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
Mythic 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 G1 (10 days) and G2 (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 G1 and for ICD in G2, with F1 and F5 (identified by molecular biology). The results showed that G1 (WAN) increased the biodegradation of TE and G2 (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 microorganismos foram cultivados nos tempos de crescimento inicial, C1 (10 dias) e C2 (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 C1, e para CIC em C2, com F1 e F5 (identificados por biologia molecular). Os resultados mostraram C1 (SAN) aumentou a biodegradação do ET e C2 (SAN) favoreceu a biodegradação do CIC, em T1 (LN) sem esterilidade. A melhor atividade enzimática de laccase 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 Effluente 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, C1 (10 días) y C2 (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 biorremediadas 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 C1 y para CIC en C2, con F1 y F5 (identificados por biología molecular). Los resultados mostraron que C1 (SAN) aumentó la biodegradación de ET y C2 (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).
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 formed 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

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

Source: Autors.
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.

![Figure 2](image-url)

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-hydroxybenzotrazole) 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; Koutsatsifi & 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, glutamic acid, potassium nitrate, magnesium chloride, copper sulfate II (Alphatec, Brazil), absolute ethanol (Química Moderna, Brazil), Nucleo 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 °C 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 Panerochaete chrysosporium. Agar discs of 6 mm diameter with the grown fungi were added to Kirk medium (yeast extract 0.1 g/L; KH2PO4 0.2 g/L; MgSO4 0.05 g/L; MnSO4 0.016 g/L; CuSO4 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 °C in the absence of light and with 150 rpm of constant agitation.

In phase II of the pre-selection, the initial growth time G2 of 45 days was used, in Sabouraud agar at 30 °C 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):

$$\text{Discoloration} \% = \frac{Ai - Af}{Ai} \times 100$$  

(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 (G1 and G2). 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 °C was used, which was slowly added to 30 mL of the enzymatic extract under constant stirring. Then, the samples were refrigerated at 4 °C for 15 minutes, and then centrifuged in a centrifuge (THERMO
The enzymatic activity of the three enzymes was determined according to equation (3) (modified) by Orozco and Quesada (1995):

\[
\frac{U}{L_{\text{min}}} = \frac{\Delta \text{Abs} \cdot V_T}{\epsilon \cdot d \cdot \Delta t \cdot V_m}
\]

Where \(\Delta \text{Abs}\) is the absorbance differential (Absf - Abso); \(VT (\mu\text{L})\) is the total reaction volume; \(\epsilon (\text{M}^{-1} \cdot \text{cm}^{-1})\) is the extinction coefficient for the oxidation of ABTS; \(d (\text{cm})\) is the path length of the reaction cell; \(\Delta t (\text{min})\) is the reaction time; and \(V_m (\mu\text{L})\) is the final volume of the enzymatic extract.
extinction coefficient for the reaction catalysis; \(d\) (cm) is the distance that passes the light incidence radius through the sample; \(\Delta t\) (min) is the reaction time differential (\(t_f - t_0\)); \(V_m\) (\(\mu\)L) 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 \(\mu\)M 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 \(\mu\)L 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 \(\mu\)L, 25 \(\mu\)L, 50 \(\mu\)L and100 \(\mu\)L, measured in the spectrophotometer of Elisa at 665 nm for 4 hours, and project to 309 hours for curve tendency obtain.

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

\[
y = ax + b
\]  

(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:

\[
V = \frac{V_{MAX} \cdot [S]}{K_M + [S]}
\]  

(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):

\[
\frac{1}{V} = \frac{K_M}{V_{MAX} \cdot [S]} + \frac{1}{V_{MAX}}
\]  

(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 °C, 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 - G1);
2. Textile effluent tested in fungi with initial growth time of 20 days (TE - G1);
3. Indigo carmine dye 50 ppm tested on fungi with an initial growth time of 45 days (ICD - G2);
4. Textile effluent tested on fungi with an initial growth time of 45 days (TE - G2).

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 (665 nm).

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

<table>
<thead>
<tr>
<th>FERMENTATION TIMES WITH NITROGEN LIMITATION (NL)</th>
<th>FUNGUS</th>
<th>DISCOLORATION (%)</th>
<th>DAY ON WHICH MAXIMUM DISCOLORATION WAS ACHIEVED</th>
<th>FUNGUS</th>
<th>DISCOLORATION (%)</th>
<th>DAY ON WHICH MAXIMUM DISCOLORATION WAS ACHIEVED</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 (1 day)</td>
<td>F5</td>
<td>68.07</td>
<td>10</td>
<td>F7</td>
<td>70.34</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>F2</td>
<td>64.92</td>
<td>10</td>
<td>F3</td>
<td>65.25</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>F1</td>
<td>58.25</td>
<td>10</td>
<td>F1</td>
<td>63.58</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>F4</td>
<td>51.58</td>
<td>10</td>
<td>F4</td>
<td>58.27</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>F6</td>
<td>47.02</td>
<td>10</td>
<td>F2</td>
<td>51.93</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>F7</td>
<td>44.91</td>
<td>10</td>
<td>F6</td>
<td>45.16</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>F3</td>
<td>35.44</td>
<td>7</td>
<td>F5</td>
<td>44.33</td>
<td>10</td>
</tr>
<tr>
<td>T2 (4 days)</td>
<td>F5</td>
<td>63.16</td>
<td>4</td>
<td>F1</td>
<td>78.98</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>F2</td>
<td>59.65</td>
<td>7</td>
<td>F4</td>
<td>65.14</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>F6</td>
<td>58.25</td>
<td>7</td>
<td>F5</td>
<td>64.31</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>F4</td>
<td>54.04</td>
<td>7</td>
<td>F6</td>
<td>63.97</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>F1</td>
<td>45.61</td>
<td>7</td>
<td>F7</td>
<td>55.15</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>F3</td>
<td>45.61</td>
<td>4</td>
<td>F2</td>
<td>47.45</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>F7</td>
<td>38.60</td>
<td>7</td>
<td>F3</td>
<td>42.25</td>
<td>10</td>
</tr>
<tr>
<td>T3 (7 days)</td>
<td>F2</td>
<td>62.46</td>
<td>4</td>
<td>F6</td>
<td>96.15</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>F5</td>
<td>60.35</td>
<td>4</td>
<td>F3</td>
<td>95.63</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>F6</td>
<td>60.35</td>
<td>4</td>
<td>F7</td>
<td>95.53</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>F1</td>
<td>56.14</td>
<td>4</td>
<td>F5</td>
<td>95.11</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>F7</td>
<td>54.04</td>
<td>10</td>
<td>F4</td>
<td>95.11</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>F4</td>
<td>51.58</td>
<td>10</td>
<td>F2</td>
<td>92.82</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>F3</td>
<td>40.70</td>
<td>10</td>
<td>F1</td>
<td>92.51</td>
<td>10</td>
</tr>
</tbody>
</table>

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 G1 and G2.

In the initial growth time G1 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 G2, 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 G1, 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 G2 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 metabolities in fungi by the WAN (Moreira, 2006).

The initial growth time influenced the TE discoloration, showing that G2 is more efficient to biodegrade dyestuffs.
and G1 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 (G2), ligninolytic enzymes are found in a higher proportion for long periods (45 days) (Henn et al., 2020).

In shorter periods of growth and WAN (G1, 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>
<thead>
<tr>
<th>FUNGUS</th>
<th>DISCOLORATION FOR ICD (%)</th>
<th>DAY ON WHICH MAXIMUM DISCOLORATION WAS ACHIEVED</th>
<th>DISCOLORATION FOR TE (%)</th>
<th>DAY ON WHICH MAXIMUM DISCOLORATION WAS ACHIEVED</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>84.08</td>
<td>25</td>
<td>84.91</td>
<td>13</td>
</tr>
<tr>
<td>F2</td>
<td>64.31</td>
<td>22</td>
<td>81.05</td>
<td>13</td>
</tr>
<tr>
<td>F5</td>
<td>47.76</td>
<td>16</td>
<td>86.67</td>
<td>13</td>
</tr>
<tr>
<td>F6</td>
<td>48.60</td>
<td>13</td>
<td>81.40</td>
<td>13</td>
</tr>
</tbody>
</table>

The highest percentages found by each fungus at times G1 and G2 are shaded. Source: Authors.

Quanto ao planejamento fatorial estatístico experimental aplicado (4²), 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.

<table>
<thead>
<tr>
<th>FUNGUS</th>
<th>CONCENTRATION OF TOTAL PROTEINS</th>
<th>LACCASE</th>
<th>LIGNIN PEROXIDASE</th>
<th>MANGANESE PEROXIDASE</th>
</tr>
</thead>
<tbody>
<tr>
<td>FM</td>
<td>15.162 µg/mL</td>
<td>4.444 x 10^{-7} U/L</td>
<td>2.115 x 10^{-7} U/L</td>
<td>-1.410 x 10^{-6} U/L</td>
</tr>
<tr>
<td>F1</td>
<td>1.924 µg/mL</td>
<td>1.481 x 10^{-8} U/L</td>
<td>1.763 x 10^{-7} U/L</td>
<td>-6.936 x 10^{-5} U/L</td>
</tr>
<tr>
<td>F5</td>
<td>20.019 µg/mL</td>
<td>5.674 x 10^{-6} U/L</td>
<td>2.233 x 10^{-7} U/L</td>
<td>-1.821 x 10^{-5} U/L</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>FUNGUS</th>
<th>10 µL ENZYMATIC EXTRACT</th>
<th>25 µL ENZYMATIC EXTRACT</th>
<th>50 µL ENZYMATIC EXTRACT</th>
<th>100 µL ENZYMATIC EXTRACT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{MAX}$ (ppm/h)</td>
<td>$K_M$ (ppm)</td>
<td>$V_{MAX}$ (ppm/h)</td>
<td>$K_M$ (ppm)</td>
</tr>
<tr>
<td>FM</td>
<td>0.0039</td>
<td>117,330</td>
<td>0.0043</td>
<td>135,646</td>
</tr>
<tr>
<td></td>
<td>0.0003</td>
<td>117,330</td>
<td>0.0043</td>
<td>135,646</td>
</tr>
<tr>
<td>F1</td>
<td>0.0015</td>
<td>432,084</td>
<td>0.0058</td>
<td>266,585</td>
</tr>
<tr>
<td></td>
<td>0.0001</td>
<td>432,084</td>
<td>0.0058</td>
<td>266,585</td>
</tr>
<tr>
<td>F5</td>
<td>0.0075</td>
<td>577,900</td>
<td>0.0060</td>
<td>213,587</td>
</tr>
<tr>
<td></td>
<td>0.0001</td>
<td>577,900</td>
<td>0.0060</td>
<td>213,587</td>
</tr>
</tbody>
</table>

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 G2, and above 80% in G1 (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).

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 G1 and G2, 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 G1, 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 G1 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 G1 (A) for the textile effluent (TE) and (B) for the indigo carmine dye (ICD).

![Graph A: % Survival of Artemia sp. nauplii in G1 for textile effluent (TE)](image)

![Graph B: % Survival of Artemia sp. nauplii in G1 for the indigo carmine dye (ICD)](image)

Source: Authors.

In Figure 7 (A) on the first day 100% survival was recorded for all TE samples in G2, 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 G2 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.,
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


