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Effects of pH, Temperature and Agitation on the Decolourisation of Dyes by Laccase-Containing Enzyme Preparation from *Pleurotus sajor-caju*

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HIGHLIGHTS

- Laccase-containing *Pleurotus sajor-caju* broth has potential for decolourising dyes.
- Thirteen dyes presented colour reduction exceeding 50% after enzymatic treatment.
- The best results for decolourisation were obtained at pH 3.2 and 35°C.
- The agitation of the reaction medium promoted negative effect on decolourisation.

Abstract: (1) Background: In this study, the effects of different pH values (2.4, 3.2, 4.4 and 5.0), temperatures (30, 35, 40, 45 and 50°C) and agitation (100 rpm) on the enzymatic decolourisation of twenty-two dyes belonging to the chromophore groups anthraquinone, azo and triphenylmethane were assessed. (2) Methods: In all conditions, it was used a crude enzyme broth containing 30 U mL^{-1} laccases produced by *Pleurotus sajor-caju*

PS-2001 in submerged process. (3) Results: Regarding the effects of pH values, the best results were obtained at pH 3.2 and 30°C, in which bleaching was observed for all dyes evaluated. In assays conducted at different temperatures, highest levels of decolourisation were observed at 35°C and pH 3.2 for nineteen of the dyes assessed. Thirteen dyes presented colour reduction exceeding 50% after the enzymatic treatment, including all acid and all disperse dyes evaluated. The reciprocal agitation of 100 rpm promoted negative effect on decolourisation. (4) Conclusion: From the results achieved, one can conclude that the laccase-containing preparation of *P. sajor-caju* PS-2001 has potential for the decolourisation of some dyes widely used in different industrial sectors, especially in the textile industry, and therefore could be used in future strategies for the biotreatment of coloured wastes.

Keywords: *Pleurotus sajor-caju*; laccases; dye decolourisation; anthraquinone; azo; triphenylmethane.

INTRODUCTION

Worldwide, about 10,000 different dyes and pigments are manufactured for commercialization, with annual production exceeding 7 x 10^5 tons [1,2,3,4]. Synthetic dyes are widely used for dyeing fabric, printing paper, colour photography and as additives in petroleum-based products. Based on the chemical structure of group chromophore, dyes are classified as anthraquinone, azo, triphenylmethane, indigo, heterocyclic and polymeric. Among these, azo, anthraquinone and triphenylmethane dyes are the most extensively produced and used in textile industries, the first group corresponding to approximately 50% of the total [5].

Dyes are identified as the most problematic compounds present in textile effluents due to their high solubility in water and low degradability [6]. It is estimated that the traditional textile finishing industry consumes about 100 L of water for 1 kg of textile material, and that 10 to 15% of the compounds used in dyeing processes are found in industrial effluents [7,8]. Dyes are usually stable to factors such as light and temperature, and visible in water even at relatively small concentrations (10 to 50 mg L^{-1}) [1]. Besides the visual impact, the presence of these compounds in water reduces its transparency, hindering the absorption of light and therefore affecting aquatic plants and algae [3,6,9,10]. Furthermore, the solubility of oxygen in water is also reduced, with adverse effects in terms of chemical oxygen demand (COD) and biological oxygen demand (BOD). In addition, dyes are potentially toxic, carcinogenic, mutagenic and allergenic compounds, and their efficient removal from industrial wastes is absolutely mandatory [5,11].

Because of their resistance to microbial attack, the elimination of these coloured substances from liquid effluents is mainly based on physical or chemical procedures, such as adsorption, coagulation, flocculation, membrane filtration, irradiation, concentration, and chemical transformation. However, these methods are expensive, what limits their applicability [2,5,8,12]. In this context, the development of unconventional processes for dye-containing effluents treatment is required.

The possibility of using white-rot fungi in biotreatment strategies comes from their capacity to produce a non-specific enzyme system able to metabolize a wide range of pollutants to CO_2 and H_2O [13,14]. These fungi tolerate concentrations considerably high of

pollutants and can transform polycyclic aromatic hydrocarbons (PAHs), polyphenols, dioxins, chlorinated pesticides, organophosphate insecticides, anilines and dyes, showing potential for use in industrial segments as processing of coal, oil refining, resins and plastics, electroplating, chemical, textile dyes, mining and pulp and paper industries [13,15,16].

Fungi of the genus *Pleurotus* represent a cosmopolitan group of mushrooms that have great nutritional value, therapeutic, medicinal properties and several environmental and biotechnological applications, due to their enzyme complex [17]. The ligninolytic enzymes produced by *Pleurotus* and other Basidiomycetes include manganese peroxidases (MnP), lignin peroxidases (LiP) and laccases (Lac), which are secreted to the growth medium in response to low levels of nutrients [18]. Some fungi produce Lac, MnP and LiP, while others produce only one or two of these enzymes [19]. These organisms are enzymatically equipped to oxidize compounds with similar structures to lignin, breaking the aromatic ring and forming compounds that may suffer further degradation, being mineralised [17].

Laccases are multi-copper polyphenol oxidases, which oxidize phenolic compounds reducing oxygen to water by removing an electron from the aromatic substrate [16]. They are present in most white-rot fungi and also found in other types of fungi, plants, some bacteria and insects [20,21]. Fungal laccases are of particular interest due to of their capability to oxidize a wide range of industrially relevant substrates as phenolic and aromatic amines. Thus, these enzymes represent a promising alternative for biotechnological processes of environmental interest such as bleaching and delignification of cellulose pulp, decolourisation of textile dyes, oxidation of PAHs, phenol removal, detoxification of effluent and environmental pollutants, and degradation of recalcitrant compounds [8,16,21,22,23,24]. Laccases can also be used in cosmetics, chemical, pharmaceutical, food and beverage industries, as biosensor of phenolic compounds in environmental, pharmaceutical and industrial areas, in nanobiotechnology, synthetic chemistry and soil bioremediation [22,25,26,27,28].

Development of aerobic bacteria to be used for the decolourisation of dyes often results in strains with ability limited to attack a single chemical structure. Many azo dyes can be broken down in potentially mutagenic and/or carcinogenic amines in anaerobiosis, due to the action of very specific azo-reductases [1]. However, laccases act by oxidation and are less specific with respect to the substrate [16,20]. Due to the fact of using synthetic dyes with a wide variety of chemical structures, it is interesting the development of biocatalytic processes able to act on this diversity [29]. The use of laccases for developing enzyme-based treatment processes is particularly interesting because it can be produced with less demanding induction conditions than those observed for LiP and MnP [30].

Given this, the aim of this work was to evaluate the decolourisation of twenty-two dyes belonging to the chromophore groups anthraquinone, azo and triphenylmethane, using laccase-containing preparation produced by *Pleurotus sajor-caju* PS-2001 in submerged process, with respect to the influence of the parameters pH, temperature and agitation.

MATERIAL AND METHODS

Organism and Culture Conditions

Pleurotus sajor-caju strain PS-2001, from the microorganism culture collection of the Institute of Biotechnology of the University of Caxias do Sul (Brazil), was grown and maintained in a medium containing (per litre): 20 g *Pinus* spp. sawdust, 20 g wheat bran, 2.0 g $CaCO_3$, and 20 g agar-agar. Petri dishes with the fungus were incubated at 28°C until complete mycelial growth and subsequently stored at 4°C [31].

The inoculum for bioreactor cultivations were prepared in 500-mL Erlenmeyer flasks containing 100 mL of medium containing (per litre): 5 g glucose, 1.5 g pure casein (Synth®) and 100 mL of a mineral solution (composition per litre: 20 g KH_2PO_4, 14 g $(NH_4)_2SO_4$, 3.0 g $MgSO_4.7H_2O$, 3 g urea, 3.0 g $CaCl_2$, 15.6 g $MnSO_4.H_2O$, 50 mg $FeSO_4$, 14 mg $ZnSO_4$, and 20 mg $CoCl_2$) [31]. After autoclaving at 1 atm for 15 minutes, three mycelial disks with 1.5 cm in diameter were scraped from the stored Petri dishes containing *P. sajor-caju* mycelium and added to the flasks [30]. Inoculum growth occurred under reciprocal agitation of 180 min^{-1}, at 28±2ºC, for 6 days. Volumes of 400 mL of inoculum suspension (10% v/v) were used to start bioreactor cultivations.

For the production of laccase-containing enzyme broth, the following cultivation medium was used (per litre): glucose, 5.0 g; pure casein (Synth®), 1.5 g; $CuSO_4$, 100 mg; benzoic acid, 100 mg; mineral solution, 100 mL [32]. The cultivations were carried out in a stirred-tank bioreactor B. Braun Biotech model Biostat®B with 4.0 L of working volume. The bioreactor containing the cultivation medium was autoclaved for 20 minutes at 1.5 prior to inoculation. During the initial hours of cultivation the bioreactor was kept at an impeller speed of 200 min^{-1} and an air flow rate of 2 L min^{-1}). Afterwards, when the dissolved oxygen concentration (DO) decreased to about 30% of saturation, DO was maintained at this level by automatically varying the air flow rate [30]. Before inoculation, under the initial aeration and agitation conditions, the volumetric oxygen transfer coefficient (K_La) was determined by the method described by Moo-Young and Blanch [33] as 12 h^{-1}. Silicone-based antifoam was used when necessary and the pH was automatically controlled at 6.5 by adding 2 mol L^{-1} NH_4OH or 2 mol L^{-1} H_2SO_4 at 28±1ºC [31].

Dye decolourisation assays

In decolourisation assays, a total of twenty-two dyes were assayed as follows: ten from the azo chromophore group, four from the anthraquinone group, and eight from the triphenylmethane group (Table 1). The basic chemical structures of the different groups of dyes used in this work are presented in Figure 1.

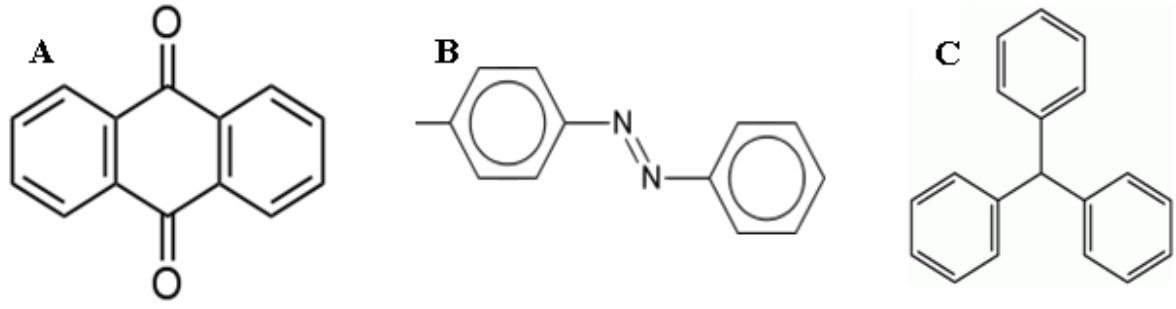

Figure 1. Basic chemical structures of dyes of chromophore groups anthraquinone (A), azo (B) and triphenylmethane (C).

Decolourisation assays were done in 25-mL test tubes containing 7.0 mL of 50 mg L^{-1} dye solution, 7.0 mL of Mc'Ilvaine buffer (sodium hydrogen phosphate and citric acid), at an adequate pH value according to the condition evaluated, and 7.0 mL of crude *P. sajor-caju* cultivation broth with 30 U mL^{-1} laccases, corresponding to 10 U mL^{-1} in the reaction medium as defined by Schmitt et al. [24]. The tubes containing the mixtures were kept in a thermostatic bath, and 0.4 mL samples were collected in triplicate each 24 hours, during 168 or 240 hours of reaction. The absorbance of samples was read in spectrophotometer at a wavelength between 350 to 750 nm, as previously defined for each particular dye (Table 1).

Table 1. Dyes, respective chromophore group, and wavelength used in decolourisation assays carried out with laccase-containing enzyme broth produced by *Pleurotus sajor-caju* PS-2001.

Dye	Group	Wavelenght (nm)
Acid Blue 80	Anthraquinone	628
Acid Green 28	Anthraquinone	685
Reactive Blue 220	Anthraquinone	609
Remazol Brilliant Blue R	Anthraquinone	591
Acid Red 315	Azo	493
Congo Red	Azo	494
Disperse Blue 79	Azo	539
Disperse Orange 30	Azo	464
Disperse Red 324	Azo	470
Levafix Brilliant Red E-4BA	Azo	513
Levafix Golden Yellow E-G	Azo	434
Orange G	Azo	478
Reactive Red 198	Azo	518
Reactive Yellow 15	Azo	411
Brilliant Green	Triphenylmethane	610
Bromocresol Green	Triphenylmethane	616
Bromophenol Blue	Triphenylmethane	590
Coomassie Brilliant Blue G-250	Triphenylmethane	579
Gentian Violet	Triphenylmethane	582
Malachite Green	Triphenylmethane	610
Methyl Violet	Triphenylmethane	584
Phenol Red	Triphenylmethane	433

λ = wavelength of the maximum absorption for dyes obtained from scanning assays (350 to 750 nm) with solutions of dyes in concentration of 50 mg L^{-1}.

The decolourisation reaction was evaluated at pH values of 2.4, 3.2, 4.4 and 5.0, at 30°C and without agitation [30].

For the assessment of the effect of temperature on decolourisation, the reaction tubes were kept at 35, 40, 45, and 50°C in a bath without agitation [30].

Decolourisation assays under agitation were performed at pH 3.2 and temperatures of 30 and 35ºC, under reciprocal agitation of 100 min^{-1}.

Determination of Laccases Activity

Laccases (Lac) activity was determined at 25°C using 0.45 mmol L^{-1} 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS - Sigma®) as substrate in reaction mixtures containing 90 mmol L^{-1} of pH 5.0 sodium acetate buffer solution and an appropriate amount of culture supernatant. ABTS oxidation was estimated by measuring increase in absorbance at 420 nm (ε_{420} = 3.6 x 10^4 cm^{-1} mol^{-1}) for 90 seconds [34]. One enzyme unit corresponds to the quantity in μmol of product released per minute per mL of sample.

Determination of Degree of Decolourisation

Decolourisation degree was calculated by comparing the initial absorbance of the reaction mixture and those at the different assay times, the results being expressed in terms of percentage of reduction in absorbance [24].

RESULTS

Dye decolourisation assays were carried out under different pH values at 30ºC for up to 168 hours. The pH values evaluated were 2.4, 3.2, 4.4, and 5.0. In a previous work of our group [30], these pH values were defined as the optimum for the activity of three possible laccase isoforms which were identified in *P. sajor-caju* cultivation broth. The results obtained in the decolourisation assays with the twenty-two dyes evaluated are shown in Table 2. From these data, one can be seen that the best results in terms of percentage of decolourisation were mostly obtained at pH 3.2, the only condition under which all dyes were affected by the enzymatic treatment. The highest decolourisation levels were observed for the dyes *Acid Blue* 80, *Acid Green* 28, *Brilliant Green*, *Bromocresol Green*, *Coomassie Brilliant Blue* G-250, *Disperse Blue* 79 and *Reactive Red* 198, showing percentages of decolourisation between 23% (*Disperse Blue* 79) and 46% (*Brilliant Green*) after different reaction times. Among these dyes, three of them belong to the triphenylmethane group, two to the azo group and two to the anthraquinone group, as shown in Table 1.

At pH 2.4, only five of the dyes assayed showed no decolourisation after 168 hours (*Bromocresol Green*, *Congo Red*, *Disperse Red* 324, *Levafix Brilliant Red* E-4BA and *Reactive Blue* 220), three of them belonging to the azo group (Table 1). Treatments of solution of *Acid Blue* 80, *Acid Green* 28, *Brilliant Green* and *Reactive Red* 198 presented removal percentages above 20%.

In contrast to the results obtained at pH 3.2 and 2.4, pH 4.4 and 5.0 have shown to be ineffective with regard to enzymatic decolourisation of dyes under the conditions evaluated. Only thirteen dyes had decrease in colour after 168 hours at pH 4.4, with results above 20% of decolourisation for *Acid Blue* 80 and *Reactive Blue* 220 (anthraquinone), *Congo Red* (azo) and *Gentian Violet* (triphenylmethane). The less satisfactory results were obtained at pH 5.0, since only six dyes showed decolourisation after the treatment (*Acid Blue* 80, *Brilliant Green*, *Gentian Violet*, *Malachite Green, Methyl Violet* and *Reactive Blue* 220), four of them belonging to the triphenylmethane group and two to anthraquinone group (Table 1).

In the previous assays, pH 3.2 was identified as the most suitable for the decolourisation of dyes at 30ºC. Therefore, the set of experiments carried out to assess the influence of the reaction temperature (35, 40, 45 and 50ºC) on the decolourisation of the dyes under evaluation were done at this pH value. Such temperature values were defined from previous studies that have shown increasing laccases activities from 30 to 50°C [30]. The results are presented in Table 3.

Table 2. Maximum dye decolourisation after enzymatic treatment carried out at different pH values and 30ºC, without agitation.

pH	**2.4**		**3.2**		**4.4**		**5.0**	
Decolourisation	**%**	**t (h)**	**%**	**t (h)**	**%**	**t (h)**	**%**	**t (h)**
Acid Blue 80	26.6	168	28.6	168	23.2	24	28.0	24
Acid Green 28	25.5	48	27.1	168	ND	---	ND	---
Reactive Blue 220	ND	---	3.56	168	23.0	168	19.1	24
Remazol Brilliant Blue R	17.8	168	10.8	72	ND	---	ND	---
Acid Red 315	5.58	168	13.8	168	ND	---	ND	---
Congo Red	ND	---	14.4	24	28.8	168	ND	---
Disperse Blue 79	16.7	24	23.5	168	ND	---	ND	---
Disperse Orange 30	5.39	168	9.87	96	10.5	72	ND	---
Disperse Red 324	ND	---	13.4	48	0.31	24	ND	---
Levafix Brilliant Red E-4BA	ND	---	2.86	168	ND	---	ND	---
Levafix Golden Yellow E-G	3.66	168	5.17	168	0.68	24	ND	---
Orange G	13.5	168	5.57	168	ND	---	ND	---
Reactive Red 198	23.6	168	25.5	168	ND	---	ND	---
Reactive Yellow 15	9.16	168	16.8	168	1.42	96	ND	---
Brilliant Green	48.8	48	45.9	168	13.0	48	19.0	168
Bromocresol Green	ND	---	26.5	168	ND	---	ND	---
Bromophenol Blue	0.08	168	4.48	168	9.43	48	ND	---
Coomassie Brilliant Blue G-250	15.7	168	35.8	168	0.18	24	ND	---
Gentian Violet	6.01	168	8.73	168	33.6	168	20.9	168
Malachite Green	1.73	96	0.79	168	1.22	24	10.3	168
Methyl Violet	15.9	168	16.8	168	16.2	96	7.64	24
Phenol Red	6.68	168	0.21	168	ND	---	ND	---

ND – Decolourisation not observed.

The data obtained at pH 3.2 and 30ºC showed decolourisation in different percentage levels for all dyes in test (Table 2). With regard to the results obtained under the further temperatures evaluated (Table 3), it was observed that at 35ºC the percentage of decolourisation was generally higher than those achieved with the other temperatures tested (30, 40, 45 and 50ºC). At 35ºC and pH 3.2, only three dyes, belonging to triphenylmethane chromophore group showed no reduction in colour (*Gentian Violet*, *Malachite Green* and *Methyl Violet*). With the exception of *Phenol Red*, all other dyes showed percentages of decolourisation above 20%, and some of them reached values

above 60% (*Acid Blue* 80, *Congo Red*, *Disperse Blue* 79, *Disperse Red* 324 and *Orange* G). Among the temperatures evaluated (Table 3), 40ºC has shown to be inadequate for this treatment, given that only ten dyes showed decolourisation under this condition, although in some cases decolourisation has been greater than 30%. At 45ºC, fourteen dyes responded positively to the enzymatic treatment, some of them showing relatively high percentage of decolourisation. However, only nine dyes were decolourised at 50ºC, with reduction in colour lower than those obtained under the other conditions.

Table 3. Maximum dye decolourisation after enzymatic treatment carried out at different temperatures and pH 3.2, without agitation.

Temperature (ºC)	**35**		**40**		**45**		**50**	
Decolourisation	**%**	**t (h)**	**%**	**t (h)**	**%**	**t (h)**	**%**	**t (h)**
Acid Blue 80	65.7	168	36.3	72	47.6	168	34.3	96
Acid Green 28	54.5	48	39.3	96	50.0	168	43.3	168
Reactive Blue 220	27.6	168	ND	---	17.8	168	ND	---
Remazol Brilliant Blue R	55.9	168	24.0	96	34.4	168	29.8	48/72
Acid Red 315	54.0	168	32.6	96	24.9	72	11.7	168
Congo Red	64.5	168	42.8	96	50.6	168	20.5	24
Disperse Blue 79	66.6	96	ND	---	36.3	168	ND	---
Disperse Orange 30	57.6	168	32.8	72	46.5	168	30.3	96
Disperse Red 324	62.2	96	29.9	96	39.4	72	19.5	24
Levafix Brilliant Red E-4BA	53.2	168	21.5	96	34.1	168	18.9	168
Levafix Golden Yellow E-G	44.1	168	ND	---	ND	---	ND	---
Orange G	67.7	168	ND	---	ND	---	ND	---
Reactive Red 198	57.3	168	26.7	72	42.6	168	15.6	72
Reactive Yellow 15	20.1	168	ND	---	24.6	168	ND	---
Brilliant Green	56.4	48	ND	---	7.94	168	ND	---
Bromocresol Green	20.8	168	ND	---	ND	---	ND	---
Bromophenol Blue	31.7	168	ND	---	ND	--	ND	---
Coomassie Brilliant Blue G-250	56.3	168	ND	---	6.99	72	ND	---
Gentian Violet	ND	---	ND	---	ND	---	ND	---
Malachite Green	ND	---	0.11	168	ND	---	ND	---
Methyl Violet	ND	---	ND	---	ND	---	ND	---
Phenol Red	4.09	168	ND	---	ND	---	ND	---

ND – Decolourisation not observed.

Figure 2 depicts the profile of the absorbance readings, performed every 24 hours, of the enzymatic treatment of the thirteen dyes which showed more than 50% decolourisation at pH 3.2 and 35ºC (Table 3), comprising eight dyes belonging to the azo group, three to the anthraquinone group, and two to the triphenylmethane group. It is interesting to remark that all acid dyes (*Acid Blue* 80, *Acid Green* 28 and *Acid Red* 315) and all disperse dyes (*Disperse Blue* 79, *Disperse Orange* 30 and *Disperse Red* 324) evaluated are included among these thirteen dyes.

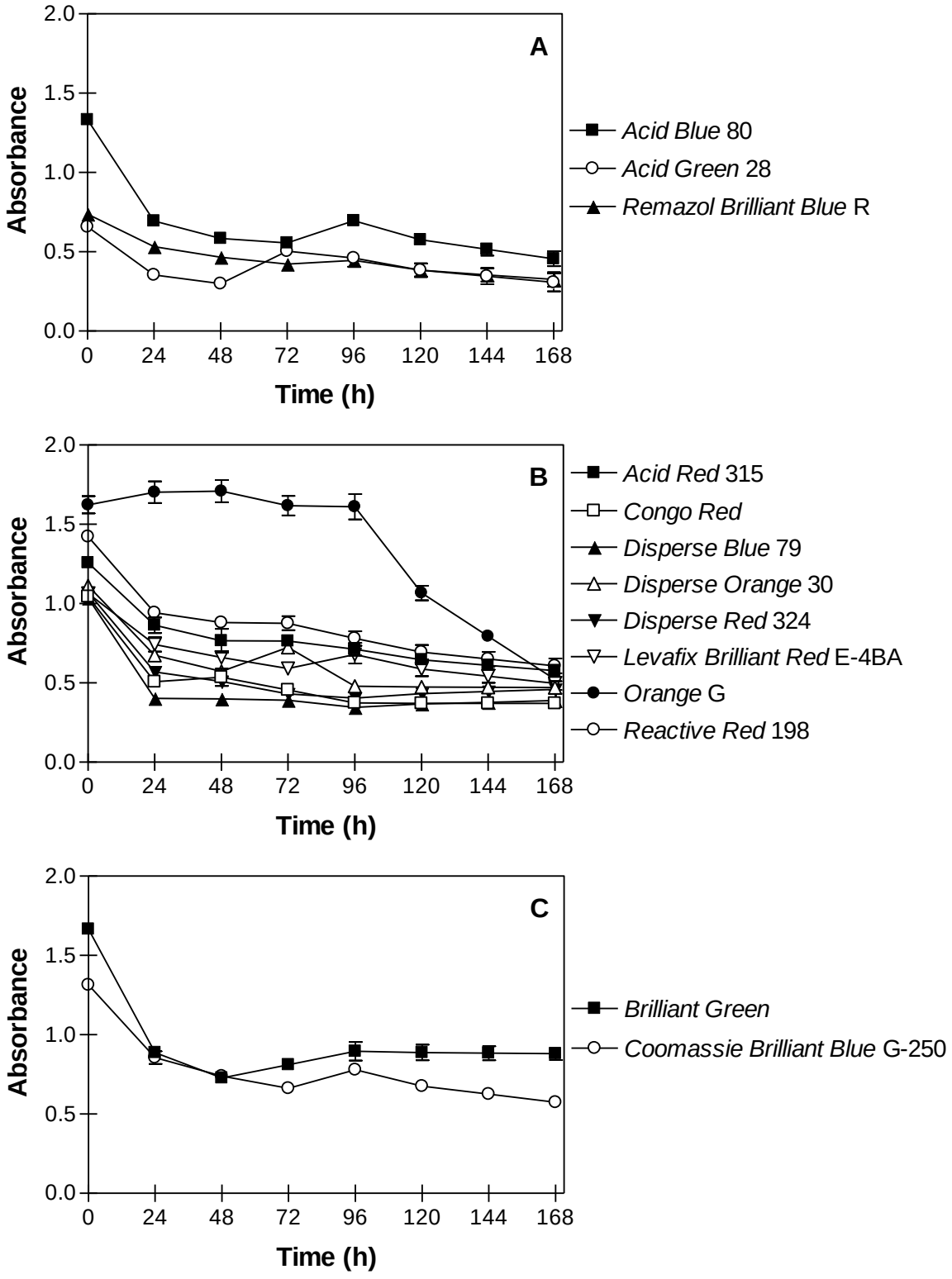

Figure 2. Decolourisation of dyes as a function of time during enzymatic treatment with laccase-containing *Pleurotus sajor-caju* PS-2001 cultivation broth at pH 3.2 and 35ºC. Dyes of chromophore groups anthraquinone (A), azo (B) and triphenylmethane (C).

As shown in Tables 2 and 3, pH 3.2 and temperatures of 30 and 35ºC were adequate conditions for decolourisation of dyes of different chromophore groups. Thus, the assays under reciprocal agitation were performed using this value of pH in both temperatures during 240 hours of incubation. In Table 4, the percentages of decolourisation of the dyes studied obtained in experiments performed under reciprocal agitation of 100 rpm are presented. The data indicate that the use of agitation led to a remarkable effect on the decolourisation of dyes. At 30°C, only three dyes showed decrease in colour (*Brilliant Green*, *Malachite Green* and *Methyl Violet*), all from the triphenylmethane group. At 35ºC, decolourisation was observed for five dyes, being one from the anthraquinone group (*Acid Blue* 80) and four from triphenylmethane group (*Brilliant Green*, *Gentian Violet*, *Malachite Green* and *Methyl Violet*). In general, the percentages of removal observed under agitation (Table 4) were much lower than those obtained in static conditions (Tables 2 and 3) using the same values of pH and temperature. In fact, only *Gentian Violet* solution, at 35°C and after 240 hours of incubation, was decolourised in a level over 40%.

Table 4. Maximum dye decolourisation after enzymatic treatment carried out at different temperatures and pH 3.2, under agitation.

Temperature (ºC)	**30**		**35**	
Decolourisation	**%**	**t (h)**	**%**	**t (h)**
Acid Blue 80	ND	---	15.5	96
Acid Green 28	ND	---	ND	---
Reactive Blue 220	ND	---	ND	---
Remazol Brilliant Blue R	ND	---	ND	---
Acid Red 315	ND	---	ND	---
Congo Red	ND	---	ND	---
Disperse Blue 79	ND	---	ND	---
Disperse Orange 30	ND	---	ND	---
Disperse Red 324	ND	---	ND	---
Levafix Brilliant Red E-4BA	ND	---	ND	---
Levafix Golden Yellow E-G	ND	---	ND	---
Orange G	ND	---	ND	---
Reactive Red 198	ND	---	ND	---
Reactive Yellow 15	ND	---	ND	---
Brilliant Green	13.0	96	16.7	240
Bromocresol Green	ND	---	ND	---
Bromophenol Blue	ND	---	ND	---
Coomassie Brilliant Blue G-250	ND	---	ND	---
Gentian Violet	ND	---	41.1	240
Malachite Green	1.54	96	3.06	240
Methyl Violet	0.38	240	19.6	216
Phenol Red	ND	---	ND	---

ND – Decolourisation not observed.

Considering the development of an enzymatic technology for dye decolourisation to be applied to large-scale effluent treatment systems, the results of this set of experiments are quite interesting, since the use of agitation implies in a high demand for energy, which would raise the costs of the process.

DISCUSSION

The data obtained in the present study have shown that is possible to attain significant decolourisation of dyes of different chromophore groups by using laccase-containing preparations from *P. sajor-caju* PS-2001 under controlled conditions. In general, as occurs in any enzymatic process, decolourisation of dyes is affected by parameters such as characteristics of the substrate, pH, temperature, agitation, and incubation time, as shown in this work, together with other factors like dye and enzyme concentrations, and use of mediators that have also been described by different authors [24,35,36,37]. These and other works found in the specialised literature, which may serve to corroborate in some extent our findings, are focused in the sequence.

With respect to the effect of the temperature on the decolourisation of the different dyes, it was observed a negative effect on the process by temperatures over 35°C, despite the fact that laccases from *P. sajor-caju* PS-2001 present increasing activities up to 50°C [30], as already mentioned. The explanation for this behaviour lies in the stability of these enzymes as a function of temperature and time. In studies on the laccases of *P. sajor-caju* PS-2001, these same authors have shown that enzyme activities were practically unaltered after pre-incubation at 20°C for 168 hours, and 30ºC for 72 hours. At 40ºC, however, less than 20% of the original activity remained after 72 hours of pre-incubation, whereas at 50 and 60ºC, laccases were almost totally inactivated after approximately 10 hours [30].

Unlike the methodology adopted in the present work, which used crude enzymatic broth produced in stirred-tank bioreactor, Munari et al. [23] studied the decolourisation of various dyes during the growth of *P. sajor-caju* PS-2001 in solid and liquid cultures. In that work, satisfactory results were noticed for the decolourisation of dyes belonging to the anthraquinone group (*Acid Blue 80* and *Reactive Blue 220*), whereas azo dyes presented resistance to degradation under the assayed conditions. Schmitt et al. [24] evaluated the potential of three oxidoreductases, including laccase of *P. sajor-caju* PS-2001 and two peroxidases, for the decolourisation of textile disperse dyes (*Disperse Red* 343, *Disperse Red* 167 and *Disperse Blue* 148). In this study, which was carried out in aqueous solutions, the influence of different concentrations of dyes and enzymes, pH, temperature, and addition of mediators – hydroxybenzotriazole (HBT) and syringaldazine (SYR) – was assessed. Tests with *Disperse Red* 167 and *Disperse Blue* 148 showed percentages of decolourisation of 15% and 25%, respectively, and under the conditions evaluated, the use of only 10 U mL^{-1} laccases of *P. sajor-caju* PS-2001 has shown to be more efficient in colour removal than horseradish and microbial peroxidases.

Similarly to *P. sajor-caju*, other species of the genus *Pleurotus* are also able to discolour industrial dyes. According to Rodríguez et al. [11], this ability in *Pleurotus ostreatus* is related to the activity of laccases. Zilly et al. [38] have shown that the fungus *Pleurotus pulmonarius*, which produces only laccases, can discolour synthetic dyes of azo and

triphenylmethane groups. *Pleurotus calyptratus* was applied to the decolourisation of anthraquinone (*Remazol Brilliant Blue* R) and azo (*Orange* G) dyes, but showed limited capacity for degrading triphenylmethane dyes, as *Crystal Violet* and *Malachite Green* [39].

By using laccases from *Paraconiothyrium variabile*, Ashrafi et al. [40] achieved 60% of decolourisation of the dye *Reactive Red* 120, after 30 minutes of treatment, and 90% of decolourisation of *Disperse Blue* 56, after 60 minutes, at pH 5.0 and 40°C. However, in this case, purified laccases were used, while in the present work we have used a crude enzyme preparation without any purification procedure. Yang et al. [41] studied a strain of *Trametes* able to discolour efficiently a variety of synthetic dyes, including some from azo, triphenylmethane and anthraquinone groups, after 5 days of cultivation. The same dyes were also treated with the purified laccases produced by the fungus, in the absence of redox mediators, but few of them have been completely decoloured. Superior results were obtained by Lu et al. [42] that achieved 80% of decolourisation of *Remazol Brilliant Blue* R by using 5 U mL^{-1} purified laccase from *Pycnoporus sanguineus* at pH 3.0 and 40°C.

In addition to oxidation-reduction reactions, Durán and Esposito [25] reported that laccases also participate in polymerization of compounds. This was also observed by Moldes et al. [43] in studies on the decolourisation of dyes in the absence of mediators. The occurrence of that kind of reaction could be an explanation for the poor results obtained in assays under agitation (Table 4). In fact, in these experiments, few dyes were decolourised and, in most of the cases, it was observed an increase in absorbance of the reaction media, suggesting that the enzymes present in the crude broth have catalysed polymerization reactions of dyes as a consequence of the intensification of the mixing conditions. In this work, in which a crude laccase-containing preparation was used, agitation caused negative effects on the decolourisation of dyes. On the other hand, Kaushik and Malik [36] observed increasing dye decolourisation when the process was carried out simultaneously with fungal growth in flasks under agitation in comparison to a stationary culture, a result that was surely associated to the more intense oxygen transfer and nutrient distribution in the first case.

Besides the already cited fungi, several other species produce different isoforms of extracellular laccases. Champagne et al. [44] showed that laccases of *Trametes versicolor* were able to remove colour of the anthraquinone dye *Reactive Blue* 19. Niebisch et al. [12] applied laccases of *Lentinus crinitus* for degrading the textile dye *Reactive Blue* 19. Khelifi et al. [6] reported the ability of the fungus *Aspergillus alliaceus* to discolour *Indigo* and *Congo Red* dyes during their growth in liquid media, with activities of laccases, manganese peroxidises, and lignin peroxidises being detected in the cultivation broth. Cantele et al. [45], using 10 U mL^{-1} laccases from *Marasmiellus palmivorus* in the reaction medium, the same activity used in the present work, have shown that these enzymes were able to efficiently discolour only *Acid Blue* 80 and *Reactive Blue* 220 among eleven different dyes. Similar results were observed even when the enzyme activity was increased up three times.

According to Ashrafi et al. [40], azo dyes are recalcitrant compounds, less susceptible to enzymatic action, while those of the anthraquinone group are more easily oxidised by laccases. This was also reported by Eichlerová et al. [39] after evaluating results of assays conducted with *P. calyptratus*, suggesting that azo dyes are more resistant to decolourisation due to its chemical structure. For the biodegradation of reactive dyes of anthraquinone and azo classes, Forss and Welander [46] evaluated the use of a continuous

system, observing colour removal for the dyes *Reactive Black* 5, *Reactive Red* 2 and *Reactive Blue* 4.

In a study of Jarosz-Wilkolazka et al. [47], 115 fungus strains were compared regarding the ability of removing colour from anthraquinone and azo dye solutions. White-rot fungi were the fastest in the colour removal, and *Acid Red* 183 proved to be more resistant to decolourisation. Among the species tested, sixty-nine showed ability to decolourise the dye *Basic Blue* 22 (anthraquinone) and only sixteen discoloured *Acid Red* 183 (azo). Chagas and Durrant [48] stated that the white-rot fungi *Phanerochaete chrysosporium* and *P. sajor-caju* could be used in bioprocesses for removing colour from industrial effluents. *P. sajor-caju* bleached 50% of the azo dye *Orange* G, indicating that laccases have greater participation in the process of decolourisation than MnP. Laccases also showed ability to decolourise *Remazol Brilliant Blue* R during the growth of *Trametes pubescens* in bioreactor with decolourisation of 55% in 4 hours and 70% in 24 hours, without the addition of redox mediators [5].

Laccases of *T. versicolor* play an important role in the attack on the structure of triphenylmethane dyes. However, the identification of degradation products is also a relevant issue, since the metabolites produced after processing can be highly toxic [49]. Champagne and Ramsay [50] used immobilised laccases for treating anthraquinone and azo dyes. Although anthraquinone dyes have been discoloured more quickly than azo dyes, their reaction derivatives have shown to be much more toxic than the original form of the dye, whereas azo dyes discolouration products did not show toxicity after enzymatic treatment. Selvam et al. [15] reported that laccases of *Thelephora* sp. were able to discolour the azo dyes *Orange* G and *Congo Red* in the absence of redox mediators, showing greater efficiency than MnP and LiP enzymes in colour removal process. However, Murugesan et al. [51] observed that the presence of the mediator HBT was essential for the decolourization of the dye *Reactive Black* 5 by purified laccases from *P. sajor-caju*. In addition, Zeng et al. [10] suggest that anthraquinone dyes can act as mediators in processes of decolourisation of azo dyes, using laccases of *Trametes troggi*.

Several studies have shown that white-rot fungi are able discolour a wide range of dyes with different chemical structures, such as anthraquinone, azo, triphenylmethane and heterocyclic, in which is also reported partial mineralization of dyes by enzymatic and non-enzymatic systems [52,53]. Spectrophotometric and microscopic analyses of *Funalia trogii* pellets showed that the process of decolourisation occurs due to microbial metabolism, but not by biosorption [35]. In addition to the factors already mentioned (pH, temperature, use of redox mediators, type and initial concentration of dye), ionic strength and redox potential also affect the decolourisation of dyes by fungi [36,37]. Laccases and peroxidases are eco-friendly biocatalysts for the removal of wide spectrum of textile and non-textile dyes [54]. However, laccases from different sources exhibit a wide range of redox potentials, which interfere in its potential of application in decolourisation processes [16].

Table 5 summarises some relevant works found in the specialised literature about the decolourisation of dyes by fungal enzymes under both *in vivo* and *in vitro* conditions.

Table 5. Examples of studies on the decolourisation of dyes belonging to different chromophore groups by enzymatic and microbial processes.

Producing fungi	Dyes	Enzymes	Assay conditions	Reference
Aspergillus alliaceus	*Congo Red* *Indigo*	Laccases and lignin peroxidases (LiP)	Microbial process: solid medium in agar plates	[6]
Aspergillus sp.	*Reactive Blue* 114 *Reactive Red* 239 *Reactive Yellow* 15	Laccases	Enzymatic process: commercial enzyme formulation	[55]
Dichomitus squalens	*Orange* G *Remazol Brilliant Blue* R	Laccases and manganese peroxidases (MnP)	Microbial process: static liquid cultivation	[56]
Lentinus crinitus	*Reactive Blue 220*	Laccases	Enzymatic process: extracellular extract	[12]
Lentinula edodes	*Amido Black* *Brilliant Cresyl Blue* *Congo Red* *Ethyl Violet* *Methyl Green* *Methyl Violet* *Methylene Blue* *Poly* R478 *Remazol Brilliant Blue* R *Trypan Blue*	MnP, LiP and Laccases	Microbial process: solid state cultivation	[19]
Marasmiellus palmivorus	*Acid Green* 28 *Reactive Blue* 220	Laccases	Enzymatic process: lyophilised crude enzyme extract	[57]
Marasmiellus palmivorus	*Acid Blue* 80 *Acid Red* 315 *Dianix Yellow* *Disperse Orange* 30 *Foron Rubine* *Navy Blue* *Reactive Blue* 220 *Reactive Red* 198 *Reactive Red* 4BL *Reactive Yellow* 15 *Remazol Black* B	Laccases	Microbial process: submerged cultivation in shake flasks (180 rpm)	[45]
Phanerochaete chrysosporium	*Amaranth* (red) *New Coccine* (red) *Orange* G (orange) *Tartrazine* (yellow)	MnP and β-glucosidase	Microbial process: liquid cultivation under shaking conditions	[48]

Phanerochaete chrysosporium and *Pleurotus ostreatus*	*Acid Blue* 62 *Acid Red* 299 *Direct Black* 38 *Direct Blue* 1 *Direct Red* 81 *Disperse Blue* 1 *Disperse Yellow* 3 *Reactive Black* *Reactive Blue* 19 *Reactive Red* 4 *Reactive Yellow* 81	Laccases and MnP	Microbial process: liquid cultivations in flasks (rotary shaker at 120 rpm)	[58]
Pleurotus calyptratus	*Orange* G *Remazol Brilliant Blue* R	Laccases, MnP and aryl-alcohol oxidase (AAO)	Microbial process: static liquid cultivation	[39]
Pleurotus ostreatus	*Acid Black* 194 *Acid Blue* 185 *Reactive Blue* 158	Laccases	Enzymatic process: crude extract	[11]
Pleurotus pulmonarius	*Amido Black* *Brilliant Cresyl Blue* *Congo Red* *Ethyl Violet* *Methyl Green* *Methyl Violet* *Remazol Brilliant Blue R* *Trypan Blue*	Laccases	Microbial process: solid and submerged cultivations Enzymatic process: crude extracellular extract	[38]
Pleurotus sajor-caju	*Amaranth* (red) *New Coccine* (red) *Orange* G (orange) *Tartrazine* (yellow)	Laccases and glucose-oxidase (GOD)	Microbial process: liquid cultivation under shaking conditions	[48]
Pleurotus sajor-caju	*Acid Blue* 80 *Acid Green* 28 *Acid Red* 315 *Disperse Blue* 79 *Disperse Orange* 30 *Disperse Red* 324 *Reactive Blue* 220 *Reactive Red* 198 *Reactive Yellow* 15	Laccases, MnP, LiP and veratryl-alcohol oxidase (VAO)	Microbial process: solid and submerged cultivations Enzymatic process: crude extracellular extract	[23]
Pleurotus sajor-caju	*Reactive Black* 5	Laccases	Enzymatic process: purified enzyme and HBT as mediator	[51]
Pycnoporus sanguineus	*Remazol Brilliant Blue* R	Laccases	Enzymatic process: ultrafiltration-purified enzyme	[42]

Thelephora sp.	Amido Black 10B *Congo Red* *Orange* G	Laccases, LiP and MnP	Enzymatic process: purified enzymes	[15]
Trametes hirsuta	*Acid Blue* 225 *Acid Blue* 74 *Basic Red* 9 *Direct Blue* 71 *Reactive Black* 5 *Reactive Blue* 19 *Reactive Blue* 221	Laccases	Enzymatic process: purified and immobilised enzyme	[7]
Trametes hirsuta	*Acid Green* 26 *Acid Red* 97	Laccases-mediator system	Enzymatic process: crude enzyme (violuric acid as redox mediator)	[59]
Trametes pubescens	*Remazol Brilliant Blue* R	Laccases	Microbial process: temporary immersion bioreactor	[5]
Trametes sp.	*Acid Red* *Amido Black* 10B *Congo Red* *Coomassie Brilliant Blue* G250 *Bromphenol Blue* *Cresol Red* *Crystal Violet* *Fast Blue* RR *Malachite Green* *Orange* G *Remazol Brilliant Blue* R	Laccases	Enzymatic process: purified enzyme	[41]
Trametes sp.	*Eriochrome Black* T *Malachite Green* *Remazol Brilliant Blue* R	Laccases	Enzymatic process: purified enzyme and redox mediators	[60]
Trametes trogii	*Acid Blue* 129 *Acid Red* 1 *Reactive Black* 5 *Reactive Blue* 4 *Remazol Brilliant Blue* R	Laccases	Enzymatic process: purified enzyme	[10]
Trametes versicolor	*Acid Blue* 74 *Acid Red* 27 *Disperse Blue* 3 *Reactive Black* 5 *Reactive Blue* 19	Laccases	Enzymatic process: immobilised enzyme on porous glass beads	[50]
Trametes versicolor	*Amaranth* *Cibacron Brilliant Yellow* *Reactive Black* 5 *Remazol Brilliant Blue* R	Laccases and MnP	Enzymatic process: purified enzymes	[61]

Trametes versicolor	*Reactive Blue* 19	Laccases	Enzymatic process: lyophilised enzyme, ABTS as mediator and non-ionic surfactant (Merpol)	[44]
Trametes versicolor	*Reactive Blue* 19	Laccases	Enzymatic process: immobilised enzyme on controlled-porosity-carrier silica beads	[62]
Trametes versicolor	*Phenol Red*	Laccases	Enzymatic process: crude enzyme and 1-hydroxybenzotriazole (HBT) as redox mediator	[43]
Trametes versicolor	*Acid Green* 27 *Acid Violet* 7 *Indigo Carmine*	Laccases	Enzymatic process: purified enzyme and redox mediators	[1]
Trametes versicolor	*Acid Fuchsin* *Acid Green* 16 *Basic Fuchsin* *Brilliant Green* 1 *Methyl Green*	Laccases	Enzymatic process: commercial purified enzyme	[49]

The results of the present work as well as those found in the literature clearly indicate that the success of the enzymatic decolourisation of dyes is dependent on the particular characteristics of both coloured compound and enzyme preparation. As such, taking in account the large number of dyes used all over the world, a continuous search for new producing fungi and their enzymes might be done in order to make this technology widely applicable in the industrial activity.

CONCLUSION

From the data obtained in this work, it is possible to establish that the laccase-containing enzymatic preparation from *P. sajor-caju* PS-2001, produced in submerged process in bioreactor, is able to unspecifically oxidize a wide range of dyes with different chemical structures, including compounds from anthraquinone, azo, and triphenylmethane chromophore groups. For each dye, different incubation times for decolourisation are required, an aspect probably related to the affinity between enzyme and substrate, as well as to the higher or lower recalcitrance of the particular compound.

The results obtained in this study indicate that pH 3.2 and temperatures of 30 and 35^{o}C, without agitation, are adequate conditions for the decolourisation of aqueous solution of dyes belonging to different chromophore groups. The ideal values for temperature are related, below the best for laccases activity as reported in the literature, are dependent on the thermal stability of the enzymes. Agitation does not favour the removal of colour from the reactional mixture evaluated. On the contrary, increasing colour intensity is observed when

the reaction is carried out under mixing, possibly due to the occurrence of polymerization reactions of dyes mediated by the laccases themselves.

The findings of this work are important because they reinforce the technical feasibility of using crude fungal enzyme extracts, without any procedure of purification. That possibility could lead to a significant decrease in the costs of the process envisaging future large-scale application of this biotreatment technology in different sectors of the industry.

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