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Bio-Removal of Methylene Blue from Aqueous Solution by *Galactomyces geotrichum* KL20A

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Abstract: The conventional treatments used to remove dyes produced as a result of different industrial activities are not completely effective. At times, some toxic by-products are generated, affecting aquatic ecosystems. In this article, an efficient use of microorganisms is presented as a biodegradation technique that is a safe environmental alternative for the benefit of aquatic life. A strain of the yeast *Galactomyces geotrichum* KL20A isolated from Kumis (a Colombian natural fermented milk) was used for Methylene Blue (MB) bioremoval. Two parameters of the bioremediation process were studied at three different levels: initial dye concentration and growth temperature. The maximum time of MB exposure to the yeast was 48 h. Finally, a pseudo-first-order model was used to simulate the kinetics of the process. The removal percentages of MB, by action of *G. geotrichum* KL20A were greater than 70% under the best operating conditions and in addition, the kinetic simulation of the experimental results indicated that the constant rate of the process was 2.2×10^{-2} h^{-1} with a half time for biotransformation of 31.2 h. The cytotoxicity test based on the hemolytic reaction indicated that by-products obtained after the bioremoval process reached a much lower percentage of hemolysis (22%) compared to the hemolytic activity of the negative control (100%). All of these results suggest that the strain has the capacity to remove significant amounts of MB from wastewater effluents.

Keywords: bioremediation; dyes; *Galactomyces geotrichum*; methylene blue

1. Introduction

Most of the industrial effluents in developed countries have a high load of xenobiotic compounds, such as dyes. These compounds are recalcitrant, difficult to biodegrade, and toxic to aquatic species, animals, and human beings. Most of the dyes used in the textile industry are stable, resistant to biological, physical, and chemical treatments, implying that their complete removal is difficult. Due to their high solubility in water, they cause contamination in local aquatic systems when they are discharged directly without prior treatment [1]. In particular, Azo dyes are the largest synthetic chemical class of dyes that are characterized by the presence of one or more azo bonds (-N=N-). They are used to a great degree in textile, pharmaceutical, leather, food, cosmetic, painting, and large-scale printing industries due to their ease of synthesis and stability [2]. MB is an aromatic heterocyclic used in different applications (e.g., medicine [3], biological [4], and textile industries [5]). Currently, MB dye is one of the most commonly used substances for dyeing cotton, wood, and silk [6]. The dye is commonly found in industrial wastewater, which causes devastating effects on the environment [7]. Given the characteristics of solubility and stability of azo dyes, traditional methods of flocculation, sedimentation, or adsorption are not efficient in the removal of these compounds because they only

transfer matter between different phases without effective degradation of the compounds [8]. Currently, such physicochemical methods are the most important ones used for the treatment of wastewater produced by the textile industry, resulting in effluents of acceptable quality, while at the same time generating waste products with high levels of toxicity. For this reason, research on the application of biological processes as a method of dye degradation has been intensified in recent years due to the resulting low toxicity. Their high efficiency [9,10], which is due in part to the inherent capacity of the microorganisms to break down organic pollutants by using them as a source of carbon and energy, or by co-metabolism [11,12], has also contributed to intensified research. The use of yeasts for the bioremoval of toxic compounds has many advantages in comparison to filamentous bacteria and fungi, since they grow rapidly and have the capacity to adapt and resist unfavorable environments [13]. The *Geotrichum* spp. is a filamentous fungus (Phylum Ascomycota, Order Saccharomycetales) that has transparent spores by segmentation of vegetative filaments [14]. *G. geotrichum* is a holomorphic organism, which means that it presents two forms of reproduction (sexual and asexual): the asexual form of multiplication gives rise to the anamorph (*Geotrichum candidum*) or imperfect state, while the sexual form gives rise to the teleomorph (*G. geotrichum*) or perfect state. These fungal species are capable of alternating a unicellular phase (yeast growth) with another mycelium (hyphae) in response to changes in various environmental factors (nutrients, CO_2 pressure, pH changes, temperature). They are called "dimorphic fungi" and tend to proliferate as yeasts in tissues. However, they assume filamentous forms at room temperature in the environment [15]. This fungus is present in diverse habitats. It is found as a component of the natural flora in milk and is used as a ripening agent for cheese. Furthermore, it presents colonies of fast growth (four days of maturation), and of unlimited size. *G. geotrichum* is white and has a moist, yeast-like, appearance, and is easy to obtain. This yeast is characterized by true hyphae, thick (macrosiphonated) forming numerous arthroconidia, hyaline, and rectangular. The absence of blastoconidia differentiates it from the genus *Trichosporon* spp. [16]. Their lipases and proteases produce fatty acids and peptides that can be metabolized by other microbial populations, promoting the development of different aromas [17]. *G. geotrichum* can be considered one of the fungi with a large number of biotechnology applications. In fact, 38% of the publications which refer to this organism mention its capacity to biodegrade toxic compounds and its application in bioremediation processes in the treatment of wastewater for the improvement of the environment [18]. For *G. geotrichum* MTCC (registered at Microbial Type Culture Collection and Gene Bank, MTCC) a 100% efficiency in methyl red discoloration was reported after one hour at 30 °C. The composition of malt yeast medium used for decolorization studies was malt extract (3.0 g/L), yeast extract (3.0 g/L), peptone (5.0 g/L), and glucose (10.0 g/L) (pH 7.0) [19], while Waghmode et al. reported the decolorization of the azo dye Rubine by *G. geotrichum* MTCC 1360 within 96 h from exposure at 30 °C and pH 7.0 [20]. Govindwar et al. also reported a reduction of 86% via discoloration of the RY-84A dye at 50 mg L^{-1} after 30 h of exposure [21]. Despite extensive studies on different microorganisms for the development of biotechnological applications, this is the first time that removal of MB with *G. geotrichum* KL20A has been reported.

In this work, the removal of MB dye with *G. geotrichum* KL20A was studied in order to search for alternative methods of water treatment that would be environmentally friendly and safe.

2. Materials and Methods

2.1. Yeast Isolation and Culture

The yeast *G. geotrichum* KL20A was isolated from traditional Kumis samples collected in Valle del Cauca (in the southwest of Colombia) as reported by Chaves-López et al. [22]. The identification was carried out by sequencing of the D1/D2 region of the 26S rRNA gene and by random amplification of polymorphic DNA (RAPD-PCR). Subsequently, it was sequenced and compared with the data available in The European Molecular Biology Laboratory (EMBL) nucleotide sequence database. The strain was maintained in Petri dishes containing 12 mL of YPD-agar medium (10 g/L yeast extract,

20 g/L dextrose, 20 g/L peptone, 20 g/L agar) and periodically reactivated for 24 h at 30 °C in the same growth medium [19,21]. A suspension cell solution was then prepared in YPD-broth (without agar) and a chamber count was performed using a Neubauer chamber to control the concentration of the yeast (approximately 3.2×10^7 cel/mL) to be used in the removal tests. The Neubauer chamber remains the most common method for cell counting around the world, also known as hemocytometer.

2.2. Removal Tests

2.2.1. Effects of the Methylene Blue Concentration and Temperature

Methylene Blue dye (MB CI 52015 from Merck KGa, Darmstadt, Germany) was used to prepare a stock solution of 3.13 M (1000 ppm, pH 7.3) by dissolving the required amount of the dye in sterile distilled water. Concentrations of 50, 100, and 200 ppm were used for the removal tests. To this end, sterilized tubes containing 1 mL of YPD-broth medium (pH 7.0) were added to the appropriate MB concentrations (50 ppm, 100 ppm, and 200 ppm) and inoculated with 3.2×10^7 cells/mL of *G. geotrichum* KL20A. Decolorization of MB was studied at 25 °C, 30 °C and 35 °C for 48 h. Periodically, 160 µL of the samples were transferred into a 96 well microplate and the absorbance at 660 nm was read using Elisa SPR-960 reader [23]. The removal tests were carried out in triplicate with their respective targets, under the strictest biosecurity conditions to preserve the asepsis of the experiments.

2.2.2. Effects of the pH

In order to study the effect of the pH on the discoloration of the MB, we performed the experiment using the optimal conditions of concentration and temperature to degrade MB (50 ppm, 35 °C). Thus, we modified the above-mentioned pH of the medium to achieve values of 5, 7, and 9 using HCl/NaOH 0.1 N solutions. The pH was measured with a Thermo-Fisher Scientific pH meter, previously calibrated with buffer solutions of pH 4, 7, and 10. Also in this pH study, the inoculum was yeast suspension containing 3.2×10^7 cells/mL.

Finally, biodegradation kinetics were investigated during the 48 h and a pseudo-first-order model was used to simulate the data and derive the biodegradation rate constant.

2.3. Citotoxicity Analysis

The evaluation of the toxicological impact of the MB dye and its metabolites, was carried out through a hemolytic activity test following the methodology cited by Zhen [24] with some modifications as follows: the negative control was phosphate-buffered saline (PBS) (pH 7.2) treated erythrocytes (this buffer protects the erythrocytes against lysis), and the positive control was a solution of 0.1% of Triton-X-100 (Sigma, Milwaukee, WI, USA), a surfactant reagent that is well known to cause cell lysis. The solution was prepared according to the manufacturer's recommendations). Spectrophotometric analysis was used to measure the absorbance of free hemoglobin (545 nm) in a Thermo Genesys 10S UV/VIS spectrophotometer. The hemolytic activity was reported as percentage of hemolysis (% Hem) evaluated using the following equation:

$$\%\text{Hem} = \frac{A_M - A_{CN}}{A_{CP} - A_{CN}} * 100 \quad (1)$$

where: A = absorbance, M = sample, CN = negative control, CP = positive control.

The results were plotted to obtain the hemolytic activity curve (%) vs. concentration of yeast extract.

2.4. Statistical Analysis

The experimental design is based on the factorial method 2^3, which consists of two independent variables: MB concentration and the growth temperature of the removal process with a study of three levels for each variable according to the values reported in Table 1.

Table 1. Levels and variables involved in the study.

Variable Factor	Low Level	Medium Level	High Level
MB * concentration (ppm)	50	100	200
Temperature (°C)	25	30	35

* MB: Methylene blue.

In order to evaluate the interactions of the variables in relation to the MB bioremediation, the experimental data obtained were processed using the analysis of variance (ANOVA). For the ANOVA study, we used Microsoft Excel statistics. This methodology examines data from repeated assays during which both factors and levels are varied.

3. Results and Discussions

3.1. Effect of MB Concentration and the Growth Temperature on the Removal Process

The results of the removal experiments are presented in Figure 1. Furthermore, in Appendix A (Figure A1) we show the photographic tracking of the biodegradation test of MB dye for 48 h (50 ppm and 30 °C). In all cases examined, we determined that there was a reduction, depending on the temperature and concentration combination used; however, different percentages of these reductions were observed. For high concentrations (100 and 200 ppm) during the first 12 h, the removal percentage was lower (12% and 3%, respectively), compared to MB concentration of 50 ppm (45% of dye removal). The initial MB concentration plays a determinant role for optimal removal, since the increase in the concentration of the dye decreases the rate of degradation, probably due to the toxic effect of the dye on the organism [25,26]. In addition, high concentrations of dyes cause inhibition of the metabolic processes of microorganisms [27]. Nevertheless, the toxic effect could be modified (i.e., decreased) if more yeast is added to the solution, as a result of the constant ratio between the MB concentration and the yeast content.

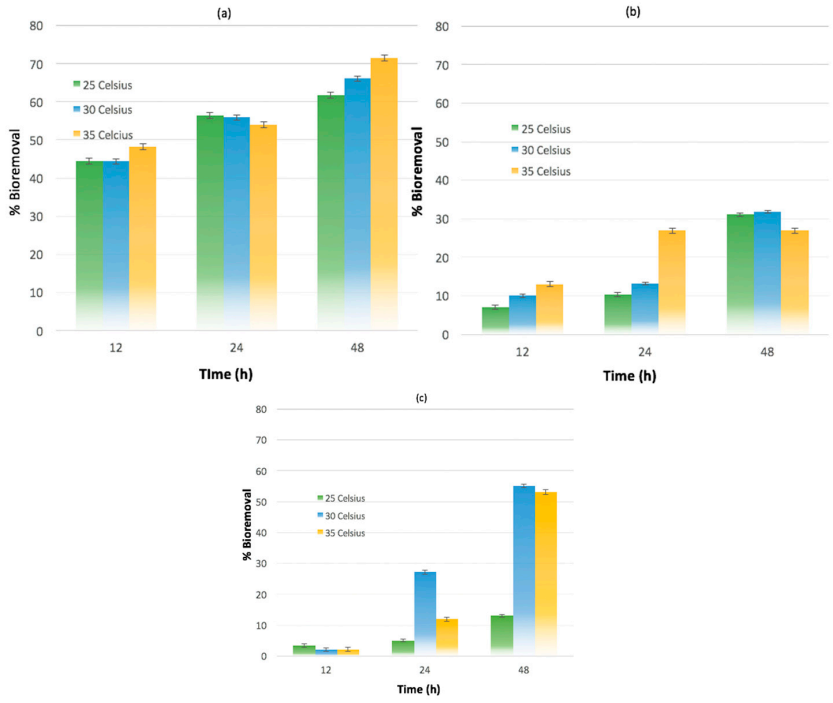

Figure 1. Removal percentages of MB with initial concentration of (**a**) 50 parts per million (ppm); (**b**) 100 ppm; (**c**) 200 ppm by exposition to yeast *G. geotrichum* KL20A at three different temperatures during the 48 h.

Previously, it has been determined that decolorization of a mixture of dyes by *G. geotrichum* was greatly affected by the addition of various carbon and nitrogen sources, instead of the yeast load [23]. Also, it has been reported that carbon and nitrogen sources have an important influence on the extent of discoloration using microorganisms. Different metabolic characteristics in the microorganisms lead to differences in the uptake of sources, and this also will have an effect on the azo dye discoloration [28–30]. On the other hand, dyes are generally deficient in carbon, and biodegradation without an extra carbon source would be very difficult. For that reason, we used YPD-broth to support cell growth, as evidenced by the hemocytometer count. Carbon sources have two purposes: as sources of energy for the growth of the microorganisms and as electron donors, which are necessary to break bonds [31]. All of these observations suggest that in the future, it will be very important to vary carbon and nitrogen sources to be able to study the effect of the discoloration of different dyes, as well as the effect of the dye structure. However, we found that based on the conditions used in this study, there was an initial efficient load of yeast for a discoloration higher than 70%. Additionally, Figure 1 shows that the best biodegradation results are obtained at 35 °C (71.5% +/− 0.8%). This result may be correlated with two effects. Firstly, to the optimum temperature for the enzymatic activity in many cells, which is normally placed between 35 and 40 °C. Secondly, the result could be related to the mass transfer rate of the dye, since at higher temperatures the viscosity of the solution containing the dye is decreased, increasing the diffusion rate of the dye molecules through the boundary of the outer layer, while at low temperatures this process is slower, decreasing the removal percentage [32].

All the results shown in Figure 1 verify that under the above-mentioned conditions, the yeast *G. geotrichum* presents bioremediation properties towards MB dye.

In order to statistically confirm the observed results, a quantitative study was carried out to determine the respective influence of each independent parameter on the response variable (percentage removal). For this study, the analysis of variance of two factors with three replicates per level was used. The combined effects and the percentage of removal (%) obtained from Figure 1 are listed in Table 2.

Table 2. Effect of the initial dye concentration variable and temperature on the percentage (%) in MB bioremediation from data of Figure 1.

Concentration MB (ppm)	25 °C	30 °C	35 °C
200	9.1 [1]	62.0	58.9
	23.7	71.4	56.1
	6.2	57.0	44.3
100	27.7	27.7	26.5
	32.4	32.4	27.4
	35.1	35.1	26.7
50	58.2	70.7	66.2
	63.6	64.7	71.7
	63.4	62.8	76.6

[1] This value corresponds to methylene blue (MB) % bioremoval after 48 h to exposition to *G. geotrichum* KL20A.

We used ANOVA assay to study the effect of each factor (MB concentration and growth temperature), and the F-distribution (F) was used to determine the statistical effect of each factor. If F Value > F critical Value, then the studied parameter had a statistically significant effect on removal percentage. Table 3 lists the ANOVA results obtained from data of Figure 1; theoretical aspects can be found in reference [33].

Table 3. Results of analysis of variance (ANOVA) obtained from data of Figure 1.

Origin of Variations	Sum of Squares	Degrees of Freedom	Average of Squares	F-Value	Probability	F-Critical Value
Sample *	6132.4	2	3066.2	98.6	2.0×10^{-10}	3.5
Level **	1618.5	2	809.3	26.0	4.9×10^{-6}	3.5
Interaction ***	2586.1	4	646.5	20.8	1.5×10^{-6}	2.9

* Factor: This parameter determines if each factor (temperature and BM concentration) had a statistically significant effect on removal percentage; ** Level: This parameter determines if each level (25, 30, and 35 °C for temperature) and (50, 100, and 250 ppm for BM concentration) had a statistically significant effect on removal percentage; *** Interaction: This parameter determines if the interaction between two factors (temperature and MB concentration) had a statistically significant effect on removal percentage.

The results indicate that in all cases the factor F was significantly higher than the critical value, indicating that both factors studied (temperature and MB concentration) and the levels of variation for each factor had a statistically significant effect on the results of the biodegradation tests. The results validated the observations made in the previous sections and it is confirmed that the optimum removal conditions were achieved for a load of 50 ppm of MB dye and a temperature of 35 °C, which does not deactivate the enzymatic system of the yeast, reaching a percentage of 71.5% removal of the MB.

3.2. Effect of pH in the Removal Process

Once the optimum conditions of operation for the bioremoval process were established (50 ppm, 35 °C), we carried out a study of the pH medium effect under these conditions. Three pH values (5, 7, and 9) were evaluated. Figure 2 shows the effects of the pH based on the percentage of biodegradation. As shown, the pH is a critical factor for MB biodegradation. It has been suggested that microbial cells are significantly affected by the pH of their immediate environment because they apparently have no mechanism for adjusting their internal pH [34].

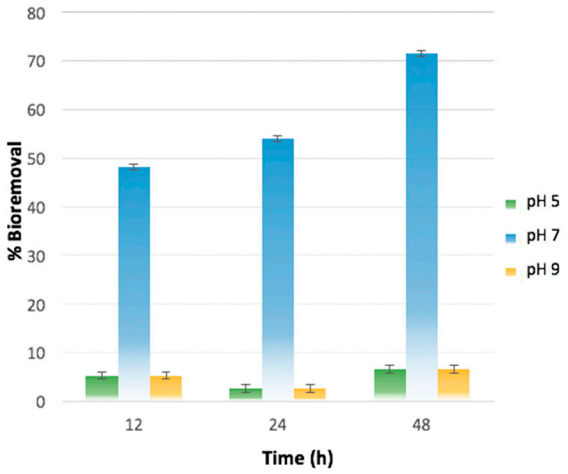

Figure 2. Effect of the pH on the biodegradation of methylene blue (MB) dye at the initial MB concentration (50 ppm), and at the temperature of 35 °C.

The pH of dyes discharged in wastewater varies greatly as a result of their pH dependent nature. It is well known that the interaction between sorbate and sorbent is also affected by the pH of an aqueous medium in two ways: firstly, dyes are complex aromatic organic compounds having different functional groups and different ionization potentials at varying pH, which generates a net charge that depends on the pH of the solution. Secondly, the surface of the biosorbent consists of biopolymers with many functional groups, so the net charge on biosorbent is also pH dependent. Therefore, the interaction between dye molecules and biosorbent is a combined result of charges on dye molecules and the surface of the biosorbent [35]. With an increase in pH, the net negative charge of the biosorbent

increases due to deprotonation of different functional groups [35–37]. However, it has been previously reported that *G. geotrichum* can decolorize Brilliant blue G dye at a pH between 5 to 9, while at pH 3 it has a very low activity, probably due to a loss of the active conformation in the enzymes at low pH (protonation of functional groups by hydrogen ions, losing their activity) [38]. Adsorption and enzymatic activity are dependent on the pH [39]. It has been evidenced that the extent of discoloration is influenced by the pH of the media, as well as that the pH affects the color of the solution and the solubility of the dye [40]. For example, Aspergillus fumigatus decolorates 90% of a real textile effluent containing reactive textile dyes including Reactive Black RC, Reactive Yellow HF2-GL, Reactive Blue BGFN, Reactive Black B-150 and Reactive Red A-6BF at values of 3, 78% at pH 5 and 55% at pH 8 [41]. For these reasons, we selected the pH range between 5 and 9 for the study of the optimal pH solution.

At pH 7 a higher bioremoval percentage (71.5%) is reached than at pH 5 and 9 (6% and 12%), respectively. These results are in agreement with other reports; for example, discoloration of mixtures of structurally different dyes at pH 7 and at 30 °C in 24 h by action of *G. geotrichum* MTCC 1360 was reported by Waghmode et al. [23]. Furthermore, these authors reported 87% discoloration of the azo Rubine GFL dye (50 ppm) at 30 °C, and pH of 7 in 96 h using *G. geotrichum* MTCC 1360, (3.0 g malt extract, 3.0 g yeast extract, 5.0 g peptone, and 10.0 g glucose, per liter) [42].

3.3. Kinetic of the Bioremoval Process

In order to simulate experimental results of MB kinetic removal, we used a pseudo-first-order model according to Equation (2):

$$r = -\frac{dC}{dt} = k_t[C] \quad (2)$$

Here, r is the rate of dye removal, k_t is the constant rate of the process (h^{-1}), [C] is the MB concentration (ppm). Integration of (1) yields:

$$[C] = [C]_o e^{-k_t * t} \quad (3)$$

$$\ln\left(\frac{[C]_t}{[C]_o}\right) = -k_t * t \quad (4)$$

Figure 3a shows the results of the kinetic study and the percentages of removal of MB with an initial concentration of 50 ppm by action of the yeast *G. geotrichum* KL20A at 35 °C. A steep slope is observed for the first 24 h, indicating a faster rate in the removal, while the slope changes to a constant value close to the maximum removal. It is known that the removal is faster at low concentrations of the dye [39], in this case MB, thus in this investigation we used the following conditions for the removal of the MB dye: a temperature of 35 °C and a MB concentration of 50 ppm, using a cell concentration of 3.2×10^7 cells/mL of the yeast. Reports in the literature suggest that the several biological species that are studied from the physicochemical point of view follow a pseudo-first order model [43]. Therefore, using such a model, the regression analysis was performed (Figure 3) and the process rate constant was obtained. Figure 3b shows the results after utilizing the kinetic model (Equation (4)). The value obtained for the kinetic constant of the MB removal process by *G. geotrichum* KL20A at 35 °C was 2.2×10^{-2} h^{-1} corresponding to a half time for biotransformation of 31.2 h. This value of the kinetic constant is considerably higher than that obtained for other species for the degradation of pollutants: (a) the degradation of polyethoxylates by bacteria, which reported a k_t value of 7.2×10^{-3} h^{-1} a half time for biotransformation of 96 h [44]; (b) the removal of disperse blue with Klebsiella sp., yields a k_t value of 1.0×10^{-3} h^{-1} for a reaction time of 160 h [45].

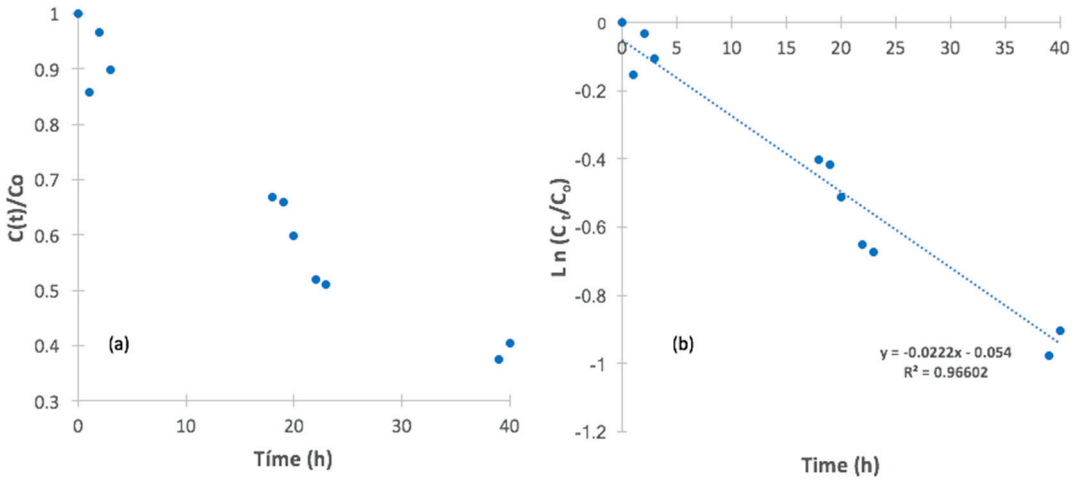

Figure 3. (**a**) Kinetics of bioremediation of methylene blue (MB) at an initial concentration of 50 ppm per action of the yeast *G. geotrichum* KL20A at 35 °C. (**b**) Model L-H on kinetic data of MB removal for the yeast at an initial concentration of 50 ppm, temperature at 35 °C and pH 7.0.

3.4. Citotoxicity Test

The cytotoxicity test based on the hemolytic reaction is a highly reliable test in the determination of the cytotoxicity of extracts that, in one way or another, may come into contact with human beings [46]. It can be observed in Figure 4 that the percentage of hemolysis of the final extracts obtained after the discoloration of MB dye experiments was lower than what was obtained for MB solutions, indicating a lower cytotoxicity property for the extracts as compared to the MB solutions. It is important to assess cytotoxicity of the final extracts, since the metabolites produced from dye degradation are, in many cases, more toxic than the parent dye. For example, the products of the oxidation of indigo blue via electro-incineration and chemical coagulation with $Al_2(SO_4)_3$ are more toxic than the parent dye [31]. It is important to note that the extract without purification procedures (to remove medium and yeast) is much less cytotoxic (less percentage of hemolysis) compared to the original solution of the MB. The metabolites produced from dye degradation are, in many cases, more toxic than the parent dye [39]. Several azo dyes and the amines from their degradation have shown mutagenic responses in Salmonella and mammalian assay systems, and their toxicity depends on the nature and position of the substituents in the molecule [47]. Therefore, it is very important for any bioremediation technology to evaluate the toxicity of the pollutants and metabolites formed after dye degradation in order to study the feasibility of the method [48]. Toxicity should be evaluated using various methodologies with respect to phytotoxicity, ecotoxicity, genotoxicity, mutagenicity, acute toxicity, microbial toxicity, and toxicity on invertebrates. Normally, the test chosen corresponds to those that are more economic or accessible in terms of the time and feasibility to test the samples [39]. However, we did not investigate the mechanism of dye degradation, which will be the subject of a forthcoming study, with the identification of some metabolites produced after the biodegradation process and different toxicity assays, including the oxidative stress response. Nevertheless, the cytotoxicity analysis of the final extracts resulting from the degradation process (without any further purification or extraction process) demonstrated a substantial reduction of the cytotoxicity to human erythrocytes, a result that deserves a deeper investigation, since normally it is the phytotoxicity of the extracts that is evaluated. Phytotoxicity of the final extracts has been observed to decrease for *G. geotrichum* bioremediation processes. For example, a consortium-GB containing two microorganisms, *G. geotrichum* MTCC 1360 and Bacillus sp. VUS, was able to degrade the sulfur-containing dye Brilliant Blue G optimally at pH 9 and at 50 °C, and the phytotoxicity test revealed the nontoxic nature of the metabolites resulting from the degradation process. In another study, *G. geotrichum* MTCC 1360, showed 88% ADMI (American Dye Manufacturing Institute) removal of the mixture of structurally different dyes

(Remazol red, Golden yellow HER, Rubine GFL, Scarlet RR, Methyl red, Brown 3 REL, Brilliant blue) (70 mg L^{-1}) within 24 h at 30 °C and pH 7.0 under shaking conditions (120 rpm). The phytotoxicity study indicated the conversion of the complex dye molecules into simpler oxidizable products having a less toxic nature.

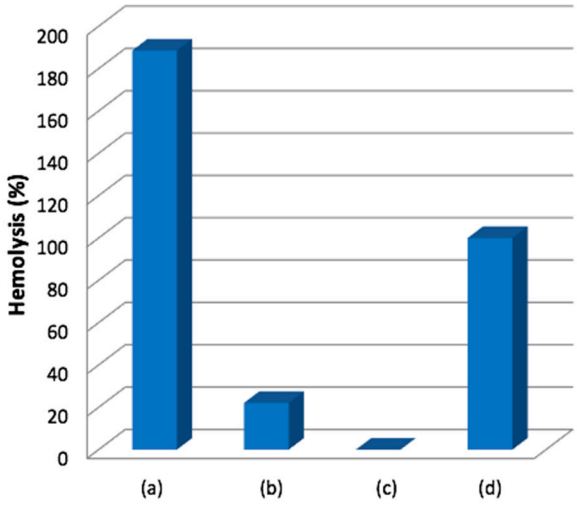

Figure 4. Comparison of hemolytic activity (%) to: (**a**) methylene blue (MB), (**b**) yeast extracts obtained from MB removal tests, (**c**) negative control (phosphate-buffer-treated erythrocytes, PBS) and (**d**) positive control (Triton-X-100).

4. Conclusions

The removal of the MB dye at different initial loads (50 ppm, 100 ppm, and 200 ppm) was evaluated using a yeast strain *G. geotrichum* KL20A with an initial concentration of 3.2×10^7 cells/mL for a reaction time of 48 h at 25, 30, and 35 °C. Results indicated that the increasing temperature in the study range had a positive effect on the removal process; 76.6% removal was obtained at the highest temperature of 35 °C, with a dye concentration of 50 ppm. On the other hand, the MB concentration had an adverse effect on the removal process. The highest removal percentage was obtained at the lowest MB concentration, as other authors have already demonstrated; this is presumed to be due to intoxication of the microorganism once the concentration increases. The study of kinetics allowed us to calculate the value of the kinetic constant of the MB removal process by *G. geotrichum* KL20A at optimal conditions. The kinetic study yielded a rate constant 2.2×10^{-2} h^{-1} and a half time for biotransformation of 31.2 h. The hemolytic reaction test is considered acceptable and favorable, since the hemolytic activity (% hem) was the lowest percentage reported for the positive control and for the initial MB. It is important to note that the final sample of MB obtained after the yeast treatment is much less cytotoxic (less% hemolysis) compared to the initial MB solution without any yeast treatment, which demonstrated that *G. geotrichum* KL20 was effective to remove MB with a substantial reduction of the cytotoxicity to human erythrocytes.

All of the results confirmed that *G. geotrichum* KL20 can be used for the removal of effluents with MB dye loads, but it would be valuable in future studies to compare the concentrations used here with the concentrations of MB in real streams of wastewater.

Author Contributions: Conceptualization, M.C., C.D.G.-T., W.V. and C.C.-L.; methodology, M.C., C.D.G.-T. and W.V.; validation, M.C., C.D.G.-T. and W.V.; formal analysis, M.C.,C.D.G.-T. and W.V.; M.C.,C.D.G.-T. and W.V.; C.D.G.-T.,W.V. and C.C.-L.; data curation, M.C.,C.D.G.-T. and W.V.; writing—original draft preparation, M.C.,C.D.G.-T. and W.V.; writing—review and editing, M.C.,C.D.G.-T.,W.V. and C.C.-L.; visualization, X.X.; supervision, C.D.G.-T. and W.V.; project administration, C.D.G.-T. and W.V.; funding acquisition, C.D.G.-T.,W.V. and C.C.-L.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

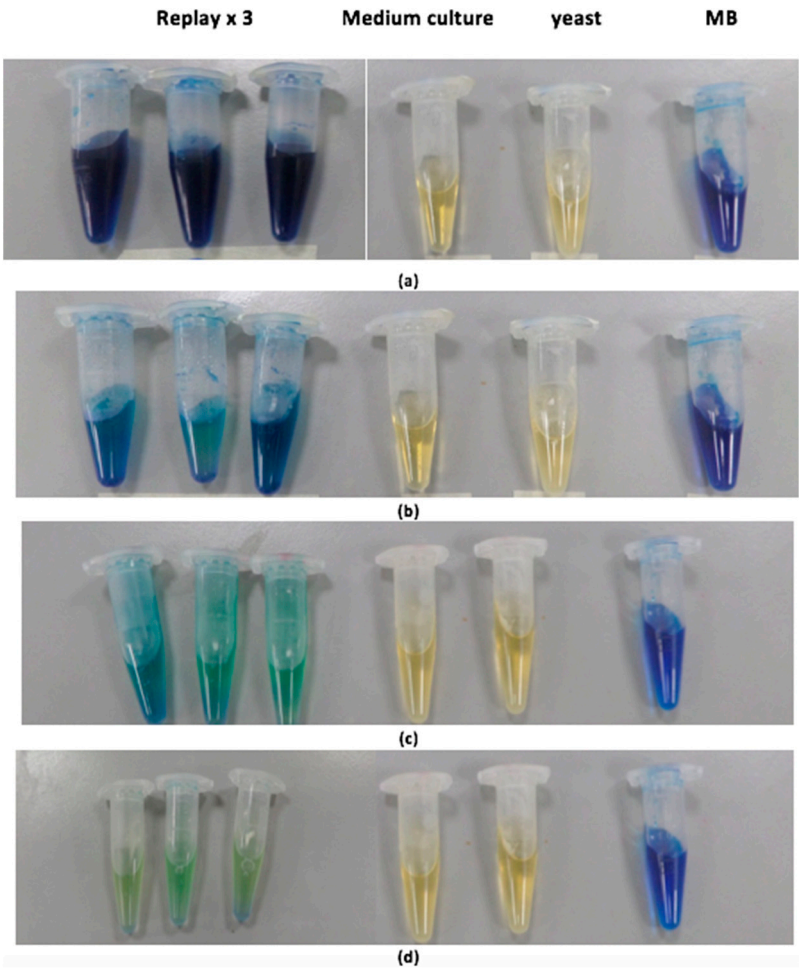

Figure A1. Tests of biodegradation of MB dye by the action of the yeast *Galactomyces geotrichum* KL20A. (**a**) Time zero hours, (**b**) Time 12 h, (**c**) Time 24 h, (**d**) Time 48 h, (MB concentration was 50 ppm and temperature was 30 °C). The images on the left correspond to replays (×3) of the degradation experiments. The images on the right correspond to the controls used in order of yeast, culture medium (YPD-broth).

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