption was performed with the mineral kaolinite (raw kaolin, pure kaolin, calcined raw kaolin, calcined pure kaolin, NaOH-treated raw kaolin, NaOH-treated pure kaolin). It was observed that kaolinite clay, that presented relatively large adsorption capacity, was quite effective for the basic dye, methylene blue removal even at low concentration (15 mg/L) [40]. This system, however, uses a quite alkaline pH (8.0–10.0). Other adsorbents that showed high adsorption capacity up to 900 mg/L include the commercially activated carbons and indigenously prepared activated carbons from bamboo dust, coconut shell, groundnut shell (GNSC), rice rusk (RHC) and straw (SC), whose relative adsorption capacity is as follows: CAC > SC > RHC > CSC > GNSC > BDC. Kannan and Sundaram concluded that IPACS (indigenously prepared activated carbons) could be employed as low-cost adsorbents, being five times cheaper than CACs (commercially activated carbons) [39]. Nevertheless their adsorption capacity, effectiveness and low cost, these adsorption methods do not convey degradation of the pollutant. Thus additional procedures are necessary for its final disposal. On the other hand, chemical oxidation is very efficient to cleave and destroy the dyes' native structure. The oxidative mineralization of MB with H_2O_2 was studied using supported alumina catalysts: copper(II), cobalt(II), manganese(II), and nickel(II) ions [10]. The authors observed that the rate of colour removal depended on the concentration of reactants, pH, ionic strength and surfactant concentration and also that the supported catalysts were very stable and could be reused. This process, however, requires low MB concentrations and a quite high MB:H_2O_2 ratio (1:52), being quite difficult to control the final products in these reactions.

Considering advanced oxidation process (AOP), heterogeneous photocatalysis appears as an emerging destructive technology leading to the total mineralization of a great variety of organic pollutants. Houas and co-workers [6] who used MB as a model to establish the degradation pathway in coloured aqueous media, observed that MB could be successfully decolorized and degraded by titanium-based photocatalysis at room temperature. Other experiments using solar pilot devices

Table 3
Methodologies applied for methylene blue removal/degradation

Methodology	Concentration range (mg/L)	Temperature (°C)	pH	Time reaction (min)	MB:H_2O_2	Reference
Biocatalysis (LiP)	10–50	30	4.0	30	1:5	Present work
Metals supported on alumina (Cu, Co, Mn, Ni)	8	30	7–10	240	1:52	[10]
Adsorption on kaolinite	15	27	8.0–10.0	180	–	[40]
Activated carbon (groundnut shell, coconut shell, bamboo dust, rice husk, straw)	100–900	30	7.2	35	–	[41]
Photocatalyzed solar light/TiO_2	1.5–10	25–35	7.0	360	–	[42]
Photocatalyzed UV/TiO_2	5–30	25	3.0; 6.7 and 9	60	–	[43]
Fenton reaction	1–10	25–30	2.2–2.6	60	1:14	[44]

showed that advanced oxidation technology, that is also of low cost, can be envisaged to clean coloured effluents in semi-arid countries [6]. Another photocatalytic colour removal system equipped with immobilized TiO_2 and illuminated by solar light was studied to remove colour from wastewater. The results revealed that the efficiency for methylene blue solution colour removal was higher with solar light irradiation than with artificial UV light irradiation [42]. Photocatalytic degradation of MB on $CaIn_2O_4$ under visible light irradiation has also been studied [43]. It was reported that the high photocatalytic activity could be kept in a wide visible light region up to 580 nm. The photoelectrochemical degradation of MB in aqueous solution was investigated by Tain-Cheng using a three dimensional electrode photoreactor. It was found that MB could be degraded more efficiently by photoelectrochemical process than by photocatalytic oxidation or electrochemical oxidation alone. Oxidation by Fenton reactions is proven and is an economically feasible process for destruction of a variety of hazardous pollutants in wastewater [44]. Dutta and coworkers reported MB oxidation using Fenton-like reactions. The effects of different parameters like dye initial concentration, Fe^{2+}, H_2O_2, pH, temperature and added electrolytes were evaluated. The degradation rate of the dye was found to be very fast in the initial reaction period, leading to almost 90% of conversion in the first 10 min.

Considering the foregoing and the collection of data presented in Table 3, the use of lignin peroxidase for MB degradation presents advantages related to the high efficiency of the catalyst that in a concentration of 1.1 μM was able to degrade, at room temperature and within 30 min, 50 mg/L MB using a ratio LiP:peroxide of 1:900. Moreover, previous results from our laboratory showed that non-toxic or undesirable by-products are formed [12,34]. As the biocatalyst was quite stable at temperatures up to 60 °C and its stability was not affected by LiP:hydrogen peroxide molar ratios up to 1:1500, when using stepwise peroxide addition, there is still room for improvement of this reaction efficiency. Moreover, further studies related to the biocatalyst reduction cost, its stabilization and recycling through immobilization and the development of industrial products based on the biocatalyst formulation could favour its use at industrial scale. Indeed, as shown in Tables 1 and 2 there is a great potential for environmental biocatalysis. It is worth to emphasize the paramount importance of the biodegradability of the biocatalysts. Combined techniques using biocatalysts and chemical or physical treatments may also be an interesting and promising alternative.

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