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"DECOLORIZATION AND KINETICS OF REACTIVE DYES BY NATIVE LACCASES FROM NORTHEAST MEXICO"

TESIS

PRESENTADA COMO REQUISITO PARCIAL PARA

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ELABORADA POR:

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- A Miguel, Consuelo, Marco, Stephy y Erick, porque son el motor de mi vida.
- A Dios porque sin él nada de ésto hubiera sido posible.

Abstract

Laccase isoforms Lac-I and Lac-II produced by the basidiomicete *Pycnoporus sanguineus* were investigated. Both enzymes presented high stability during prolonged storage under freezing conditions (-20 ^{o}C) with no significant activity reduction.

For RBBR, decolorization efficiencies range from 82 to 88% after 3 hours of incubation for both isoforms at 1 and 8 U mL^{-1}. However, with 8 U mL^{-1} the decolorization ranges between 70 to 80% during the first 5 minutes of incubation. A significant lower decolorization pattern was observed with RB-5 reaching a maximum decolorization efficiency of 50% after 15 hr of incubation. For this time, the enzymatic decolorization of RB-5 with 1 U mL^{-1} was between 14 to 27% and at the high laccase activity level, 8 U mL^{-1}, was from 33 to 53%.

The use of laccase mediators was applied to improve RB-5 decolorization rates using violuric acid and N-hydroxypthalamide. Violuric acid reduced decolorization time from 15 hr to 25 min with 1 mM and 1 U mL^{-1} of enzyme activity and reaching up to 80% decolorization. The maximum decolorization efficiency obtained with N-HPT was 71% after 15 hr of incubation.

Kinetic parameters of the two laccases were determined using RBBR as substrate: K_m was 0.243 and 0.117 mM and V_{max} was 1.233 and 1.012 mM sec^{-1} for Lac-I and Lac-II respectively. Both enzymes have high decolorization power for dye decolorization although Lac-I was slightly more active against RBBR and RB-5 than Lac-II throughout the experimentation. These enzymes demonstrated high potential against both dyes and have significant differences in comparison to other reported enzymes.

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1. Introduction

Synthetic dyes are extensively used in various industrial processes in the textile, paper, cosmetics, pharmaceutical, and food industries (Mazmanci & Ünyayar, 2005). The textile industry is the largest user of these dyes, consuming about 56% of the approximately 0.7 million tons produced annually worldwide (Park, et al., 2007). Many dyes are difficult to decolorize due to their complex structure and synthetic origin. There are many structural varieties, such as acidic, basic, disperse, azo, diazo, anthroquinone-based, and metal complex dyes (Robinson, *et al.,* 2001); about 50% of the industrial dyes produced in the world are azo dyes.

Synthetic dyes are preferred to their natural counterparts because of their superior fastness in fabric, high photolytic stability, and resistance to microbial decolorization (Murugesan, Dhamija, Nam, Kim, & Chang, 2007). However, they are considered xenobiotic compounds, and they are highly recalcitrant to biodegradation. At least 10% of the dyestuff used enters the environment through wastewater, which is markedly colored, causing severe damages to the ecosystem. The dyes significantly affect photosynthesis due to the reduction of light penetration, and they are considered toxic for aquatic life and living organisms drinking from these waters because of the presence of compounds such as metals and chlorides (Pajot, de Figueroa, & Fariña, 2007).

2. Types of dyes

Synthetic dyes exhibit considerable structural diversity, such as acidic, basic, disperse, azo, diazo, anthroquinone based and metal complex dyes. The chemical classes of dyes employed more frequently on industrial scale are azo, anthraquinone, sulfur, indigoid, triphenylmethyl (trityl), and phthalocyanine derivatives (Figure 1) (Forgacs, Cserháti, & Oros, 2004).

According to certain common chemical structural features, dyes can also be classified as cationic, nonionic and anionic. A characteristic feature of anionic dyes is the presence of one or more sulfonate ($-SO_3^-$) groups, usually as sodium (Na^+) salts. These groups impart negative charge to the dyes and features such as acidity and reactivity. Cationic dyes are positively charged and are also known as basic dyes. Nonionic or disperse dyes do not ionize in an aqueous medium (Robinson, *et al.*, 2001). The chromophores in anionic and nonionic dyes are mostly azo groups or antraquinones. The reductive cleavage of azo linkages is responsible for the formation of toxic amines in the effluent. Anthraquinone-based dyes are more resistant to degradation due to their

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fused aromatic structures and thus remain colored for a longer time in wastewater. Basic dyes have high brilliance and intensity of colors and are highly visible even in a very low concentrations. Most of the metal complex dyes are based on chromium, a well-known carcinogen (Fu Y. , 2001).

Many dyes are cause for concern because they are made from known carcinogens, such as benzidine and other aromatic compounds (Robinson, *et al.*, 2001). Weber and Wolfe (1987) demonstrated that azo and nitro compounds are reduced in sediments resulting in the formation of toxic amines similarly, Chung et al. (1978) illustrated their reduction in the intestinal environment. The ability of some disperse dyes to bioaccumulate has also been demonstrated (Robinson, *et al.*, 2001). RBBR (C.I. 61,200) is an important industrial dye used in the production of polymeric dyes (Palmieri, Cennamo, & Sannia, 2005). It is an anthracene derivative and as a result, its chemical structure is related to an important group of toxic and recalcitrant organopollutants (Machado, Matheus, & Bononi, 2005). RB-5 (C.I. 20,505) is another industrially important dye (Lucas, Amaral, Sampaio, Peres, & Dias, 2006). RB-5 is a diazo dye (i.e., it contains two azo bonds, -N=N-) which is highly recalcitrant to conventional wastewater treatment processes (Lucas, Amaral, Sampaio, Peres, & Dias, 2006). These two dyes were used in this study as models to test the potential for dye decolorization of the newly isolated enzymes.

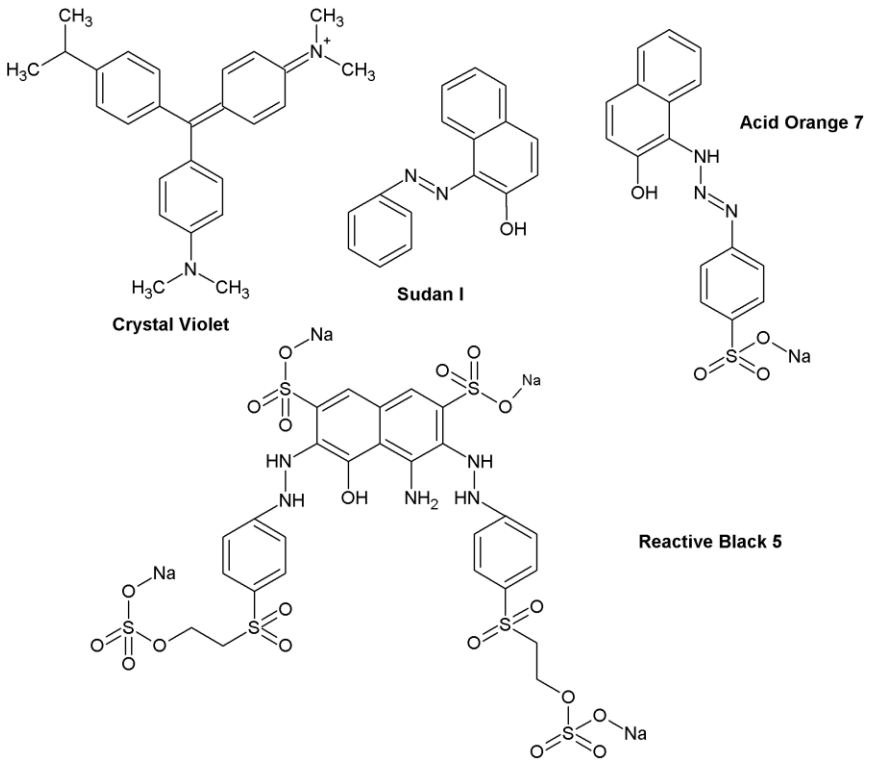

Figure 1. Chemical structure of the synthetic dyes most frequently studied in decolorization experiments (Forgacs, Cserháti, & Oros, 2004).

2.1 Methods for dye removal

Color can be removed by chemical and physical methods including adsorption, coagulation-flocculation, ion-exchange, oxidation and electrochemical methods, but these are expensive because of the high chemical usage, costly infrastructure and high operating expenses (Kunamneni, Ghazi, Camarero, Ballesteros, Plou, & Alcalde, 2008). In addition, many of these compounds are highly resistant to microbial attack and as a result, they are hardly removed from effluents by conventional biological processes, such as activated sludge treatment (Palmieri, Cennamo, & Sannia, 2005). Table 1 lists the common methods for textile dye treatment by chemical and physical means.

Adsorption has been observed to be an effective process for color removal from dye wastewaters. but it is too expensive. Many studies have been undertaken to find low-cost adsorbents, which include peat, bentonite, steel-plant slag, fly ash, China clay, maize cob, wood shavings, and silica (Fu & Viraraghavan, 2001). However, these low-cost adsorbents generally have low sorption

capacities, which mean that a large amount of the adsorbent is needed. Some of the adsorbents for dye removal are described in Table 1 .

In recent years, several studies have focused on microorganisms capable of biodegrading and biosorbing dyes in wastewaters. These microorganisms include bacteria, fungi and algae.

Table 1 Chemical and physical methods for dye removal from industrial effluents (Robinson, *et al.,* 2001).

Type	Method	Advantages	Disadvantages
Chemical	Fenton's reagent	Effective decolorization of both soluble and insoluble dyes	Sludge generation
Chemical	Ozonation	Applied in gaseous state: no alteration of volume	Short half-life (20 min)
Chemical	Photochemical	No sludge production	Formation of by-products
Chemical	NaOCl	Initiates and accelerates azo-bond cleavage	Release of aromatic amines
Chemical	Cucurbituril	Good sorption capacity of various dyes	High cost
Chemical	Electrochemical destruction	Breakdown compounds are non-hazardous	High cost of electricity
Physical	Activated carbon	Good removal of wide variety of dyes	High cost
Physical	Peat	Good adsorbent due to cellular structure	Specific surface areas for adsorption are lower than activated carbon
Physical	Wood chips	Good sorption capacity for acidic dyes	Requires long retention times
Physical	Silica gel	Effective for basic dye removal	Side reactions prevent commercial application
Physical	Membrane filtration	Removes all dye types	Concentrated sludge production
Physical	Ion exchange	Regeneration: no adsorbent loss	Not effective for all dyes
Physical	Irradiation	Effective oxidation at lab scale	Requires a lot of dissolved O_2
Physical	Electrokinetic coagulation	Economically feasible	High sludge production

2.2. Biological treatments

Due to the lack of effective wastewater treatments for the degradation of these recalcitrant compounds, and the need for improved biological remediation techniques, enzyme technology has received increased attention. However, the role of microorganisms is wrought with problems. The accumulation in the environment of highly toxic pollutants only emphasizes the fact that

microorganisms, by themselves, are insufficient to protect the biosphere form anthropogenic pollution. A limiting factor in bioremediation of polluted contaminated sites is the very slow rate of degradation that limits the use of bacteria during these processes (Whiteley & Lee, 2006). Despite the disadvantages described above, the use of other microorganisms, primarily fungi, has been explored as an alternative to degrade aromatic and recalcitrant compounds such as dyes (Lucas, Amaral, Sampaio, Peres, & Dias, 2006). The basidiomycete group, in particular white-rot fungi (WRF), has been reported to be capable of degrading a diverse group of contaminants, including dyes (Nilsson, Moller, Mattiasson, Rubindamayugi, & Welander, 2006). WRF are responsible for wood decay through extracellular ligninolytic enzymes, which include laccases, lignin peroxidases (LiP), and manganese peroxidases (MnP) (Eichlerova, Homolka, & Nerud, 2007). Some biological treatments are described in Table 2, with their advantages and disadvantages.

Table 2. Biological treatments for dye removal from wastewater (Whiteley & Lee, 2006).

Name	Advantages	Disadvantages
Aerobic digestion	Aerobic bacteria are very efficient in breaking down waste products. The aerobic pathway releases a substantial amount of energy.	Production of large amounts of sludge, which contains volatile organics, organic solids, nutrients, pathogens, heavy metals, toxic organic chemicals.
Anaerobic digestion -Hydrolysis -Acidogenesis -Acetogenesis -Methanogenesis	Proven to be unique and the most beneficial stabilization technique as it optimizes cost effectiveness, is environmentally sound, minimizes the amount of final sludge disposal and has the ability to produce a net energy gain in the form of methane gas.	It is only capable of partially treating waste in a conventional wastewater treatment system with high levels of degradation requiring longer retention times and further treatment methods.
Enzymatic degradation	There are over 1000 enzymes reported to be involved in the biodegradation of aromatic dyes. It does not usually produce toxic by-products. Reduced effluent toxicity and pollution load, reduced consumption of bleaching chemical.	More research is needed for locations with complex mixtures of contaminants. Unknown degradation efficiencies and kinetic parameters for contaminants such as dyes.
Bioleaching	Cheaper to operate and maintain, more environmentally friendly than traditional extraction methods.	Very slow compared to the others. Toxic chemicals are sometimes produced in the process.

Production of these enzymes varies among species. For instance, *Pycnoporus cinnabarinus* has been reported to produce only laccases (Eggert, Temp, & Eriksson, 1996). In contrast, *Pleurotus ostreatus* was reported to produce only MnP and manganese-independent peroxidase (MIP) (Shrivastava, Christian, & Vyas, 2005). Laccases from white-rot fungi are receiving increasing attention as potential industrial enzymes in various applications such as pulp delignification, wood fiber modification, dye or stain bleaching, chemical and medicinal synthesis, and contaminated water or soil remediation (Kunamneni, Ghazi, Camarero, Ballesteros, Plou, & Alcalde, 2008). In addition, laccases offer the possibility to reduce costs, protect the environment, address health and safety, and improve quality and functionality of the processes (Chen, Wang, Hua, & Du, 2007).

3. Laccase Production and Mechanisms

3.1. Discovery

Laccase is one of the few enzymes studied since the 19th century. They were first discovered by Yoshida in 1883 in the exudates of the Japanese lacquer tree, *Rhus vernicifera*, and it was subsequently shown that they produced by fungi as well (Thurston, 1994). Laccase is one of a small group of enzymes called the large blue copper proteins or blue copper oxidases. The other members of this group are the plant ascorbate oxidases and the mammalian plasma protein ceruloplasmin (Thurston, 1994).

The blue copper oxidases have been intensively studied because they share with the terminal oxidases of aerobic respiration the ability to reduce molecular oxygen to water (equation 1) (Kunamneni, Ghazi, Camarero, Ballesteros, Plou, & Alcalde, 2008) (Widsten & Kandelbauer, 2008).

$$4\,PhOH + O_2 \xleftrightarrow{Laccase} 4PhO\cdot + 2H_2O \qquad (1)$$

Laccase-catalyzed oxidation of phenolic hydroxyl groups to phenoxy radicals (Widsten & Kandelbauer, 2008).

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3.2. Localization

Laccases (EC 1.10.3.2; benzenediol: oxygen oxidoreductase) are blue multi-copper-containing enzymes that are widely distributed among plants, fungi and bacteria (Kalme, Jadhav, Jadhav, & Sanjay, 2009). These oxidative enzymes are particularly abundant in white rot fungi, which are well known for their ability to produce extracellular oxidative enzymes such as the laccases themselves, lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (VP). These enzymes have the ability to degrade lignin as well as other wood components and a wide variety of recalcitrant compounds, including synthetic dyes (Erkurt, Ünyayar, & Kumbur, 2007).

3.2.1. Lignin

Lignin is a rigid organic polymer which functions as cementing material in wood cells. Harsh physicochemical conditions have to be applied to attack or modify this compound such as defibration, bleaching, etc (Call & Mücke, 1997). Lignin consists of *p*-hydroxyphenyl, guaiacyl, and syringyl-type phenylpropane units, in which the aromatic ring has 1 to 3 free or etherified hydroxyl groups, respectively (Figure 2) (Widsten & Kandelbauer, 2008).

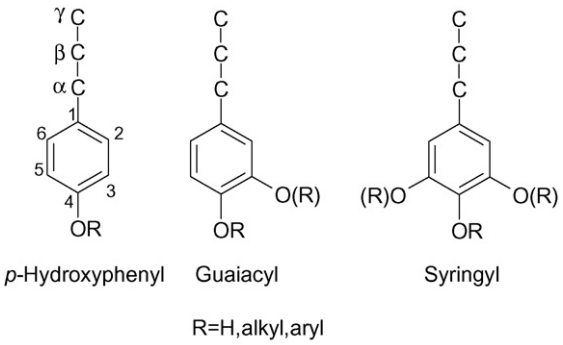

Figure 2. Lignin model compounds (Widsten & Kandelbauer, 2008).

3.3. Reactions

Substrate oxidation by laccases is due to the reaction of one electron that generates a free radical (Figure 3). The redox process takes place with the assistance of a cluster of four copper atoms that form the catalytic core of the enzyme; they also confer the typical blue color to these enzymes because of the intense electronic absorption of the Cu-Cu linkages. The overall outcome of the catalytic cycle is the reduction of one molecule of oxygen to two molecules of water and the concomitant oxidation of four substrate molecules to produce four radicals (Riva, 2006). The initial product is unstable and can suffer a second catalytic oxidation by the enzyme or a non-enzymatic

reaction (hydration, polymerization, etc.). The phenylpropane units in lignin are linked together by ether and carbon-carbon bonds, which the enzyme breaks (Widsten & Kandelbauer, 2008).

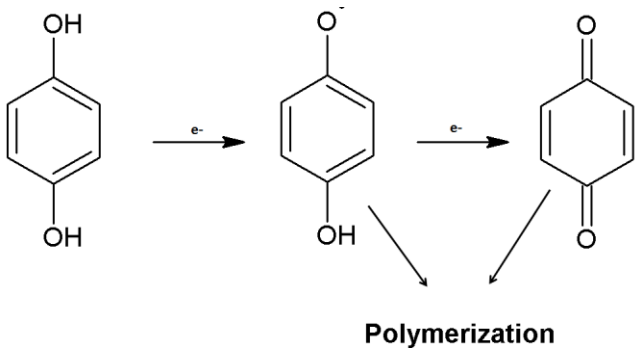

Figure 3. The typical laccase reaction, where a diphenol undergoes a one-electron oxidation to form an oxygen-centered free radical (Thurston, 1994).

Frequently the substrates of interest cannot be oxidized directly by laccases, either because they are too large to penetrate into the enzyme active site, or because they have a particular high redox potential. It is possible to overcome this limitation with the addition of chemical mediators (Kurniawati & Nicell, 2007).

3.4. Laccase Mediator Systems (LMS)

Mediators allow laccases to oxidize non-phenolic compounds, expanding the range of substrates that can be oxidized by these enzymes. Laccase Mediator Systems (LMS) were first described by Boubonnais and Paice in 1990 and they were first developed to solve problems of pulp bleaching, an important biotechnical application of a process based on a single lignolytic enzyme (Soares, Pessoa de Amorim, & Costa-Ferreira, 2001).

The mediator is first oxidized by the laccase, and then it diffuses into the cell wall, oxidizing lignin inaccessible to laccase (Figure 4). Laccase-mediated oxidation of non-phenolic lignin units can follow an electron transfer, a radical hydrogen atom transfer, or an ionic mechanism, depending on the mediator.

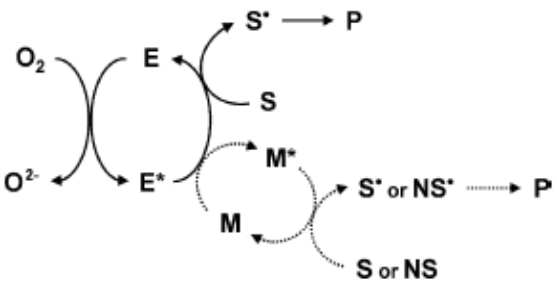

Figure 4. Laccase acting either directly on substrates or indirectly on substrates and non-substrates via mediators. Abbreviations: native laccase (E); oxidized laccase (E*); substrate (S); substrate radical (S*); non-substrate (NS); non-substrate radical (NS*); end products (P); mediator (M); oxidized mediator (M*). (Soares, Pessoa de Amorim, & Costa-Ferreira, 2001).

The first synthetic mediator found to be able to oxidize high-redox potential non-phenolic lignin compounds is ABTS (2,2´-azinobis-3-ethylbenzenethiazoline-6-sulfonic acid). Other widely investigated synthetic mediators are HBT (1-hydroxybenzotriazole) and VA (violuric acid). However, the cost of synthetic mediators tends to be prohibitive for implementation. In addition, high concentrations may not be appropriate for some applications such as wastewater treatment (Kurniawati & Nicell, 2007). As a result, interest in mediators derived from plants or industrial by-products has been generated. One of the first natural laccase mediators discovered was syringaldehyde. Figure 5 shows some natural and synthetic laccase mediators (Widsten & Kandelbauer, 2008).

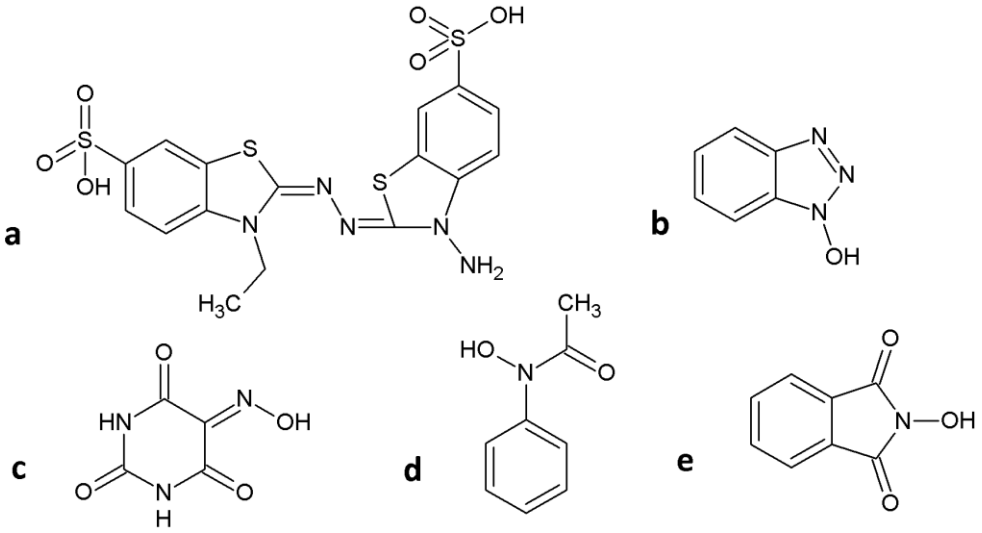

Figure 5. Laccase mediators; (A) 2,2´-azinobis-3(ethylbenzenthiazoline-6-sulfonic acid) (ABTS); (B) 1-hydroxybenzotriazole (HBT); (C) violuric acid (VA); (D) N-hydroxyacetanilide (NHA); (E) N-hydroxyphthalimide.

3.5. Laccase Structure

Fungal laccases often occur as isoenzymes that oligomerize to form multimeric complexes. The molecular mass of the monomer ranges from about 50 to 100 kDa.

Table 3 shows the properties of some purified laccases. An important feature is a covalently linked carbohydrate moiety, which may contribute to the high stability of the enzymes (Claus H. , 2004).

Table 3. Properties of some purified laccases

Organism	MW (Da)	Reference
Pycnoporus sanguineus	58 000	Lithauer *et al*., 2007
Botryotinia fuckeliana	73 000 - 80 000	Saito *et al*., 2003
Pleurotus ostreatus(a)	64 000	Pozdnyakova *et al*. 2006
Pleurotus ostreatus(b)	40 000	Liu *et al.*, 2009
Polyporus versicolor	60 000	Thurston, 1994
Neurospora crassa	65 000	Thurston, 1994
Pycnoporus cinnabarinus	81 000	Eggert *et al,* 1996
Pycnoporus sanguineus	54 000 (Lac-I)	This work
Pycnoporus sanguineus	48 000 (Lac-II)	This work

A minimum of four copper atoms per active protein unit is needed for catalytic activity:

- Type 1: paramagnetic “blue” copper
- Type 2: paramagnetic “non-blue” copper.
- Type 3: diamagnetic spin-coupled copper-copper pair.

Type 1 copper confers the typical blue color to multicopper proteins, which results from the intense electronic absorption caused by the covalent copper-copper cysteine bond. Due to its high redox potential of ca. +790 mV, type 1 copper is the site where substrate oxidation takes place. Type 2 copper shows no absorption in the visible spectrum and reveals paramagnetic properties in EPR studies. It is strategically positioned close to the type 3 copper, a binuclear center spectroscopically characterized by an electron adsorption of 330 nm (oxidized form) and by the absence of an EPR signal as the result of the anti-ferromagnetic coupling of the copper pair. The

type 3 copper center is also the common feature of another protein superfamily, which includes the tyrosinases and haemocyanins (Claus H. , 2004).

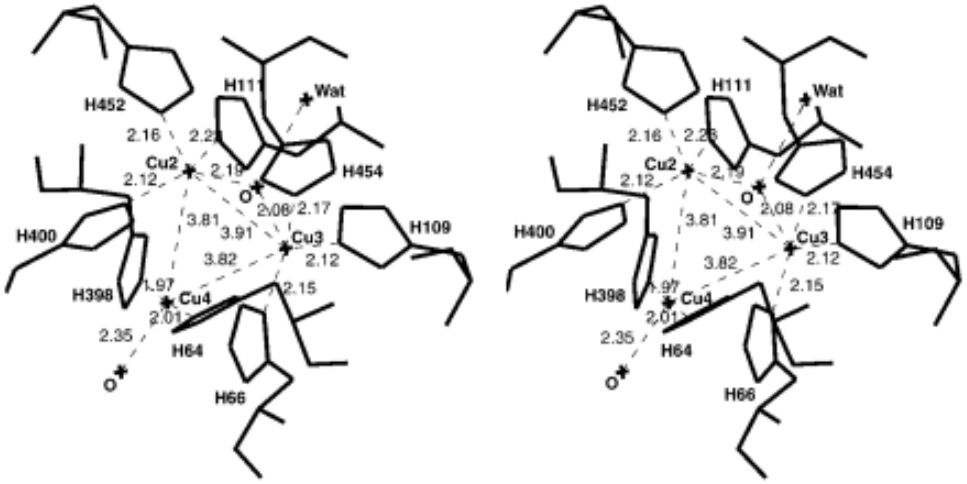

Figure 6. Stereo view of the T2/T3 coppers and their close environment in *Trametes versicolor*. Bonds are represented by thin dashed lines and lengths are given in Å (Piontek, Antorini, & Choinowski, 2002).

4. Objectives

4.1. General objective

To evaluate the decolorization kinetics of two textile dyes, Ramazol Brilliant Blue R (RBBR) and Reactive Black 5 (RB-5), by laccase isoenzymes produced by *Pycnoporus sanguineus* isolated in northeast Mexico.

4.2. Specific objectives

1. To evaluate dye decolorization using different laccase isoforms on two dyes (RBBR y RB-5) at different enzyme and dye concentrations.
2. To determine the kinetic parameters (V_{max} & K_m) of the laccase isoforms using RBBR as substrate.
3. To compare the catalytic power for dye decolorization of the newly isolated laccase isoenzymes.
4. To analyze the effect of mediators on the decolorization of recalcitrant dyes in order to extend the use of laccases to other types of chemicals.

5. Materials and Methods

5.1. Screening of native fungal strains and enzyme production

A screening process to isolate WRF was conducted in the mountains of the State of Nuevo Leon, in northeast Mexico. From the 92 fungal isolates obtained, 15 showed potential for dye decolorization (Hernández-Luna, Gutierrez-Soto, & Salcedo-Martínez, 2008). *Pycnoporus sanguineus* (*cinnabarinus)* was selected for this study as its two isoforms (Lac-I and Lac-II) showed ligninolytic activity and decolorizing potential during preliminary assessments.

Enzyme production was conducted in Petri disheswith yeast-malt-glucose agar medium (YMGA), inoculated with the local fungal strains and incubated for 1-2 weeks. Three 0.5 cm diameter plugs of growing mycelium were taken from the periphery of the culture and used as inocula for liquid cultures. A modified Dhouib medium supplemented with an inducer was used to propagate *P. sanguineus* (Zouari-Mechichi, Mechichi, Dhouib, Sayadi, Martínez, & Martínez, 2006). This medium consisted of glucose (10 g L^{-1}), peptone (5 g L^{-1}), yeast extract (1 g L^{-1}), ammonium tartrate (2 g L^{-1}), potassium phosphate (1 g L^{-1}), potassium chloride (0.5 g L^{-1}), magnesium sulfate (0.5%), trace elements (EDTA, $FeSO_4$, $ZnSO_4$, $MnCl_2$, H_3BO_4, $CoCl_2$, $CuCl_2$, and $NaMoO_4$) in aqueous solution (1 mL), and copper sulfate (0.35 mM) as inducer. The medium was sterilized at 121 ^oC for 15 min. Three days after initial inoculation, ethanol (3%) was added as a secondary inducer.

Cultures were incubated at 25 ^oC on a rotary shaker at 150 rev min^{-1}. Enzyme activity in the liquid cultures was measured every other day by taking 1 mL of liquid broth. A 100 µL sample aliquot was centrifuged at 14,000 rev min^{-1} for 10 min (Eppendorf Centrifuge Model 5415c). The peaks of enzyme production were established by plotting enzyme activity versus time. After approximately 15 days of fungal growth, when the enzyme production was at its maximum, the broth was filtered to remove the biomass. The supernatant was frozen at -20 ^oC for 24 h, then thawed and filtered again. The supernatant was concentrated by ultrafiltration to about 5% of the original volume. Several 0.1 mL aliquots were taken for the analysis of components.

5.2. Laccase purification

Enzyme purification is a series of processes intended to isolate a single type of enzyme from a complex mixture. Purification is vital for the characterization of the function, structure and

interactions of the enzyme of interest. Separation of one protein from all others is typically the most laborious aspect of enzyme purification. Separation steps exploit differences in size, physico-chemical properties and binding affinity. Chromatography and other methods along with adjusted conditions of pH, salt concentration, etc., are chosen based on the types of enzymes being purified.

5.2.1. Ionic exchange

During the first step of the purification process, the samples that were previously concentrated were loaded on a DEAE-Sepharose column (2.5 cm x 17 cm) equilibrated with 20 mM sodium phosphate buffer, pH 6.0. The enzyme fractions were eluted with a linear concentration gradient from 20 to 150 mM potassium phosphate. Fractions containing laccase activity were collected and further concentrated in an Amicon cell. Several aliquots (0.1 mL) were taken for the analysis of components. Ion exchange chromatography showed two distinct laccase activity peaks that were detected and labeled Lac-I and Lac-II according to elution time (Hernández-Luna, Gutierrez-Soto, & Salcedo-Martínez, 2008).

5.2.2. Gel filtration

Concentrated samples of Lac-I and Lac-II were applied and eluted independently on a Sephadex G-200 column (1.5 cm x 71 cm) equilibrated with 100 mM sodium phosphate buffer, pH 6.0. Fractions containing laccase activity were collected and concentrated. Aliquots (0.1 mL) were taken for analysis of components. In both cases, an apparently homogeneous single peak with laccase activity was detected. Lac-I and Lac-II preparations showed specific activities of 60 U mg^{-1} and 58 U mg^{-1} after gel permeation and a main band of 54 kDa and 48 kDa by SDS-PAGE analysis, respectively (Hernández-Luna, Gutierrez-Soto, & Salcedo-Martínez, 2008).

5.2. Determination of laccase activity

According to Beer's Law (equation 2), the absorbance or fluorescence or whatever optical parameter is being used, is directly proportional to the concentration of the reagent:

$$A = \varepsilon l c \qquad (2)$$

Where A is the absorbance, ε the extinction coefficient, l the path length and c is the concentration usually in µmol mL^{-1}. In the case of enzymes, this concentration is per unit time and it is the same as activity, i.e. µmol mL^{-1} min^{-1}. Consequently, if the extinction coefficient of a

substance at a particular wavelength is known, and the change in an optical parameter is measured over time, it is possible to determine the activity of the enzyme (equation 3):

$$\frac{dV}{dt} = \frac{dA}{dt}\frac{1}{\varepsilon l} \times d \qquad (3)$$

Where dV/dt are the units of activity in μM min^{-1}, dA/dt is the slope in min^{-1}, ε is the molar extinction coefficient in mM^{-1} cm^{-1} (for ABTS is 36), l the light's path length through the well in cm and d the dilution factor.

The extinction coefficient for the substance under investigation can be calculated from the slope of a linear plot, usually by linear regression, between the absorbance and several concentrations of pure substance. In the case of substances that do not absorb strongly, they are usually reacted with a dye to produce color that is measured at some specific wavelength in the visible spectrum. Specific activity is defined as units of enzyme activity per mass of protein; the amount of protein in the biomass may be determined by different methods, depending on the concentration of protein (Whiteley & Lee, 2006).

Laccase activity was determined spectrophotometrically by the oxidation of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid). The reaction medium contained 2 mM ABTS in 100 mM acetate buffer, pH 4.0, and was assayed at 25 ^{o}C. Oxidation of ABTS was followed by absorbance increase at 420 nm. One unit of laccase activity was defined as the amount of enzyme transforming 1 μmol of its target substrate per minute.

5.3. Determination of V_{max} and K_m

The kinetics of simple enzyme-catalyzed reactions was first characterized in 1919 by two chemists, Michaelis and Menten, who derived a hyperbolic equation (equation 4) relating reaction rate to substrate concentration (Whiteley & Lee, 2006):

$$v_1 = \frac{V_{max}[S]}{K_m + [S]} \qquad (4)$$

where v_1 is the rate of the enzyme-catalyzed reaction, $[S]$ the concentration of substrate, K_m the Michaelis-Menten constant, and V_{max} is the maximum reaction rate. It can quickly be established that K_m is also equal to the substrate concentration that would give the 50% V_{max}. At low substrate concentrations, the rate of an enzymatic reaction follows a first-order kinetics model; i.e., it is directly proportional to substrate concentration ($v=k[S]$) (Figure 7, region a). In contrast, at high

substrate concentration, the rate becomes independent of substrate concentration, a zero order kinetics model ($v = V_{max}$) (Figure 7, region b). In this region, almost all of the enzyme units are bound to substrate.

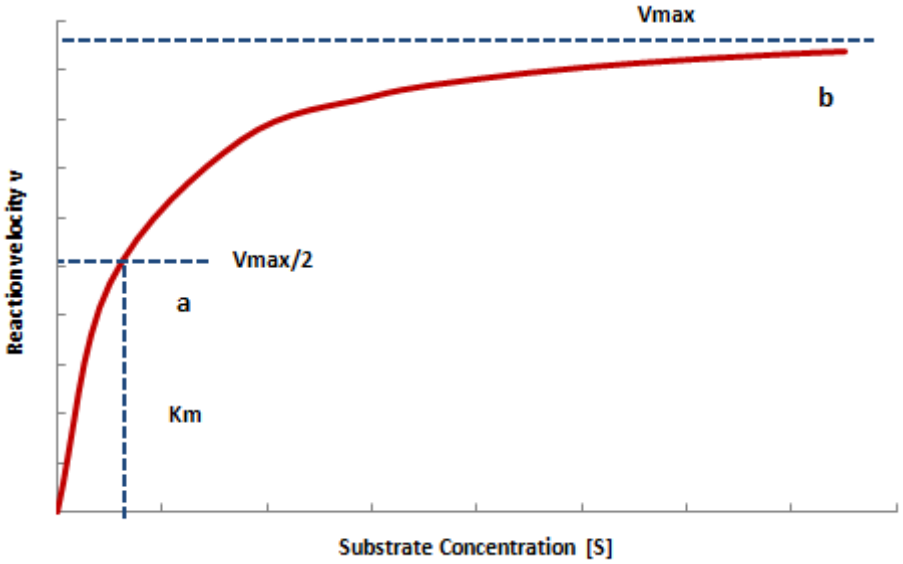

Figure 7. A typical Michaelis Menten curve representing change in velocity of an enzyme catalyzed reaction with respect to substrate concentration (Whiteley & Lee, 2006).

To avoid this curvilinear plot, Lineweaver and Burk used a linearized version of the Michaelis-Menten equation to calculate the kinetic parameters. They used a double reciprocal plot, $1/v_1$ versus $1/[S]$ (Figure 8) with a slope of K_m/V_{max} and an intercept at $1/V_{max}$ (Whiteley & Lee, 2006). Table 4 shows additional methods for the calculation of the kinetic parameters used in this work.

Table 4. Calculation methods for the Michaelis-Menten kinetic parameters

Method	Form
Lineweaver-Burk	$\frac{1}{V} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}}$
Eadie-Hofstee	$V = -K_m\frac{V}{[S]} + V_{max}$
Scatchard	$\frac{V}{[S]} = -\frac{1}{K_m}V + \frac{V_{max}}{K_m}$
Hanes-Woolf	$\frac{[S]}{V} = -\frac{1}{V_{max}}[S] + \frac{K_m}{V_{max}}$

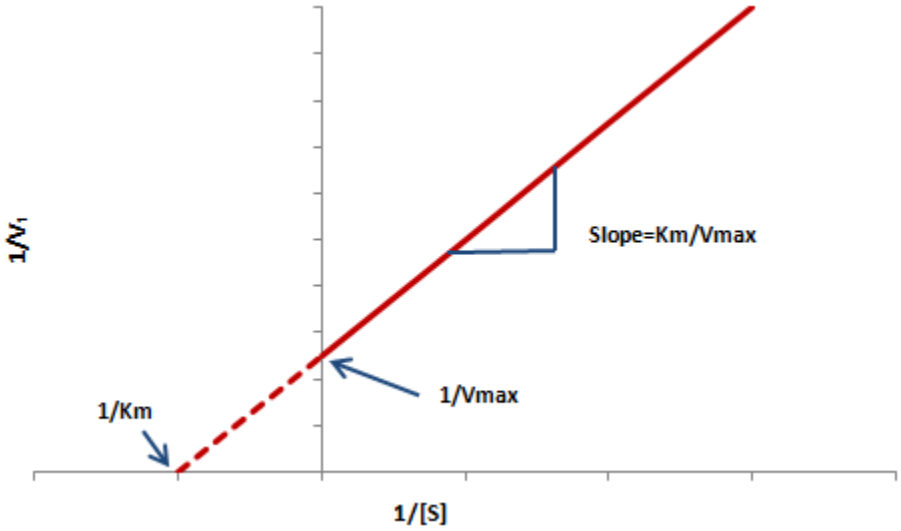

Figure 8. Lineweaver-Burk double reciprocal plot

5.3. Chemicals

The anthraquinone-based dye Remazol Brilliant Blue R (CAS 2580-78-1), the 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonate (ABTS), as well as the laccase mediators violuric acid (VA) and N-hydroxyphthalimide (N-HPT) were obtained from Sigma-Aldrich (St. Louis, MO). The diazo dye Reactive Black 5 was courteously supplied by Pyosa, S.A. de C.V., a local dye and pigments company, and used as provided without further purification. Chemical structures of both dyes are shown in Figure 9 and 10.

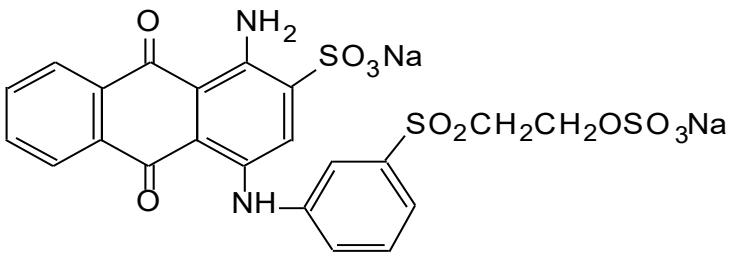

Figure 9. Remazol Brilliant Blue R (C.I. 61200, Reactive Blue 19)

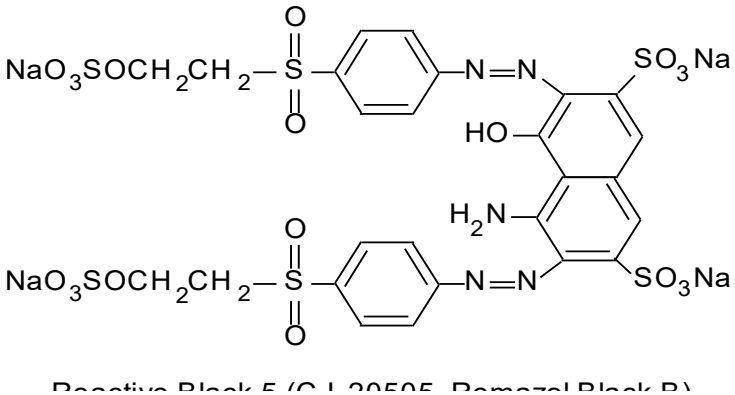

Figure 10. Reactive Black 5 (C.I. 20505, Remazol Black B)

5.4. Experimental Design

5.4.1. Decolorization Assessment

A full factorial method was used for the design of experiments. Initially, the two isoforms (Lac-I and Lac-II) obtained from *P. sanguineus* were assessed. For each isoform, the concentrations of enzyme tested were 1 and 8 U mL^{-1}. Decolorization of two reactive dyes (Remazol Brilliant Blue R, RBBR and Reactive Black 5, RB-5) was evaluated at two concentrations 50 and 100 mg L^{-1}. All the experiments were carried at pH 4.0 and 25 ^{o}C. A set of two control groups was used to evaluate the no-effect baseline. The first control group contained dyes at both concentrations but no enzymes. The second group contained the enzymes prepared at both concentrations but no dye. The dye or enzyme volumes were substituted with tartrate buffer (0.1 M, pH 4.0). Table 5 shows the factors and levels used in this study. All experiments and controls were conducted in triplicate, and the values shown are averages ± standard deviations.

Table 5. Variables used for the experimental design for RBBR and RB-5

Variable	Units	Coded Value	
		High	Low
		1	-1
Enzyme Isoform	-	Lac-I	Lac-II
Isoform Concentration	U mL^{-1}	8	1
Dye Concentration	mg L^{-1}	100	50

The analyses were performed at the maximum absorbance spectra (λ_{max}) of the dyes used (595 nm for RBBR and 596 nm for RB-5) using a FLUOstar Omega microplate reader set at 25°C. The decolorization efficiency was expressed as percentage according to the following equation:

$$Decolorizaton\ (\%) = \frac{A_0 - A_f}{A_0} \times 100 \qquad (5)$$

Where A_0 = initial absorbance and A_f = final absorbance. The final absorbance for the dye RBBR was reported after 3 h incubation and for RB-5 after 15 h. The statistical analyses were performed using the statistical software Minitab 15.1. Treatment effects were declared statistically significant if $P < 0.001$, unless otherwise noted.

5.4.2. RB-5 with mediators

A full factorial method was also used for the design of these experiments. The two isoforms (Lac-I and Lac-II) were evaluated, for each isoform the concentration of enzyme used was 1 U mL^{-1}. Decolorization of the Reactive Black 5 (RB-5) was evaluated at concentrations of 50 and 100 mg L^{-1}. Only RB-5 was studied because it is the most recalcitrant of the tested dyes. Two mediators were selected: Violuric Acid (VA) and N-hydroxyphthalimide (N-HPT) and they were tested at 1 and 5 mM. All the analyses were performed at pH 4.0 and at 25 °C. Table 6 shows the variables used for this study. Control samples without enzyme or without dye were run in parallel under identical conditions. All experiments and controls were performed in triplicate and values shown are the average of the samples ± standard deviation. The statistical analyses were performed using the statistical software Minitab 15.1. Treatment effects were declared statistically significant if $P < 0.001$, unless otherwise noted.

Table 6. Variables used for the experimental design of mediators

Variable	Units	Coded Value	
		High	Low
		1	-1
Enzyme Isoform	-	Lac-I	Lac-II
Dye Concentration	mg L^{-1}	100	50
LMS	-	VA	N-HPT
Mediator Concentration	mM	1	5

5.4.3. V_{max} and K_m assessment

For the calculation of the kinetic parameters, initial concentrations of colorant of 10, 20, 30, 40, 50 60, 70, 80, 90 and 100 mg L^{-1} were used. A kinetic window of 6 minutes was used for the measurement; each enzyme was tested at different concentrations (0.04, 0.6, 1 and 8 U mL^{-1}) using trial and error to obtain the linear part of the decolorization for initial velocities. For the calculation of the parameters, the MARS Data Analysis Software was used and five methods were applied: Michaelis-Menten, Lineweaver-Burk, Eadie-Hofstee, Scatchard, and Hanes-Woolf.

6. Results and Discussion

6.1. Laccase activity

Laccase activity in the enzyme stock solution was evaluated before every experiment; the activity was also determined after diluting the enzyme according to the experimental plan. Laccase activity was assayed using ABTS oxidation on a window of 2.5 min, using triplicates in every case. Table 7 lists laccase activity for each experiment carried with Lac-I and Lac-II, and it shows that Lac-I had higher activity than Lac-II during the different stages of the experimental work, although this difference is not statistically significant. Moreover, it also proves that both enzymes had high stability, with no apparent activity reduction while this project was conducted.

Table 7. Laccase activity per run

Experiment #	Activity (U L^{-1})	
	Lac-I	Lac-II
1	2721	2538
2	2670	2362
3	2758	2500
4	2746	2532
5	2670	2286
Average	**2713 ± 41**	**2443 ± 113**

Appendix A shows the average slope for Lac-I and Lac-II, from which the average laccase activity was calculated and the slope per run for Lac-I and Lac-II respectively.

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6.2. Decolorization assays

Dye decolorization ability of the two purified laccase isoforms produced by *P. sanguineus* was assayed on RBBR and RB-5 at 50 and 100 mg L^{-1}. Dye decolorization was evaluated with 1 and 8 U mL^{-1} of enzyme without redox mediators.

For RBBR, no significant difference in decolorization was observed after 3 hours for both activity levels. Decolorization efficiency ranged from 82 to 87% at 1 U mL^{-1} and between 83 to 88% for 8 U mL^{-1}. However, the difference between 1 and 8 U mL^{-1} is evident at shorter reaction times; the measurement at 10 minutes shows a difference in decolorization of approximately 35% between 1 and 8 U mL^{-1}. Figure 11 shows the time course of dye decolorization for RBBR with 50 mg L^{-1}, and Figure 12 with 100 mg L^{-1}. For high laccase activities with RBBR, maximal value of decolorization sets at 5 minutes, whereas at lower activity, a similar value is only reached after 50 minutes. A similar pattern was observed at higher concentrations of RBBR, 6 and 50 minutes for 1 and 8 U mL^{-1} respectively. Table 8 summarizes the decolorization percentages for RBBR under different experimental conditions. Both enzymes reach similar decolorization percentages at the same activity level. In order to make evident the initial velocities (V_o) relevant to the Michaelis and Menten model, Figure 13 shows a close-up of the fast kinetics occurring in the first five minutes of decolorization with 8 U mL^{-1} of Lac-I and Lac-II; during this time, a significant decolorization between 70 to 80% decolorization is reached for both isoforms.

Decolorization of RBBR (50 mg L^{-1}) using laccase from *G. lucidum* has been reported to degrade 40.5% after 1 h incubation and 98.5% after 12 h (Murugesan, Nam, & Chang, 2007). At a higher concentration, 100 mg L^{-1}, decolorization percentages were 39.6 and 94.0% after 1 and 12 h, respectively (Murugesan, Nam, & Chang, 2007). Although these decolorization levels are similar to ours, the concentration of enzyme used was 25 U mL^{-1}, compared to 1 and 8 U mL^{-1} used in this study. This translates into three times more decolorization capacity for the enzymes studied in this work. Moreover, other authors have reported a maximum 70% decolorization of RBBR after 2 h incubation with enzyme activity of 20 or 100 U mL^{-1} of crude laccase obtained from *P. ostreatus* (Palmieri, Cennamo, & Sannia, 2005). Soares *et al*. reported that a pure commercial laccase obtained from *Aspergillus* did not decolorize RBBR in the absence of a redox mediator. The differences in decolorization extent may be related to species variation in laccase structure and/or its redox potential.

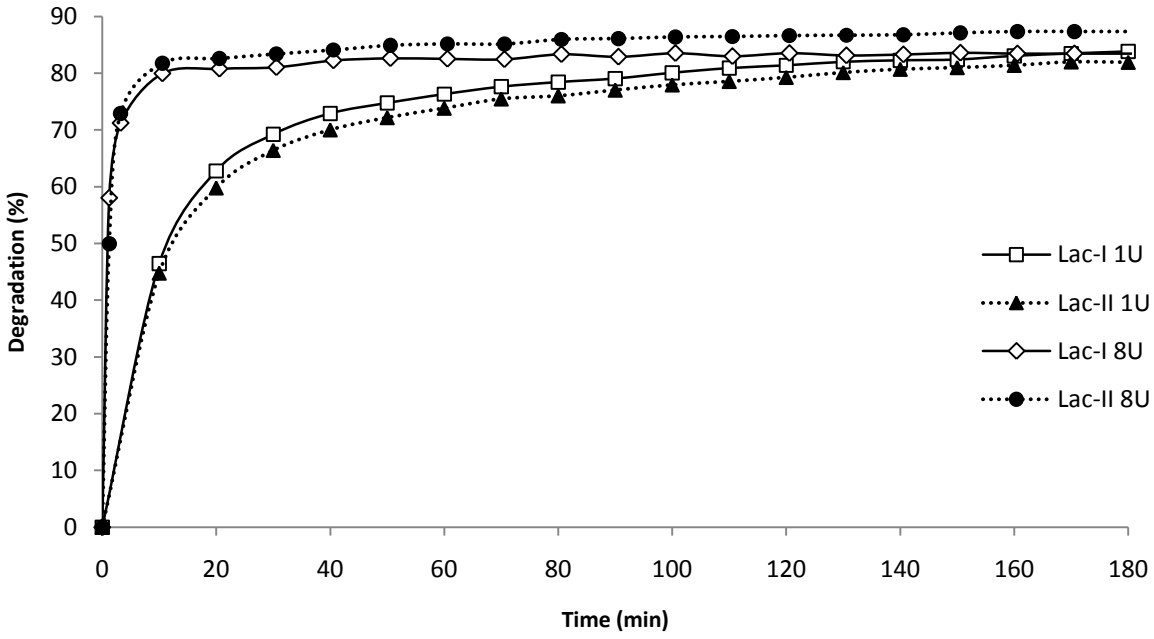

Figure 11. Decolorization of RBBR at 50 mg L^{-1}

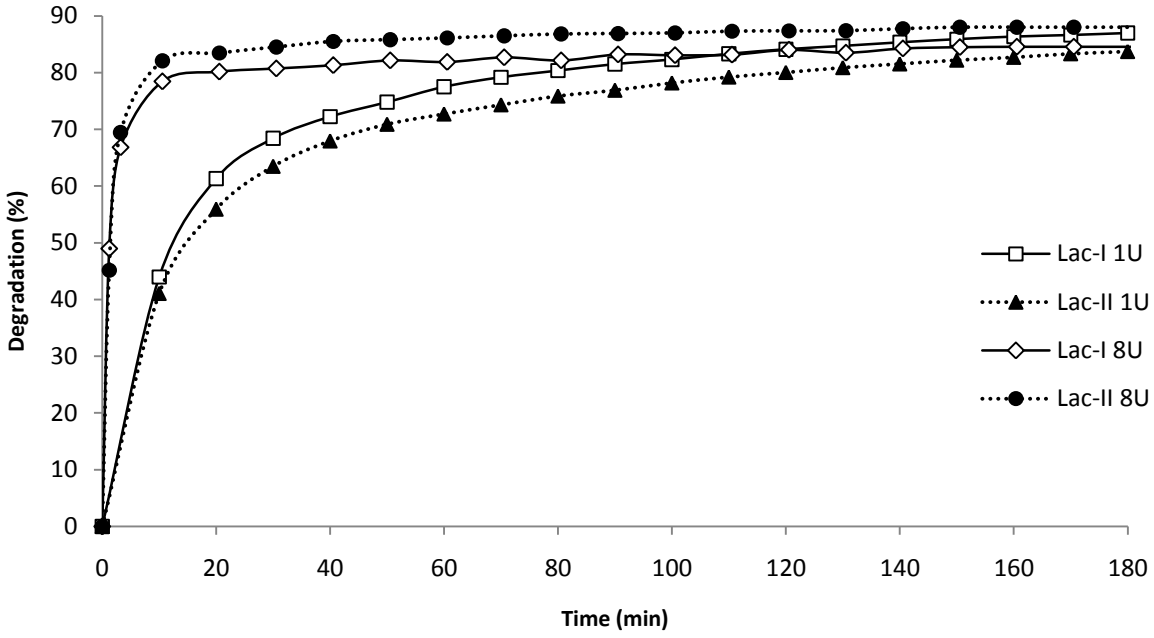

Figure 12. Decolorization of RBBR at 100 mg L^{-1}

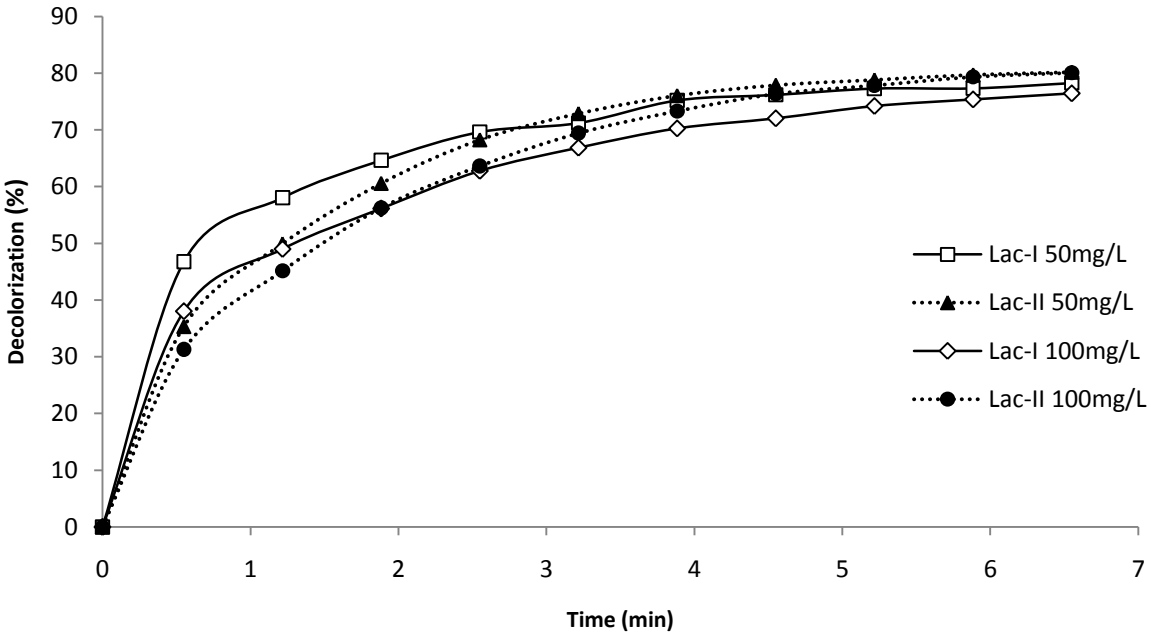

Figure 13. RBBR decolorization with 8 U mL^{-1} of Lac-I and Lac-II laccases

Based on the factorial statistical analysis, the most significant factor on dye decolorization was the enzyme concentration, followed by the interaction between the enzyme isoform and enzyme concentration (Table 9) which explains the difference in decolorization efficiency between 1 and 8 U mL^{-1} of enzyme. The main effects were significant at $P<0.001$ up to 10 min of decolorization progress.

Table 8. RBBR decolorization after 10 min and 3 h

Enzyme Isoform	Enzyme Concentration (U ml^{-1})	Dye Concentration (mg L^{-1})	Decolorization @ 10 min (%)	Decolorization @ 3 h (%)
LAC-I	1	50	46.4 ± 0.51	83.8 ± 1.12
LAC-I	1	100	43.9 ± 1.02	86.9 ± 0.38
LAC-I	8	50	79.8 ± 0.90	83.4 ± 0.65
LAC-I	8	100	78.2 ± 4.46	84.5 ± 3.43
LAC-II	1	50	44.7 ± 2.62	81.9 ± 2.24
LAC-II	1	100	41.0 ± 0.76	83.7 ± 0.51
LAC-II	8	50	81.4 ± 0.75	87.3 ±0.36
LAC-II	8	100	81.8 ± 0.47	87.9 ± 0.47

Table 9. Estimated effects and coefficients for the decolorization of RBBR at 10 min

Term	Coef	P
Constant	62.158	0.000
EnzIsof	-0.067	0.869
EnzConc	18.133	0.000
ColConc	-0.917	0.034
EnzIsof*EnzConc	-1.208	0.008
EnzIsof*ColConc	-0.092	0.820
EnzConc*ColConc	0.625	0.135
EnzIsof*EnzConc*ColConc	-0.400	0.328

The statistical analysis for the RBBR decolorization at 3 hr showed that the most significant factor in dye decolorization was the interaction between the enzyme isoform and enzyme concentration (Table 10). The two-way interaction were significant at $P<0.001$, opposite to what was observed in the short incubation times.

Table 10. Estimated effects and coefficient for the decolorization of RBBR at 3 hr

Term	Coef	P
Constant	84.942	0.000
EnzIsof	-0.250	0.437
EnzConc	0.850	0.016
ColConc	0.808	0.020
EnzIsof*EnzConc	-1.592	0.000
EnzIsof*ColConc	-0.200	0.533
EnzConc*ColConc	-0.367	0.260
EnzIsof*EnzConc*ColConc	-0.092	0.774

The laccase isolated from *P. sanguineus* show higher activity against RBBR in comparison to RB-5. Laccase decolorization was in the range of 82-88% for RBBR at 3 h. A significant lower decolorization pattern was observed with RB-5, ranging from 2-10% and 14-50% at 3 and 15 h respectively. Figure 14 shows the time course of dye decolorization for RB-5 with 50 mg L^{-1}. Although both laccases yield similar decolorization patterns, Lac-I shows slightly higher activity in comparison to Lac-II at the same enzyme concentration. At a higher dye concentration (100 mg L^{-1}) the decolorization capacity from Lac-I was higher than Lac-II (Figure 15). The difference of the two experiments, at 50 and 100 mg L^{-1}, may be due to mass transfer limitations in the system resulting

from increasing dye concentration and therefore an increase on reaction rate purely due to higher Lac-I catalytic power.

The decolorization time course as well as the overall decolorization efficiency was significantly different for RB-5 compared to RBBR. After 15 hours of incubation, enzymatic decolorization of RB-5 with 1 U mL^{-1} varied from 14 to 27%; at the high laccase activity level, 8 U mL^{-1}, decolorization ranged from 33 to 53%. For the first three hours, dye decolorization was minimal (4-10%) at both enzyme and dye concentrations. Table 11 summarizes the decolorization percentages for RB-5 under different experimental conditions.

RB-5 has been reported to be resistant to decolorization using purified laccase from *Pleurotus sajor-caju* in the absence of mediators (Murugesan, Dhamija, Nam, Kim, & Chang, 2007). Using laccase produced by *Ischoderma resinosum*, RB-5 was not decolorized by the enzyme, without using redox mediators (violuric acid) (Kokol, Doliska, Eichlerov, Baldrian, & Nerud, 2007). Similarly, Murugesan et al. (2007) did not observe decolorization of RB-5 using crude enzyme alone without redox mediators. A decolorization of 62% was achieved after 1 h incubation when 1 mM *N*-hydroxybenzotriazole (HBT) was added. Photographs of the decolorization of RBBR and RB-5 discussed in this section are shown in Appendix B and C. Overall, Lac-I showed a higher dye decolorization capacity at both dye concentrations tested. At the high colorant concentration, doubling laccase activity resulted in doubling decolorization efficiency.

Table 11. RB-5 decolorization after 3 and 15 h

Enzyme Isoform	Enzyme Concentration (U mL^{-1})	Dye Concentration (mg L^{-1})	Decolorization @ 3 h (%)	Decolorization @ 15 h (%)
LAC-I	1	50	4.4 ± 0.1	27.6 ± 0.3
LAC-I	1	100	4.7 ± 0.4	18.9 ± 0.2
LAC-I	8	50	10.4 ± 1.0	53.8 ± 3.4
LAC-I	8	100	7.9 ± 1.0	37.8 ± 0.9
LAC-II	1	50	3.7 ± 0.3	23.8 ± 0.7
LAC-II	1	100	2.4 ± 0.6	14.3 ± 0.7
LAC-II	8	50	8.2 ± 0.8	52.0 ± 4.3
LAC-II	8	100	5.6 ± 0.5	33.3 ± 0.8

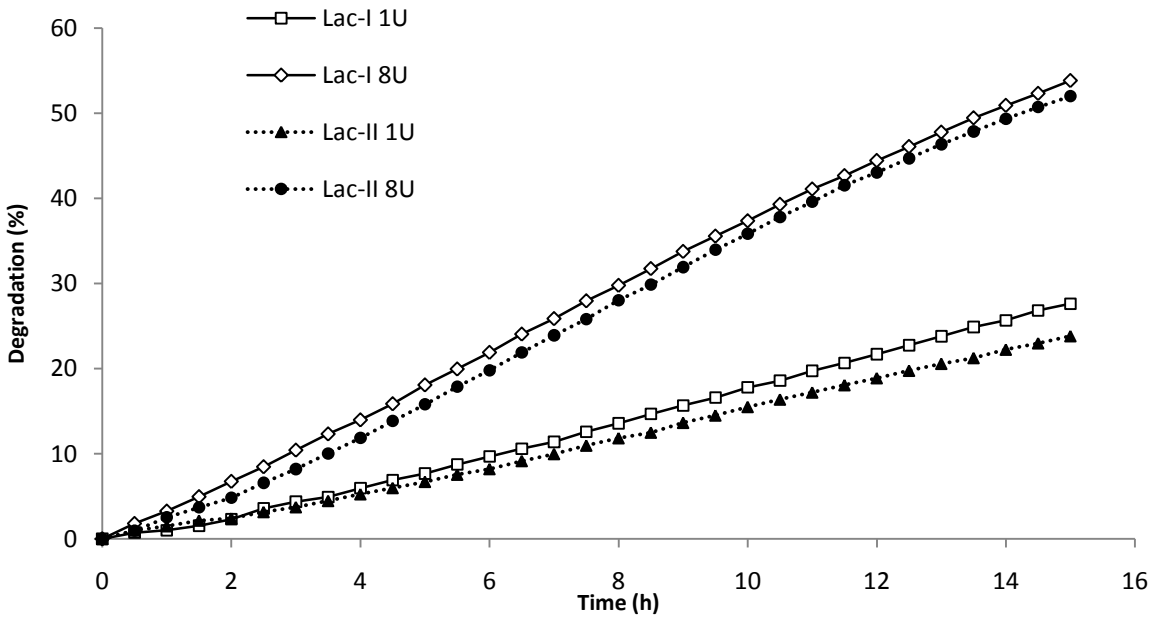

Figure 14. Decolorization of RB-5 at 50 mg L^{-1}

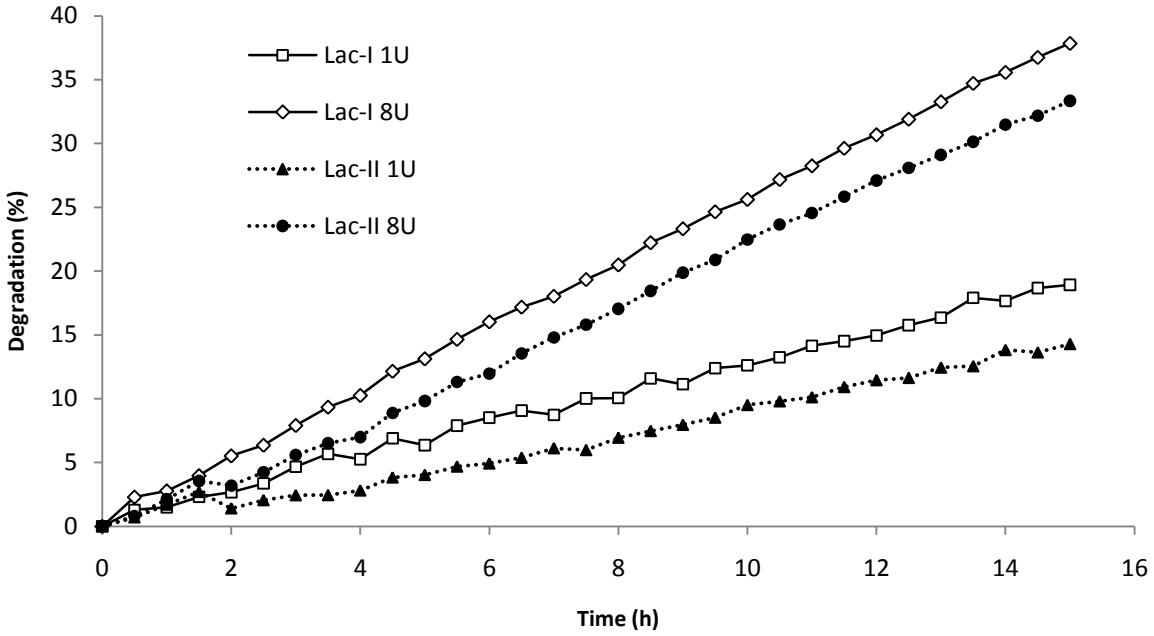

Figure 15. Decolorization of RB-5 at 100 mg L^{-1}

Based on the factorial statistical analysis, the most significant factor in the decolorization of RB-5 was enzyme concentration, followed by the enzyme isoform (Table 12). The main effects were significant at P<0.001 for a 3 hr decolorization period.

Table 12. Estimated effects and coefficients for the decolorization of RB-5 at 3 hr

Term	Coef	P
Constant	6.1815	0.000
EnzIsof	1.1904	0.001
EnzConc	1.8490	0.000
ColConc	-0.9849	0.003
EnzIsof*EnzConc	-0.0563	0.842
EnzIsof*ColConc	-0.0121	0.966
EnzConc*ColConc	-0.2929	0.307
EnzIsof*EnzConc*ColConc	0.0354	0.900

The most significant factors on the decolorization of RB-5 at 15 hr were the enzyme concentration and colorant concentration (Table 13). The main effects were significant at P<0.001 for the 3 hr decolorization. This shows that a long incubation time, the colorant concentration is an important factor due to the mass transfer limitations.

Table 13. Estimated effects and coefficients for the decolorization of RB-5 at 15 hr

Term	Coef	P
Constant	33.982	0.000
EnzIsof	3.116	0.017
EnzConc	10.271	0.000
ColConc	-7.486	0.000
EnzIsof*EnzConc	-1.536	0.207
EnzIsof*ColConc	-0.440	0.711
EnzConc*ColConc	-1.171	0.331
EnzIsof*EnzConc*ColConc	1.112	0.356

6.3. Mediators

As shown above, decolorization efficiency for RB-5 was very low for Lac-I and Lac-II; because of this, two mediators were tested to decrease decolorization time and increase decolorization percentage, violuric acid (VA) and N-hydroxyphtalamide (N-HPT). Figure 16 shows that violuric acid

has a pink color and N-hydroxyphtalamide a light yellow. Each mediator was tested at 1 and 5 mM for colorant concentrations of 50 and 100 mg L^{-1}, the enzyme concentration selected was 1 U mL^{-1}.

Figure 16. N-Hydroxyphtalamide (left) and violuric acid (right)

The decolorization profile for RB-5 with Lac-I in the presence of violuric acid is shown in Figure 17. It is observed that the fastest decolorization was achieved when 50 mg L^{-1} of RB-5 and 5mM of VA were used. However, higher decolorization percentages at 25 min and 15 hr were obtained at lower mediator concentrations at both 50 and 100 mg L^{-1} of RB-5. Constant values were obtained after 5 minutes of incubation except for 100 mg L^{-1} and 1mM, where stabilization occurred after 10 min of incubation.

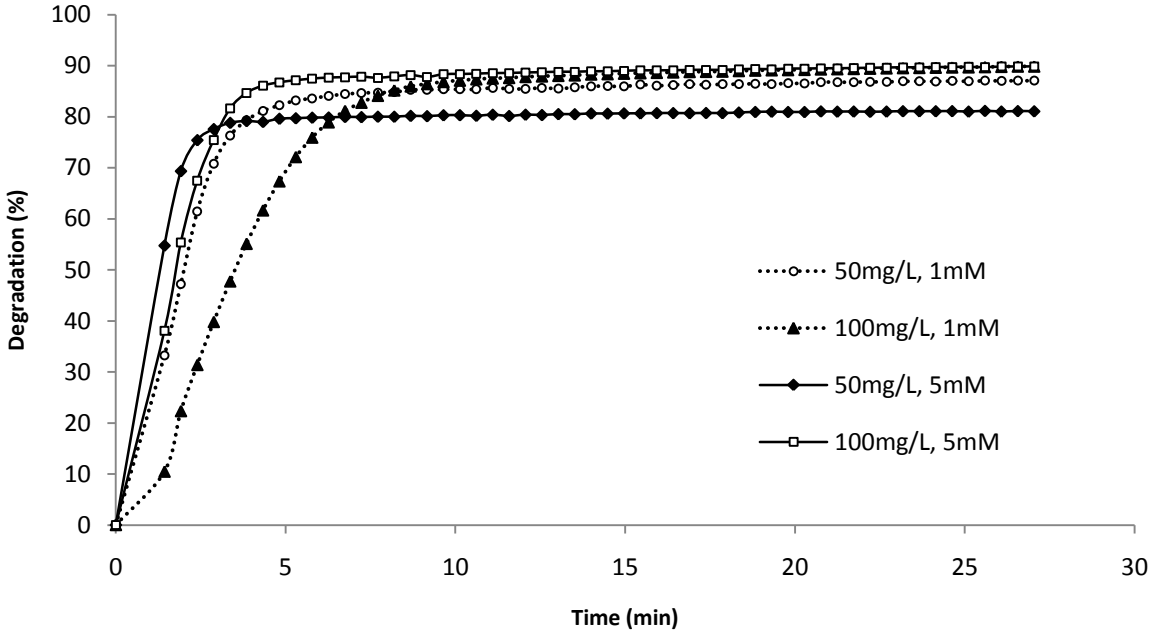

Figure 17. Decolorization of RB-5 with violuric acid as mediator for Lac-I

Figure 18 shows the decolorization kinetics for RB-5 with Lac-I in the presence of N-hydroxyphtalamide, where higher decolorization percentages were obtained at lower colorant concentrations. In addition to this, higher decolorization efficiency was obtained at lower mediator concentrations. The maximum decolorization efficiencies obtained after 15 hr of incubation were 71 and 67% at 50 and 100 mg L^{-1} of colorant respectively with 1 U mL^{-1} of enzyme. These values are significantly lower to those obtained with violuric acid in shorter times (25 min).

For Lac-II, the RB-5 decolorization profile with violuric acid is shown in Figure 19. It is observed that the slowest decolorization was found when 100 mg L^{-1} of RB-5 and 51mM of VA were used, similar to Lac-I. Constant values were obtained after 5 minutes of incubation except for 100 mg L^{-1} and 1mM where stabilization occurs after 10 min of incubation. Decolorization efficiencies are around 80 to 90% after 3 hr of incubation.

Figure 20 shows the decolorization kinetics for Lac-II with N-hydroxyphtalamide, where the mediator behavior is similar to that with Lac-I, higher decolorization efficiencies are obtained at lower mediator concentrations. In addition to this, the differences between colorant concentrations are more remarkable. The maximum decolorization efficiencies obtained after 15 hr of incubation were 67 and 37% for 50 and 100 mg L^{-1} of colorant respectively, both with 1 U mL^{-1} of enzyme.

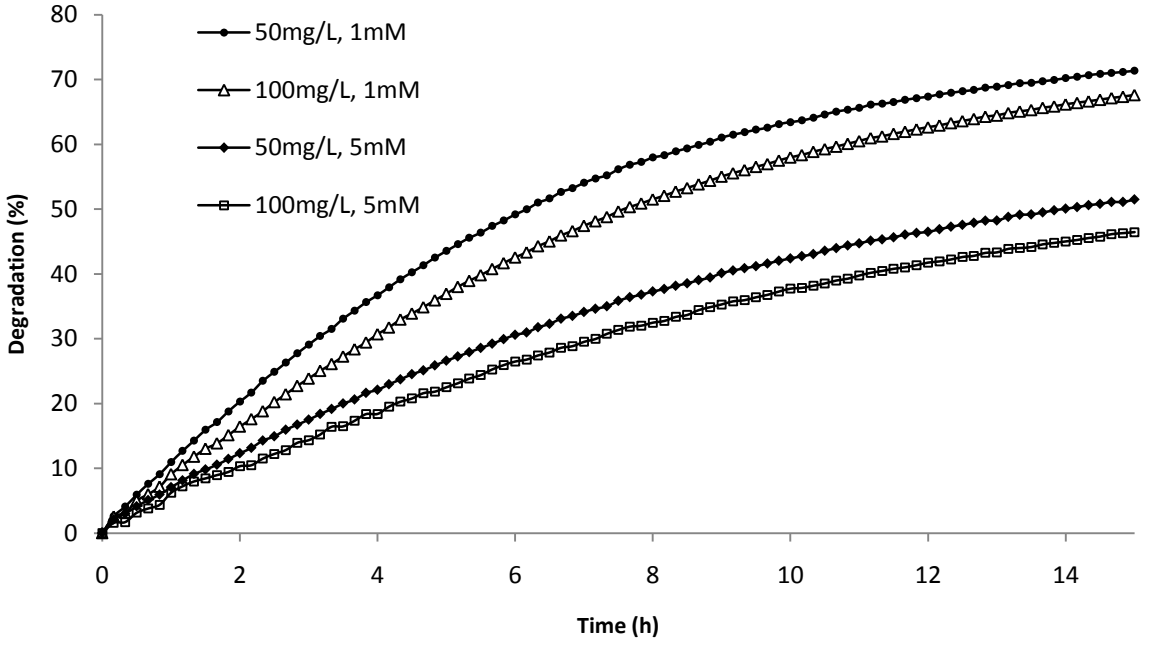

Figure 18. Decolorization of RB-5 with N-Hydroxypthalamide as mediator for Lac-I

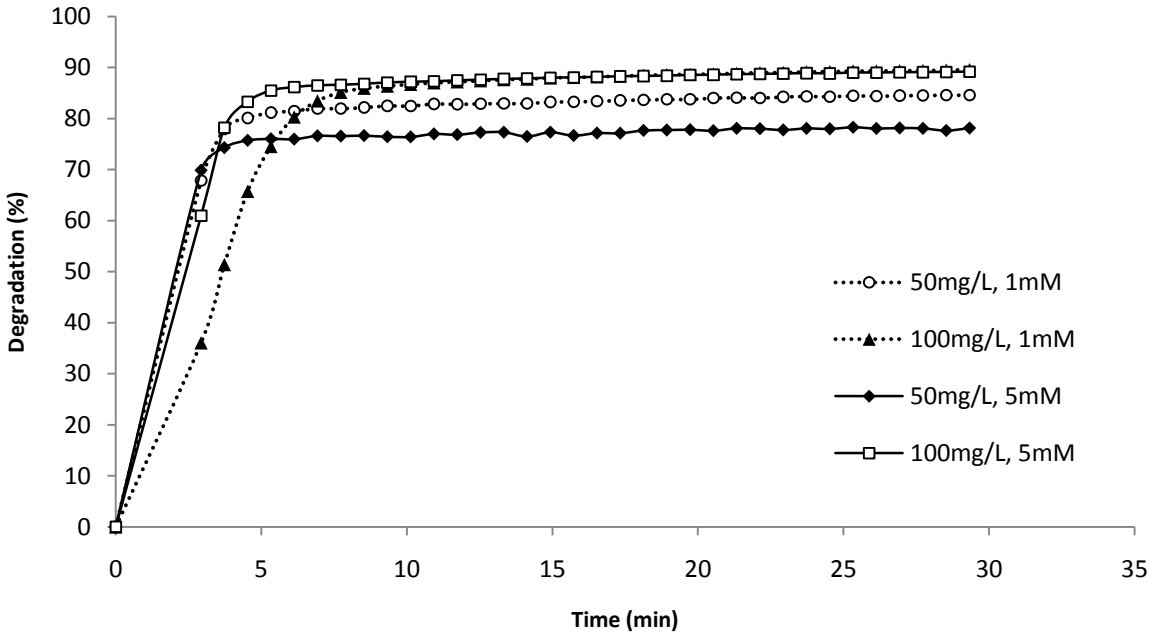

Figure 19. Decolorization of RB-5 with violuric acid as mediator for Lac-II

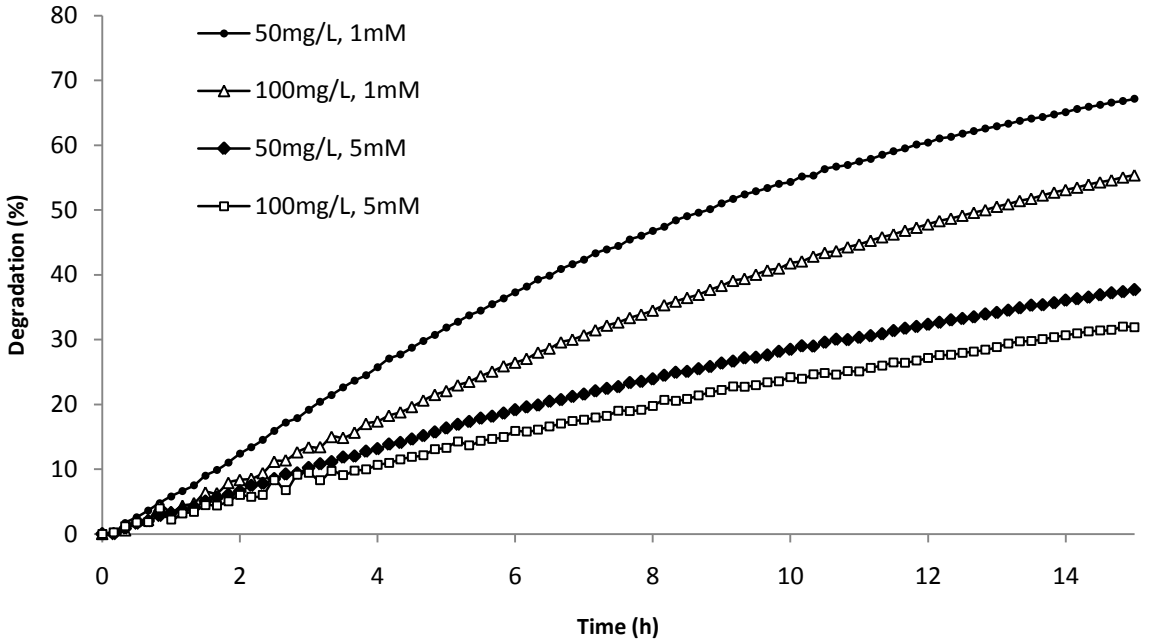

Figure 20. Decolorization with N-Hydroxypthalamide as mediator for Lac-II

Decolorization of RB-5 by both Lac-I and Lac-II was significantly increased with violuric acid as a mediator, reaching approximately 80% decolorization after 10 min. In contrast, N-HPT does not affect decolorization efficiency significantly; moreover, higher concentrations of N-HPT seem to inhibit decolorization, yielding higher decolorization percentages at 1 mM than at 5 mM. Table 14 summarizes decolorization efficiencies for each mediator under different experimental conditions.

The most common mediator used for the decolorization of RB-5 is hydroxybenzotriazole (HBT) although it was not tested in this study because it is not available on the Mexican of the market. An 84% decolorization of RB-5 has been reported with *P. sajor caju* laccases after 36 h with 2.5 U mL^{-1} of enzyme, 65 mg L^{-1} of colorant and 1.5 mM of HBT (Murugesan, Dhamija, Nam, Kim, & Chang, 2007). For *P. pinisitus* with 200 mg L^{-1} of colorant and 2 mM of HBT, 70% decolorization was achieved after 16 h (Claus, Faber, & König, 2002). For *G. lucidum*, 62 to 77% decolorization was obtained during 1-2 h of reaction using N-HPT 1mM and 50 mg L^{-1} of colorant; no decolorization occurred without mediator. The decolorization percentage increased for *I. resinosum* (25U mL^{-1}) with the use of violuric acid 5 mM from 5 to 60% after 1 h and 100 mg L^{-1} of colorant (Kokol, Doliska, Eichlerov, Baldrian, & Nerud, 2007).

Table 14. Decolorization of RB-5 after 25 min and 15 h for different mediators with 1 U mL^{-1} of enzyme

#	Mediator	Enzyme Isoform	Mediator Concentration (Mm)	Dye Concentration (mg L^{-1})	Decolorization ± SD @ 25 min	Decolorization ± SD @ 15 h
1	VA	Lac-I	1	50	87.3 ± 0.59	89.0 ± 0.53
2	VA	Lac-I	1	100	89.9 ± 0.04	93.2 ± 0.01
3	VA	Lac-I	5	50	81.2 ± 0.20	82.5 ± 0.31
4	VA	Lac-I	5	100	89.9 ± 0.02	92.7 ± 0.06
5	VA	Lac-II	1	50	84.6 ± 0.59	86.9 ± 0.51
6	VA	Lac-II	1	100	89.6 ± 0.06	92.7 ± 0.02
7	VA	Lac-II	5	50	78.1 ± 0.80	80.4 ± 0.81
8	VA	Lac-II	5	100	89.2 ± 0.01	91.9 ± 0.04
9	N-HPT	Lac-I	1	50	4.12 ± 0.30	71.3 ± 1.14
10	N-HPT	Lac-I	1	100	3.13 ± 0.35	67.6 ± 1.23
11	N-HPT	Lac-I	5	50	3.00 ± 0.13	51.4 ± 0.93
12	N-HPT	Lac-I	5	100	1.87 ± 0.51	46.8 ± 1.03
13	N-HPT	Lac-II	1	50	2.58 ± 0.93	67.1 ± 0.76
14	N-HPT	Lac-II	1	100	2.16 ± 0.64	55.6 ± 1.18
15	N-HPT	Lac-II	5	50	1.61 ± 0.18	37.6 ± 0.34
16	N-HPT	Lac-II	5	100	1.79 ± 0.84	31.9 ± 2.35

The factorial statistical analysis showed that main effects, 2 and 3-way interactions were statistically significant at *P*<0.001 at 25 min and 15 hr of incubation time.

Based on the factorial statistical analysis, a model was build with all the significant factors and interactions with a fit data of R_{adj}^2= 99.99 %. The most significant factor on dye decolorization analysis was the mediator type, followed by mediator concentration. The model equation was based on the statistical parameters obtained in the experimental design at 25 min of incubation:

Percentage of dye decolorization (25 min of incubation) = 39.8107 X $_{Mediator}$+ 1.1079X $_{EnzIsof.}$ -1.7138 X $_{MedConc}$ +0.0063 X $_{ColConc}$ +1.0892 X $_{Mediator}$ X $_{EnzIsof}$ -1.2779 X $_{Mediator}$ X $_{MedConc}$ -0.0397 X $_{Mediator}$ X $_{ColConc}$ +0.1138 X $_{EnzIsof}$ X $_{MedConc}$ -0.0028 X $_{EnzIsof}$ X $_{ColConc}$ +0.0165 X $_{MedConc}$ X $_{ColConc}$ -0.0886 X $_{Mediator}$ X $_{EnzIsof}$ X $_{MedConc}$ -0.0177 X $_{Mediator}$ X $_{EnzIsof}$ X $_{ColConc}$ +0.0130 X $_{Mediator}$ X $_{MedConc}$ X $_{ColConc}$ -0.0020 X $_{MedConc}$ X $_{EnzIsof}$ X $_{ColConc}$ +0.0022 X $_{Mediator}$ X $_{EnzIsof}$ X $_{MedConc}$ X $_{ColConc}$

6.4. Determination of V_{max} and K_m

As mentioned in section 5.4.3, several enzyme concentrations were tested for the calculation of the kinetic parameters. The concentration selected for the calculation was 0.6 U mL^{-1} to ensure that enzyme concentration was much lower than substrate concentration, as indicated by the Michaelis-Menten constraint of [E] <<[S].

Kinetic parameters of the two laccases were determined using RBBR as substrate: K_m was 0.243 and 0.117 mM, and V_{max} 1.233 and 1.012 mM sec^{-1} for Lac-I and Lac-II, respectively. Values for Lac-I are slightly higher than those for Lac-II, but still very similar. Therefore, decolorization efficiency of the two laccases is almost identical. For *P. ostreatus,* K_m values of 0.054 and 0.051 mM for POXA3 and POXC were obtained using the same substrate while the catalytic efficiency (K_{cat}/K_m) was 15 x 10^4 and 25 x 10^3 mM^{-1} s^{-1} respectively (Pozdnyakova *et al*. 2006). The kinetic fit calculations for RB-5 are shown in appendix E.

Table 15 and Table 16 summarize the enzyme kinetic fit with different methods for Lac-I and Lac-II respectively. The best fit (Lineweaver-Burk) is plotted in Figure 21 and Figure 23 for each laccase. Figure 22 and Figure 24 show the initial velocity (V_o) calculated with the slope and used to estimate the kinetic parameters. For Lac-II concentration of 30 and 50 mg L^{-1} of colorant were taken away of the calculation due to an error in the laccase concentration. The rest of the kinetic methods are shown in Appendix D. The kinetic fit calculations for RB-5 are shown in appendix E.

Table 15. V_{max} and K_m for RBBR decolorization with Lac-I.

	V_{max} [mM sec^{-1}]	K_m [mM]	R	R^2
Michaelis- Menten	1.446	0.300	0.994	0.988
Lineweaver- Burk	1.233	0.243	0.999	0.998
Eadie-Hofstee	1.260	0.248	0.941	0.886
Scatchard	1.381	0.280	0.941	0.886
Hanes-Woolf	1.352	0.273	0.956	0.915

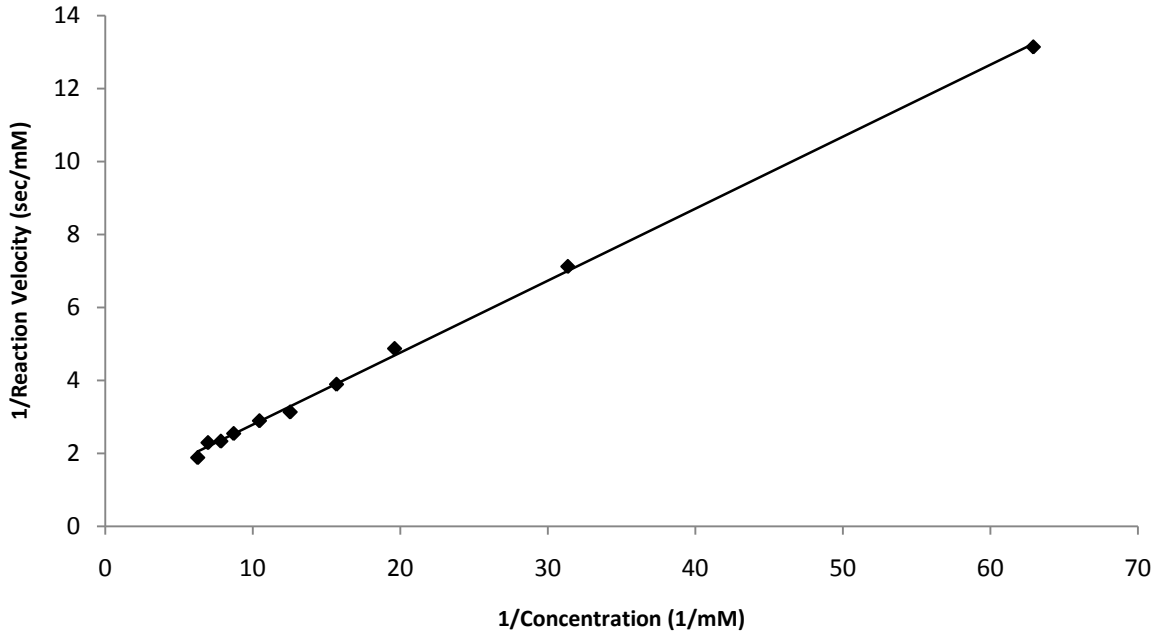

Figure 21. Lineweaver-Burk plot for RBBR decolorization with Lac-I

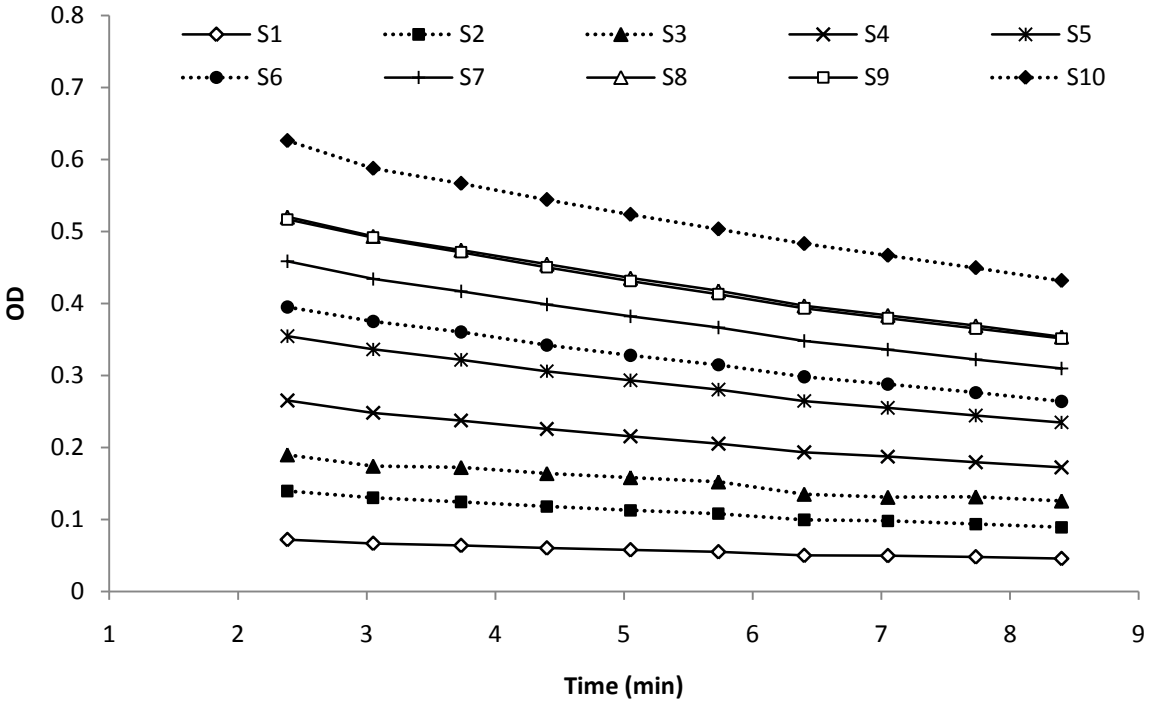

Figure 22. Initial slope for Lac-I

Table 16. V_{max} and K_m for RBBR decolorization with Lac-II.

	V_{max} [mM sec^{-1}]	K_m [mM]	R	R^2
Michaelis- Menten	1.090	0.132	0.989	0.979
Lineweaver- Burk	1.012	0.117	0.998	0.996
Eadie-Hofstee	1.024	0.118	0.952	0.907
Scatchard	1.085	0.130	0.952	0.907
Hanes-Woolf	1.066	0.127	0.972	0.945

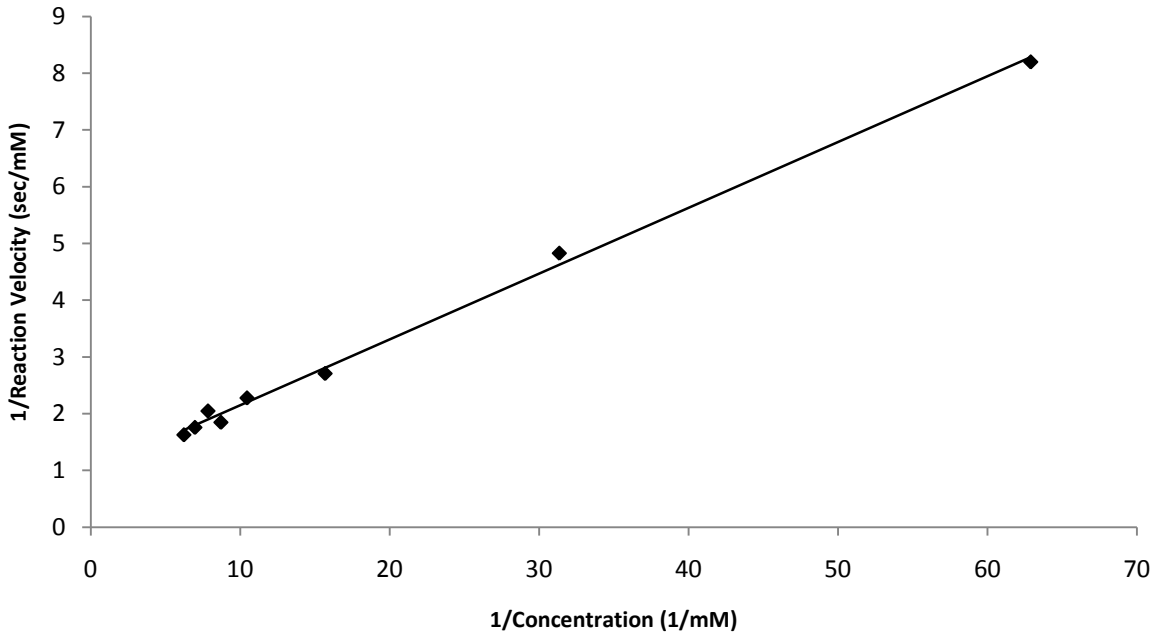

Figure 23. Lineweaver-Burk fit for Lac-II

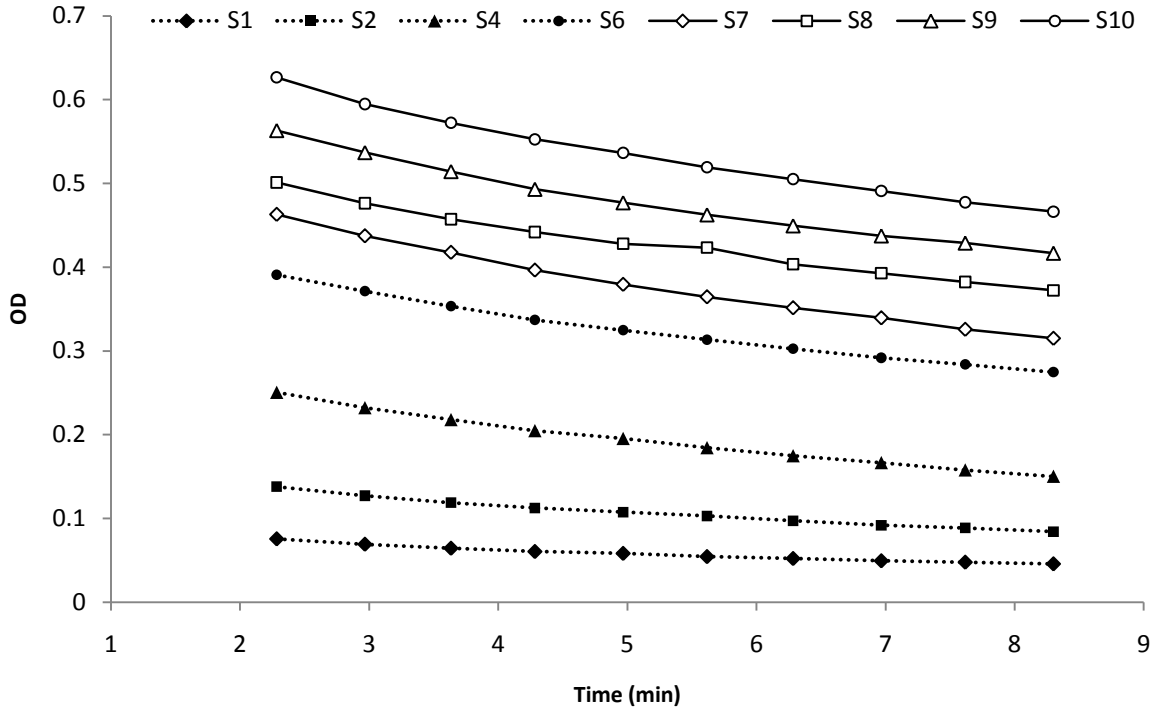

Figure 24. plot for RBBR decolorization with Lac-II

7. Conclusions

- Both newly isolated laccase isoforms demonstrated high capability to decolorize reactive dyes. These new enzymes offer an opportunity to treat wastewater effluents that contain dyes with local technology.
- The use of synthetic mediators improved the decolorization of RB-5. Violuric acid had a better performance than N-hydroxypthalamide, reducing incubation time from 15 hr to 25 min.
- The value for Lac-II K_m was half of the value for Lac-I, implying that only half the concentration of the substrate is needed to reach 50% of V_{max} for Lac-II. However, both isoforms showed approximately the same V_{max}.

8. Recommendations

- The possible relationship between the molecular weight difference (11%) of both enzymes and the difference in decolorization suggests an interesting new research topic on protein structure and its effect on decolorization capacity.
- These enzyme isoforms could also be useful to degrade other recalcitrant pollutants, such as hormones and organic pesticides, because their molecular structures are closely related to the tested dyes.
- Because of the potential for decolorization, the study of scaling-up processes for industrial applications are recommended, and they should include the design of optimum growth media, bioreactor optimization, and downstream processing.

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Appendixes

Appendix A. Activity calculation

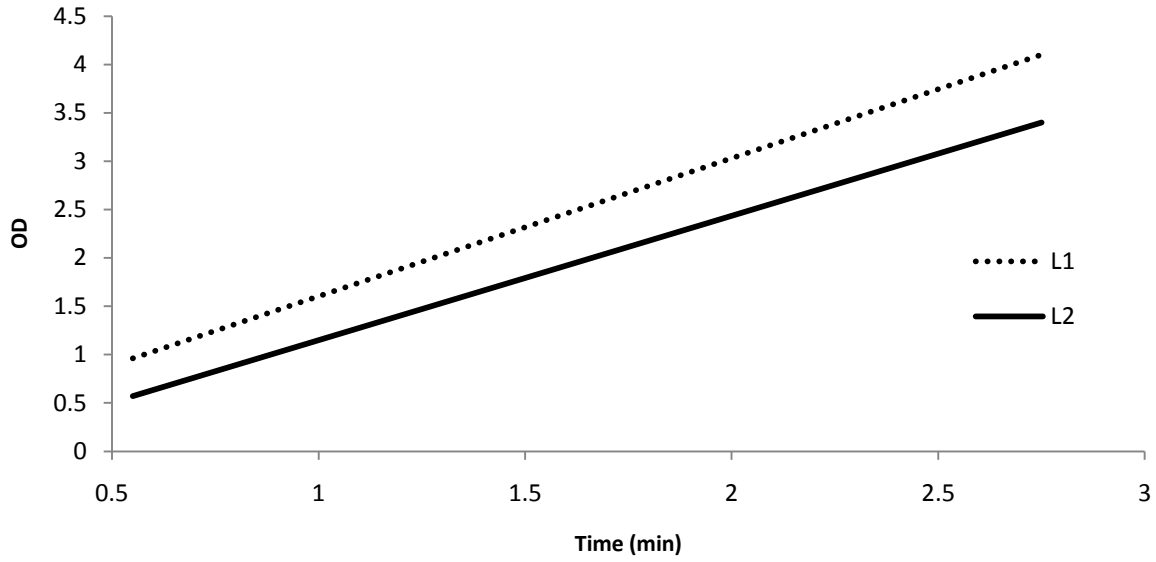

Figure A1. Average slope for Lac-I and Lac-II

Laccase activity of *P. sanguineus* has been reported as 2820 U L^{-1} (Litthauer, van Vuuren, van Tonder, & Wolfaardt, 2007), slightly greater than our enzyme. For *C. unicolor*, activity has been reported as 3600-4000 U L^{-1} (Michniewicz, Ledakowicz, Ullrich, & Hofrichter, 2008), and for *I. resinosum*, 34.5 U L^{-1} (Kokol, Doliska, Eichlerov, Baldrian, & Nerud, 2007). All activity measurements used ABTS oxidation.

Appendix B. RBBR decolorization

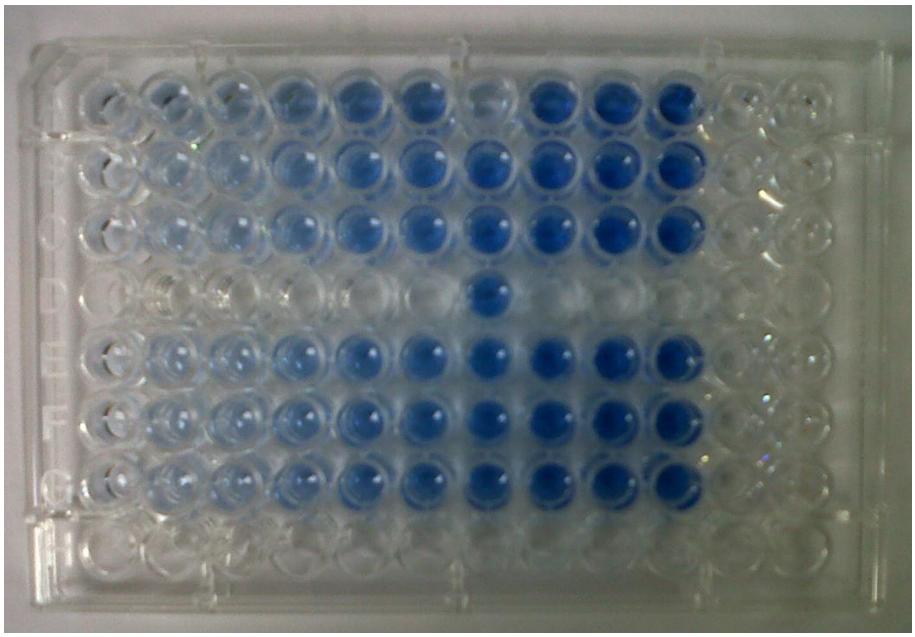

Figure B1. RBBR from 10-100 mg L^{-1} at time zero.

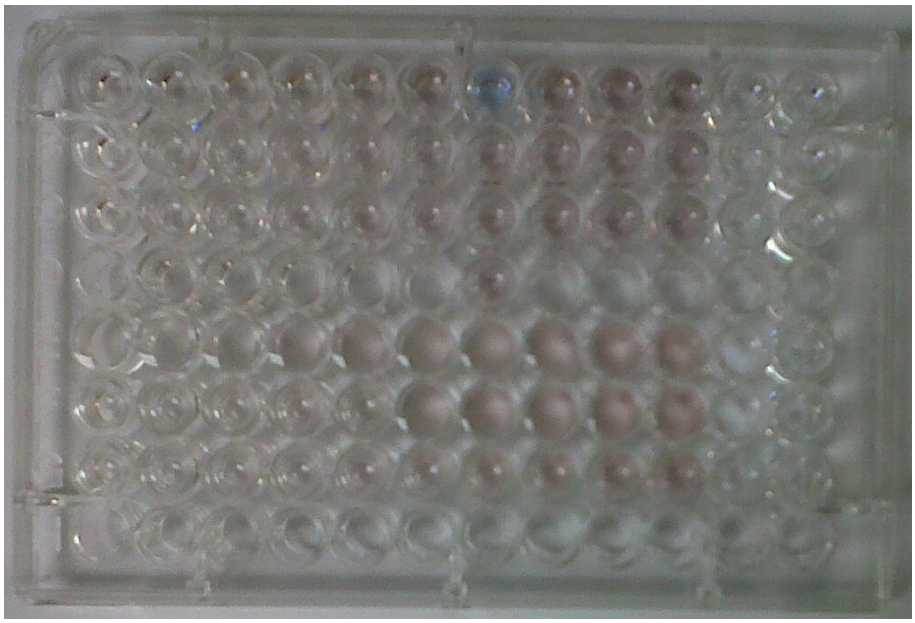

Figure B2. Decolorization of RBBR after 15 h.

Appendix C. RB-5 decolorization

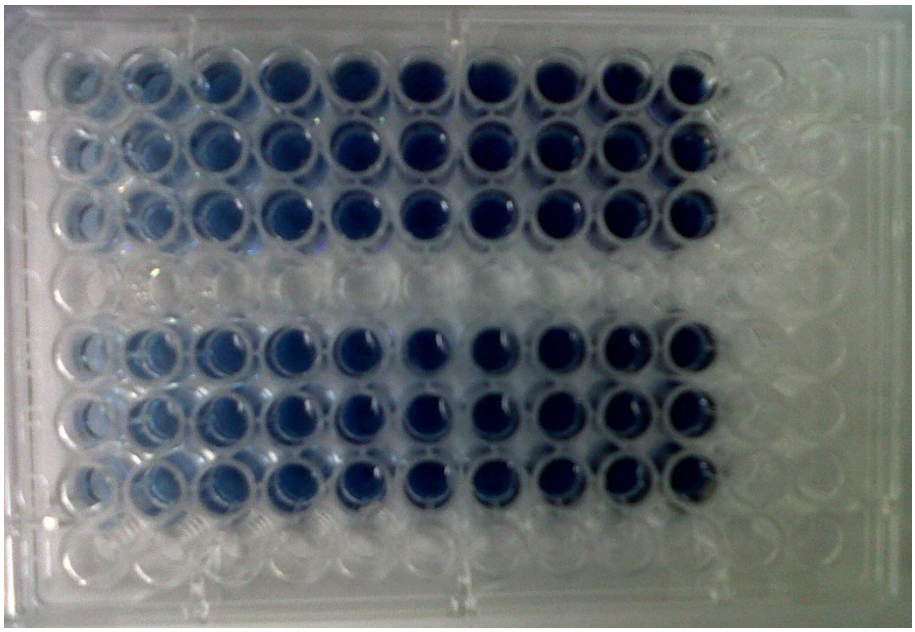

Figure C1. RB-5 from 10-100 mg L^{-1}

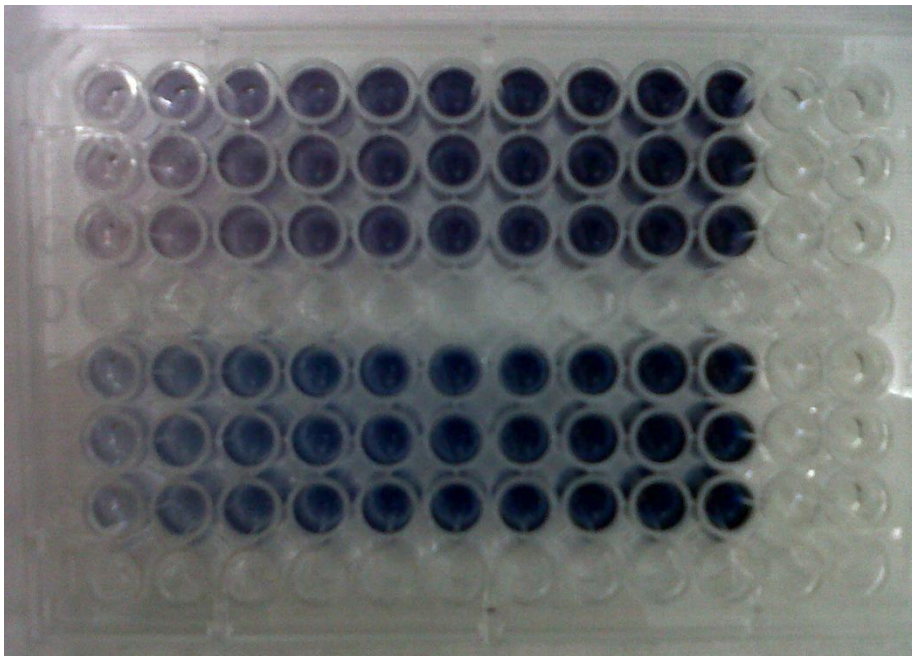

Figure C2. RB-5 decolorization after 3 h (Lac-I in the upper wells, and Lac-II in the lower wells)

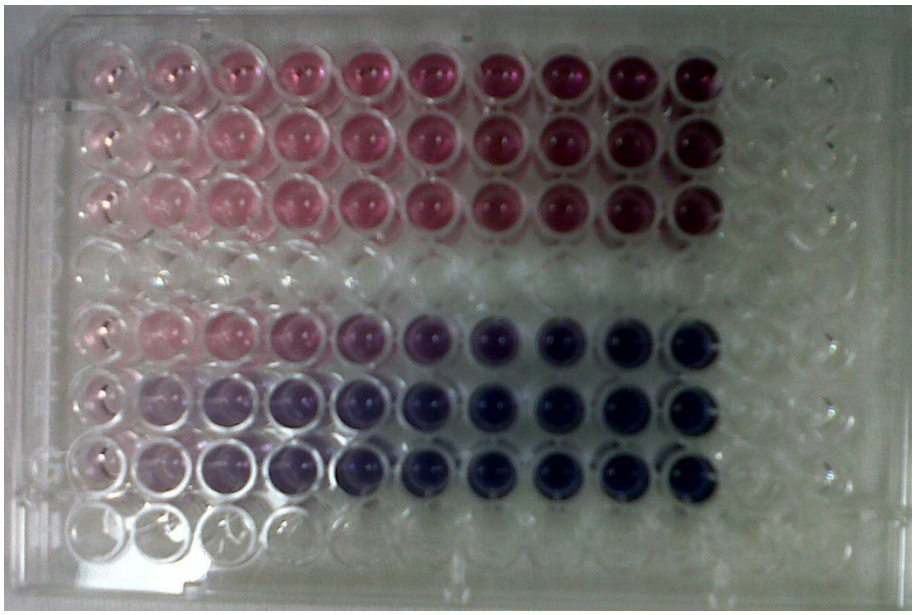

Figure C3. RB-5 decolorization after 15 h (Lac-I in the upper wells, and Lac-II in the lower wells)

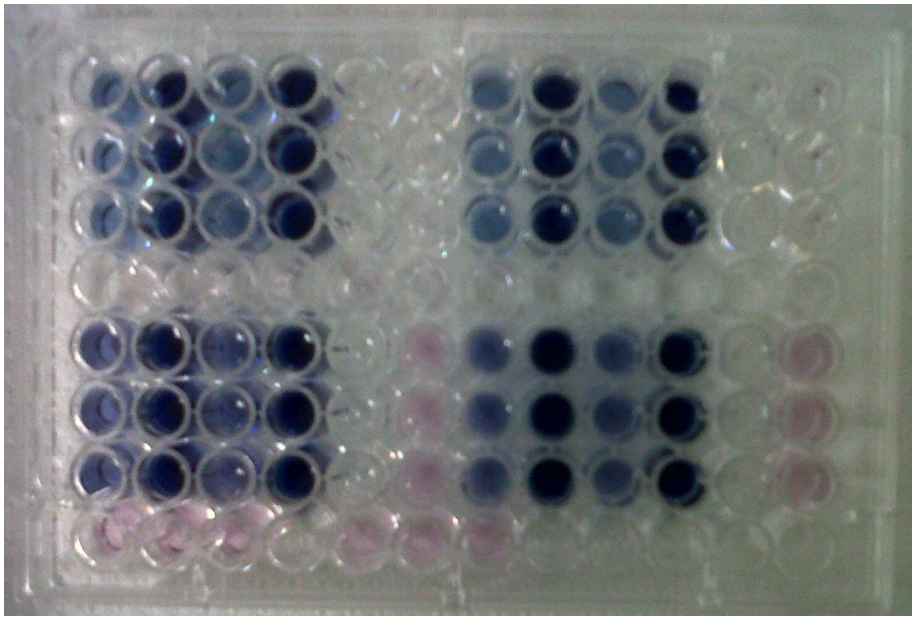

Figure C4. Experimental design for RB-5 with redox mediators at time cero

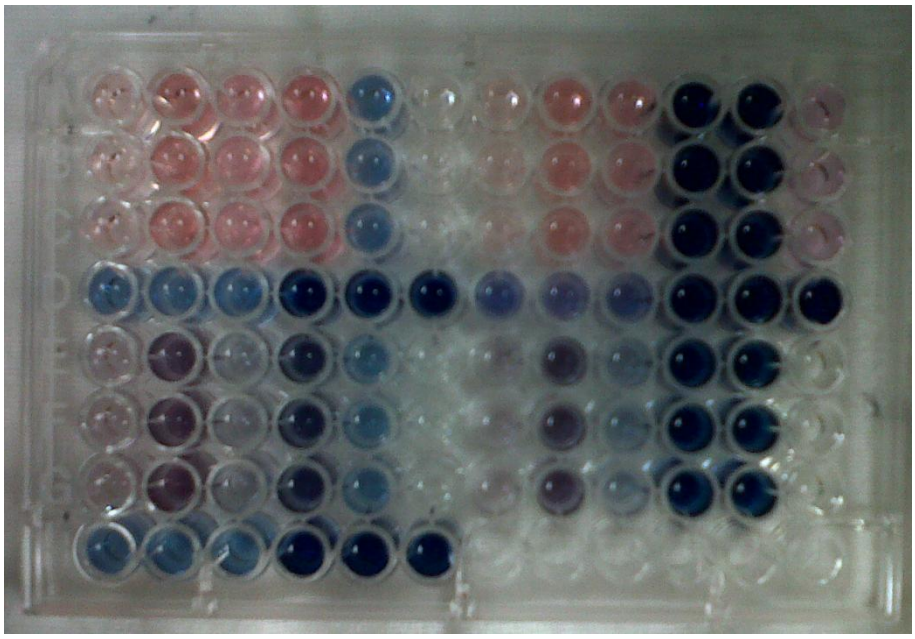

Figure C5. RB-5 decolorization with mediators after 15 h (up VA, down N-HPT)

Appendix D. Enzyme kinetic fits for RBBR

Laccase 1

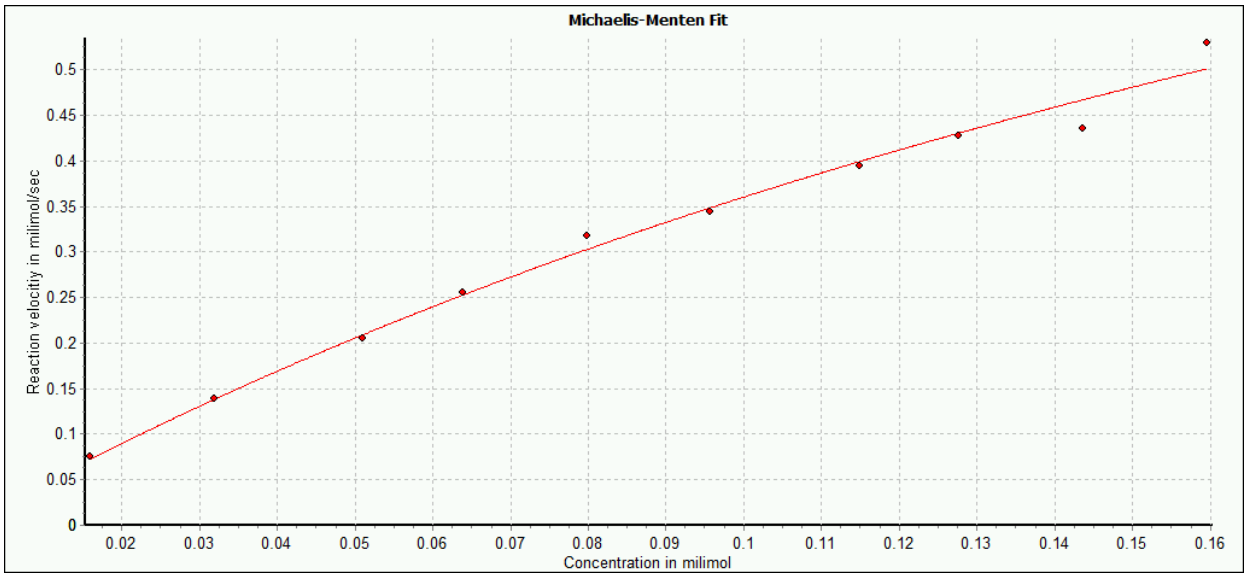

Figure D1. Michaelis-Menten fit for Lac-I with RBBR

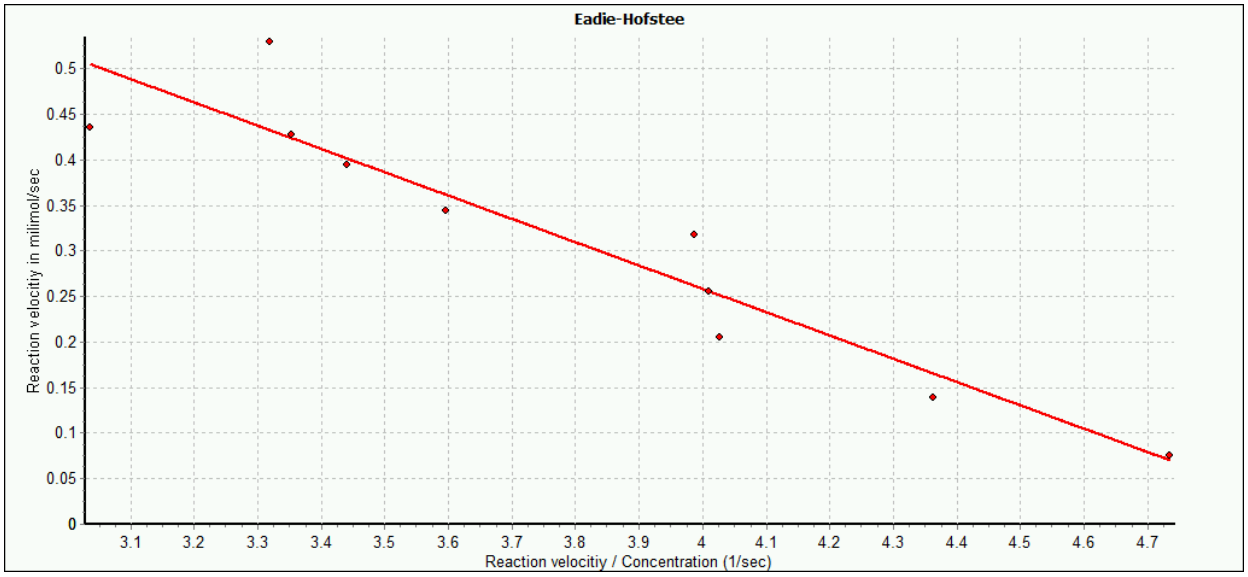

Figure D2. Eadie-Hofstee fit for Lac-I with RBBR

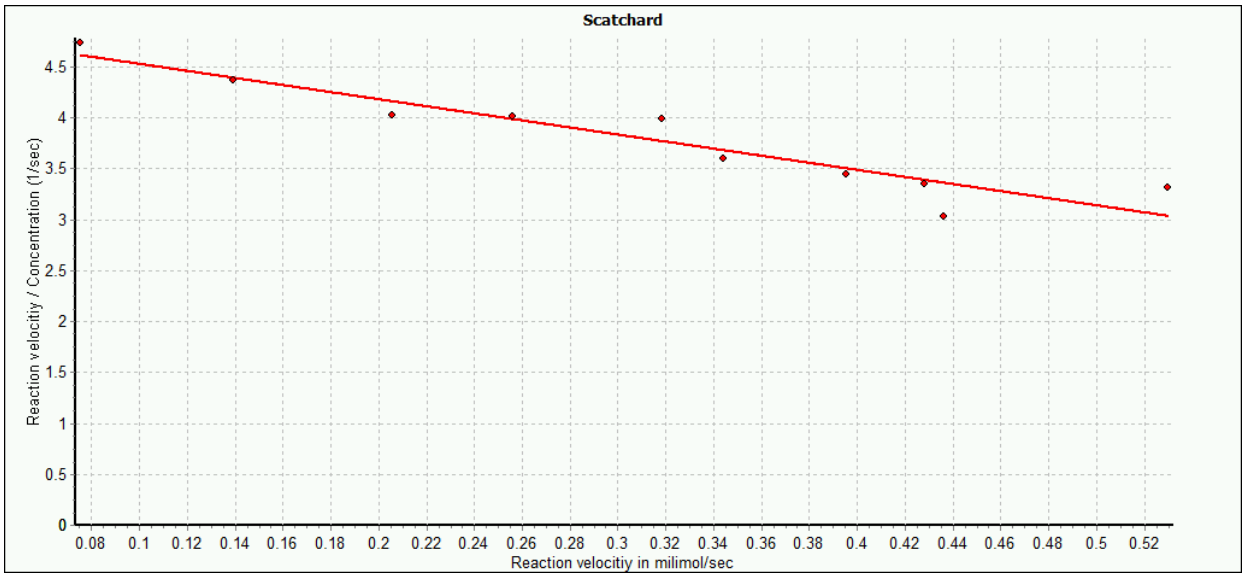

Figure D3. Scatchard fit for Lac-I with RBBR

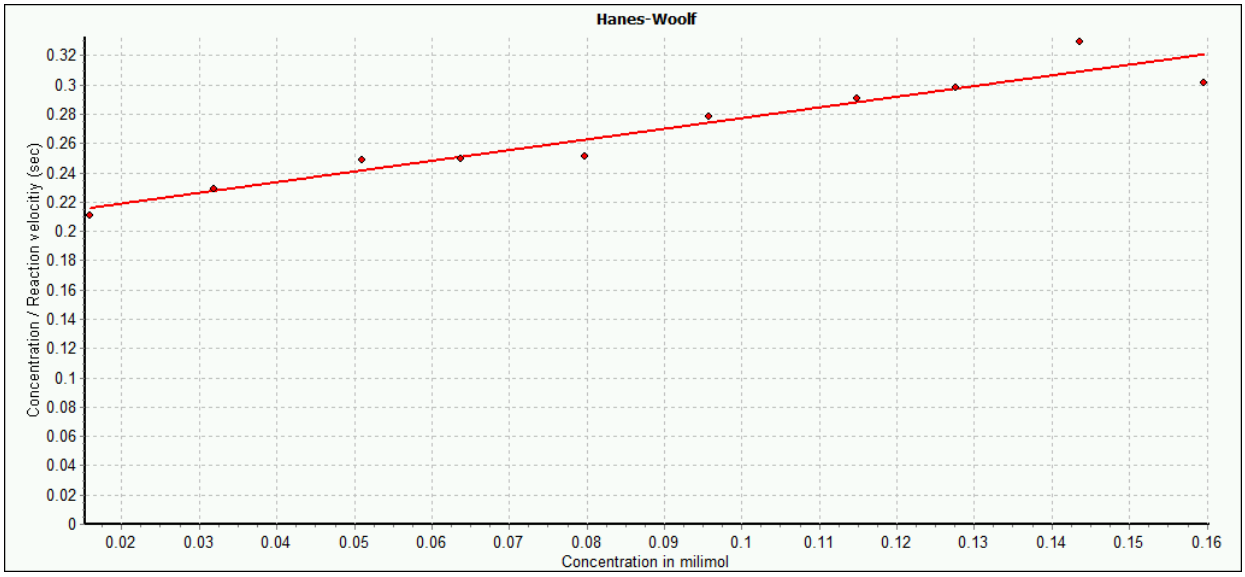

Figure D4. Hanes- Woolf fit for Lac-I with RBBR

Laccase 2

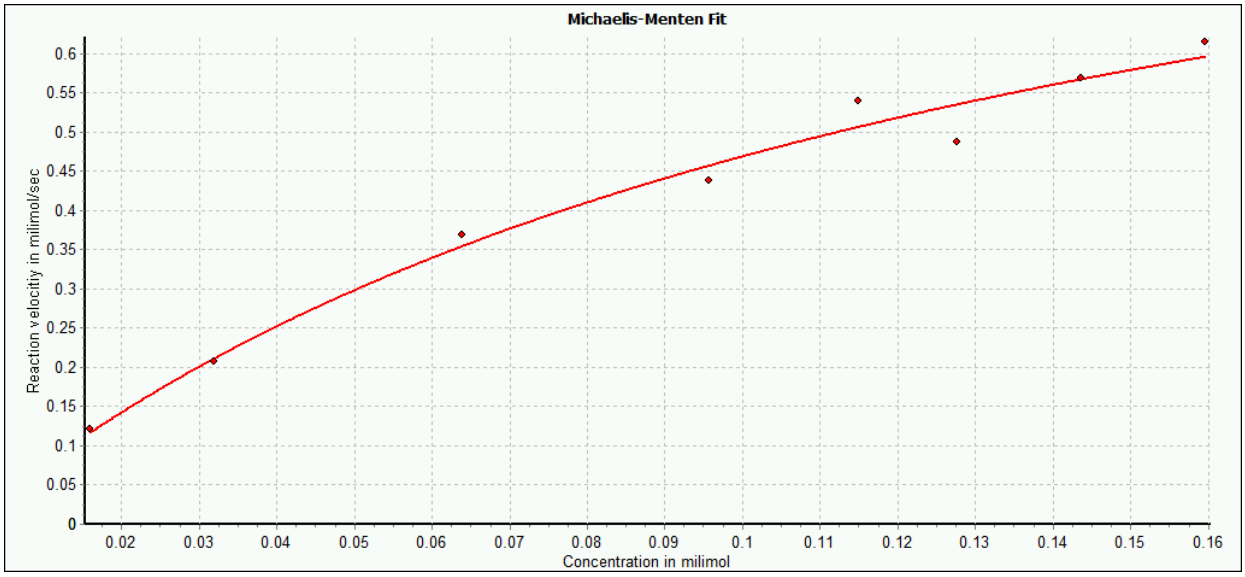

Figure D5. Michaelis-Menten fit for Lac-II with RBBR

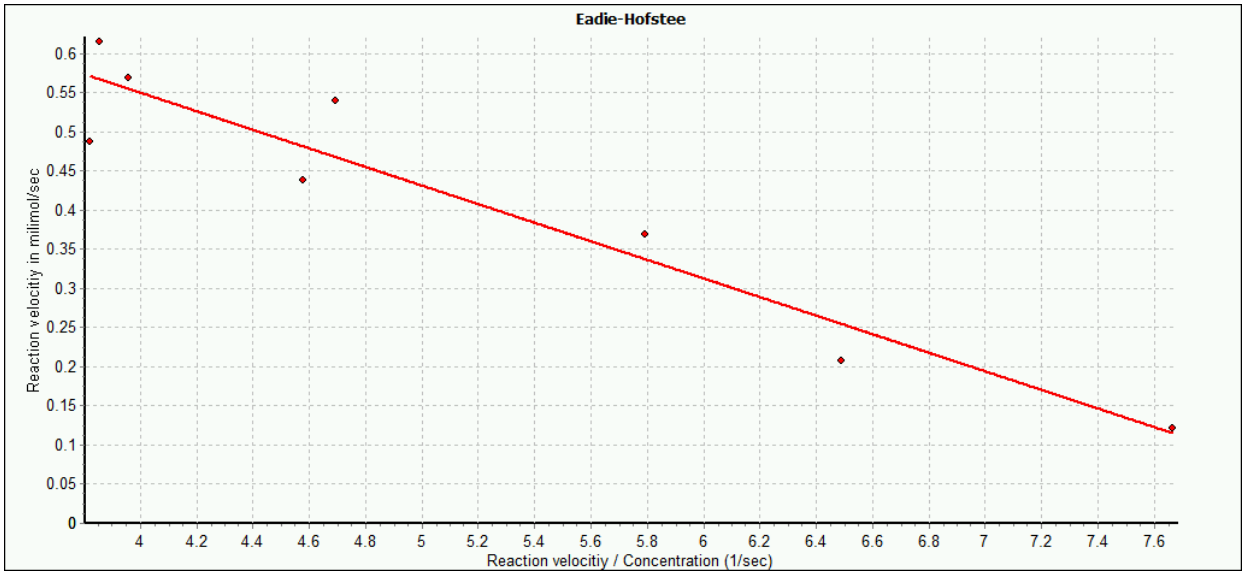

Figure D6. Eadie-Hofstee fit for Lac-II with RBBR

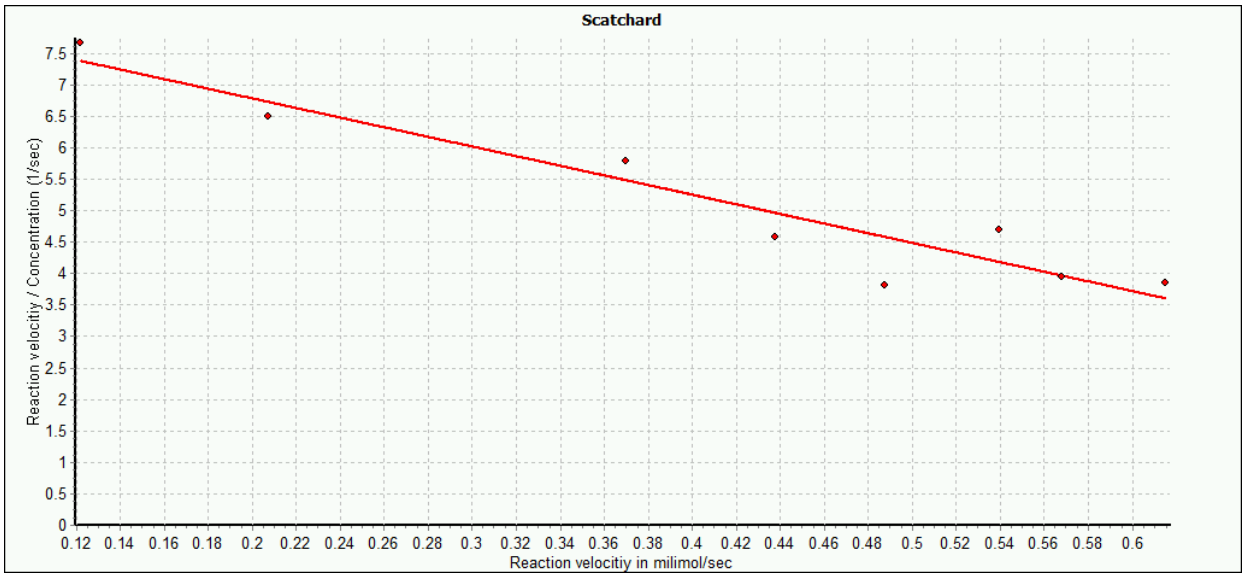

Figure D6. Scatchard fit for Lac-II with RBBR

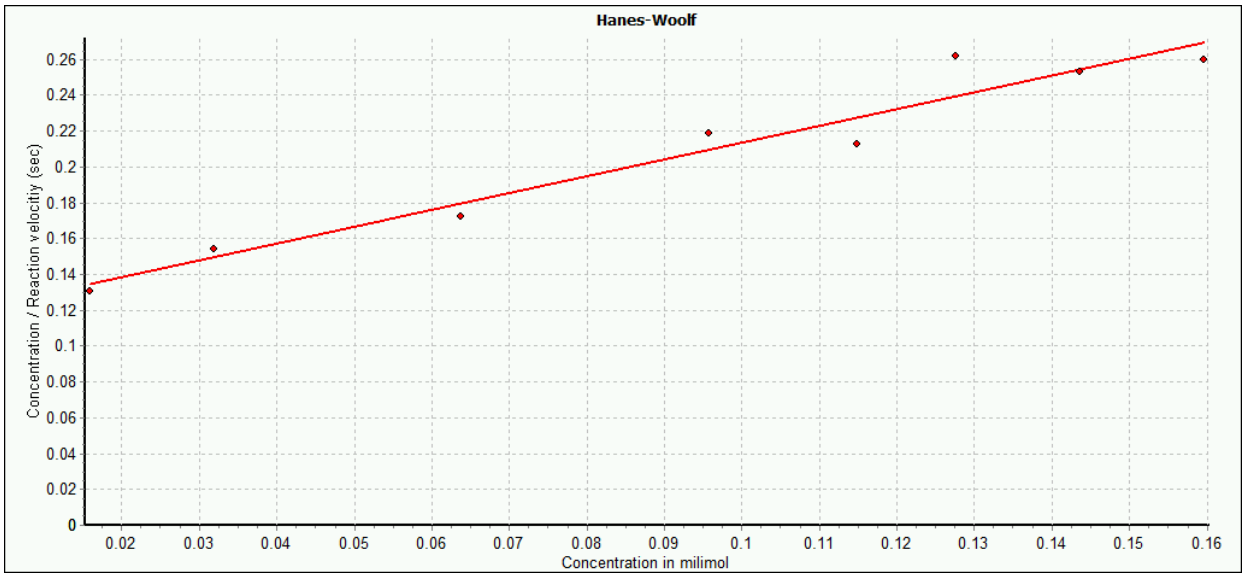

Figure D6. Hanes-Woolf fit for Lac-II with RBBR

Appendix E. Enzyme kinetic fits for RB-5

RB-5 decolorization follows a first order kinetic model, *V=k[S].* Figures E1 and E2 below show the calculation of *k* for Lac-I, and Figures E3 and E4 for Lac-II.

Laccase 1

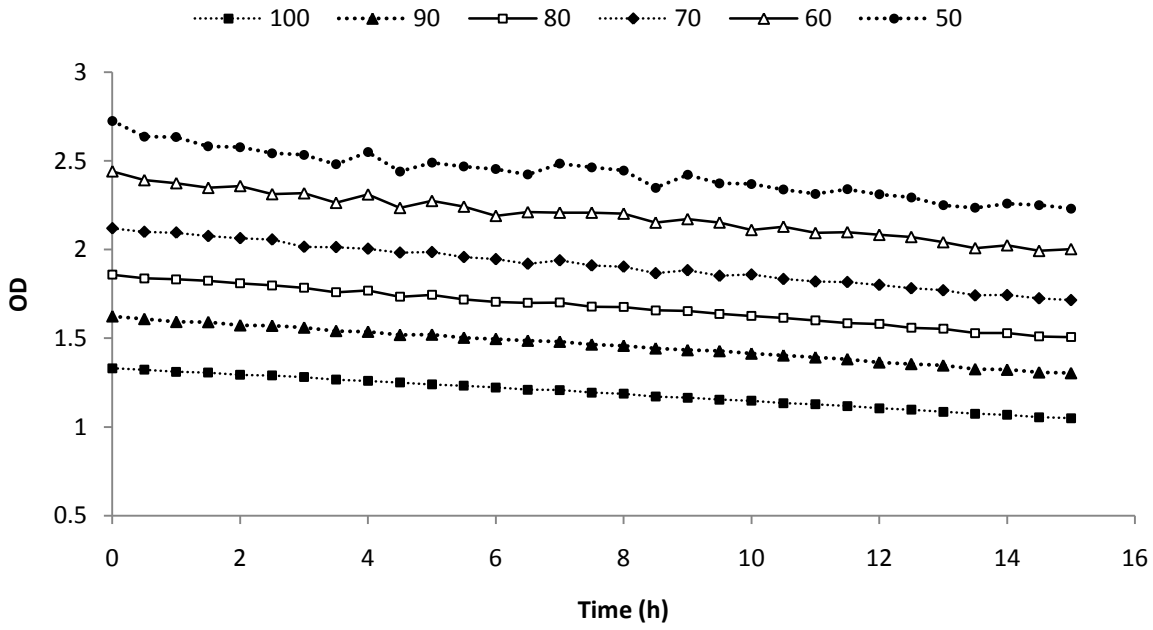

Figure E1. Initial velocities for Lac-I using RB-5 as substrate

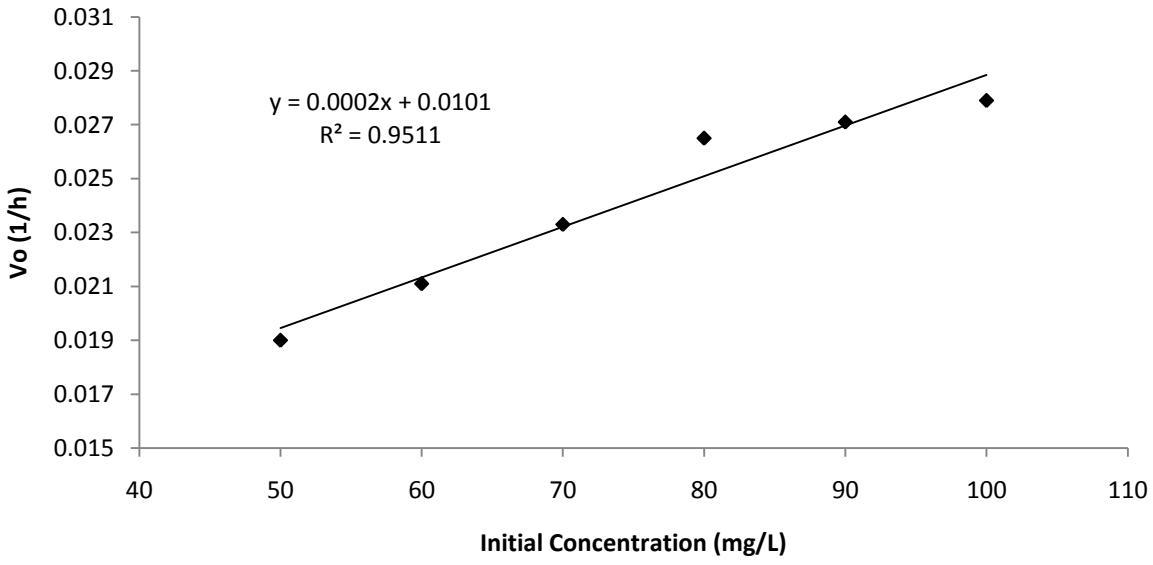

Figure E2. K calculation for Lac-I using RB-5 as a substrate

Laccase 2

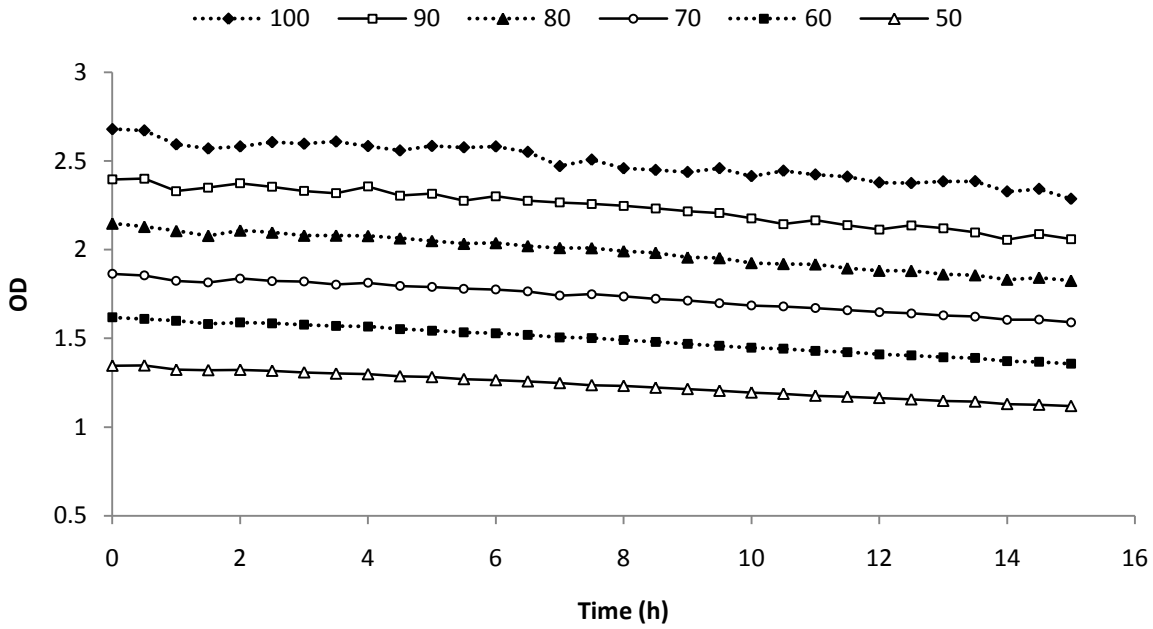

Figure E3. Initial velocities for Lac-II using RB-5 as substrate

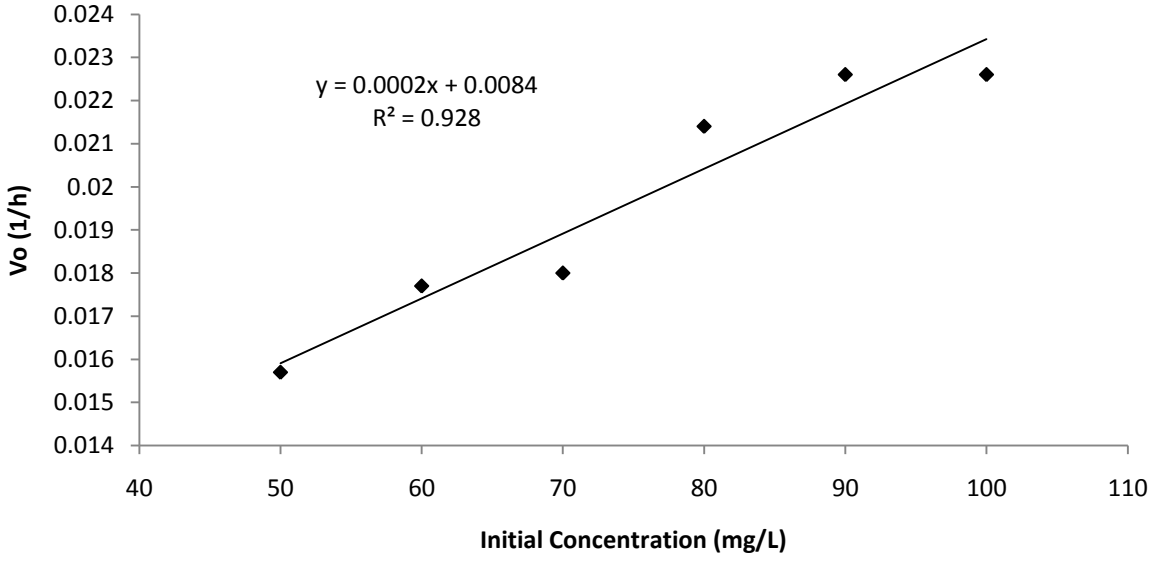

Figure E4. K calculation for Lac-II using RB-5 as a substrate