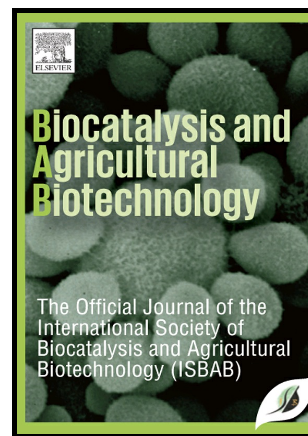
Author's Accepted Manuscript



BIODEGRADATION OF INDUSTRIAL DYES BY A SOLVENT, METAL AND SURFACTANT-STABLE EXTRACELLULAR BACTERIAL LACCASE

Claudia N. Britos, Julian E. Gianolini, Hugo Portillo, Jorge A. Trelles

PII: S1878-8181(17)30593-5
DOI: https://doi.org/10.1016/j.bcab.2018.03.015
Reference: BCAB727

To appear in: *Biocatalysis and Agricultural Biotechnology*

Received date: 16 November 2017
Revised date: 17 March 2018
Accepted date: 19 March 2018

Cite this article as: Claudia N. Britos, Julian E. Gianolini, Hugo Portillo and Jorge A. Trelles, BIODEGRADATION OF INDUSTRIAL DYES BY A SOLVENT, METAL AND SURFACTANT-STABLE EXTRACELLULAR BACTERIAL LACCASE, *Biocatalysis and Agricultural Biotechnology,* https://doi.org/10.1016/j.bcab.2018.03.015

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting galley proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

BIODEGRADATION OF INDUSTRIAL DYES BY A SOLVENT, METAL AND SURFACTANT-STABLE EXTRACELLULAR BACTERIAL LACCASE

Claudia N. Britos[1], Julian E. Gianolini[1], Hugo Portillo[1] and Jorge A. Trelles[1,2] *

[1] *Laboratory of Sustainable Biotechnology* (LIBioS), National University of Quilmes, Roque Sáenz Peña 352, Bernal B1876BXD, Argentina

[2] *National Scientific and Technical Research Council* (CONICET), Godoy Cruz 2290 CABA C1425FQB, Argentina

* Corresponding author. Tel.: +054 1143657100 (ext. 5645); fax: +54 11 43657132.
E-mail address: jtrelles@unq.edu.ar (Jorge A. Trelles)

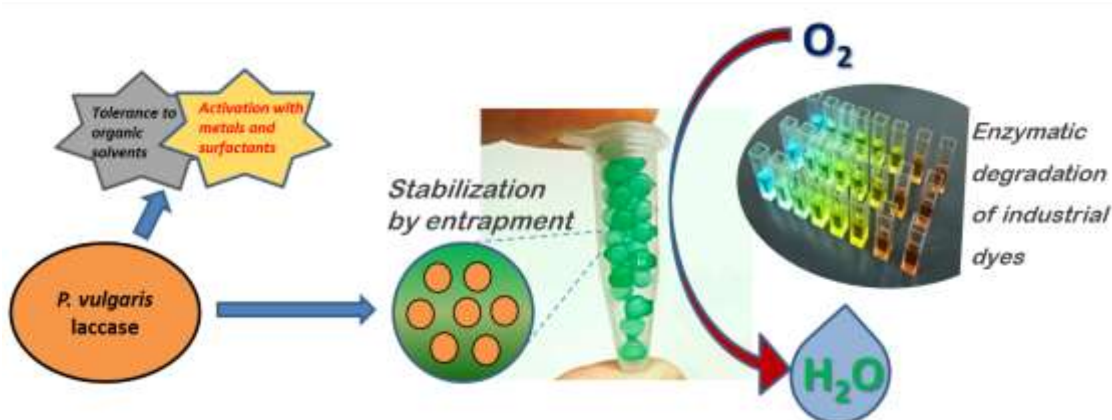
ABSTRACT

Several industrial activities release highly recalcitrant and dangerous dyes into the environment. The use of laccases to catalyze biooxidations represents an important alternative to dye effluent treatments. This study reports on the characterization of extracellular laccase of *P. vulgaris* ATCC 6896. This new enzyme was stable for more than 6 h at 60 °C and retained its activity completely under acidic and alkaline conditions. The *P. vulgaris* laccase showed a high tolerance to enzymatic inhibitors such as sodium azide and organic solvents (acetonitrile, ethanol and methanol), and its activity increased up to 5 times with the addition of Fe^{2+}, Cu^{2+}, Zn^{2+} or surfactants. Finally, the *P. vulgaris* laccase was stabilized by immobilization in Cu-alginate gels. The derivatives showed significantly higher thermostability than the free enzyme, and extended shelf life of up to 500 h. This biocatalyst was used to decolorize bromothymol blue (59%), Coomassie brilliant blue R (72%), methyl violet 10B (52%), Remazol brilliant blue R (51%) and trypan blue (85%) at short reaction times without the addition of mediators, and reused up to 160 cycles without loss of efficiency. This enzymatic biocatalyst could be effectively used in sewage treatment due to its ability to decolorize recalcitrant dyes without the addition of redox mediators, and its high thermal and chemical stability.

Accepted manuscript

ACCEPTED MANUSCRIPT

Graphical abstract



KEYWORDS

Biodegradation, Dyes, Laccase, Biocatalysis, Cu-alginate, Immobilization.

1. INTRODUCTION

The paper, leather and textile industries use more than 10,000 different synthetic dyes, besides huge amounts of water. The dyeing processes usually have low yields, less than 50%, and it is estimated that about 280,000 tons of dyes are discharged into effluents per year in the world (Rodríguez Couto and Toca Herrera, 2006). Dyes are recalcitrant in nature, many of them are toxic to the aquatic biota, and some of them have been reported to be carcinogenic and mutagenic agents (Mathur *et al.*, 2006). The concentration of dyes in effluents is extremely variable and depends on the type of dye and the dyeing process. In textile industries, colorants are applied in concentrations from 0.01 to 0.25 g/L, and it is estimated that between 2% and 20% is directly discharged into liquid residues (Ghaly *et al.*, 2014).

Currently, dye removal from effluents is by physicochemical means, including adsorption, precipitation, coagulation-flocculation, oxidation, filtration and

Accepted manuscript

ACCEPTED MANUSCRIPT
photodegradation (Tychanowicz *et al.*, 2004). These technologies often have several limitations, such as low decolorization yields, generation of large amounts of sludge for disposal or high implementation costs (Molina-Guijarro *et al.*, 2009).

Therefore, the great interest in effective technologies for the treatment of colored effluents makes biocatalytic processes an economical and environmentally friendly alternative. The use of laccases (EC 1.10.3.2) for industrial biooxidations has surged in recent years since, unlike peroxidases, they do not require hydrogen peroxide addition (Loera Corral *et al.*, 2006). Besides, this enzyme displays remarkably low substrate specificity, catalyzing the oxidation of a fairly broad range of chemicals, such as phenolic compounds, diamines and aromatic amines with the concomitant reduction of oxygen to water as the sole by-product (Mendoza *et al.*, 2014). This makes laccases suitable enzymes for a variety of 'green' oxidation processes (Riva, 2006). Although these enzymes are widespread in nature, fungal laccases have been the most widely characterized. Bacterial laccases have several properties superior to those of their fungal partners, such as stability under high temperature (Miyazaki, 2005), alkaline pH (Singh *et al.*, 2007) and tolerance to high salt concentrations (Britos and Trelles, 2016). Additionally, bacteria usually have higher growth rates than fungi and are more susceptible to improvements in levels of activity, selectivity and expression through protein engineering (Santhanam *et al.*, 2011). However, bacterial laccases have not been exploited extensively on an industrial scale as most of the reported enzymes are intracellular or spore bound, which makes their production and purification extremely difficult (Sondhi *et al.*, 2014).

In order to be used in biotechnological processes, several cost reduction strategies can be applied, immobilization being the most important.

The immobilization process generally helps to stabilize the structure of the enzymes and, as a consequence, maintains their activities. Thus, in comparison to the free enzymes in solution, immobilized enzymes are more robust and more resistant to environmental changes (Homaei *et al.*, 2013). Heterogeneous systems of immobilized

Accepted manuscript

ACCEPTED MANUSCRIPT

enzymes allow the easy recovery of enzymes and products, their reuse and application in continuous processes, and a greater control of the reaction. In addition, immobilization increases the stability of the enzyme under storage and operating conditions. All of this results in a notable reduction in the cost of the enzyme and the reaction products (Mohamad *et al.*, 2015).

This study describes the development of a strong enzymatic biocatalyst based on a novel extracellular laccase of *Proteus vulgaris* ATCC 6896, which is stable in the presence of heavy metals, detergents and organic solvents, and also showed great ability to decolorize industrial dyes.

2. MATERIALS AND METHODS

2.1. Materials

Chemicals for culture media were provided by Britania S.A. (Argentina). 2, 2´-Azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) and dyes were purchased from Sigma Chem. Co. (Brazil). Matrices and supports were provided by Sigma Chem. Co. (Brazil), Biodynamics (Argentina), Britania S.A. (Argentina) and Stanton (Argentina). Other reagents of analytical grade were supplied by Anedra (Argentina) or Sintorgan S.A. (Argentina).

2.2. Microorganism and laccase production

Proteus vulgaris ATCC 6896 was grown in Luria-Bertani (LB) medium (5 g/L meat extract, 10 g/L peptone, 5 g/L NaCl pH 7.0) at 37 ºC with shaking (200 rpm). Culture supernatant was collected by centrifugation for 30 min at 4ºC and 11,000 *g*, incubated for 16 h in a centrifugal vacuum concentrator (SpeedVac®) until 5-fold volume reduction, clarified once more by centrifugation and stored at 4 ºC until use. This cell-free laccase extract was characterized by total protein content by the adapted Bradford method (Britos and Trelles, 2016) and laccase activity test.

2.3. Laccase activity test

Accepted manuscript

Laccase activity was measured spectrophotometrically at 420 nm (ε_{420nm} 3.6 x10^4 $M^{-1}cm^{-1}$) (Childs and Bardsley, 1975) using ABTS as substrate. Reaction conditions were 20% (v/v) cell-free laccase extract, 5 mM ABTS, 0.05 M $CuSO_4$, 0.1 M sodium acetate buffer pH 4.6. Unless otherwise stated, reactions were carried out at 30 °C and 200 rpm. The initial reaction rates were obtained from the linear portion of the progress curve. One enzyme unit (EU) was defined as the quantity of enzyme that catalyzes the oxidation of 1 μmol of ABTS per min.

2.4. Laccase characterization

ABTS reaction was performed at different temperatures to determine maximum activity. Additionally, the stability of crude laccase with temperature and pH was studied. Fractions of enzyme were incubated at different temperature (30-80 ºC) or pH (4.0-9.0) and used to catalyze the activity test.

The effect of additives on laccase activity was determined. Metal ions (Na^+, K^+, Mg^{2+}, Ca^{2+} , Sr^{2+} , Ba^{2+} , Fe^{2+} , Zn^{2+} , Cu^{2+} at 1, 10 and 50 mM), inhibitors (EDTA, sodium azide and sodium citrate at 0.1, 1 and 5 mM), surfactants (CTAB, Pluronic® F-68, Sarkosyl™, SDS, Triton® X-100 and Tween® 80, each at 0.1, 1 and 5 mM) and organic solvents (methanol, ethanol, acetone, acetonitrile, each at 5% and 20% (v/v)) were mixed with the laccase and incubated at 30 ºC. Lacasse activity was determined by ABTS reaction and compared with the control without additive treatment.

2.5. Synthetic dye decolorization by laccase

P. vulgaris supernatant was used to decolorize four groups of synthetic dyes: anthraquinone, azo, indigoid, and triarylmethane. The reaction medium contained 0.1 M sodium acetate buffer, 0.05 M $CuSO_4$ pH 4.6 and 0.011 EU of free or immobilized laccase in a total volume of 2.5 mL. The decolorization processes were performed at 30 °C and 200 rpm and periodically withdrawn to measure the reduction of each dye absorbance. The concentration of each dye was selected so that the initial absorbance, at its maximum wavelength, was about 1 unit. The maximum wavelength of dyes was determined by full spectrum scan among 400-700 nm of reaction mixture at the initial

Accepted manuscript

ACCEPTED MANUSCRIPT

and final time. The selected dyes and their values of λ_{max} (in nm) were: Amido black 10B (660), Bromothymol blue (425), Coomassie brilliant blue R (595), Indigo carmine (595), Malachite green (660), Methyl orange (487), Methyl red (550), Methyl violet 10B (595), Remazol brilliant blue R (595) and Trypan blue (595). Controls in which laccase was replaced by distilled water or a clean support/matrix were conducted in parallel. The decolorization ability of each dye was expressed in percentage calculated as follows:

$$Decolorization\ (\%) = \frac{A_0 - A_t}{A_0} \times 100$$

Where A_0 is the initial absorbance value and A_t is the absorbance after each time interval, both at maximum wavelength for each dye. The values reported correspond to average values of experiments performed in triplicate and corrected by variations of the reagent or support controls without enzyme, when appropriate.

2.6. Laccase immobilization

Laccase preparation was immobilized on different supports according to the following procedures. Immobilization yields were determined as the difference in protein content of the mixture before and after incubation with the supports (Ramirez-Tapias *et al*, 2017). Protein determinations were carried out by the adapted Coomassie blue method. The derivatives obtained were evaluated for laccase activity by ABTS oxidation (Britos and Trelles, 2016).

2.6.1. Immobilization by adsorption

Two hundred mg of support (DEAE-sepharose or IDA-Cu-agarose) was incubated with 0.1 mg of laccase in 0.1 M sodium acetate buffer pH 4.6. The immobilization mixture was gently stirred for 16 h at 4 ºC, washed, dried by vacuum filtration and stored at 4 ºC until use (Britos *et al*, 2016).

2.6.2. Entrapment in thermogels

Agar or agarose (4% and 3% (w/v) final concentration, respectively) was mixed with 0.1 mg of laccase (Trelles and Rivero, 2013). The mixture was added dropwise to cold

Accepted manuscript

vegetal oil and incubated for 5 min. The gel beads were washed with N-hexane, distilled water and 0.1 M sodium acetate pH 4.6 and stored at 4 ºC until use.

2.6.3. Entrapment in Cu-alginate gels

Sodium alginate (8% (w/v) final concentration) was mixed with 0.1 mg of crude laccase. The mixture was added dropwise to a stirred solution of 0.1 M $CuSO_4$ and incubated for 5 min at room temperature. The resulting gel beads were washed with 0.1 M sodium acetate pH 4.6 and stored at 4 ºC until use.

2.7. Optimization of immobilization parameters

Laccase (0.02 EU) was mixed with sodium alginate (8% (w/v)), added to 0.1 M $CuSO_4$ and incubated for 0.5, 1 or 2 h to determine the optimal polymerization time. The cross-linker concentration was determined by adding the laccase-alginate mixture at 0.1, 0.2 or 0.5 M $CuSO_4$. Finally, laccase was mixed with different concentrations of sodium alginate (5%, 8% or 10% (w/v)) and added to $CuSO_4$ solution. After washing, the obtained beads were used to catalyze ABTS reaction.

2.8. Stability and operational profile of immobilized laccase

The inactivation profile was determined by incubating the derivatives at 30 or 50 ºC and 200 rpm, and assessing activity until deactivation or mechanical destabilization.
In addition, the retention of activity under storage conditions was evaluated. For this purpose, immobilized derivatives were stored at 4 ºC and periodically used to catalyze ABTS reaction until activity loss.
Finally, the biocatalysts were used repeatedly until the residual activity was 50% of the initial activity, and their corresponding half-life was determined.

Accepted manuscript

3. RESULTS AND DISCUSSION

3.1. Laccase production

Extracellular enzymes represent an interesting option for industrial applications, because production costs are reduced by simplification of downstream operations. However, most of the bacterial laccases identified are expressed intracellularly (Singh

ACCEPTED MANUSCRIPT

et al., 2011). In previous studies in our laboratory, several laccase-producing bacteria of different genera were identified. Activity assays on intracellular preparations and cell-free culture supernatants allowed us to identify those that express the enzyme extracellularly. From those that were positive, *Proteus vulgaris* ATCC 6896 was noted for its high activity in the early hours of culture. The enzyme was excreted during culture growth, and laccase activity increased due to biomass accumulation, reaching a maximum concentration of 50 EU/L in only 5 h of culture (data no shown). Similar laccase titers were obtained with *Streptomyces* strains at 8 (Margot *et al.*, 2013) or 14 days of culture (Moya *et al.*, 2010). Additionally, cell-free supernatants were concentrated 5 to 6 times without significant activity loss, obtaining preparations with titers of 100 ± 8 EU/L. The extracellular production of this laccase allowed us to use simple operations of protein recovery and concentration, obtaining preparations that could be used directly as biocatalyst, avoiding costly and time-consuming techniques of overexpression and purification.

Few *Proteus* laccases were reported; however, the most relevant biochemical characteristics for their subsequent stabilization and application in biodegradation processes have not been studied until now.

3.2. Laccase biochemical characterization

Besides being easy to produce, some other properties of enzymes such as thermal and chemical stability are required for them to be exploited on an industrial scale. The maximum activities of *P. vulgaris* laccase were observed between 45 °C and 60 °C, with reaction rates 8 times higher than those corresponding to 30 °C (Fig. 1A). Moreover, the thermal inactivation profile of the enzyme was determined (Fig. 1B). *P. vulgaris* laccase was more heat resistant than other bacterial laccases reported (Sondhi *et al.*, 2014). The half-life of this enzyme at 60°C was of 6 h, and it retained activity for more than 10 h at 40 ºC. At 30 ºC, this bacterial laccase was stable for more than 12 h of incubation.

Accepted manuscript

ACCEPTED MANUSCRIPT

The effect of pH on the enzymatic activity is shown in figure 1C. *P. vulgaris* laccase was completely stable at pH values between 5.0 and 8.0 for more than 16 h. Also, at pH 4.0, the enzyme retained activity completely after 8 h and started to slowly inactivate until 16 h of incubation. In general, fungal laccases are stable only at acidic pH (Baldrian, 2006), and this feature can be a drawback in the treatment of effluents, which generally have pH values above 7.0 (D'Souza *et al.*, 2006). *P. vulgaris* laccase showed high activity and stability at acid and alkaline pH, being compatible for applications in sewage treatment and bioremediation.

Besides synthetic dyes, industrial wastewater may contain various inorganic chemicals such as sulfides, sulfates, chlorides, carbonates, peroxides, chlorine bleach, and heavy metals (D'Souza-Ticlo *et al.*, 2009), which could have an effect on the enzyme activity. Therefore, the stability of laccase against various metal compounds usually present in wastewater was examined (Table 1). Alkali metal ions such as Na^+ and K^+ inhibited the enzyme, and the alkaline earth metals produced a variable effect depending on the atomic radius. Mg^{2+} had no effect on activity, whereas Ca^{2+} partially inhibited the enzyme. Moreover, strontium and barium ions, of higher metal character, completely inhibited the enzyme in all concentrations tested.

Conversely, the enzyme was strongly activated (up to 3 times) by transition metals such as Fe^{2+} and Cu^{2+}, and poor metals such as Zn^{2+}. The activation by certain metals, especially Cu^{2+}, has also been observed in other laccases, and may be due to the filling of type II copper binding sites with copper ions. Additionally, laccase folding experiments indicated that Zn^{2+} ions could replace copper restoring 100% of activity, indicating the nonessential role of copper in this enzyme (Salony *et al.*, 2008).

It has been reported that binding of NaN_3 to type II and III copper sites blocks internal electron transfer inhibiting laccase activity (Ryan *et al.*, 2003), and that chelating agents, such as EDTA, could sequester the copper of the relatively unstable type II binding site. The enzyme was also resistant to potent inhibitors of metalloenzymes

Accepted manuscript

ACCEPTED MANUSCRIPT

(Table 2). EDTA, citrate and NaN_3 had inhibitory effects, but significantly lower than those observed for other laccases (Niladevi and Prema, 2008).

On the other hand, the anionic surfactant SDS inhibited *M. verrucaria* laccase (Zhao *et al.*, 2012) and CTAB, a cationic detergent, was identified as competitive inhibitor of *C. bulleri* laccase (Vasdev *et al.*, 2005). Conversely, *Bacillus sp.* (Dalfard *et al.*, 2006) and *F. solani* (Wu *et al.*, 2010) laccases were slightly activated by the same compounds. A surface interaction between the surfactant and laccase has been suggested (Ji *et al.*, 2009). In this study, *P. vulgaris* laccase was highly stable in the presence of nonionic surfactants (Triton® X-100, Tween® 80, and Pluronic® F-68), and a strong stimulatory effect (up to 500%) on laccase activity was observed with harsh cationic and anionic detergents, showing the potential of this novel enzyme to be used in wastewater treatments. The improvement in activity was, as in cooperative interaction models (Gayatridevi *et al.*, 2016), concentration-dependent.

Several fungal and bacterial laccases can be inactivated by organic solvents (Kaushik and Thakur, 2013), which is a limitation for applications with complex effluents and poorly water-soluble substrates. *P. vulgaris* laccase was found to be stable at different concentrations of acetonitrile, and partially retained its activity in the presence of alcohols (Table 3). This tolerance can allow the use of *P. vulgaris* laccase in co-solvent systems for the treatment of pollutants of low water solubility (Hautphenne *et al.*, 2016).

Finally, the ability of *P. vulgaris* laccase to decolorize different dyes was studied (Table 4). Due to the enormous structural diversity of the dyes, some model molecules that can be representative and that serve to prove the low specificity of the enzyme under study were selected. High rates of decolorization were obtained for all groups tested, showing the wide range of substrates that recognize the enzyme. These results are superior to those reported so far using modified enzymes (Pardo *et al.*, 2013), addition of mediators (Mendoza *et al.*, 2014), and long reaction times (Razak and Annuar, 2014). In this work, *P. vulgaris* laccase was able to decolorize azo-, diazo-,

Accepted manuscript

ACCEPTED MANUSCRIPT

anthraquinone and triarylmethane dyes at 3 h of incubation without any mediator, using an enzymatic cell-free preparation.

3.3. Development of an immobilized biocatalyst

3.3.1. Support selection

Two strategies were studied to stabilize *P. vulgaris* laccase: adsorption and entrapment in hydrogel matrices. As mentioned above, Cu^{2+} ions interact with specific sites of the enzyme, and this property has been exploited to recover laccases from crude extracts by chelating chromatography (Kataoka *et al.*, 2007). Alternatively, several examples of laccase recovery from concentrated preparations by ion exchange chromatography on DEAE-sepharose have been described (Telke *et al.*, 2011). Therefore, these matrices could also serve as supports for immobilization. On the other hand, laccase entrapment in natural thermogels, such as agar or agarose, has been explored to a limited extent, unlike ionotropic gels such as alginate (Niladevi and Prema, 2008).

In general, divalent cations (Cd^{2+}, Co^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}) are suitable cross-linking agents for alginate gel preparations. Although calcium is the most commonly used ion in polymerization, Ca-alginate gels are more porous, less flexible and have lower chemical stability than Cu-alginate (Brandi *et al.*, 2006). It has been proposed that copper interacts nonspecifically with the alginate, and forms inter- or intramolecular chelates with carboxyl and hydroxyl groups of the guluronate segments of the macromolecule (Goh *et al.*, 2012).

Table 5 lists the results of different immobilized laccase derivatives. The enzyme could be immobilized efficiently in all materials tested. The activity of the ones immobilized in thermogels was significantly lower than that of those immobilized in hydrogel such as Cu-alginate or by adsorption, and can be related to the reduction in the transport of reagents and products due to the matrix network or immobilization methodology used. Only the DEAE-agarose and Cu-alginate derivatives retained the enzyme after a 24-hour washout procedure. However, the DEAE-agarose derivative was discarded for

Accepted manuscript

ACCEPTED MANUSCRIPT

future experiments because a strong interaction of ABTS and dyes with the support was observed, which affects the performance of the reaction of interest. Therefore, entrapment in Cu-alginate was the enzymatic stabilization method selected for *P. vulgaris* laccase.

3.3.2. Optimization of immobilization parameters

The physical, mechanical and chemical characteristics of the alginate gels depend not only on the composition of the material (proportion and length of the monomeric segments GG and MM) (Lee and Mooney, 2012), but also on the identity of the polymerizing ion and its concentration (Moreira *et al.*, 2006), and on other parameters of their preparation process. The time of contact of the alginate with the cross-linking ion significantly affected the activity of laccase. Longer residence times resulted in lower reaction rates; so the incubation time of beads was determined at 0.5 h at room temperature (Fig. 2A). It has been shown that the concentration of the cross-linking ion has little effect on the mechanical force. However, high concentrations of polymerizing ion cause a greater degree of cross-linking and, as a consequence, a reduction in the permeability of the matrix (Cappa *et al.*, 2014). The activity of *P. vulgaris* laccase immobilized in Cu-alginate was not affected significantly for copper concentrations between 0.1 and 0.2 M (Fig. 2B). Only at concentrations of 0.5 M a negative effect on the activity was observed, possibly due to reduction in the rate of diffusion of products and reagents. Therefore, a Cu^{2+} concentration of 0.1 M was selected for immobilization. An increase in matrix concentration produces gels with smaller, dense and stronger internal pores, providing greater mechanical stability (Wright *et al.*, 2012). Here, a 10% (w/v) alginate produced the best biocatalyst, with a reaction rate that duplicated the one corresponding to 8% (w/v) (Fig. 2C). This biocatalyst, with an enzymatic load of 0.05 EU/g, was selected for thermal stability assays.

Entrapment in Cu-alginate gels significantly stabilized *P. vulgaris* laccase (Fig. 3). The biocatalyst could be stored for 1600 h without being deactivated, retaining even 70% of

Accepted manuscript

the initial activity. In addition, the derivative showed high thermoresistance, retaining activity for 500 h of incubation at 55 °C, 50 times greater than the stability of the free enzyme and the double of those reported for other alginate-based biocatalysts (Phetsom *et al.*, 2009).

3.3.3. Biodegradation of dyes by the immobilized biocatalyst

The biocatalyst developed was used to test dye removal (Table 6). The immobilized laccase efficiently catalyzed the discoloration of the 6 substrates in only 3 h of reaction, with higher yields than previously reported (D'Souza *et al.*, 2006; Osma *et al.*, 2010). Also, the biocatalyst was periodically used to degrade the dyestuffs under study until deactivation. The half-life of the biocatalysts improved significantly with respect to other similar ones (Niladevi and Prema, 2008; Phetsom *et al.*, 2009), with an enzymatic and structural stability of up to 500 h under operating conditions. These biocatalysts were active during 160 cycles of degradation without loss of efficiency, demonstrating the strength of the developed derivative. Finally, the effect of the addition of the activators identified above on the decolorization performance was studied. For this purpose RBBR, an industrial dye identified as toxic and recalcitrant, with an estimated half-life of more than 40 years, was used (Osma *et al.*, 2010).

P. vulgaris laccase was incubated with Cu^{2+}, Zn^{2+} (10 mM), CTAB or SDS (1 and 5 mM respectively) and immobilized in alginate-Cu gels under optimized conditions. The biocatalysts were used to decolorize RBBR as described before, and the efficiency was compared with nonactivated derivatives. Copper pre-activation was the most effective for this purpose, and allowed us to obtain 50% of RBBR decolorization at 2 h, improving both the yield and productivity of the reaction.

4. CONCLUSIONS

P. vulgaris was able to produce a heat-resistant extracellular laccase that has improved activity at alkaline pH. This activity could be increased by the presence of common

Accepted manuscript

contaminants of industrial and domestic effluents, such as heavy metals and detergents. Moreover, the enzyme retained its activity in the presence of chemical inhibitors and organic solvents in high concentrations.

The *P. vulgaris* laccase recognized a wide range of dyes with high percentages of decolorization without costly purification and concentration operations. This enzyme was stabilized by immobilization in Cu-alginate, obtaining a biocatalyst with extended thermal, mechanical and chemical stability.

ACKNOWLEDGEMENTS

This research was supported by Agencia Nacional de Promoción Científica y Tecnológica (PICT 2013-2658 and PICT 2014-3438), Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 2014-KA5-00805) and Universidad Nacional de Quilmes (PUNQ 1409/15).

REFERENCES

Baldrian, P., 2006. Fungal laccases – occurrence and properties. FEMS microbiology reviews 30, 215-242.

Brandi, P., D'Annibale, A., Galli, C., Gentili, P., Pontes, A.S.N., 2006. In search for practical advantages from the immobilisation of an enzyme: the case of laccase. Journal of Molecular Catalysis B: Enzymatic 41, 61-69.

Britos, C.N., Trelles, J.A., 2016. Development of strong enzymatic biocatalysts for dye decolorization. Biocatalysis and Agricultural Biotechnology 7, 228-233.

Cappa, V.A., Rivero, C.W., Britos, C.N., Martinez, L.M., Lozano, M.E., Trelles, J.A., 2014. An efficient biocatalytic system for floxuridine biosynthesis based on Lactobacillus animalis ATCC 35046 immobilized in Sr-alginate. Process Biochemistry 49, 1169-1175.

Childs, R.E., Bardsley, W.G., 1975. The steady-state kinetics of peroxidase with 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid) as chromogen. Biochem. J. 145, 93-103.

D'Souza, D., Tiwari, R., Sah, A.K., Raghukumar, C., 2006. Enhanced production of laccase by a marine fungus during treatment of colored effluents and synthetic dyes. Enzyme and microbial technology 38, 504- 511.

D'Souza-Ticlo, D., Sharma, D., Raghukumar, C., 2009. A thermostable metal-tolerant laccase with bioremediation potential from a marine-derived fungus. Marine Biotechnology 11, 725-737.

Accepted manuscript

ACCEPTED MANUSCRIPT

Dalfard, A.B., Khajeh, K., Soudi, M.R., Naderi-Manesh, H., Ranjbar, B., Sajedi, R.H., 2006. Isolation and biochemical characterization of laccase and tyrosinase activities in a novel melanogenic soil bacterium. Enzyme and microbial technology 39, 1409-1416.

Drzewiecka, D., 2016. Significance and Roles of Proteus spp. Bacteria in Natural Environments. Microbial Ecology 72, 741-758.

Gayatridevi, S., Jayalakshmi, S.K., K., S., 2016. Activation of purified polyphenol oxidase and laccase of chickpea cv. ICCV10 by sodium dodecyl sulfate. The Bioscan 11, 69-72.

Ghaly, A., Ananthashankar, R., Alhattab, M., Ramakrishnan, V., 2014. Production, characterization and treatment of textile effluents: a critical review. J Chem Eng Process Technol 5, 1-18.

Goh, C.H., Heng, P.W.S., Chan, L.W., 2012. Alginates as a useful natural polymer for microencapsulation and therapeutic applications. Carbohydrate Polymers 88, 1-12.

Hautphenne, C., Penninckx, M., Debaste, F., 2016. Product formation from phenolic compounds removal by laccases: A review. Environmental Technology & Innovation 5, 250-266.

Homaei, A.A., Sariri, R., Vianello, F., Stevanato, R., 2013. Enzyme immobilization: an update. Journal of Chemical Biology 6, 185-205.

Ji, G., Zhang, H., Huang, F., Huang, X., 2009. Effects of nonionic surfactant Triton X-100 on the laccase-catalyzed conversion of Bisphenol A. Journal Environmental Science (Ch) 21, 1486-1490.

Kataoka, K., Komori, H., Ueki, Y., Konno, Y., Kamitaka, Y., Kurose, S., Tsujimura, S., Higuchi, Y., Kano, K., Seo, D., Sakurai, T., 2007. Structure and function of the engineered multicopper oxidase CueO from Escherichia coli--deletion of the methionine-rich helical region covering the substrate-binding site. Journal of molecular biology 373, 141-152.

Kaushik, G., Thakur, I.S., 2013. Purification, characterization and usage of thermotolerant laccase from *Bacillus sp.* for biodegradation of synthetic dyes. Applied Biochemistry and Microbiology 49, 352-359.

Lee, K.Y., Mooney, D.J., 2012. Alginate: properties and biomedical applications. Progress in polymer science 37, 106-126.

Loera Corral, O., Pérez Pérez , M.C.I., Barbosa Rodríguez, J.R., Villaseñor Ortega, F., 2006. Laccases. In: Torres-Pacheco, R.G.G.-G.a.I. (Ed.), Advances in Agricultural and Food Biotechnology. Research Signpost, Kerala, India, pp. 323-340.

Margot, J., Bennati-Granier, C., Maillard, J., Blanquez, P., Barry, D.A., Holliger, C., 2013. Bacterial versus fungal laccase: potential for micropollutant degradation. AMB Express 3, 63.

Mathur, N., Bhatnagar, P., Bakre, P., 2006. Assessing mutagenicity of textile dyes from Pali(Rajasthan) using Ames bioassay. Applied Ecology and Environmental Research 4, 111-118.

Mendoza, L., Ibrahim, V., Álvarez, M.T., Hatti-Kaul, R., Mamo, G., 2014. Laccase production by *Galerina* sp. and its application in dye decolorization. Journal of Yeast and Fungal Research 5, 13-22.

Miyazaki, K., 2005. A hyperthermophilic laccase from *Thermus thermophilus* HB27. Extremophiles 9, 415-425.

Mohamad, N.R., Marzuki, N.H.C., Buang, N.A., Huyop, F., Wahab, R.A., 2015. An overview of technologies for immobilization of enzymes and surface analysis

Accepted manuscript

ACCEPTED MANUSCRIPT

techniques for immobilized enzymes. Biotechnology, Biotechnological Equipment 29, 205-220.

Molina-Guijarro, J.M., Pérez, J., Muñoz-Dorado, J., Guillén, F., Moya, R., Hernández, M., Arias, M.E., 2009. Detoxification of azo dyes by a novel pH-versatile, salt-resistant laccase from *Streptomyces ipomoea*. Int. Microbiol. 12, 13-21.

Moreira, S.M., Moreira-Santos, M., Guilhermino, L., Ribeiro, R., 2006. Immobilization of the marine microalga Phaeodactylum tricornutum in alginate for in situ experiments: Bead stability and suitability. Enzyme and microbial technology 38, 135-141.

Moya, R., Hernández, M., García-Martín, A.B., Ball, A.S., Arias, M.E., 2010. Contributions to a better comprehension of redox-mediated decolouration and detoxification of azo dyes by a laccase produced by *Streptomyces cyaneus* CECT 3335. Bioresource Technology 101, 2224-2229.

Niladevi, K.N., Prema, P., 2008. Immobilization of laccase from *Streptomyces psammoticus* and its application in phenol removal using packed bed reactor. World Journal of Microbiology & Biotechnology 24, 1215-1222.

Osma, J.F., Toca-Herrera, J.L., Rodríguez-Couto, S., 2010. Transformation pathway of Remazol Brilliant Blue R by immobilised laccase. Bioresource Technology 101, 8509-8514.

Pardo, I., Chanagá, X., Vicente, A.I., Alcalde, M., Camarero, S., 2013. New colorimetric screening assays for the directed evolution of fungal laccases to improve the conversion of plant biomass. BMC biotechnology 13, 90.

Phetsom, J., Khammuang, S., Suwannawong, P., Sarnthima, R., 2009. Copper-alginate encapsulation of crude laccase from Lentinus polychrous Lev. and their effectiveness in synthetic dyes decolorizations. Journal of Biological Sciences 9, 573-583.

Razak, N.N.A., Annuar, M.S.M., 2014. Thermokinetic comparison of Trypan Blue decolorization by free laccase and fungal biomass. Applied Biochemistry and Biotechnology 172, 2932-2944.

Riva, S., 2006. Laccases: blue enzymes for green chemistry. Trends in Biotechnology 24, 219-226.

Rodríguez Couto, S., Toca Herrera, J.L., 2006. Industrial and biotechnological applications of laccases: A review. Biotechnology Advances 24, 500-513.

Ryan, S., Schnitzhofer, W., Tzanov, T., Cavaco-Paulo, A., Gübitz, G.M., 2003. An acid-stable laccase from *Sclerotium rolfsii* with potential for wool dye decolourization. Enzyme and microbial technology 33, 766-774.

Salony, J.L., Garg, N., Baranwal, R., Chhabra, M., Mishra, S., Chaudhuri, T.K., Bisaria, V.S., 2008. Laccase of *Cyathus bulleri*: structural, catalytic characterization and expression in *Escherichia coli*. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics 1784, 259-268.

Santhanam, N., Vivanco, J.M., Decker, S.R., Reardon, K.F., 2011. Expression of industrially relevant laccases: prokaryotic style. Trends in Biotechnology 29, 480-489.

Saratale, G.D., Saratale, R.G., Chang, J.S., Govindwar, S.P., 2011. Fixed-bed decolorization of Reactive Blue 172 by Proteus vulgaris NCIM-2027 immobilized on Luffa cylindrica sponge. International Biodeterioration & Biodegradation 65, 494-503.

Singh, G., Bhalla, A., Kaur, P., Capalash, N., Sharma, P., 2011. Laccase from prokaryotes: a new source for an old enzyme. Rev Environ Sci Biotechnol 10, 309-326.

Accepted manuscript

Singh, G., Capalash, N., Goel, R., Sharma, P., 2007. A pH-stable laccase from alkali-tolerant γ-proteobacterium JB: purification, characterization and indigo carmine degradation. Enzyme and microbial technology 41, 794-799.

Sondhi, S., Sharma, P., Saini, S., Puri, N., Gupta, N., 2014. Purification and characterization of an extracellular, thermo-alkali-stable, metal tolerant laccase from *Bacillus tequilensis* SN4. PLoS ONE 9, e96951.

Telke, A.A., Ghodake, G.S., Kalyani, D.C., Dhanve, R.S., Govindwar, S.P., 2011. Biochemical characteristics of a textile dye degrading extracellular laccase from a Bacillus sp. ADR. Bioresource Technology 102, 1752-1756.

Tychanowicz, G.K., Zilly, A., de Souza, C.G.M., Peralta, R.M., 2004. Decolourisation of industrial dyes by solid-state cultures of *Pleurotus pulmonarius*. Process Biochemistry 39, 855-859.

Vasdev, K., Dhawan, S., Kapoor, R.K., Kuhad, R.C., 2005. Biochemical characterization and molecular evidence of a laccase from the bird's nest fungus *Cyathus bulleri*. Fungal Genetics and Biology 42, 684-693.

Wright, B., Cave, R.A., Cook, J.P., Khutoryanskiy, V.V., Mi, S., Chen, B., Leyland, M., Connon, C.J., 2012. Enhanced viability of corneal epithelial cells for efficient transport/storage using a structurally modified calcium alginate hydrogel. Regenerative Medicine 7, 295-307.

Wu, Y.-R., Luo, Z.-H., Kwok-Kei Chow, R., Vrijmoed, L.L.P., 2010. Purification and characterization of an extracellular laccase from the anthracene-degrading fungus *Fusarium solani* MAS2. Bioresource Technology 101, 9772-9777.

Zhao, D., Zhang, X., Cui, D., Zhao, M., 2012. Characterisation of a novel white laccase from the deuteromycete fungus *Myrothecium verrucaria* NF-05 and its decolourisation of dyes. PLoS ONE 7, e38817.

Figure 1. Characterization of *P. vulgaris* laccase. (A) Influence of temperature on its activity. The ABTS reaction was performed at different temperatures. (B) Thermal inactivation. Laccase was incubated at different temperatures and its residual activity was determined until deactivation. (C) Laccase chemical stability. The enzyme was incubated at different pH values (4.0 to 9.0) and its residual activity was determined. Data points represent the means of three replicates ± SD.

Figure 2. Immobilization of *P. vulgaris* laccase in Cu-alginate. Parameters such as incubation time (A), cross-linker concentration (B) or alginate concentration (C) were studied. The efficiency of derivatives was determined by ABTS assay. Data points represent the means of three replicates ± SD.

Figure 3. Stability of the immobilized biocatalyst. The mechanical and enzymatic stability of *P. vulgaris* laccase entrapped in Cu-alginate was studied at 30 or 50 °C and 200 rpm (operating conditions), and at 4 °C (storage conditions). The residual activity was determined by comparison with the initial activity.

Accepted manuscript

ACCEPTED MANUSCRIPT

Table 1. Effect of ions on *P. vulgaris* laccase. The enzyme was incubated with different ions, and its activity was measured using ABTS as substrate at 25 ºC and pH 4.5. Relative activity was calculated with respect to the control reaction (ra=1). Results represent the mean ± SD (n=3).

	Relative activity			
	0 mM	1 mM	10 mM	50 mM
Control	1.00			
NaCl		0.62 ± 0.02	0.60 ± 0.03	0.63 ± 0.01
Na_2SO_4		0.54 ± 0.01	0.52 ± 0.02	0.53 ± 0.02
$NaNO_3$		0.58 ± 0.03	0.60 ± 0.02	0.56 ± 0.08
KCl		0.55 ± 0.05	0.61 ± 0.02	0.59 ± 0.01
KNO_3		0.57 ± 0.04	0.60 ± 0.01	0.27 ± 0.06
$MgSO_4$		1.00 ± 0.01	1.06 ± 0.02	1.04 ± 0.01
$CaCl_2$		0.68 ± 0.02	0.67 ± 0.03	0.65 ± 0.01
$SrCl_2$		0	0	0
$BaCl_2$		0	0	0
$ZnSO_4$		0.96 ± 0.10	2.72 ± 0.12	2.95 ± 0.15
$CuSO_4$		1.00 ± 0.02	2.86 ± 0.10	3.34 ± 0.13
$FeSO_4$		2.86 ± 0.05	3.34 ± 0.08	3.14 ± 0.05

Table 2. Effect of inhibitors and surfactants on *P. vulgaris* laccase. Enzyme activity was measured using ABTS as substrate at 25 ºC and pH 4.5. Relative activity was calculated with respect to the control reaction (ra=1). Results represent the mean ± SD (n=3).

		Relative activity			
		0 mM	0.1 mM	1 mM	5 mM
	Control	1.00			
Inhibitors	**NaN_3**		0.54 ± 0.01	ND[1]	ND[1]
	EDTA		1.03 ± 0.04	0.97 ± 0.02	0.76 ± 0.01

Accepted manuscript

ACCEPTED MANUSCRIPT

	Sodium citrate	0.84 ± 0.02	0.70 ± 0.01	0.62 ± 0.02
Surfactants	**SDS[2]**	1.34 ± 0.05	1.61 ± 0.03	5.83 ± 0.10
	Sarkosyl®[3]	1.16 ± 0.02	1.43 ± 0.04	4.17 ± 0.08
	CTAB[4]	1.04 ± 0.05	4.70 ± 0.12	3.04 ± 0.05
	Triton™ X-100[5]	1.33 ± 0.06	1.26 ± 0.02	0.87 ± 0.05
	Tween® 80[6]	1.11 ± 0.02	ND[1]	1.20 ± 0.03
	Pluronic® F-68[7]	1.02 ± 0.04	0.99 ± 0.02	1.13 ± 0.09

[1] not determined, [2] sodium lauryl sulfate (anionic); [3] N-lauryl sarcosine (anionic); [4] alkyltrimethylammonium bromide (cationic), [5] polyethylene glycol tert-octylphenyl ether (nonionic); [6] polyethylene glycol sorbitan monooleate (nonionic); [7] polyoxyethylene-polyoxypropylene block copolymer (nonionic surfactant).

Accepted manuscript

Table 3. Stability of *P. vulgaris* laccase in organic solvents. Laccase activity was measured using ABTS as substrate at 25 ºC and pH 4.5. Relative activity was calculated with respect to the control reaction (ra=1). Results represent the mean ± SD (n=3).

	Concentration (% (v/v))	*Residual activity*
Control	0	1.00
Acetone	5	0
	20	0
Acetonitrile	5	0.85 ± 0.02
	20	0.71 ± 0.01
Ethanol	5	0.72 ± 0.03
	20	0.47 ± 0.02
Methanol	5	0.70 ± 0.02
	20	0.52 ± 0.01

ACCEPTED MANUSCRIPT

Table 4. Substrate specificity of free *P. vulgaris* laccase. Reaction yields at 3 h. Results represent the mean ± SD (n=3).

Dye	*Dye class*	*Concentration (μM)*	*Decolorization (%)*
Amido black 10B	Diazo	200	31 ± 1
Bromothymol blue	Triarylmethane	40	46 ± 3
Coomassie brilliant blue R	Triarylmethane	40	58 ± 6
Indigo carmine	Indigoid	10	15 ± 3
Malachite green	Triarylmethane	20	24 ± 1
Methyl orange	Sulfonated monoazo	40	26 ± 3
Methyl red	Azo	40	20 ± 1
Methyl violet 10B	Triarylmethane	40	22 ± 2
Remazol brilliant blue R	Anthraquinone	40	17 ± 2
Trypan blue	Diazo	20	65 ± 1

Table 5. Immobilization of *P. vulgaris* laccase. The enzyme was incubated with different matrices and supports. Immobilized derivatives were assayed by ABTS reaction, and its activity retention was evaluated after a washing procedure.

		Enzyme load (EU/g)	Immobilization yield (%)	Activity retention (%)
Supports				
	DEAE-agarose	0.055	31	95
	Cu-IDA-agarose	0.088	33	38
Matrices				
	Agar	0.015	100	5
	Agarose	0.022	100	7
	Cu-alginate	0.033	99	98

Accepted manuscript

ACCEPTED MANUSCRIPT

Table 6. Performance of Cu-alginate biocatalysts. The immobilized laccase was used for the biotransformation of dyes by 3 h reaction cycles at 30 °C and 200 rpm until deactivation.

	Bromothymol blue	Coomassie brilliant blue R	Methyl red	Methyl violet 10B	Remazol brilliant blue R	Trypan blue
Decolorization yield (%)	59	72	32	52	40	85
Half-life (h)	360	ND	ND	ND	300	500
Cycles of reuse	120	ND	ND	ND	100	160

ND: not determined

HIGHLIGHTS

P. vulgaris produces a heat- and alkaline-resistant extracellular laccase.

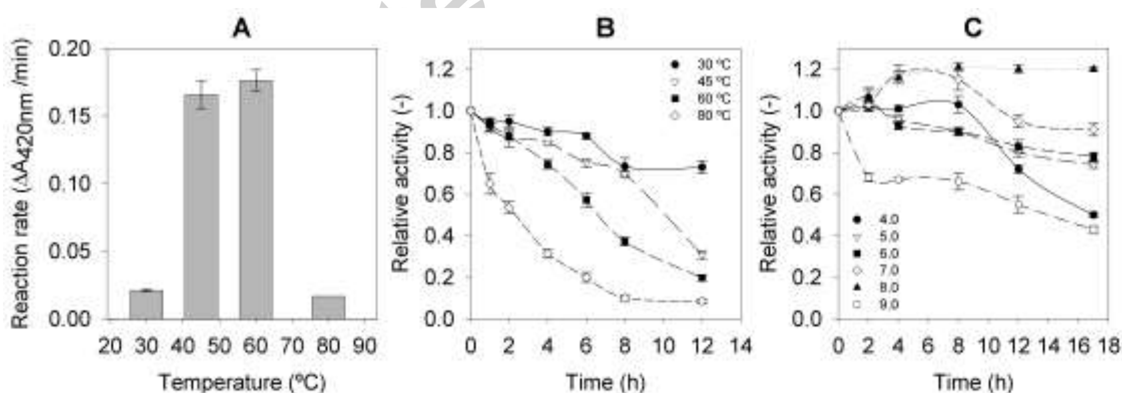
Laccase activity is significantly improved by metals and ionic surfactants.

The enzyme was tolerant to inhibitors and solvents present in sewage effluents.

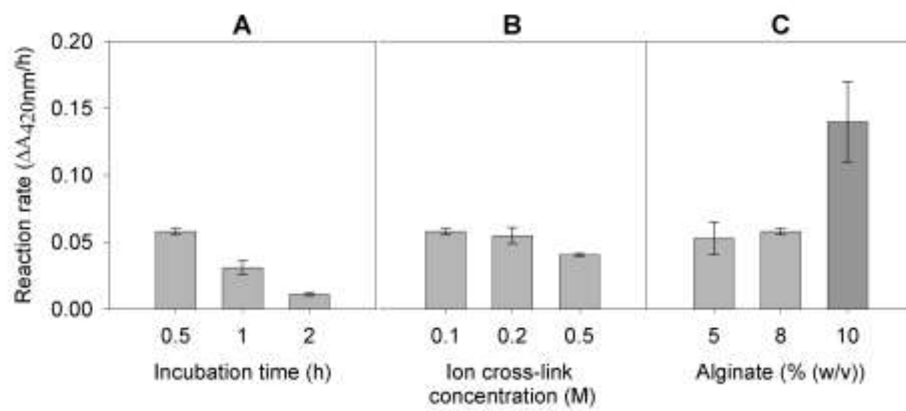
Immobilized laccase decolorized dyes at only 3 h of reaction without redox mediator.

The biocatalyst developed was thermostable and can be reused up to 160 cycles.

Accepted manuscript



ACCEPTED MANUSCRIPT



pted