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The Use of Heavy Metals in Mycoremediation of Synthetic Dyes

Research Article

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Abstract: Synthetic dyes represent a major class of toxic pollutants that are resistant to biological degradation, and heavy metals play a special role in this process. In this study, two isolates of lignicolous macromycetes, *Lenzites betulina* as well as another species less frequently studied, *Trametes gibbosa,* were tested in order to assess their remediation potential against three different synthetic dyes under specific conditions. The effect of heavy metal ions in the discoloration of synthetic dyes process, and the optimal concentration of manganese ions necessary were evaluated. The dyes' discoloration efficiency of the fresh isolates were compared to the isolates maintained by refrigeration, isolates that were repeatedly sub-cultivated and isolates that were previously grown on dye-supplemented media, and then were assessed. The discoloration process was evaluated in liquid nutrient media, 10 replicates were used for each working version. Evaluation of discoloration rate was obtained by using a UV-VIS spectrometer. The results were interpreted statistically by the Kruskal-Wallis test and by use of a new complex method, Multiple Factor Analysis. The fresh isolates showed the highest discoloration capacity while the isolates previously grown on the dye-supplemented media presented a low discoloration rate. Manganese ions gave a positive effect on enzyme induction while copper and cobalt ions inhibited the process.

Keywords: *Bioremediation • Synthetic dyes • Lenzites betulina • Trametes gibbosa • Heavy metals • Inoculum*

List of abbreviations

AF – Acid fuchsine
MB – Methyl blue
L. betulina R, *T. gibbosa* R – isolates maintained by refrigeration
L. betulina 2, *T. gibbosa* 2 – fresh isolates
L. betulina 100, *T. gibbosa* 100 – isolates pre-grown on media supplemented with 100 mg L^{-1} dye
L. betulina 250, *T. gibbosa* 250 – isolates pre-grown on media supplemented with 250 mg L^{-1} dye
TB – Toluidine blue

1. Introduction

Remediation of xenobiotics is not only a necessity but also a great challenge nowadays. Of all types of xenobiotics, synthetic dyes represent a major class of pollutants, due to their large production, their diverse chemical structure and their resistance to biological discoloration. Thus, because of their toxicity, many of them are a threat to aquatic organisms [1]. Synthetic dyes are chemical compounds with varied structure, and are widely used in numerous industrial activities, particularly in the textile industry, where large amounts of effluents result, with the production of dyes requiring proper treatment. Triarylmethane dyes are used in textile dying, paper printing or as biocide agents [2]. The triarylmethane dyes are compounds derived from methane with three of the hydrogen atoms substituted by aryl rings. Thiazine dyes are heterocyclic aromatic chemical compounds, containing nitrogen and sulphur.

Although many physicochemical techniques have been developed for treatment of dyes, implementation is usually quite costly and its effectiveness is limited

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[3,4]. Biological methods are an attractive alternative because of the low cost of implementation and lack of adverse effects to the environment [5,6]. Some lignicolous basidiomycetes, also known as white rot fungi, are capable of degrading a wide range of xenobiotics, including synthetic dyes, due to their versatile enzyme system. The enzymes produced by these organisms are involved not only in hydrolysis of macromolecular compounds, but also in various redox reactions, thereby neutralizing toxic chemicals [7]. The enzymes commonly associated with the synthetic dye degradation are part of the ligninolytic enzyme system, involving laccases [8], manganese-dependent peroxidases, manganese-independent peroxidases, aryl alcohol oxidases [9], versatile peroxidases [10], glyoxal oxidases [11] *etc*.

Numerous studies have reported the discoloration of synthetic dyes by employing lignicolous macromycetes, and some authors have optimized the culture conditions in order to obtain an increased rate of the process [12], although many questions remain unanswered about the factors involved in the process.

The ability of fungi to degrade the dyes differs with the isolate or the fungal species used, the dye tested, and the conditions in the culture. Heavy metal ions have had a positive effect on induction of the ligninolytic enzyme system [13], most particularly the manganese-dependent peroxidase and laccase, and some of them are required for the metalloproteins synthesis [9]. High concentrations of heavy metals can completely inhibit fungal development [14]. The industrial effluents resulting from the different activities in which dyes are used, such as in textile industry, may have a high content of heavy metal ions, added as salts in the dye baths, and as a result, the effect of heavy metals is quite important. Synthetic dyes are toxic to fungi under conditions in the environment where fungi tend to grow [15]. Under the action of oxidative stress, fungi react in neutralizing the toxic agent by stimulating the ligninolytic enzyme system [8,16].

The study has assessed the effect of metal ions in the synthetic dyes mycoremediation process. The Multiple Factors Analysis was employed for the first time. It is a complex statistical method used to assess the effect of metal ions and to establish correlations between the effect of these ions and the dye class and the particularly species involved. The fungi used were isolated prior to the experimental trials, and were subjected to preliminary screening regarding to their ability to mycoremediate synthetic dyes [17]. The strains were chosen on the basis of their efficiency as observed in the screening process, and also on their similar patterns of growth, enabling the results to be readily compared and analyzed. Common dyes were chosen from the structural classes of dyes that are frequently used in many industrial activities. The concentrations of heavy metals were chosen according to the tolerance limit of the fungi [18,19], similar to the ones that can be found in the majority of the industrial effluents.

The effects of growing fungi on dye-supplemented media in the discoloration of the same synthetic dye in a later process, were examined. Thus, the hypothesis that these procedures will positively influence the discoloration of dyes by inducing the enzyme system compared with non-treated samples was tested for the first time.

The biochemical properties of the fungal strains may be affected by preservation methods, and some enzymes may not be synthesized with maximum efficiency. In this manner, the efficiency of discoloration of isolates previously maintained by refrigeration, has been tested and sub-cultivated prior to the test compared with the efficiency of fungal isolates repeatedly sub-cultivated (or fresh isolates), in order to assess how preservation by refrigeration affects the discoloration efficiency of the fungi. The tested isolates belong to species less studied in the mycoremediation processes. *Trametes gibbosa* has not been studied very much in synthetic dye mycoremediation, with some authors reporting that this species has low efficiency [20]. In our experiments, the potential of this species was higher than other isolates from species known as efficient in these processes, such as *Trametes versicolor* [21].

The selection of fungal isolates that can tolerate heavy metals is of particular importance in the elaboration of strategies for the mycoremediation of industrial effluents.

2. Experimental Procedures

2.1 Fungal strains and inocula

Two fungal strains: *Lenzites betulina* (L.) Fr. and *Trametes gibbosa* Pers. Fr. were previously isolated using dikaryotic mycelium [22] and then the isolates were deposited in the collection of microorganisms, MIUG from „Dunarea de Jos" University of Galati, (MIUG B95 and MIUG B96). Fresh isolates were also tested, one isolate for each species. Fungal isolates were previously tested for the ability of synthetic dyes's mycoremediation, and were maintained by sub-culturing procedure. The identification of the selected species was performed using classical macroscopical and microscopical methods [23-25] and later confirmed by analysis of the characteristics of the cultures [26].

2.2 Dyes

Two different classes of dyes have been used: Methyl blue (MB) and Acid Fuchsine (AF): Triarylmethane and Toluidine blue (TB): Thiazine. All the reagents are of analytical grade and were acquired from Merck or Sigma-Aldrich.

2.3 Media and culture conditions

Organic liquid media, containing malt extract (malt extract - 15 g L^{-1}, dextrose - 10 g L^{-1}) supplemented with MB, AF and TB at a final dye concentration of 100 mg L^{-1}, have been distributed in test tubes, 10 ml each. The pH was adjusted at 5.00 using HCl, 0.1 mM L^{-1}. The inoculation was performed with square agar plugs of 1 cm^2, covered with mycelium, cropped from the periphery of an actively growing colony, using a modified version of the method described by Levin and collaborators [27]. Media were sterilized by autoclaving at 120ºC in a 75 litre upright model autoclave (Raypas, Barcelona, Spain), and the samples were incubated in the dark, at 25ºC, for 10 days in an orbital shaker, at 180 rpm (GFL 3500, Germany). Ten replicates were examined for each combination (species-type of inoculums-variant of media-concentration of heavy metal ions) and non-inoculated controls. Samples that gave very diverse values of discoloration, were classified as errors and were redone.

2.4 The effect of pre-cultivation on discoloration processes

Five types of inoculum were employed in the experiment: the mycelium was repeatedly sub-cultivated, and over monthly periods (*L. betulina* and *T. gibbosa*); the mycelium was maintained for eight months by refrigeration at 4ºC and sub-cultivated prior to inoculation (*L. betulina* R and *T. gibbosa* R); the mycelium of fresh isolates (*L. betulina* 2 and *T. gibbosa* 2); the mycelium grown on a nutrient media (malt extract - 20 g L^{-1}, glucose - 10 g L^{-1}, agar - 15 g L^{-1}), supplemented with 100 mg L^{-1} dye (*L. betulina* 100 and *T. gibbosa* 100) and 250 mg L^{-1} dye (*L. betulina* 250 and *T. gibbosa* 250).

2.5 The influence of heavy metals

To test the effect of heavy metals on the discoloration process, nutrient media containing the amounts per litre of the following were used: 0.2 g $CuSO_4 \cdot 5H_2O$, 0.2 g $MnSO_4 \cdot 4H_2O$, 0.2 g $ZnSO_4 \cdot 7H_2O$; 0.2 g $FeSO_4 \cdot 7H_2O$, 0.2 g $Co(NO_3)_2 \cdot 6H_2O$. Each of the various media concentrates were enriched with heavy metal ions and supplemented with one of three tested dyes, then inoculated with *L. betulina* and *T. gibbosa*, resulting in 36 combinations. The effect of manganese ion concentration was analyzed by cultivating *T. gibbosa* on media added to 100 mg L^{-1} AF, TB and $MnCl_2$ in final concentrations of 0.1, 0.25, 0.4, 0.55, 0.7 and 0.85 mM L^{-1}.

2.6 Spectrophotometric studies

At the end of the experiment, the liquid media was centrifuged at 5000 rpm (MICRO 22 R, Hettich) for ten minutes, and the supernatant obtained was photometrically evaluated using an UV-VIS spectrophotometer (Pharmaspec UV-1700 model, Shimadzu). The absorbance was measured at the wavelength corresponding to the maximum absorption for each dye. The discoloration rate was calculated according to the equation: $\%\ discoloration = (Ai - Af) / Ai * 100$, where Ai is the initial absorbance at λmax and Af is the absorbance after incubation period [28]. All the calculations were verified using a calibration curve.

2.7 Statistical analysis

To compare the results, a Kruskal-Wallis test was run, grouping data sets based on the dye used and the species tested. The categories of inoculum, the medium variant and metal ions were used as variables. The test allows comparison of both sets of data to highlight the significant differences, and were organized by rank. In addition, for overall comparison of the data obtained, a Multiple Factor Analysis was carried out for each data set. All statistical tests were performed using XLSTAT 2013 software (AddinSoft, Trial version).

3. Results and Discussions

3.1 The effect of pre-cultivation on dye supplemented media

The results obtained were statistically analyzed to see if there were significant differences between the different working trials. The Kruskal-Wallis test not only allows ready comparison of the data in order to identify differences but also allows the ranking the observed results (depending on the magnitude of differences observed).

Fresh isolates (*L. betulina* 2 and *T. gibbosa* 2) showed greater efficiency in the discoloration of the three dyes (Table 1), and the isolates maintained by refrigeration presented different results, which varied according to the species employed and the dye tested; in all these instances, the discoloration rate was moderate. The appearance of diverse groups (A, B and C) proves a significant difference among the results obtained, indicating that the treatment produced a varied response.

Fungal mycelium pre-grown on the supplemented media and used as a source of inoculum showed

DYE	INOCULA	SUM OF RANKS	GROUPS	INOCULA	SUM OF RANKS	GROUPS
	LB_{250}	79.50	A	TG_{100}	137.50	A
	LB_r	191.50	A. B	TG_{250}	157.00	A
MB	LB_{100}	273.50	B	TG	196.50	A
	LB	278.00	B	TG_r	358.50	B
	LB_2	452.50	C	TG_2	425.50	B
	LB	151.00	A	TG	125.50	A
	LB_r	175.00	A	TG_{100}	192.00	A. B
TB	LB_{250}	219.50	A	TG_{250}	219.50	A. B
	LB_{100}	286.50	A	TG_r	306.50	B. C
	LB_2	443.00	B	TG_2	431.50	C
	LB_{100}	154.00	A	TG_{100}	145.50	A
	LB_r	182.00	A	TG_{250}	167.50	A
AF	LB	228.00	A	TG	172.50	A
	LB_{250}	276.00	A. B	TG_2	350.50	B
	LB_2	435.00	B	TG_r	439.00	C

Table 1. Influence of inoculum type in the discoloration of dyes - multiple pairwise comparison using Steel-Dwass-Critchlow-Fligner procedure, $P<0,0001$ (Kruskal-Wallis Test, XLSTAT 2012) (AF - Acid fuchsine, MB – Methyl blue, TB – Toluidine blue, R – isolates maintained by refrigeration, 2 – fresh isolates, 100 and 250 – isolates pre-grown on media supplemented with 100 mg L^{-1} dye and 250 mg L^{-1} dye respectively)

a slower development and a reduced rate of discoloration. In this instance, fungal development is delayed so that the induction of enzyme system prior to inoculation (if present), does not provide an advantage, and the mycelium develops anaemically, with fewer hyphae because of the toxic effect of the dye. A slightly higher discoloration were observed for the isolates pre-grown on media with 250 mg L^{-1} dye to those pre-grown 100 mg L^{-1} dye added media. This may be due to an induction of the enzyme system.

The TB discoloration was slightly elevated compared with the discoloration of MB, although in previous experiments [17] the second dye was rapidly discoloured. A possible explanation is that MB is a triarylmethane dye and despite rapid discoloration by fungi, it shows a strong toxicity in high concentrations. Based on this effect, triarylmethane dyes are used as antifungal compounds [2].

The results were different for the three dyes and even for the two species. For this reason, global comparison of the data was performed using a complex statistical procedure that analyzes multiple categories of factors. Multiple Factor Analysis is a procedure that combines Principal Component Analysis with Multiple Correspondence Analysis, and allows simultaneous comparison of data series taking into account both quantitative and qualitative variables by applying the two subsequent procedures.

The variability of the results is mainly due to the category of inoculum (Figure 1A) and dye type. Species were found to exert less influence on the discoloration process. Therefore, the inoculum category played a very important role in the discoloration of dyes.

In these experiments, the species and dye type as qualitative variables and the dye discoloration rate as quantitative variables for the five categories of inoculum were considered, expressed as averages of values for the ten replicates of each sample. A circle of correlation (Figure 1B) graphically represents the position of the variables (in this case, the category of the inoculum), and the relationship between them. The plane of the variables and the factors that produce them, in this case 91% of the information, indicates that these projections are accurate.

There is a very strong correlation between the isolates pre-grown on media with 100 and 250 mg L^{-1} dye, demonstrating approximately identical effects overall for the two treatments; *i.e.*, cultivation of fungi on dyes supplemented media induces similar changes in terms of fungal behaviour even if their concentrations are different. The orthogonal position of fresh isolates (denoted by 2), indicates a lack of correlation between them and the first, while the position in quadrant I indicates the increased discoloration of synthetic dyes. Isolates that were maintained by refrigeration discoloured the dyes

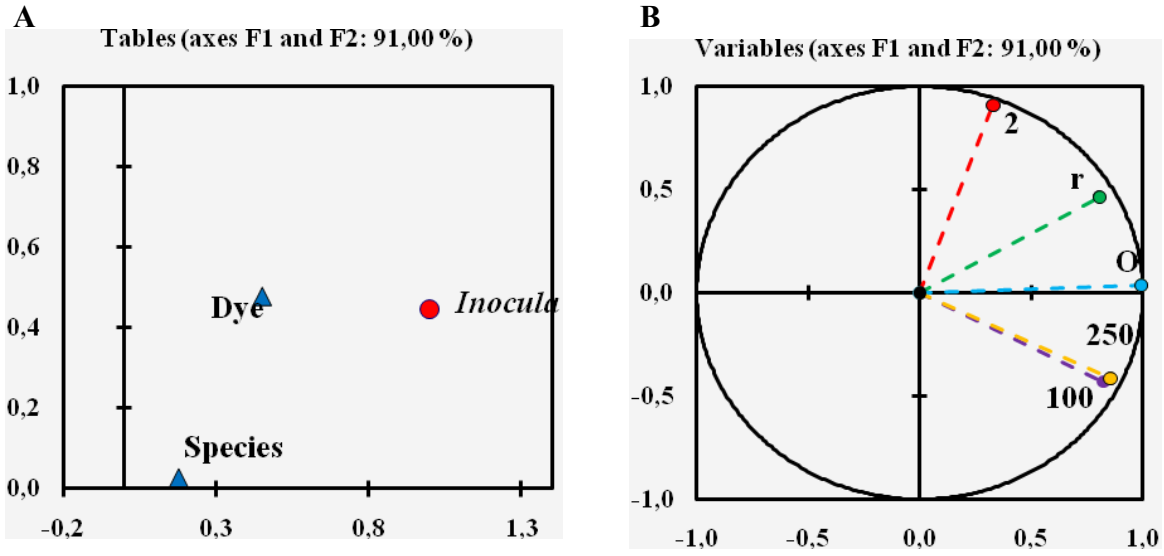

Figure 1. A. Table of coordinates - the Lg coefficient of relationship between variables; B. Circle of correlation between factors and variables (2 - fresh isolates; r - isolates maintained by refrigeration; O - isolates repeatedly sub-cultivated; 100, 250 - isolates pre-grown on media supplemented with 100 or 250 mg L^{-1}).

moderately; their position mainly given by *Trametes gibbosa* isolates (*T. gibbosa* R) (Table 1). The isolates pre-grown on dye-supplemented media (*L. betulina* 100, *L. betulina* 250, *T. gibbosa* 100, *and T. gibbosa* 250), discoloured with a low efficiency.

The results obtained calls into question the efficacy of using a single long-term isolate. These results may be attributed to the phenomenon of culture senescence or fungal metabolic adaptation to a nutrient-rich culture medium. Under these circumstances, the activity of some enzymes may not be required for fungal nutrition. Anastasi and co-workers [12] found that discoloration of dyes is faster on media with low nitrogen content. To avoid the occurrence of these phenomena, fungi might be sub-cultivated on media containing an inducer of various types of enzymes, without necessarily exhibiting high toxicity towards fungi. Therefore, the best results were recorded for fresh isolates and not for pre-grown on dyes supplemented media.

3.2 The effect of heavy metals addition

The addition of heavy metals in the nutritive medium presented different effects depending on the metal ions involved, the fungal isolate and the synthetic dye used. The results obtained for the metals-supplemented media were compared to results obtained using the heavy metal-free media. The discoloration of the dyes was significantly affected by the addition of heavy metals, either by increasing the discoloration efficiency or by inhibiting the fungal development, depending on the metal (Table 2).

The ions of Mn^{2+} and Fe^{2+} had a positive effect in the discoloration of MB and AF in both tested isolates, whereas ions of Co^{2+}, Cu^{2+} and Zn^{2+} clearly inhibited this process, for the concentration employed. Pointing and co-workers [18] observed the inhibition of synthetic dyes discoloration when these ions were added in a concentration of 0.1 mM L^{-1}. The positive effect of Mn^{2+} ions addition may be due to the involvement of manganese-dependent peroxidase in the discoloration of triarylmethane dyes [21].

For TB discoloration, the effect of the metal ions addition was different. The increased discoloration rates were recorded for heavy metals free media. For *Lenzites betulina* Zn^{2+} ions there was a strong inhibitory effect, and *Trametes gibbosa* Fe^{2+} ions inhibited the discoloration of synthetic dyes. The inhibitory effect of Zn^{2+} ions and Fe^{2+} in the dye discoloration has been reported also by Couto and co-workers [29] for lignicolous fungi species. Murugesan and co-workers [19] reported strong inhibition of laccase activity caused by the presence of Fe^{2+} ions. Therefore, the effect of heavy metals in the discoloration processes is very different based on synthetic dyes, as well as the fungal species involved [30-33]. This is due to high structural variability of synthetic dyes, and thus is due to different classes of enzymes involved in the discoloration of dyes.

Figure 2A presents the variation of the effect of the addition of heavy metals, with consideration of two factors: the species and the dye category. The effect of copper and manganese ions is strongly correlated with the type of tested dye, while the effect of zinc ions varies

DYE	*Lenzites betulina*			*Trametes gibbosa*		
	METALS	SUM OF RANKS	GROUPS	METALS	SUM OF RANKS	GROUPS
MB	Zn	141.00	A	Cu	57.00	A
	Co	200.50	A	Co	174.00	B
	Cu	229.00	A. B	-	318.00	C
	-	346.50	B. C	Zn	377.00	C
	Fe	424.00	B. C	Fe	442.00	C
	Mn	489.00	C	Mn	462.00	C
AF	Zn	102.00	A	Cu	88.00	A
	Cu	178.00	A	Zn	151.00	A
	Co	243.00	A	Co	253.00	B
	-	372.00	B	-	383.00	C
	Fe	387.00	B	Fe	424.00	C. D
	Mn	548.00	C	Mn	531.00	D
TB	Zn	63.00	A	Fe	73.00	A
	Mn	194.00	B	Zn	217.00	B
	Co	234.00	B	Cu	341.00	B
	Fe	395.00	C	Co	347.00	B
	Cu	456.00	C	Mn	361.00	B. C
	-	488.00	C	-	491.00	C

Table 2. The effect of the heavy metals addition in the discoloration of dyes - multiple pairwise comparison using Steel-Dwass-Critchlow-Fligner procedure, P<0.0001 (Kruskal-Wallis Test, XLSTAT 2012) (AF – Acid fuchsine, MB – Methyl blue, TB – Toluidine blue)

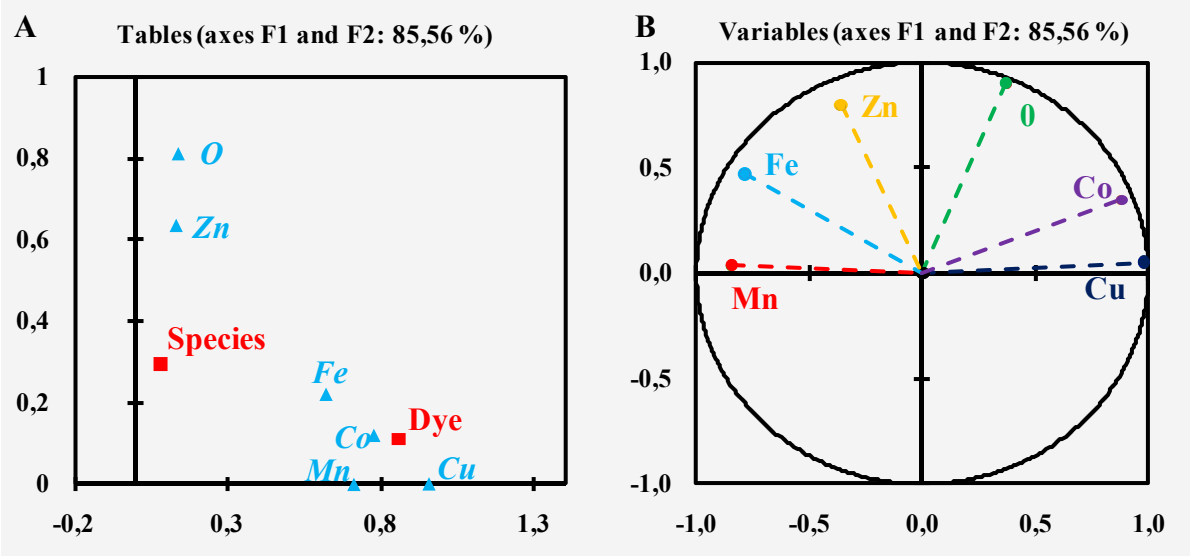

Figure 2. A. Table of coordinates - the Lg coefficient of relationship between variables; B. Circle of correlation between factors and variables (O – non-supplemented media; Cu, Mn, Fe, Zn and Co - media containing salts of heavy metals)

with the species. On the same axis the results obtained for non-supplemented media are recorded, with a strong dependence existing between the tested fungal isolate and the discoloration rate observed. This figure confirms the accuracy of the information.

Overall, the effect of the addition of metal ions varied widely and depended on the different factors examined, but an overall positive effect of the addition of Mn^{2+} and Fe^{2+} ions were observed while Cu^{2+} and Co^{2+} caused negative results, at the concentration of 0.2 g L^{-1} salt. Other authors have obtained the positive effect of Mn^{2+} ions as well [34,35]. In Figure 2B, the degree of correlation between the effects of the addition of various metal ions can be observed. The record of these results, as represented on the axis, indicates the action of the metals, from stimulation (on the left) to inhibition (on the right).

Knowledge that metals both can stimulate and can inhibit the discoloration of synthetic dyes is not sufficient. The concentration at which these metal ions produce their effects is of greater significance. Utilization of different molar concentration of Mn^{2+} ions reveals that there is a separate influence on dyes discoloration. The MB was discoloured most effectively when nutritive media was supplemented with 0.25 mM L^{-1} $MnCl_2$ (77% discoloration) and TB or AF at a concentration of 0.85 mM L^{-1} $MnCl_2$ (75% and 85%).

4. Conclusions

This study demonstrates the ability of synthetic dyes to undergo mycoremediation with the use of two different fungal isolates. The strains tested demonstrated tolerance to the moderate concentrations of heavy metal ions that were similar to the concentrations found in the dyes bath. By using a complex statistical method, the increased potential of dye mycoremediation of a very little studied species, *Trametes gibbosa* was revealed.

Pre-cultivation of fungi on media containing different concentrations of dyes had negative effects on synthetic dyes discoloration processes, while fresh isolates grown on non-supplemented media showed the highest rate of discoloration.

The repeatedly sub-cultivated isolates showed moderate activity, imposing the use of other types of nutrient media that contain substances with inductive effects on enzyme activity.

The addition of heavy metals in the culture medium showed different effects in the dye discoloration process. The main factors contributing to this variation are represented by the isolate, the fungal species and the type of dye. Ions of copper and cobalt inhibited the fungal activity, being toxic at the concentration of 0.2 g L^{-1} salt, while ions of manganese and iron, in moderate concentrations, stimulated the degradation processes.

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