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Bioremediation potential of industrial laundry effluent by agaricomycetes from brazilian tropical dry forest

Potencial de biorremediação de efluente de lavanderia industrial por agaricomycetes de floresta seca tropical brasileira

Potencial de biorremediación de efluente de lavandería industrial por agaricomycetes del bosque seco tropical brasileño

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Abstract
Mycotic bioremediation of effluents from industrial jeans laundries is a necessary biotechnological treatment to prevent contamination of water bodies. In phase I, the discoloration of Indigo Carmine Dye (ICD) and Textile Effluent (TE) by seven species of Agaricomycetes from the brazilian tropical dry forest (Caatinga) was evaluated. First, nutritional stress was caused by Nitrogen Limitation (NL) at three experimental times, T1 (1 day). T2 (4 days) and T3 (7 days). In phase II, microorganisms were cultivated in the initial growth times G_i1 (10 days) and G_i2 (25 days), Without Addition of Nutrients (WAN) and stress was induced by NL (T1). Subsequently, ICD and TE discoloration tests continued for 28 days. In the ecotoxic analysis, the biotreated samples in phase II were tested on nauplii of *Artemia* HIGH 5 without the addition of food. In phase I, the percentages of ICD and TE discoloration were greater than 55% using fungi F1, F2, F5 and F6 for 10 days without sterility. In phase II, the best percentages of discoloration were found for TE in G_i1 and for ICD in G_i2, with F1 and F5 (identified by molecular biology). The results showed that G_i1 (WAN) increased the biodegradation of TE and G_i2 (WAN) favored the biodegradation of ICD, in T1 (NL) without sterility. The best enzymatic activity of laccase and lignin peroxidase was presented in F5. The enzyme extracts had a Michaelis-Menten kinetic behavior. All samples of TE bioremediated in phase II no showed toxicity on Artemia sp. in 48 hours of experimentation.
Keywords: Nutritional stress; Initial growth time; Ligninolytic enzymes; No sterility; Ecotoxicity.

Resumo
A biorremediação micótica de efluentes de lavanderias industriais de jeans é um tratamento biotecnológico necessário para evitar a contaminação de corpos de água. Na fase I, avaliou-se a descoloração do Corante Índigo Carmim (CIC) e do Efluente Têxtil (ET) por sete espécies de Agaricomycetes da floresta seca tropical brasileira (Caatinga). Primeiro, provocou-se o estresse nutricional por Limitação de Nitrogênio (LN) em três tempos experimentais, T1 (1 dia), T2 (4 dias) e T3 (7 dias). Na fase II, os microrganismos foram cultivados nos tempos de crescimento inicial, C_i1 (10 dias) e C_i2 (25 dias), Sem Adição de Nutrientes (SAN) e induz-se o estresse por LN. Posteriormente, os testes de descoloração de CIC e ET continuaram por 28 dias. Na análise ecotóxica, as amostras biotratadas na fase II foram testadas em náuplios de *Artemia* HIGH 5 sem adição de alimento. Na fase I, as porcentagens de descoloração no CIC e ET foram maiores que 55% utilizando os fungos F1, F2, F5 e F6 por 10 dias sem esterilidade. Na fase II, as melhores porcentagens de descoloração foram encontradas para ET em C_i1, e para CIC em C_i2, com F1 e F5 (identificados por biologia molecular). Os resultados mostraram C_i1 (SAN) aumentou a biodegradação do ET e C_i2 (SAN) favoreceu a biodegradação do CIC, em T1 (LN) sem esterilidade. A melhor atividade enzimática de lacase e

lignina peroxidase apresentou-se em F5. Os extratos enzimáticos apresentaram comportamento cinético de Michaelis-Menten. Todas as amostras de ET biorremediadas na fase II, não mostraram toxicidade sobre *Artemia* sp. em 48 horas de experimentação.
Palavras-chave: Estresse nutricional; Tempo de crescimento inicial; Enzimas ligninolíticas; Sem esterilidade; Ecotoxicidade.

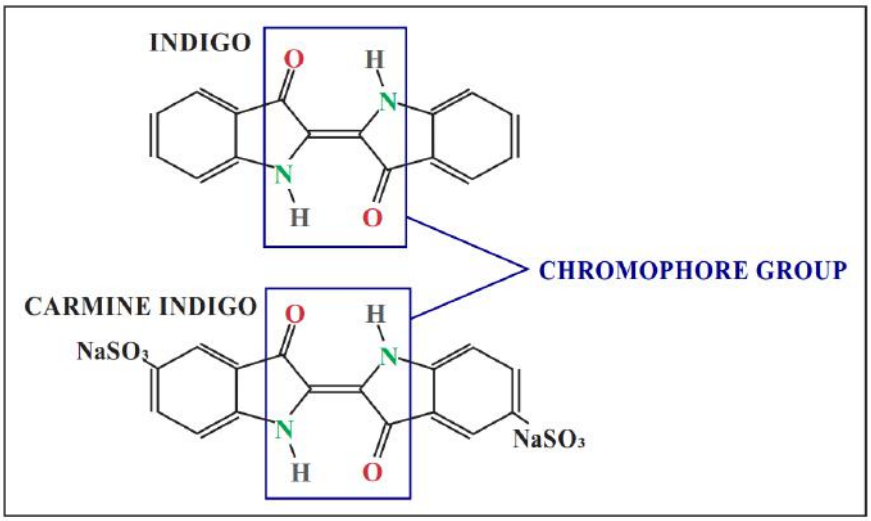
Resumen
La biorremediación micótica de efluentes de lavado industrial de jeans es un tratamiento biotecnológico necesario para evitar la contaminación de cuerpos de agua. En la fase I, se evaluó la decoloración del Colorante Índigo Carmín (CIC) y del Efluente Textil (ET) por siente especies de Agaricomycetes del bosque tropical seco brasileño (Caatinga). Primero se provocó el estrés nutricional por Limitación de Nitrógeno (LN) en tres tiempos experimentales, T1 (1 día), T2 (4 días) y T3 (7 días). En la fase II, los microorganismos se cultivaron en los tiempos de crecimiento inicial, C_i1 (10 días) y C_i2 (25 días), Sin Adición de Nutrientes (SAN) y se indujo estrés por LN. Posteriormente las pruebas de decoloración de CIC y ET continuaron durante 28 días. En el análisis ecotóxico, las muestras biotratadas en la fase II se probaron en nauplios de *Artemia* HIGH 5 sin adición de alimento. En la fase I, los porcentajes de decoloración de CIC y ET fueron superiores al 55% utilizando los hongos F1, F2, F5 y F6 durante 10 días sin esterilidad. En la fase II, los mejores porcentajes de decoloración se encontraron para ET en C_i1 y para CIC en C_i2, con F1 y F5 (identificados por biología molecular). Los resultados mostraron que Ci1 (SAN) aumentó la biodegradación de ET y C_i2 (SAN) favoreció la biodegradación de CIC, en T1 (LN) sin esterilidad. La mejor actividad enzimática de lacasa y lignina peroxidasa se presentó en F5. Los extractos enzimáticos presentaron un comportamiento cinético de Michaelis-Menten. Todas las muestras de ET biorremediadas en la fase II, no mostraron toxicidad sobre *Artemia* sp. en 48 horas de experimentación.
Palabras clave: Estrés nutricional; Tiempo de crecimiento inicial; Enzimas ligninolíticas; Sin esterilidad; Ecotoxicidad.

1. Introduction

The contamination of water bodies by the textile industry is a current problem in many parts of Brazil, such as Ipojuca and Capibaribe rivers in Pernambuco region. The fabric manufacture generates a great amount of textile effluent (TE) in the cleaning, dyeing and finishing processes, with the use of approximately 18 liters of water/ piece of clothing in addition to the use of 20 tons of dyes per year. Twenty percent of this dyes does not bond to fibers being discarded in treatment stations (Honorato el al., 2015) or in water bodies, generating a serious environmental imbalance due to its high concentration and the toxicity of each type of dye (Bedoui et al., 2015). In the "Agreste" zone of Pernambuco (a tiny region located between coastal forest area and the desertic semi-arid area from Northeast Brazil), the pieces are dyed in municipalities where there is a shortage of water. The treatment station of this TE must be efficient to increase the percentages of water reuse (Oliveira et al., 2016). For industries, the remedying process these TE require much effort and a significant cost of investment, as a result of which methodologies are needed that lower costs and they are environmentally friendly (Oliveira et al., 2016).

The indigo dye and indigo carmine dye (ICD) (Figure 1) or also known as Acid Blue 74, is widely used in the textile industry for dyeing jeans. It has highly toxic on contact with the skin or eyes, it can cause irritations even causing permanent damage to the cornea and conjunctiva. If ingested over the long term it can cause reproductive toxicity, poor anatomical development and neuronal problems (Naciri et al., 2016).

Figure 1. Molecular structure of indigo dye and indigo carmine dye.



Source: Autors.

Once time TE are discharged into water bodies without prior treatment, they cause metabolic disturbances in organisms, interfering with the life cycles of the ecosystem, and also reduce the penetration of light, which prevents the photosynthesis of organisms, and consequently the trophic chain is altered, causing the elimination of some living beings (Raman & Kanmani, 2016; Chhabra et al., 2015; Mehra et al., 2021).

In mycotic bioremediation, white-rot fungi are quite recognized for degrading cellulose and lignin, and they have been used in biodegradation processes of different compounds such as carbohydrates, aromatic compounds, hydrocarbons, synthetic dyes (Alao & Adebayo, 2022; Yesilada et al., 2018; Zhang et al., 2016).

Agaricomycetes species such as *Coriolus versicolor*, *Ganoderma lucidum*, *Ganoderma applanatum*, *Schizophyllum commune*, *Pycnoporus sanguineus*, *Pleurotus ostreatus*, *Grifola frondosa*, *Auricularia delicata*, *Trametes trogii*, *Phanerochaete chrysosporium* and *Lentinula edodes* are recognized fungi for producing ligninolytic enzymes (Shertate & Thorat, 2014).

The biological treatment of TE from jeans industrial laundries is a biotechnological treatment that besides being low cost (Bilal et al., 2017), presents promising results to discolor and remedy TE conformed by xenobiotics, through the enzymes activity such as laccase, manganese peroxidase and lignin peroxidase, which degrade them until they form water and carbon dioxide, making them harmless not only for living organisms but also avoiding water bodies contamination (Kumar & Chandra, 2020).

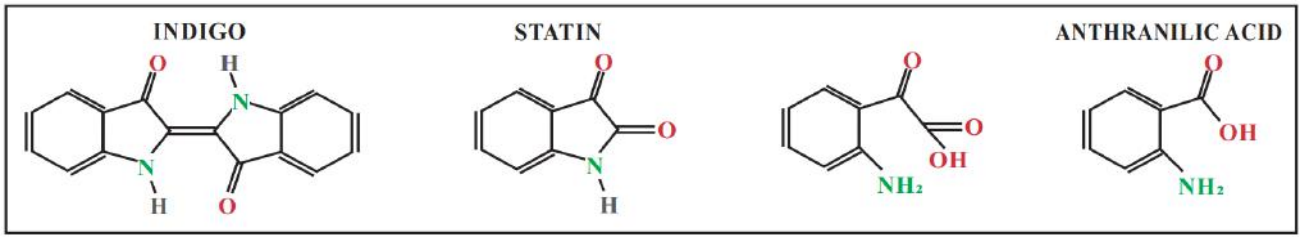
The lignin degradation occurs because of multi-enzymatic process resulting from the coordinated action of a series of intra and extracellular enzymes such as oxidoreductases, as well as other oxidases producing hydrogen peroxide and by intermediate metabolites of low molecular mass (Mattila et al., 2022; Bernal et al., 2021; Sakai et al., 2022).

The ligninolytic enzymes production is stimulated in fungi by means of the nitrogen limitation (NL) and the without addition of nutrients (WAN) in the culture medium for a long time (Ozcirak & Ozturk, 2017). The fungi are known to be resistant in difficult conditions of survival, such as those found in the Caatinga of Pernambuco, a place between the agrestal and the hinterland, where the environment is dry, but the fungi have been able to develop without problems. The ease with which these organisms can adapt in extreme environments allow them to survive in unfavorable conditions (Saroj et al., 2015).

As time goes by, the fungi exhaust the nutrients present in the culture medium, synthesizing different amounts of enzymes in different stages of life. First, they synthesize intermediate enzymes that degrade other recalcitrant xenobiotic compounds, despite their production decreases with the nutritional consumption of the medium. On the other hand, the NL

stimulates the ligninolytic enzymes production (Buswell et al., 1995), which degrade chromophore groups of dyes such as indigo dye and ICD (Fig. 1). These enzymes transfer electrons to the dye to obtain statin, and after decarboxylation occurs the anthranilic acid is produced, as shown in Figure 2. The anthranilic acid is an amino acid also known as vitamin L1 for its performance as coenzyme in the production of breast milk (Rio et al., 2015).

Figure 2. Mechanism of indigo dye degradation by *Phormidium* sp.



Source: Campos et al. (2001) - modified.

Ligninolytic enzymes could biodegrade chromophore dye compounds in sterile and non-sterile environments. The non-sterile condition is very important because of the origin of the TE (Osorio et al., 2011). A real TE without sterilization, has its own microbiota that is already adapted to these conditions, and it can present a greater degree of dyes biodegradation with influence of the ligninolytic enzymatic fungi activity, than a real sterile TE or a synthetic effluent prepared in a laboratory (Bergsten-Torralba et al., 2018).

When there is influence of a microbiota composed of fungi and bacteria, there can be an increase in the pH altering the activity of the fungi (Osorio et al., 2011), but it has also happened that a microbiota composed can be greatly favored by the presence of fungi in the discoloration of non-sterile TE, without the need to regulate the pH to acid (He et al., 2018), which reduces the process cost, because it avoids the use of chemical reagents to regulate the pH. Apart from the chromophore groups biodegradation, decrease in the toxicity of that TE has also been found after biological treatment with fungi, when the microbiota of the non-sterile TE is conserved (He et al., 2018).

The enzyme kinetics serves to determine the velocity that the enzyme is transforming a certain amount of substrate into a product, so important parameters such as the Michaelis constant (K_M) and the maximum reaction velocity (V_{MAX}) can be found. The K_M indicates the substrate concentration for which the reaction velocity is half the V_{MAX}. Therefore, the K_M allows us to find the enzyme affinity for your substrate. The laccase without purification of the fungus *Scytalidium thermophilum* presented a K_M of 2610 µM for ICD and a V_{MAX} of 613 µM/min and purified was 7440 µM and 1270 µM/min of K_M and V_{MAX} respectively (Ben Younes & Sayadi, 2013). Completely different values of parameters kinetics were determined by the purified laccases and mixed of *Trametes hirsuta* and *Sclerotium rolfsii* fungi, K_M of 9.6 µM and V_{MAX} of 96400 µM/min (Singh et al., 2007). The kinetic parameters values of laccase are very variable in ligninolytic fungi, so an approximate average of KM (500 µM) among the references is an interesting theoretical value to evaluate. This value was tested by Singh et al. (2019) using natural redox mediators such as acetosyringone and syringaldehyde, and synthetic ones such as HBT (1-hydroxylbenzotrizole) and ABTS (2,2'-azine-bis(3-ethylbenzothiazoline-6-sulphonic acid)).

After the bioremediation performed with free cells, it is of great importance to perform bioassays to confirm a possible toxicity that can be generated in an ecosystem (Dvorak et al., 2012). The evaluation is performed by the analysis of mortality, immobility, behavioral alteration, among others. This allows observing if the biologically treated material generates environmental impacts (Bergami et al., 2016). *Artemia* sp. nauplii has been used as a model in preliminary bioassays of new products since 1982 (Velayudhannair et al., 2017; Dvorak et al., 2012), and it is recommended by Food and Agriculture Organization (FAO, 2022). It has also been used in several drug toxicity tests (Epole et al., 2020), water contaminants such as

hydrocarbons (Rojo et al., 2012), cleaning products (Bartolomé-Camacho, 2007), some compounds and chemical elements (Dvorak et al., 2012; Koutsaftis & Aoyama, 2008), and polymers (Barrera et al., 2018). This microcrustacean, when newly born, is called nauplii, and at this stage of life, it is fed by non-selective filtration of the environment where it is found, filtering large amounts of different materials such as recalcitrant xenobiotic compounds (Velasco et al., 2016; Dvorak et al., 2012).

2. Methodology

2.1 Materials

Malt extract (Difco, Brazil), nutrient agar (Kasvi, Brazil), d-glucose (Neon, Brazil), peptone and bovine serum albumin (Sigma-Aldrich, Brazil), yeast extract (Biolife, Italy), monobasic potassium phosphate, manganese sulphate, indigo carmine dye, sodium acetate, sodium tartrate, hydrogen peroxide, succinic acid, sodium lactate and phenol red (Vetec, Brazil), 2,2'-azine-bis(3-ethylbenzothiazoline-6-sulfonic acid) – ABTS (Sigma-Aldrich, Brazil), Bradford reagent (Sigma, Brazil), veratryl alcohol (Aldrich, Brazil), sodium hydroxide (Cromato, Brazil), magnesium sulfate (Synth, Brazil), copper sulfate II (Alphatec, Brazil), absolute ethanol (Química Moderna, Brazil), Núcleo SAP (Cellco, Brazil), *Artemia* HIGH 5 cyst (Inve, Thailand), Staden Package 2.0 software (SourceForge, USA) and SPSS statistical software (IBM®, USA).

Liquid fermentation and discoloration were performed in the pre-selection phases I and II, in order to select the fungi with the highest percentage of discoloration.

2.2 Liquid Fermentation

In phase I of pre-selection, the 7 fungi were isolated and reactivated at 30 ºC in Petri dishes with malt culture medium (malt extract 20 g/L; glucose 20 g/L; peptone 1 g/L; agar 20 g/L; distilled water 1000 mL), this methodology was used by Gupta and Asim (2019) in solid fermentation to maintain fungal strains in the production of laccase, but in this research it was modified, so the culture medium was enriched with grass powder previously sterilized twice in an autoclave.

Later, the initial experimental growth was carried out on a Petri dishes with Sabouraud agar (40 g/L D-glucose; 10 g/L peptone; 20 g/L agar; 1000 mL of distilled water) for 20 days at the same temperature. Then, the fungi were subjected to nutritional stress by NL, according to the methodology (modified) used by Tien and Kirk (1988) in the production of lignin peroxidase by *Phanerochaete chrysosporium*. Agar discs of 6 mm diameter with the grown fungi were added to Kirk medium (yeast extract 0.1 g/L; KH_2PO_4 0.2 g/L; $MgSO_4$ 0.05 g/L; $MnSO_4$ 0.016 g/L; $CuSO_4$ 0.049 g/L; 1000 mL of distilled water), placing three discs for each fungus in 50 mL of non-sterilized medium, in 250 mL Erlenmeyer flasks. Three experimental times were used, T1, T2 and T3 of 1, 4 and 7 days respectively, at 30 ºC in the absence of light and with 150 rpm of constant agitation.

In phase II of the pre-selection, the initial growth time G_i2 of 45 days was used, in Sabouraud agar at 30 ºC and WAN. For the NL the same experimental parameters of T1 were maintained.

2.3 Discoloration

Initially, the wavelength for the ICD and for the TE was defined, through the construction of calibration curves with data obtained from a scan performed by UV-Vis spectrophotometry in the spectrophotometer (THERMO SCIENTIGIC - EVOLUTION 60). The analysis of ICD and TE color coordinates in the pigment color mode was also performed using the Spectra Lux Trial software (Santa-Cruz & Teles, 2003).

In the pre-selection phase I, after fermentation at T1, T2 and T3, the agar disks of each fungus were separated from the enzymatic extracts by vacuum filtration (qualitative filter paper), and three disks were placed in each Erlenmeyer flask (250 mL) with 50 ml of ICD 50 ppm (50mg/L). The concentration of the ICD used was modified from the methodology of Naciri et

al. (2016) which used 20 ppm of ICD. Three disks were also placed in 50 mL of industrial TE. The experiment took place for 10 days, maintaining the experimental conditions of temperature, light and agitation, without sterility. During this time, the discoloration was quantified by recording the absorbance by spectrophotometry every three days at 665nm, this wavelength was obtained according to the methodology (modified) of Ben Younes and Sayadi (2013), who performed a scan between the spectra of 200 and 800 nm, but in this research it was carried out between 200 and 1000 nm. At the end of phase I, four fungi were chosen for the best percentages of discoloration presented in the analyzed time. The discoloration percentages were calculated using equation (1), used by Mehandia et al. (2020):

$$Discoloration\ (\%) = \frac{Ai - Af}{Ai} x\ 100 \quad (1)$$

Where Ai is the initial absorbance of the ICD at its respective wavelength, and Af is the final absorbance of the ICD found in measurements every three days at the same wavelength.

In phase II of pre-selection, after 24 h (T1) fermentation, the discoloration methodology of phase I was replicated, but in an experimental time of 28 days for the four fungi chosen. At the end of phase II two fungi were chosen according to the percentages of discoloration presented in the analyzed time, and in dependence on the initial growth times (G_i1 and G_i2). The negative controls were the ICD 50 ppm or TE without fungus, and the initial controls were these fluids without fungus measured at the beginning and throughout of the test.

2.4 Identification of fungi by molecular biology

All fungi used in this test, were collected and morphologically identified between the years 2010 to 2014, by researchers from the Basidiomycota Laboratory of the "Universidade Federal de Pernambuco" (UFPE) - Brazil. In 2019, using an optical microscope (OPTON), it was observed the presence of clamp connections in the hyphae of the selected fungi (F1 and F5) in phase II of pre-selection by discoloration. The F1 and F5 fungal genera were identified by molecular biology. To continue with the next tests, a third strain was added as a model fungus (FM) for discoloration, *Phanerochaete chrysosporium*, which was also molecularly identified by the same methodology.

In the molecular genetic analyses, the fragments of the fungi basidiomata were ground with liquid nitrogen and the DNA was extracted using a method described in Góes-Neto et al. (2005). PCR amplification of the ITS and LSU regions were performed using the primer pairs ITS4-ITS5 and LR0R-LR5, respectively (White et al., 1990; Lima-Junior et al., 2014). The PCR products were purified using the enzyme Núcleo SAP CELLCO (500 preps) and followed the manufacturer's recommendations. The samples were sequenced at the "Plataforma Tecnológica de Genômica e Expressão Gênica do Centro de Biociências", UFPE, Brazil. The cycle sequencing was carried with the same primers of amplification reactions (Moncalvo et al., 2000). The electropherograms obtained were analyzed and edited in the Staden Package 2.0 software (Staden et al., 2000) and the sequences obtained were compared with those deposited in GenBank using the BLAST tool. Subsequently, the sequences were deposited on the GenBank platform.

2.5 Quantification of total proteins and ligninolytic enzymes activity

The enzymes present in the extracts obtained by the liquid fermentation of F1, F5 and FM fungi were concentrated by ethanolic precipitation of proteins according to the methodology (modified) of Costa et al. (2018). A volume of 70 mL of absolute ethanol previously refrigerated at 4 ºC was used, which was slowly added to 30 mL of the enzymatic extract under constant stirring. Then, the samples were refrigerated at 4 ºC for 15 minutes, and then centrifuged in a centrifuge (THERMO

SCIENTIGIC - JOUAN BR4i MULTIFUNCTION) at 4000 rpm for 10 min, maintaining the temperature at 4 ºC. Then, the supernatant was discarded, and the ethanol remaining in the pellet was evaporated inside the exhaust for 20 minutes at room temperature. Finally, the proteins were resuspended in 3 mL of 100 mM sodium acetate buffer at pH 4 (final precipitation ratio was 10:1 considering the initial volume of enzyme extract of 30 mL and the final volume of proteins resuspended in the buffer of 3 mL).

The determination of total protein concentration was performed in a 96-well Elisa plate (300 µL) in the UV-Vis Elisa Spectrophotometer (BIOTEK - EPOCH with GEN5 software). Based on the methodology by Bradford (1976), 50 µL of Bradford reagent, 175 µL of dissolved water and 25 µL of enzymatic extract were used for the reaction. For the control, 25 µL of 10 mM sodium acetate buffer at pH 4 were used for enzymatic extract replace. Reactions were measured in triplicate at 595 nm. The absorbances (Abs) for each fungus were determined by subtracting the value found in the control minus the absorbance values quantified in the samples. Thus, the total protein concentration was calculated based on equation (2) of the standard curve obtained with BSA ($R^2 = 0.98$):

$$y = 0.007x + 0.0412$$

$$x = \left(\frac{y - 0.0412}{0.007}\right)$$

$$Concentração\ de\ proteínas\left(\frac{\mu g}{mL}\right) = \left(\frac{Abs - 0.0412}{0.007}\right) \quad (2)$$

According to the methodology of Buswell et al. (1995), the laccase activity was determined at 420 nm at times 0 and 5 minutes, in an Elisa plate with 96 wells in triplicate. In the reaction were used 25 µL of sodium acetate buffer pH 5 100 mM, 200 µL of ABTS 0.03% (w/v) and 25 µL of enzymatic extract. The extinction coefficient for the oxidation of ABTS is ε_{420}=36000 $M^{-1}.cm^{-1}$ according to the authors. For the enzyme lignin peroxidase, the methodology of Buswell et al. (1995) was also used, the activity was determined at 310 nm at times 0 and 5 minutes. The reaction was carried out with 85.75 µL of sodium tartrate buffer pH 3 125 mM, 30.5 µl of veratryl alcohol 10 mM, 12.25 µL of hydrogen peroxide 10 mM and 122 µL of enzymatic extract. According to the authors, the extinction coefficient for the oxidation of veratryl alcohol by H_2O_2 is ε_{310}=9300 $M^{-1}.cm^{-1}$. The determination of the activity of manganese peroxidase was carried out with the methodology of Kuwahara et al. (1984), so the wavelength of 610 nm was used at times 0 and 5 minutes. In the reaction were used 12 µL of manganese sulfate 2 mM, 48 µl of bovine albumin 0.5% (w/v), 12 µL of hydrogen peroxide 2 mM prepared in sodium succinate 20 mM pH 4.5, 24 µl of sodium lactate 25 mM; 24 µL of phenol red 0.01% (w/v) and 120.25 µL of enzymatic extract. This reaction was incubated for 5 minutes at 30°C, immediately stopped with 9.5 µL of sodium hydroxide 2 M. The extinction coefficient for the oxidation of phenol red is ε_{610}=4460 $M^{-1}.cm^{-1}$ according to Lordêlo et al. (2014).

One unit (U) of enzymatic activity corresponds to the amount of enzyme (L) that oxidizes 1 mol of substrate per minute of reaction. The enzymatic activity of the three enzymes was determined according to equation (3) (modified) by Orozco and Quesada (1995):

$$\frac{U}{L_{min}} = \frac{\Delta Abs * V_T}{\varepsilon * d * \Delta t * V_m} \quad (3)$$

Where ΔAbs is the absorbance differential (Absf - Abso); VT (µL) is the total reaction volume; ε ($M^{-1}.cm^{-1}$) is the

extinction coefficient for the reaction catalysis; d (cm) is the distance that passes the light incidence radius through the sample; Δt (min) is the reaction time differential (tf - to); Vm (µL) is the sample volume in the reaction.

2.6 Enzymatic kinetics of extracts

After determining the concentration of the enzymes present in the extracts obtained from the fungi, the enzyme kinetics was calculated based on the research by Johnson and Goody (2011), thus considering the theoretical Michaelis constant K_M of laccase for ICD of 500 µM or 233 ppm with the ABTS mediator, a value determined in the research by Singh et al. (2019), but in this research the ABTS mediator was not used.

On the Elisa plate 50 µL of different concentrations of ICD as 50 ppm, 75 ppm, 117 ppm, 233 ppm, 350 ppm, 466 ppm were placed. Afterwards, the concentrated enzymes of each fungus were added in different volumes 10 µL, 25 µL, 50 µL and100 µL, measured in the spectrophotometer of Elisa at 665 nm for 4 hours, and project to 309 hours for curve tendence obtain.

The reaction velocity was obtained by the incline of the curve in the linear function (4), created between the absorbances measured in time:

$$y = ax + b \quad (4)$$

Where *y* is the absorbance measured in the spectrophotometer, *a* is the reaction velocity and *x* is the time those absorbances were measured.

Michaelis – Menten created an equation (5) (Johnson & Goody, 2011), that applies to enzyme-catalyzed chemical reactions with a single substrate:

$$V = \frac{V_{MAX} * [S]}{K_M + [S]} \quad (5)$$

The linearization of the Michaelis – Menten equation allows to know the actual value of the K_M of the enzymatic extract (enzyme complex), as a function of the laccase degradation activity on the ICD. The K_M value was calculated after linearization of equation (5) by the Lineweaver-Burk method, obtaining equation (6) (Johnson & Goody, 2011):

$$\frac{1}{V} = \frac{K_M}{V_{MAX}} * \frac{1}{[S]} + \frac{1}{V_{MAX}} \quad (6)$$

Where V is the reaction velocity at a specific ICD concentration, obtained with equation (4), V_{MAX} is the maximum reaction velocity and [S] is the ICD concentration (substrate).

Therefore, V_{MAX} is defined by the independent $1/V_{MAX}$ end and the actual K_M of the extract is defined by the dependent K_M/V_{MAX} end of equation (6).

2.7 Ecotoxicity analysis of the biologically treated samples by Agaricomycetes fungi use *Artemia* sp. (HIGH 5 - INVE)

The toxicity of the ICD 50 ppm and the TE biologically treated by the fungi, during phase II of pre-selection by decolorization, was determined using the methodology (modified) of Barrera et al. (2018), who used *Artemia franciscana* as a

biological model for the analysis of ecotoxicity. In this research, *Artemia* HIGH 5 was used to verify if the biotreated products are toxic in aquatic ecosystems. The cysts were placed for hatched in the Artemio JBL incubator. Was added 1 g of microcrustacean cysts in 500 mL of saline water 3% (w/v) pH 8.1, prepared with iodine-free coarse salt and deionized water, maintaining a constant temperature of 27 ºC, oxygenation constant with peristaltic bomb and 2000 lux of luminescence, monitored with a luxmeter (Smarter Luxmeter 1.0.1 version of Smart Tools®), for 48 h. After hatching, the nauplii (newly hatched brine shrimp) were placed in a 24-well microtiter plate, 5 nauplii per well, and 1 mL of the biotreated sample was added. As a positive control, was used the previously prepared saline water maintaining pH 8.1, the initial control was the freshly prepared ICD 50 ppm or TE, and the negative control was the ICD 50 ppm or TE that were used as negative controls in phase II pre-selection. According to Abessa et al. (2021), the survival of *Artemia* sp. nauplii was evaluated for 3 days, but in this research, it was evaluated for 5 days. The test was monitored by magnifying glasses to quantify living and dead nauplii.

2.8 Statistical analysis

The analysis below was carried out after phase II of the pre-selection of Agaricomycetes fungi by discoloration of ICD and TE.

Discoloration: ANOVA analysis of variance with factorial design 4^2 (16 treatments) was used to evaluate the combination of two types of levels: 4 species of Agaricomycetes (F1, F2, F5 and F6) versus 4 experimental conditions based on the discoloration rate measured in absorbance units, expressed in percentage, measured in wavelengths (Gutiérrez & Vara, 2012). The experimental conditions were:

1. Indigo carmine dye 50 ppm tested in fungi with initial growth time of 20 days (ICD - G_i1);
2. Textile effluent tested in fungi with initial growth time of 20 days (TE - G_i1);
3. Indigo carmine dye 50 ppm tested on fungi with an initial growth time of 45 days (ICD - G_i2);
4. Textile effluent tested on fungi with an initial growth time of 45 days (TE - G_i2).

The IBM® SPSS® statistical software was used. For this analysis considered a p_value of 0.05.

Survival of Artemia HIGH 5 (INVE): it was also used an analysis of variance ANOVA 4^2 adopting the same two types of levels indicated in the discoloration, to evaluate the survival (expressed in percentage) rate of *Artemia* sp. nauplii, during 48 hours of exposure in front of the biologically treated samples, using the IBM® SPSS® statistical software. A p_value of 0.05 was considered.

3. Results and Discussion

3.1 Phase I: Pre-selection that fungi by discoloration of ICD and TE, in T1, T2 and T3 fermentation times.

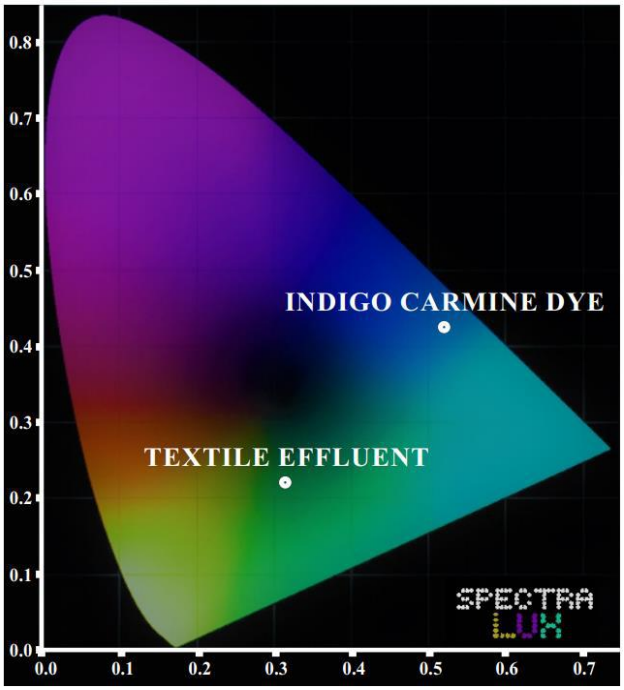
The ICD and TE sweeping showed that the maximum wavelength for ICD was 610 nm, and for the TE was 665 nm. This confirms that in the TE there are other dyes additionally ICD.

The pigmentation difference between ICD and TE was corroborated using the software Spectra Lux Trial, (Fig. 3), showing that there are more dyes besides ICD in TE, because the chromatic coordinates for the ICD were X = 0.517; Y = 0.423; Z = 0.059, and for the TE were X = 0.314; Y = 0.219; Z = 0.467. From this information, the discoloration experiments with fungus were analyzed in the wavelength found for the TE (665nm).

After phase I of the pre-selection by discoloration, the fungi that exceeded 55% of discoloration of TE were selected, in at least one of the three experimental times. In the T1 time, the percentages of TE discoloration were 68.07% for F5, 64.92% for F2, 58.25% for F1 and 47.02% for F6. The ICD percentage values of discoloration of the four fungi were 44.33%, 51.93%, 63.58% and 45.16% respectively, as shown in Table 1. In the choice of these fungi, exceptions were considered, because the degradation importance of the TE is more relevant than the degradation of the ICD for this research.

The nitrogen restriction to stimulate the production of ligninolytic enzymes, and the other microorganisms presence in the non-sterile medium, influenced the decrease of enzyme synthesis in longer stress times (T2 and T3), with the exception of the fungus F6 that reached 60.35% of TE discoloration at T3 (Osorio et al., 2011). Therefore, under these experimental conditions, the T1 experimental time was chosen to was used in the Pre-selection II phase.

Figure 3. Chromatic coordinates analysis of indigo carmine dye (ICD) and textile effluent (TE) in Spectra Lux Trial software.



Source: Autors.

Table 1. Percentage values achieved in the discoloration of ICD and TE during 10 days, after the fermentation at times T1, T2 and T3.

	TEXTILE EFFLUENT (TE)			INDIGO CARMINE DYE (ICD)		
FERMENTATION TIMES WITH NITROGEN LIMITATION (NL)	**FUNGUS**	**DISCOLORATION (%)**	**DAY ON WHICH MAXIMUM DISCOLORATION WAS ACHIEVED**	**FUNGUS**	**DISCOLORATION (%)**	**DAY ON WHICH MAXIMUM DISCOLORATION WAS ACHIEVED**
T1 (1 day)	F5	68,07	10	F7	70,34	10
	F2	64,92	10	F3	65,25	10
	F1	58,25	10	F1	63,58	10
	F4	51,58	10	F4	58,27	10
	F6	47,02	10	F2	51,93	10
	F7	44,91	10	F6	45,16	10
	F3	35,44	7	F5	44,33	10
T2 (4 days)	F5	63,16	4	F1	78,98	10
	F2	59,65	7	F4	65,14	10
	F6	58,25	7	F5	64,31	10
	F4	54,04	7	F6	63,97	10
	F1	45,61	7	F7	55,15	10
	F3	45,61	4	F2	47,45	10
	F7	38,60	7	F3	42,25	10
T3 (7 days)	F2	62,46	4	F6	96,15	10
	F5	60,35	4	F3	95,63	10
	F6	60,35	4	F7	95,53	10
	F1	56,14	4	F5	95,11	10
	F7	54,04	10	F4	95,11	10
	F4	51,58	10	F2	92,82	10
	F3	40,70	10	F1	92,51	10

Percentage values placed in descending order for each fermentation time T1, T2 and T3. Source: Authors.

3.2 Phase II: Pre-selection that fungi by discoloration of ICD and TE, in the initial growth times G_i1 and G_i2.

In the initial growth time G_i1 the discoloration percentages of the TE were 86.67% for F5, 84.91% for F1, 81.40% for F6 and 81.05% for F2, and for ICD 47.76%, 84.08%, 48.60% and 64.31% respectively. In G_i2, the percentages of discoloration for the TE were 56.14%, 63.16%, 68.42% and 71.93%, and in the discoloration of the ICD 96.12%, 93.05%, 73.83% and 75.12% respectively, values found in Table 2. The lower nutritional stress in G_i1, increased the biodegradative activity registered in the TE due to a higher production of intermediate enzymes that facilitated the degradation of xenobiotic components, however less ligninolytic enzymes were synthesized to degrade dyes, this behavior was evidenced in the ICD discoloration, except for the F1 fungus that reached a percentage value close to that registered in the degradation of the TE (Osorio et al., 2011).

Contrary, in G_i2 there was greater nutritional stress due to the prolonged time of initial growth and WAN, generating higher percentages of discoloration by ligninolytic action in the ICD, but in the TE the enzymes degraded the dyes and other few recalcitrant xenobiotic compounds present. The degradation by ligninolytic action occurs because of the production of secondary metabolites in fungi by the WAN (Moreira, 2006).

The initial growth time influenced the TE discoloration, showing that G_i2 is more efficient to biodegrade dyestuffs

and G_i1 is more efficient to biodegrade other recalcitrant compounds. The NL induces the excretion of ligninolytic enzymes that are not altered by the presence of these pollutants, therefore in prolonged growth periods and WAN (G_i2), ligninolytic enzymes are found in a higher proportion for long periods (45 days) (Henn et al., 2020).

In shorter periods of growth and WAN (G_i1, 20 days), besides the production of ligninolytic enzymes due to lack of nitrogen, the amount of carbon present stimulates the fungus in the production of intermediate enzymes that degrade other recalcitrant xenobiotic compounds, such as other oxidases producing hydrogen peroxide and intermediate metabolic compounds of low molecular mass (Kaushik & Malik, 2009; Janusz et al., 2017; Mattila et al., 2022; Kenkebashvili et al., 2012; Gupta & Asim, 2019). Therefore, a carbon source mixed with the NL accelerates the bioremediation of the TE (Gupta & Asim, 2019; Davila et al., 2020; Conceição et al., 2017). But it is necessary to realize tto evaluate toxicity of these waters to guarantee the security of the biodegraded residual by these enzymes (Henn et al., 2020).

The fungi selected for the following experimental phases were F1 and F5 for presenting the best discoloration percentages of the TE in the shortest time of experimentation (13 days), as shown in Table 2.

Table 2. Percentage values of discoloration in indigo carmine dye (ICD) and textile effluent (TE) after the experiment with the initial growth times G_i1 and G_i2.

TIME OF INITIAL GROWTH G_i1				
FUNGUS	**DISCOLORATION FOR IDC (%)**	**DAY ON WHICH MAXIMUM DISCOLORATION WAS ACHIEVED**	**DISCOLORATION FOR TE (%)**	**DAY ON WHICH MAXIMUM DISCOLORATION WAS ACHIEVED**
F1	**84.08**	25	**84.91**	13
F2	**64.31**	22	81.05	13
F5	47.76	16	**86.67**	13
F6	48.60	13	81.40	13
TIME OF INITIAL GROWTH G_i2				
FUNGUS	**DISCOLORATION FOR ICD (%)**	**DAY ON WHICH MAXIMUM DISCOLORATION WAS ACHIEVED**	**DISCOLORATION FOR TE (%)**	**DAY ON WHICH MAXIMUM DISCOLORATION WAS ACHIEVED**
F1	**93.05**	25	63.16	25
F2	75.12	28	**71.93**	25
F5	**96.12**	25	56.14	25
F6	73.83	28	**68.42**	25

The highest percentages found by each fungus at times G_i1 and G_i2 are shaded. Source: Autors.

Quanto ao planejamento fatorial estatístico experimental aplicado (4^2), a análise ANOVA indicou um valor de p_0,018, o que demonstra que há uma influência significativa das condições experimentais e do tipo de fungo Agaricomycete na descoloração do TE.

3.3 Identification of fungi by molecular biology

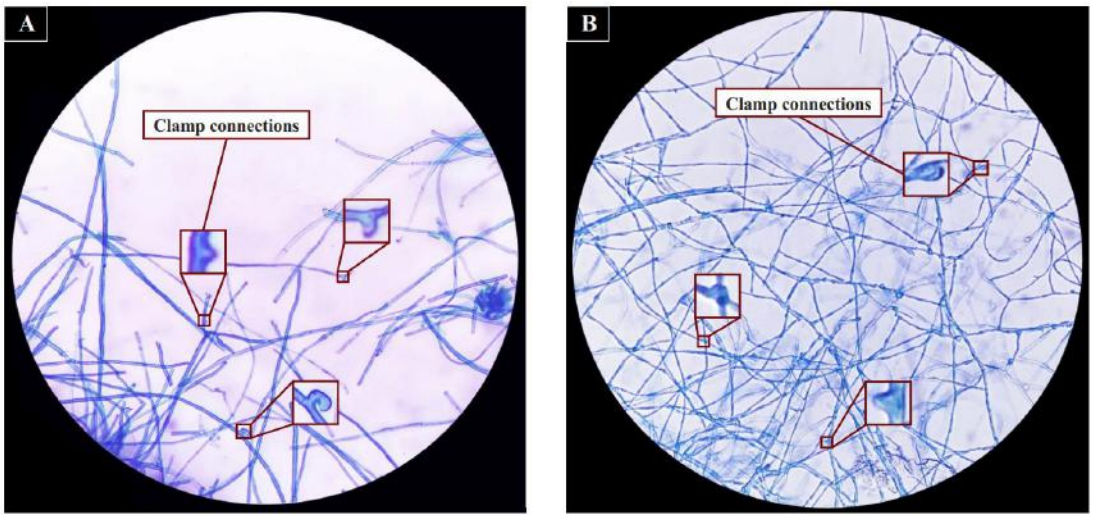
Initially, the fungi were morphologically identified under optical microscope to make sure that they belonged to

Basidiomycota phylum, thus corroborating the presence of clamp connections in the mycelium of fungi F1 (Fig. 4(A)) and F5 (Fig. 4(B)).

The genera of the selected fungi were identified with the sequences LSU and ITS in the blast. It was determined that the fungus F1 is *Phlebiopsis* sp. (ON847373, ON847375) by LSU = 99.32% and ITS = 97.57% similarity in the NCBI database. The fungus F5 is *Coriolopsis* sp. (ON847376) by ITS = 98.54% similarity, but it was not possible to generate the sequence of the LSU region for the specimen. The fungus FM (model fungus added for enzymatic analysis) is *Phanerochaete chrysosporium* (ON847372, ON847374) by LSU = 100% and ITS = 99.74% similarity.

These data are not conclusive, as they need to be confirmed by analyzing the phylogeny to conclude with the possible species of these fungi.

Figure 4. Observation of clamp connections on Agaricomycetes (A) F1 and (B) F5.



Source: Autors.

3.4 Quantification of total proteins and ligninolytic enzymes activity

The concentration of total proteins in F1 fungus extract was 1,924 µg/mL, in F5 it was 20,019 µg/mL, and for FM model fungus it was 15,162 µg/mL, as shown in Table 3. The enzymatic activity per minute of laccase reaction for F1 fungus was $1.481x10^{-8}$ U/L, for F5 fungus it was $5.674x10^{-6}$ U/L and for FM fungus it was $4.444x10^{-7}$ U/L. The lignin peroxidase activity per minute of reaction for F1 was $1.763x10^{-7}$ U/L, for F5 it was $2.233x10^{-7}$ U/L and for FM it was $2.115x10^{-7}$ U/L. The manganese peroxidase activity per minute of reaction for F1 was $-6.936x10^{-6}$ U/L, for F5 it was $-1.821x10^{-5}$ U/L and for FM it was $-1.410x10^{-5}$ U/L, as shown in Table 3. The laccase activity of *Grammothele subargentea* basidium was 170 U/L after 21 days of incubation in Czapek Dox medium, however the lignin peroxidase enzyme was not found in this work (Separrat et al., 2002). In the enzymatic extract of *P. tricholoma* the enzymatic activity of laccase was $2.65x10^{-2}$ U/L, after 6 days of incubation in minimal salt- and peptone medium (Camacho-Morales et al., 2017). In another study the lignin peroxidase and the laccase values of the fungus *Penicillium commune* reached 2.5 U/L and 1.93 U/L respectively after 5 days of incubation in Sabouraud medium (Baptista et al., 2012.

Table 3. Quantified values of total proteins and enzymatic activity in extracts of fungo FM, F1 and F5.

FUNGUS	CONCENTRATION OF TOTAL PROTEINS	LACCASE	LIGNIN PEROXIDASE	MANGANESE PEROXIDASE
FM	15,162 µg/mL	4,444 x 10^{-7} U/L	2,115 x 10^{-7} U/L	-1,410 x 10^{-6} U/L
F1	1,924 µg/mL	1,481 x 10^{-8} U/L	1,763 x 10^{-7} U/L	-6,936 x 10^{-5} U/L
F5	20,019 µg/mL	5,674 x 10^{-6} U/L	2,233 x 10^{-7} U/L	-1,821 x 10^{-5} U/L

Source: Autors.

The laccase and lignin peroxidase activity values of the enzymatic extracts in this research are low compared with the literature, but it is very important to consider the equations used in those researches to calculate those activities, since the values of the parameters affect registered values of the enzymatic activity. From all the works studied, it was chosen the equation that considered all the necessary parameters to calculate the enzymatic activity in any enzyme (Kuwahara et al., 1984). For the work developed it was necessary to make a single modification of the equation of Orozco and Quesada (1995), to obtain equation (3) and thus calculate the values found in Table 3.

Therefore, if there are parameters disregarded when calculating the enzymatic activity, the resulting values may vary widely. The discoloration percentages reached in 13 days (Table 2) by the fungi F1 and F5 in phase II of the pre-selection, are the indication that it has high discoloration capacities of the TE within the average time found in other researches, therefore, the values of enzymatic activity determined in this work, are corresponding to the percentages of discoloration reached.

As for the activity of manganese peroxidase, the values found were negative (Table 3), this demonstrates the absence of this enzyme in the enzymatic extract. The fungi need several metals such as copper, iron, manganese, molybdenum, zinc and nickel that are essential for their growth, and that also act as enzymatic co-factors inducing the ligninolytic activity (Baldrian et al., 2005), but manganese peroxidase is sensitive to these metals when they are in high amounts because they are toxic (Baldrian et al., 2005; Hatvani & Mécs, 2003), such as iron that inhibits the activity or production of this enzyme in *P. castanella* (Moreira, 2006).

3.5 Enzymatic kinetics of extracts

The enzymatic extracts of the three fungi that were tested in different volumes had behavior corresponding to the Michaelis-Menten kinetics (Fig. 5), this was evidenced when the data were linearized by Lineweaver-Burk, in order to find the kinetic parameters K_M and V_{MAX}. The recorded K_M values (Table 4) of the three fungi are in the order of magnitude of 233 ppm (500 µM K_M theoretical of the laccase) for the ICD.

Table 4. K_M and V_{MAX} values calculated at different enzymatic extract volumes in the three fungus FM, F1 and F5.

FUNGUS	10 µL ENZYMATIC EXTRACT		25 µL ENZYMATIC EXTRACT		50 µL ENZYMATIC EXTRACT		100 µL ENZYMATIC EXTRACT	
	V_{MAX} (ppm/h)	K_M (ppm)	V_{MAX} (ppm/h)	K_M (ppm)	V_{MAX} (ppm/h)	K_M (ppm)	V_{MAX} (ppm/h)	K_M (ppm)
FM	0,0039	117,330	0,0043	135,646	0,0020	58,994	0,0008	23,246
F1	0,0055	432,084	0,0058	266,585	0,0015	15,734	0,0021	68,725
F5	0,0075	577,900	0,0060	213,587	0,0021	37,261	0,0028	32,525

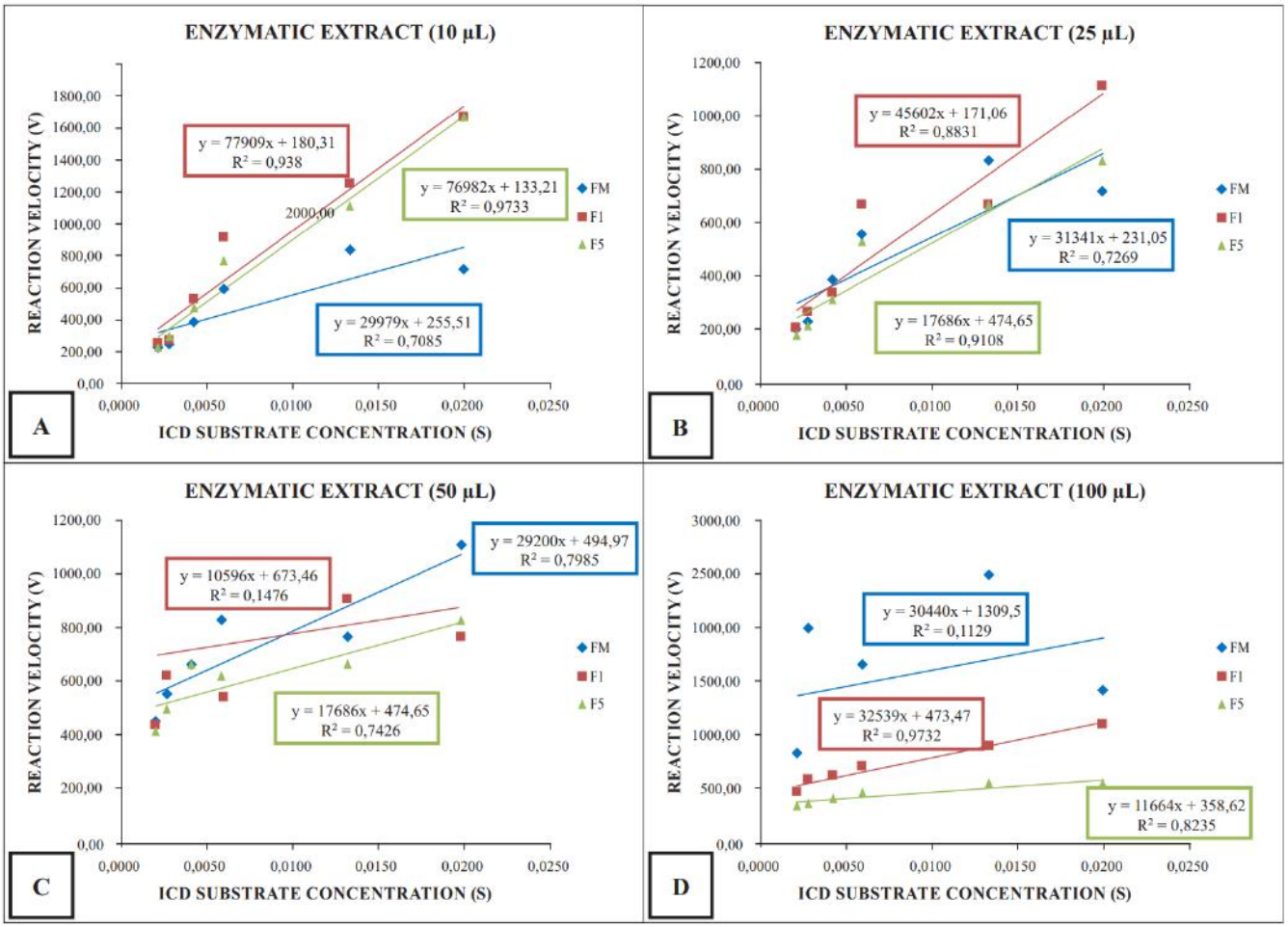
Source: Autors.

It can also be observed that in fungi FM and F5, the K_M decreased with increasing volume of extract, but fungus F1 registered the lowest K_M of the three fungi, this occurred with 50 µL of extract.

The values of V_{MAX} remained almost constant in the volumes of 10 and 25 µL of extract and decreased when was increasing the volume of extract placed in the ICD. This indicates that when the volume of the extract increased (increasing the volume of enzymes), the biodegradation rate of the ICD also increased.

Normally, the K_M should not vary much if it was a pure enzyme, but because it is an enzymatic complex, there was a synergism between the intra and extracellular enzymes present. This synergism allowed the potentiation of the complex (Mattila et al., 2022; Bernal et al., 2021; Sakai et al., 2022), obtaining an almost complete degradation of the ICD in the concentration of 50 ppm in 25 days of experimentation, above 90% in G_i2 and above 80% in G_i1 (Table 2).

Figure 5. Michaelis - Menten kinetic enzymatic behavior of the three fungi in different volumes of enzymatic extract (A) 10 µL, (B) 25 µL, (C) 50 µL and (D) 100 µL in front of different concentrations of ICD (substrate).



Source: Autors.

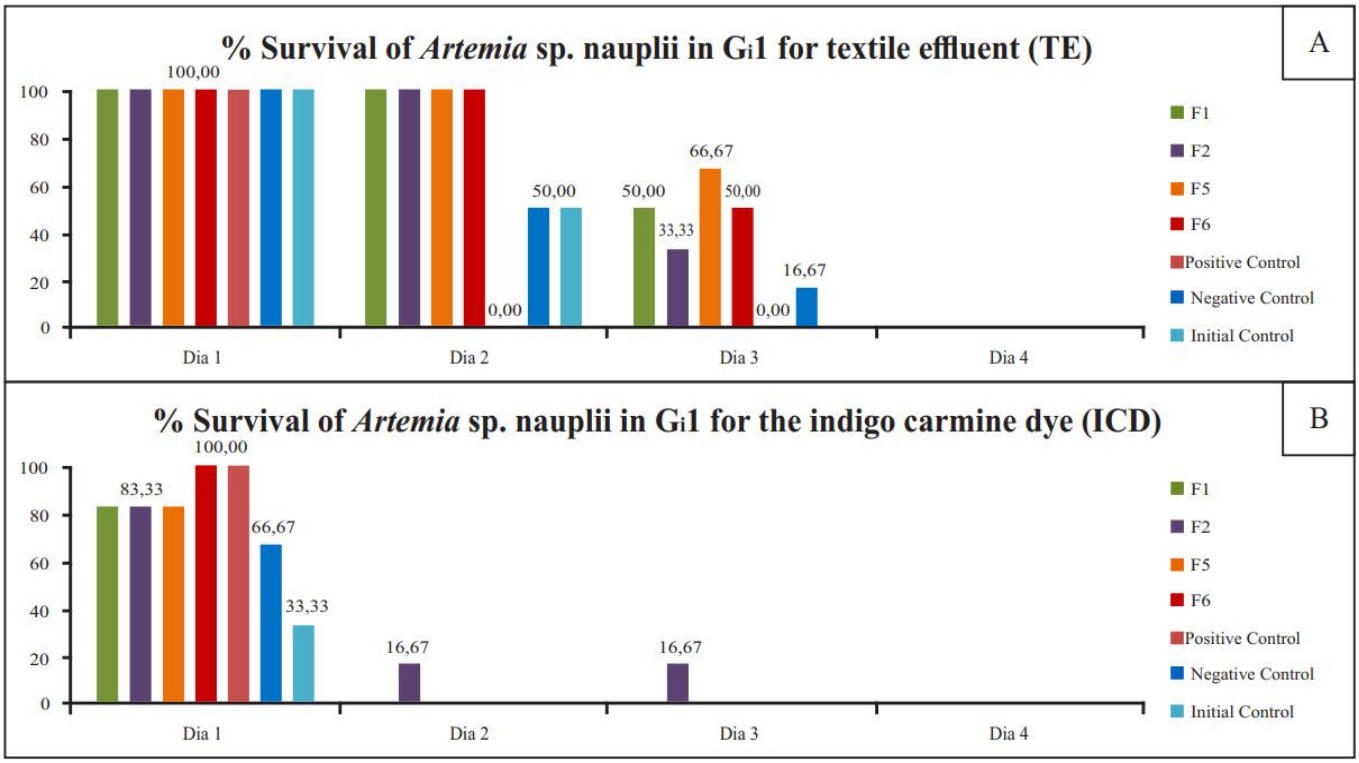
3.6 Ecotoxicity analysis of the biologically treated samples by Agaricomycetes fungi use *Artemia* sp. (HIGH 5 - INVE)

The survival percentages registered of *Artemia* HIGH 5 in direct contact with the TE and the ICD treated by the four fungi in G_i1 and G_i2, they can be observed in Figure 6. In Figure 6 (A) shows that on the first day the percentages reached were 100% for all samples, on the second day 100% was recorded for the TE samples treated by the 4 fungi in G_i1, and a decrease of 50% in live brine shrimp was observed in the initial control and in negative, and 0% in the positive control. On the third day 50% for F1, 33.33% for F2, 66.67% for F5 and 50% for F6 were registered, the negative control reached 16.67% and the initial control registered 0%. On day 4 all obtained 0% of survival.

Figure 6 (B) it is observed that in G_i1 the survival reached 100% after the first day of exposure of the brine shrimp

with the ICD treated by the fungus F6, and 83.33% for the ICD treated by F1, F2 and F3. For the positive control, the negative control and the initial control, the percentages recorded were 100%, 66.67% and 33.33% respectively. From the second and third day of exposure, only 16.67% of the ICD treated with F2 was recorded. On days 4 and 5 it was 0%.

Figure 6. % Survival of *Artemia* sp. nauplii in G_i1 (A) for the textile effluent (TE) and (B) for the indigo carmine dye (ICD).



Source: Autors.

In Figure 7 (A) on the first day 100% survivalship was recorded for all TE samples in G_i2, on the second day 100% was recorded for the samples treated by the 4 fungi and a decrease of 50% live artemias were observed in the negative control and initial control and 0% in the positive control. On the third day the samples treated by the F1, F2 and F5 fungi reached 50%, the fungus F6 reached 66.67% and the negative control was 16.67%, the other controls were quantified with 0%. On the fourth day only 16.67% of the TE treated by F1 was registered, and on the fifth day all the samples registered 0%.

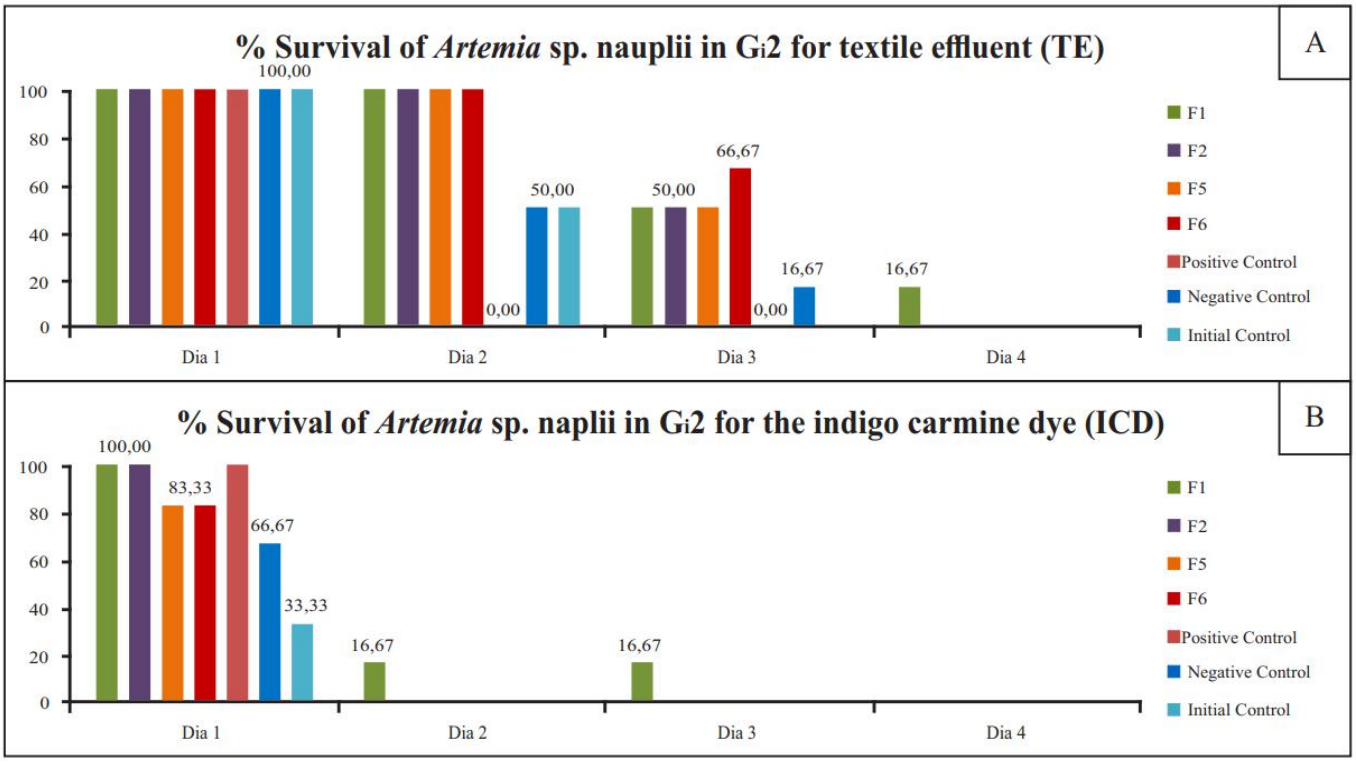
In Figure 7 (B) it can be seen that in G_i2 the survival after the first day was 100% in the ICD samples treated by the fungi F1, F2 and in the positive control. The artemias survival placed in the samples treated by fungi F5 and F6 were 83.33%, for the negative control it was 66.67% and for the initial control it was 33.33%. On the second and third day 16.67% of survival was registered only for the ICD treated by F1 fungus. On days 4 and 5 it was 0%.

According to the statistical factorial experimental design applied (4^2), to evaluate the combination toxicity of the sort of levels proposed, indicated that the samples treated biologically by the agaricomycetes fungi submitted to different initial growth times are not toxic. This result can be corroborated by the p_value of $0.0174x10^{-3}$ found in the ANOVA analysis.

As it was observed in Figures 6 and 7, the samples of the TE treated by the fungi F1, F2, F5 and F6, the *Artemia* sp. nauplii had survival until the third day, and fourth day in case of F1 fungus. In the ICD samples, microcrustaceans survived until the third day in cases of F1 and F2, hence, the pollutants degradation performed by the enzymes of the fungi generated short chains of carbon that was used by the brine shrimp to feed. The survival was not the same in the positive control (brine shrimp in saline solution without food), where 0% survival was evidenced after 24 hours of experimentation (Velasco et al.,

2016).

Figure 7. % Survival of *Artemia* sp. nauplii in G_i2 (A) for the textile effluent (TE) and (B) for the indigo carmine dye (ICD).



Source: Autors.

4. Conclusion

The study regarding the ligninolytic enzymes activity extracted from Agaricomycetes fungi from Caatinga, Pernambuco, demonstrated that these organisms have the capacity to bioremediate the textile effluent from the industrial laundry of Caruaru Municipality in conditions of non-sterility.

The initial growth time without addition of nutrients, and the time that the fungi were submitted to the nitrogen limitation, influence the bioremediation of the effluents, be it composed only the indigo carmine dye or the industrial textile effluent. The textile effluent remedied by the fungi *Phlebiopsis* sp. (Fungus 1 - F1) and *Coriolopsis* sp. (Fungus 5 - F5), proved not to be toxic on *Artemia* sp. nauplii (HIGH 5 - INVE).

It would be interesting to carry out the experiments under sterile conditions and compare them with the results of this research, this would allow us to rethink the advantages and/or disadvantages of eliminating the microorganisms found in the environment when carrying out tests in the laboratory.

It would also be necessary to carry out a phylogenetic analysis of the fungi identified at the genus level by molecular biology in this research, in order to identify the possible species involved in the biodegradation of the indigo carmine dye and industrial textile effluent.

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References

Abessa, D., Ortega, A., Pustiglione Marinsek, G., Roselli, L., Chelotti, L., & Perina, F. (2021). Acute Toxicity of Cigarette Butts Leachate on Nauplii of Artemia sp. Toxicidade aguda do lixiviado de bitucas de cigarro sobre Náuplios de Artemia sp. *Brazilian Journal of Animal and Environmental Research, 4*(1), 659-670. https://doi.org/10.34188/bjaerv4n1-055

Alao, M. B., & Adebayo, E. A. (2022). Fungi as veritable tool in bioremediation of polycyclic aromatic hydrocarbons-polluted wastewater. *Journal of Basic Microbiology, 62*(3-4), 223-244. https://doi.org/10.1002/jobm.202100376

Baldrian. P., Valášková, V., Merhautová, V., & Gabriel, J. (2005). Degradation of lignocellulose by Pleurotus ostreatus in the presence of copper, manganese, lead and zinc. *Research in Microbiology, 156*(5-6), 670-676. https://doi.org/10.1016/j.resmic.2005.03.007

Baptista, N. M. Q., Santos, A. C., Arruda, F. V. F., & Gusmão, N. B. (2012). Production of Enzymes by Lignin Peroxidase and Laccase Filamentous Fungi. *Scientia Plena, 8*(1). https://www.scientiaplena.org.br/sp/article/view/527/428

Barrera, N., Guerrero, L., Debut, A., & Santa-Cruz, P. (2018). Printable nanocomposites of polymers and silver nanoparticles for antibacterial devices produced by DoD technology. *PLOS ONE, 13*(7), e0200918. https://doi.org/10.1371/journal.pone.0200918

Bartolomé-Camacho, M. C. (2007). Valoración de la toxicidad aguda de biocidas utilizados en ambientes de la vida privada y la salud pública sobre Artemia franciscana. *Revista Latinoamericana De Recursos Naturales, 3*(1), 90-97. http://revista.itson.edu.mx/index.php/rlrn/article/view/94

Bedoui, A., Tigini, V., Ghedira, K., Varese, G. C., & Ghedira, L. C. (2015). Evaluación de una eventual ecotoxicidad inducida por efluentes textiles utilizando una batería de biotests. *Environmental Science and Polluttion Research, 22*(21), 16700-16708. https://doi.org/10.1007/s11356-015-4862-3

Ben Younes, S., & Sayadi, S. (2013). Detoxification of Indigo carmine using a combined treatment via a novel trimeric thermostable laccase and microbial consortium. *Journal of Molecular Catalysis B: Enzymatic, 87*, 62-68. https://doi.org/10.1016/j.molcatb.2012.10.007

Bergami, E., Bocci, E., Vannuccini, M. L., Monopoli, M., Salvati, A., Dawson, K. A., & Corsi, I. (2016). Nano-sized polystyrene affects feeding, behavior and physiology of brine shrimp Artemia franciscana larvae. *Ecotoxicology and Environmental Safety, 123*, 18-25. https://doi.org/10.1016/j.ecoenv.2015.09.021

Bergsten-Torralba. L. R., Giese, E., Buss, D., & Silva, M. (2018). Descoloração de efluente têxtil por fungo em condições não estéreis. *XXII Congresso Brasileiro de Engenharia Química, Blucher Chemical Engineering Proceedings, 1*(5), 1598-1601. https://doi.org/10.5151/cobeq2018-PT.0425

Bernal, S. P. F., Lira, M. M. A., Jean-Baptiste, J., Garcia, P. E., Batista, E., Ottoni, J. R., & Passarini, M. R. Z. (2021). Biotechnological potential of microorganisms from textile effluent: isolation, enzymatic activity and dye discoloration. *Annals of the Brazilian Academy of Sciences, 93*(4), e20191581. https://doi.org/10.1590/0001-3765202120191581

Bilal, M., Asgher, M., Parra-Saldivar, R., Hu, H., Wang, W., Zhang, X., & Iqbal, H. M. N. (2017). Immobilized ligninolytic enzymes: an innovative and environmental responsive technology to tackle dye-based industrial pollutants – A review. *Science of the Total Environment, 576*, 646–659. https://doi.org/10.1016/j.scitotenv.2016.10.137

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*(1-2), 248-254. https://doi.org/10.1006/abio.1976.9999

Buswell, J. A., Cai, Y., & Chang, S. T. (1995). Effect of nutrient nitrogen and manganese on manganese peroxidase and laccase production by Lentinula (Lentinus) edodes. *FEMS Microbiology Letters, 128*(1), 81-87. https://doi.org/10.1111/j.1574-6968.1995.tb07504.x

Camacho-Morales, R. L., Gerardo-Gerardo, J. L., Guillén, N. K., & Sánchez, J. E. (2017). Producción de enzimas ligninolíticas durante la degradación del herbicida paraquat por hongos de la pudrición blanca. *Revista Argentina de Microbiología, 49*(2), 189–196. https://doi.org/10.1016/j.ram.2016.11.004

Campos, R., Kandelbauer, A., Robra, K.H.A., Cavaco-Paulo, A., & Gübitz, G.M. (2001). Indigo degradation with purified laccases from Trametes hirsuta and Sclerotium rolfsii. *Journal of Biotechnology, 89*(1-2), 131–139. https://doi.org/10.1016/S0168-1656(01)00303-0

Chhabra, M., Mishra, S., & Sreekrishnan, T. R. (2015). Combination of chemical and enzymatic treatment for eficiente decolorization/degradation of textile effluent: High operational stability of the continuous process. *Biochemical Engineering Journal, 93*, 17–24. https://doi.org/10.1016/j.bej.2014.09.007

Conceição, T., Koblitz, M., Kamida, H., & Góes-Neto, A. (2017). Study of the Production of Lentinus crinitus (L.) Fr. Lignolytic Enzymes Grown on Agro-Industrial Waste. *Advances in Bioscience and Biotechnology, 8*(8), 259-272. https://doi.org/10.4236/abb.2017.88019

Costa, M. A. L., Farinas, C. S., & Miranda, E. A. (2018). Ethanol precipitation as a downstream processing step for concentration of xylanases produced by submerged and solid-state fermentation. *Brazilian Journal of Chemical Engineering, 35*(2), 477–488. https://doi.org/10.1590/0104-6632.20180352s20160502

Dávila, L., Zambrano, C., Arango, O., Betancur, J., & Arango, W. (2020). Integral use of rice husks for bioconversion with white-rot fungi. *Biomass Conversion and Biorefinery*. https://doi.org/10.1007/s13399-020-00940-6

Dvorak, P., Benova, K., & Vitek, J. (2012). Alternative Biotest on Artemia franciscana. In (Ed.), *Ecotoxicology*. IntechOpen, (pp. 51–74). Ghousia Begum. https://doi.org/10.5772/29114

Epole, N., Domínguez-Martín, E. M., Amilcar, R., Tavares, J., Vera, I., Pereira, P., Cebola, M-J., & Rijo, P. (2020). Artemia species: An Important Tool to Screen General Toxicity Samples. *Current Pharmaceutical Design, 26*(24), 2892-2908. https://doi.org/10.2174/1381612826666200406083035

Food and Agriculture Organization. FAO (2022). *Artemia spp. Cultured Aquatic Species Information Programme*. Text by Van Stappen, G.. Fisheries and Aquaculture Division [online]. Rome. Updated 2012-02-28 [Cited Thursday, June 23rd 2022]. https://www.fao.org/fishery/en/culturedspecies/Artemia_spp/en

Góes-Neto, A., Loguercio-Leite, C., & Guerrero, R. T. (2005). DNA Extraction from frozen field-collected and dehydrated herbarium fungal basidiomata: performance of SDS and CTAB-based methods. *Biotemas, 18*(2), 19-32. https://doi.org/10.5007/%25x

Gupta, A., & Asim, J. (2019). Production of laccase by repeated batch semi-solid fermentation using wheat straw as substrate and support for fungal growth. *Bioprocess and Biosystems Engineering, 42*, 499-512.. https://doi.org/10.1007/s00449-018-2053-6

Gutiérrez Pulido, H., & de la Vara Salazar, R. (2012). *Análisis y Diseño De Experimentos* (3ª ed). Mc Graw Hill.

Hatvani, N., & Mécs, I. (2003). Effects of certain heavy metals on the growth, dye decolorization, and enzyme activity of Lentinula edodes. *Ecotoxicology and Environmental Safety, 55*(2), 199-203. https://doi.org/10.1016/S0147-6513(02)00133-1

He, X. L., Song, C., Li, Y. Y., Wang, N., Xu, I., Han, E., & Wei, D. S. (2018). Efficient degradation of Azo dyes by a newly isolated fungos Trichoderma Tomentosum under non-sterile conditions. *Ecotoxicology and Environmental Safety, 150*, 232 – 239. https://doi.org/10.1016/j.ecoenv.2017.12.043

Henn, C., Monteiro, D. A., Boscolo, M., da Silva, R., & Gomes, E. (2020). Biodegradation of atrazine and ligninolytic enzyme production by basidiomycete strains. *BMC Microbiology, 20*(266). https://doi.org/10.1186/s12866-020-01950-0

Honorato, A. C., Machado, J. M., Celante, G., Borges, W. G. P., Dragunski, D.C., & Caetano, J. (2015). Biosorption of methylene blue using agro-industrial residues. *Revista Brasileira de Engenharia Agrícola e Ambiental, 19*(7), 705-710. https://doi.org/10.1590/1807-1929/agriambi.v19n7p705-710

Janusz, G., Pawlik, A., Sulej, J., Świderska-Burek, U., Jarosz-Wilkołazka, A., & Paszczyński, A. (2017). Lignin degradation: microorganisms, enzymes involved, genomes analysis and evolution. *FEMS Microbiology Reviews, 41*(6), 941–962. https://doi.org/10.1093/femsre/fux049

Johnson, K. A., & Goody, R. S. (2011). The Original Michaelis Constant: Translation of the 1913 Michaelis–Menten Paper. *Biochemistry, 50*(39), 8264–8269. https://doi.org/10.1021/bi201284u

Kaushik, P., & Malik, A. (2009). Fungal dye decolourization: Recent advances and future potential. *Environment International, 35*(1), 127–141. https://doi.org/10.1016/j.envint.2008.05.010

Kenkebashvili, N., Elisashvili, V., & Wasser, S.P. (2012). Effect of carbon, nitrogen sources and copper concentration on the ligninolytic enzyme production by Coriolopsis gallica. *Journal of Waste Conversion Bioproducts and Biotechnology, 1*(2), 22–27. https://doi.org/10.5147/jpgs.2012.0100

Koutsaftis, A., & Aoyama, I. (2008). Toxicity of Diuron and copper pyrithione on the brine shrimp, Artemia franciscana: The effects of temperature and salinity. *Journal of Environmental Science and Health, Part A, 43*(14), 1581–1585. https://doi.org/10.1080/10934520802329794

Kumar, A., & Chandra, R. (2020). Ligninolytic enzymes and its mechanisms for degradation of lignocellulosic waste in environment. *Heliyon, 6*(2), e03170. https://doi.org/10.1016/j.heliyon.2020.e03170

Kuwahara, M., Glenn, J. K., Morgan, M. A., & Gold, M. H. (1984). Separation and characterization of two extracellular H2O2 dependent oxidases from ligninolytic cultures of Phanerochaete chrysosporium. *FEBS Letters, 169*(2), 247-250. https://doi.org/10.1016/0014-5793(84)80327-0

Lima-Júnior, N., Gibertoni, T. B., & Malosso, E. (2014). Delimitation of some neotropical laccate Ganoderma (Ganodermataceae): molecular phylogeny and morphology. *Revista de Biología Tropical 62*(3), 1197–1208. https://www.redalyc.org/articulo.oa?id=44932441028

Lordêlo, C. S. M., Brito, S. V., Silva, S. V., Mitoshi, K. H., De Vasconcellos-Neto, T. J. R., Góes-Neto, A., & Bellos, K. M. G. (2014). Production of Manganese Peroxidase by Trametes villosa on Unexpensive Substrate and Its Application in the Removal of Lignin from Agricultural Wastes. *Advances in Bioscience and Biotechnology, 5*(14), 1067-1077. https://doi.org/10.4236/abb.2014.514122

Mattila, H., Österman-Udd, J., Mali, T., & Lundell, T. (2022). Basidiomycota Fungi and ROS: Genomic Perspective on Key Enzymes Involved in Generation and Mitigation of Reactive Oxygen Species. *Frontiers in Fungal Biology, 3*, e837605. https://doi.org/10.3389/ffunb.2022.837605

Mehandia, S., Sharma, S. C.,, & Arya, S. K. (2020). Isolation and characterization of an alkali and thermostable laccase from a novel Alcaligenes faecalis and its application in decolorization of synthetic dyes. *Biotechnology Reports, 25*, e00413. https://doi.org/10.1016/j.btre.2019.e00413

Mehra, S., Singh, M., & Chadha, P. (2021). Adverse Impact of Textile Dyes on the Aquatic Environment as well as on Human Beings. *Toxicology International, 28*(2), 165-176. https://doi.org/10.18311/ti/2021/v28i2/26798

Moncalvo, J. M., Lutzoni, F. M., Rehner, A. S., Johnson, J., & Vilgalys, R. (2000). Phylogenetic relationships of agaric fungi based on nuclear large subunit ribosomal DNA sequences. *Systematic Biology 49*(2), 278–305. https://doi.org/10.1093/sysbio/49.2.278

Moreira, N. S. L. (2006). *Enzimas ligninolíticas produzidas por Psilocybe castanella CCB444 em solo contaminado com hexaclorobenzeno – São Paulo*. [Dissertação de Mestrado, Instituto de Botânica da Secretaria de Estado do Meio Ambiente], Governo do Estado de São Paulo, Infraestrutura e Meio Ambiente, Memórias do Instituto de Botânica. https://www.infraestruturameioambiente.sp.gov.br/institutodebotanica/2006/01/enzimas-ligninoliticas-produzidas-por-psilocybe-castanella-ccb444-em-solo-contaminado-com-hexaclorobenzeno-sao-paulo/

Naciri, N., Farahi, A., Rafqah, S., Nasrellah, H., El Mhammedi, M. A., Lançar, I., & Bakasse, M. (2016). Effective photocatalytic decolorization of indigo carmine dye in Moroccan natural phosphate-TiO2 aqueous suspensions. *Optical Materials, 52*, 38-43. https://doi.org/10.1016/j.optmat.2015.12.011

Oliveira, G. A. R., Lapuente, J., Teixido, C. P. E., Borras, M., & Oliveira, D. P. (2016). Textile dyes induce toxicity on zebrafish early life stages. *Environmental Toxicology and Chemistry, 35*(2), 429–434. https://doi.org/10.1002/etc.3202

Orozco, K., & Quesada, S. (1995). Determinación de la actividad de la fructosa 1,6 difosfatasa en leucocitos. *Revista Médica del Hospital Nacional de Niños Dr. Carlos Sáenz Herrera, 30*(1-2), 9-18. http://www.scielo.sa.cr/scielo.php?script=sci_arttext&pid=S1017-85461995000100002&lng=en&nrm=iso

Osorio, J., Vidal, I., & Quintero, J. (2011). Decolorization of textile wastewater using the white rot fungi anamorph R1 of Bjerkandera sp. *Revista Facultad de Ingeniería Universidad de Antioquia*, *57*, 85-93. https://revistas.udea.edu.co/index.php/ingenieria/article/view/14647/12803

Ozcirak Ergun, S.,, & Ozturk Urek, R. (2017). Production of ligninolytic enzymes by solid state fermentation using Pleurotus ostreatus. *Annals of Agrarian Science*, *15*(2), 273–277. https://doi.org/10.1016/j.aasci.2017.04.003

Raman, D., & Kanmani S. (2016). Textile dye degradation using nano zero valent iron: A review. *Journal of Environmental Management*, *177*, 341-355. https://doi.org/10.1016/j.jenvman.2016.04.034

Rio, G. F., Silva, B. V., Martinez, S. T., & Pinto, A. C. (2015). Anthranilic acids from isatin: an efficient, versatile and environmentally friendly method. *Annals of the Brazilian Academy of Sciences*, *87*(3), 1525-1529. https://doi.org/10.1590/0001-3765201520140289

Rojo, N., Smith, K., Perales, J., & Mayer, P. (2012). Recreating the seawater mixture composition of HOCs in toxicity tests with Artemia franciscana by passive dosing. *Aquatic Toxicology*, *120-121*, 27-34. https://doi.org/10.1016/j.aquatox.2012.04.006

Sakai, R., Mendoza, D. M., Konadu, K. T., Cindy, Aoki, Y., Hirajima, T., Ichinose, H., & Sasaki, K. (2022). Laccase-mediator system for enzymatic degradation of carbonaceous matter in the sequential pretreatment of double refractory gold ore from Syama mine, Mali. *Hydrometallurgy*, *212*, e105894. https://doi.org/10.1016/j.hydromet.2022.105894

Santa-Cruz, P.A., & Teles, F.S. (2003). Spectra Lux Software v. 2.0 Beta. *Ponto Quântico Nanodispositivos*, *RENAMI*, *138*. https://sites.ufpe.br/vitrine/softwares/spectra-lux/

Saroj, S., Dubey, S., & Agarwal, P. (2015). Evaluation of the efficacy of a fungal consortium for degradation of azo dyes and simulated textile dye effluents. *Sustainable Water Resources Management*, *1*, 233–243. https://doi.org/10.1007/s40899-015-0027-2

Separrat, M. C. N., Martínez, M. J., Cabello, M. N., & Arambarri, A. M. (2002). Screening for ligninolytic enzymes in autochthonous fungal strains from Argentina isolated from different substrata. *Revista Iberoamericana de Micología*, *19*(3), 181-185. http://www.reviberoammicol.com/2002-19/181185.pdf

Shertate, R.S., & Thorat, P. (2014). Biotransformation of textile dyes: a bioremedial aspect of marine environment. *American Journal of Environmental Sciences*, *10*(5), 489-499. https://doi.org/10.3844/ajessp.2014.489.499

Singh, G., Capalash, N., Goel, R., & Sharma, P. (2007). A pH-stable laccase from alkali-tolerant γ-proteobacterium JB: Purification, characterization and indigo carmine degradation. *Enzyme and Microbial Technology*, *41*(6-7), 794–799. https://doi.org/10.1016/j.enzmictec.2007.07.001

Singh, G., Singh, S., Kaur, K., Kumar Arya, S., & Sharma, P. (2019). Thermo and halo tolerant laccase from Bacillus sp. SS4: Evaluation for its industrial usefulness. *The Journal of General and Applied Microbiology*, *65*(1), 26-33. https://doi.org/10.2323/jgam.2018.04.002

Staden, R., Beal, K. F., & Bonfield, J. K. (2000). The Staden Package, 1998. In: Misener, S., Krawetz, S.A. (Eds.), *Bioinformatics methods and protocols. Methods in Molecular Biology*, *132*, (pp. 115–130). Humana Press, Totowa, NJ.

Tien, M., & Kirk, T. K. (1988). Lignin peroxidase of Phanerochaete Chrysosporium. *Methods in Enzymology*, *161*, 238–249. https://doi.org/10.1016/0076-6879(88)61025-1

Velasco, S. J., Retana, O., Castro, M. J., Castro, M. G., Monroy, M., Ocampo, C., Cruz, C., & Becerril, C. (2016). Salinity effect on reproductive potential of four Artemia franciscana (Kellogg, 1906) Mexican populations grown in laboratory. *International Journal of Fisheries and Aquatic Studies*, *4*(3), 247-352. https://www.fisheriesjournal.com/archives/?year=2016&vol=4&issue=3&part=D&ArticleId=753

Velayudhannair, K., Divya, K. R., & Munuswamy, N. (2017). Quality evaluation of the invader species, Artemia franciscana from Covelong salt works, Kelambakkam, South India. *International Journal of Aquatic Biology*,*5*(4), 246-251. https://doi.org/10.22034/ijab.v5i4.316

White, T. J., Bruns, T., Lee S., & Taylor J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), *PCR protocols, a guide to methods and applications*, (pp. 315–322). Academic Press, New York. https://doi.org/10.1016/B978-0-12-372180-8.50042-1

Yesilada, O., Birhanli, E., & Geckil, H. (2018). Bioremediation and Decolorization of Textile Dyes by White Rot Fungi and Laccase Enzymes. In Prasad, R. (Eds.), *Mycoremediation and Environmental Sustainability*. (pp. 121–153). Fungal Biology. Springer, Cham. https://doi.org/10.1007/978-3-319-77386-5_5

Zhang, H., Zhang, S., He, F., Qin, X., Zhang, X., & Yang, Y. (2016). Characterization of a manganese peroxidase from white-rot fungus Trametes sp.48424 with strong ability of degrading different types of dyes and polycyclic aromatic hydrocarbons. *Journal of Hazardous Materials*, *320*, 265–277. https://doi.org/10.1016/j.jhazmat.2016.07.065