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Decolouration of industrial metal-complex dyes in successive batches by active cultures of *Trametes pubescens*

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ABSTRACT

The decolouration of the metal-complex dyes Bemaplex Navy M-T (150 mg/L) and Bezaktiv Blue BA (150 mg/L) in nine successive batches by immobilised cultures of the white-rot fungus *Trametes pubescens* was studied. Two different types of immobilisation supports were used: the commercial carriers Kaldnes™ K1 (synthetic supports) and sunflower-seed shells (SS) (natural supports). Bemaplex showed more resistance to degradation by *T. pubescens* cultures than Bezaktiv, especially in the K1 cultures. Thus, SS cultures led to decolouration percentages higher than 59% for Bemaplex in all the batches save for the last two and higher than 50% for Bezaktiv in all the batches except for the 2nd and 9th ones. K1 cultures showed decolouration percentages for Bemaplex higher than 42% in batches 1, 3, 4, 5 and 7 and for Bezaktiv higher than 70% in all the batches save for the last one. Dye decolouration was mainly due to enzyme action (biodegradation).

1. Introduction

The volume and composition of the effluents from the textile industry make them to be considered as one of the most polluting amongst all the industrial sectors. Thus, textile effluents are very difficult to treat due to their high content of suspended solids, dyes, salts, additives, detergents and surfactants, high chemical oxygen demand (COD) and high biological oxygen demand (BOD) [2,14]. In addition, most of the dyes used by the textile industry are believed to be toxic and carcinogenic [7].

Traditional technologies include various physical and chemical processes (primary treatments) coupled with a secondary biological treatment (activated sludge). These methods are often ineffective for the treatment of wastewater from the textile industry and a tertiary treatment is required (i.e. ozonation, photochemical processes). These tertiary treatments, however, are very expensive and not always solve the problem of toxicity [24]. This has impelled the search for innovative approaches to treat wastewater from the textile industry. In this regard, the white-rot fungi have been subject of an intensive research in the last years. Such fungi are the most efficient micro-organisms in breaking down synthetic dyes so far. This ability is related to the secretion of extracellular non-specific ligninolytic enzymes, mainly peroxidases and laccases. The latter have been subject of increasing research due to laccases only need molecular oxygen to bring about their catalytic action and produce water as only by-product. This feature renders them as green biocatalysts and, hence, their increasing interest.

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) are multi-copper blue oxidases, which are widely distributed in plants and fungi. They are especially abundant in white-rot fungi. Amongst them, *Trametes pubescens* is considered as a high laccase producer [5] and, consequently, it has been selected to perform the present study. Also cultivation was carried out under semi-solid-sate fermentation conditions, since it stimulates the production of ligninolytic enzymes [19]. This type of fermentation is a sort of solid-state fermentation (SSF) in which the free liquid content has been increased in order to easy the control of fermentation and increase nutrient availability [3,17].

The aim of the present study was to test the ability of *T. pubescens* grown on two different kind of supports to decolourise the recalcitrant metal-complex dyes Bemaplex and Bezaktiv in successive batches. It is important to test the reusability of the fungus for efficient industrial-scale applications. To the best of the

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author knowledge there are no decolouration studies of these dyes by white-rot fungi before this study.

2. Materials and methods

2.1. Micro-organism

T. pubescens MB89 was obtained from the Institute of Applied Microbiology, University of Natural Resources and Applied Life Sciences (Vienna, Austria) and was maintained on Petri plates containing potato dextrose agar (PDA) at 4 °C and sub-cultured every three months.

2.2. Supports

Commercial carriers Kaldnes™ K1 (Anoxkaldnes™, Sweden), made of high density polyethylene (density 950 kg/m^3; surface area 500 m^2/m^3), were used as inert supports. The carriers were autoclaved at 121 °C for 20 min until used.

Sunflower (*Helianthus annuus*) seeds were obtained from a local market and the shells were collected after normal human consumption of the seeds. The sunflower seed shells (SS) were autoclaved at 121 °C for 20 min before use. The chemical composition of the SS according to Gullón et al. [6] is 23 ± 0.15% glucan, 29.4 ± 0.0016% klason lignin, 25.8 ± 0.07% hemicelluloses, 5.40 ± 0.03% extractives and 4 ± 0.15% ash.

2.3. Cultivation conditions

Cultivation was carried out in cotton-plugged Erlenmeyer flasks (250 mL) containing 3 g of Kaldnes™ K1 carriers or 1.5 g SS, according to the experiment, and 20 mL of culture medium. The culture medium composition was the same as that of the medium M1 described in Rodriguez-Couto [16] and consisted of 10 g/L glucose, 20 g/L yeast extract, 0.9 g/L $(NH_4)_2SO_4$, 2 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L $CaCl_2 \cdot 2H_2O$, 0.5 g/L KCl and 0.5 g/L thiamine hydrochloride in 0.05 M citrate–phosphate buffer (pH 4.5). To boost laccase production 0.5 mM Cu^{+2} was added to the cultures on the 3rd cultivation day [18].

Inoculation was carried out directly in the Erlenmeyer flasks. Three agar plugs (diameter, 7 mm), from a 7-day grown fungus on PDA, per Erlenmeyer were used as inoculum. The Erlenmeyer flasks were incubated statically under an air atmosphere at 30 °C and in complete darkness.

2.4. Analytical determinations

Glucose consumption, measured as reducing sugars, was determined with the dinitrosalicylic acid reagent (DNS) using D-glucose as a standard according to the method described by Miller [11].

Laccase activity was spectrophotometrically determined as described by Niku-Paavola et al. [12] with 2,2′-azino-di-[3-ethyl-benzo-thiazolin-sulphonate] (ABTS) as a substrate. One activity unit (U) was defined as the amount of enzyme that oxidised 1 μmol of ABTS per min. The activities were expressed in U/L.

Manganese-dependent peroxidase activity was spectrophotometrically assayed at 468 nm by the method of Kuwahara et al. [9]. The reaction was started by adding 0.4 mM H_2O_2. One activity unit (U) was defined as 1 μmol of 2,6-dimethoxyphenol oxidised per minute and the activities were expressed in U/L.

Lignin peroxidase activity was spectrophotometrically determined at 310 nm according to Tien and Kirk [22]. The reaction was starting by adding 0.4 mM H_2O_2. One activity unit (U) was defined as 1 μmol of veratryl alcohol oxidised in 1 min and the activities were reported as U/L.

2.5. Decolouration of dyes in successive batches

The dyes used were the textile dyes Bemaplex Navy M-T (CI Acid Blue 193), an acid chromium-complex dye and Bezaktiv Blue BA (CI Reactive Blue 235), a reactive copper-complex dye. They were a kind gift of CTH R. Beilich GmbH (Barcelona, Spain). They were used as received, without further purification. Stock solutions in distilled water (0.1% w/v) were prepared and stored in the dark. In Fig. 1 the UV–vis spectra of the aqueous solutions of both dyes (150 mg/L) can be seen.

The dyes were aseptically added to *T. pubescens* cultures on the 5th cultivation day. The final concentration of the dyes in the flasks was 150 mg/L. Samples were taken at the beginning of the process and at determined intervals, centrifuged (8000 × *g*, 5 min) and the residual dye concentration was spectrophotometrically measured from 500 to 700 nm and calculated by measuring the area under the plot. This approach takes into account the conversion of the dye molecules to other compounds absorbing at different wavelengths and then, the ratio of the area under the visible spectrum is always equal or lower than the ratio of the absorbances at the peak. Dye decolouration was expressed in terms of percentage. Three control tests were conducted in parallel: biotic controls (without dye), abiotic controls (without fungus) and heat-killed cultures. The latter consisted of fungal cultures autoclaved on the 5th cultivation day and performed under conditions identical to those of the experimental cultures. Nine successive decolouration batches were performed. At the end of each batch, the decolourised medium was removed and 20 mL of fresh medium plus dye was added, except for the last two batches in which only dye solution was added to test the applicability of the system under more realistic conditions.

Duplicate experiments were run for comparison and the samples were analysed at least twice.

3. Results and discussion

3.1. Glucose consumption

In Fig. 2A glucose consumption, measured as reducing sugars, in both K1 and SS cultures is depicted. At the beginning of the SS cultivation, there was an initial increase in reducing sugars from the initial value (10.1 g/L) to around 12.7 g/L on day 4 (Fig. 1A), which was likely due to the release of some compounds contained in the SS after autoclaving. Then, glucose abruptly decreased until day 8 and from here onwards it was maintained at residual levels

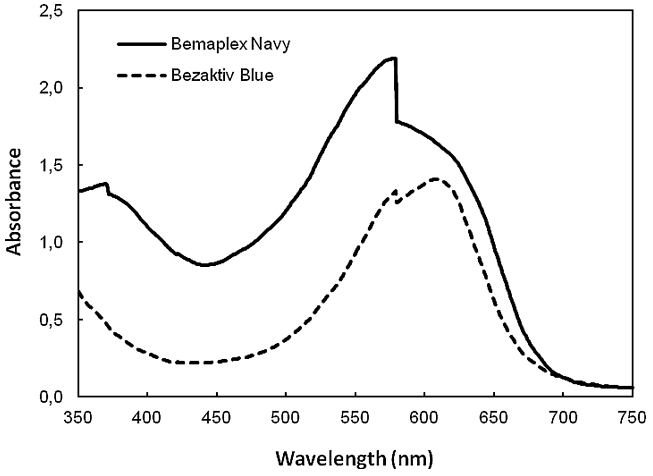

Fig. 1. Visible spectra of the aqueous solutions of the metal-complex dyes Bemaplex Navy M-T (150 mg/L) and Bezaktiv Blue BA (150 mg/L).

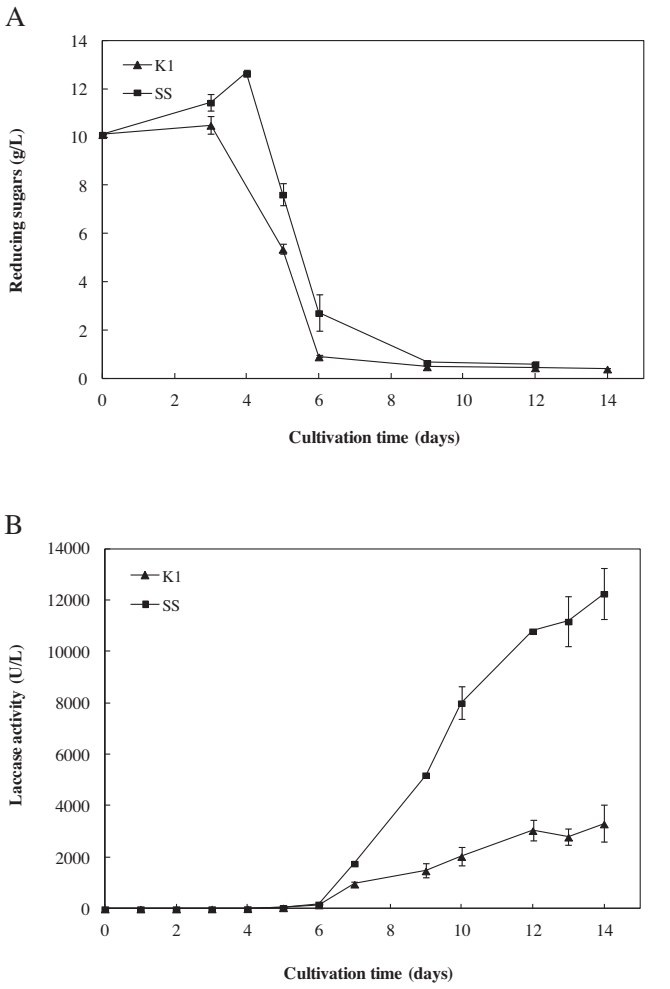

Fig. 2. Glucose evolution (A), measured as reducing sugars, and laccase production (B) by semi-solid-state cultures of *T. pubescens* grown on sunflower-seed shells (−■−) and Kaldnes™ K1 carriers (−▲−).

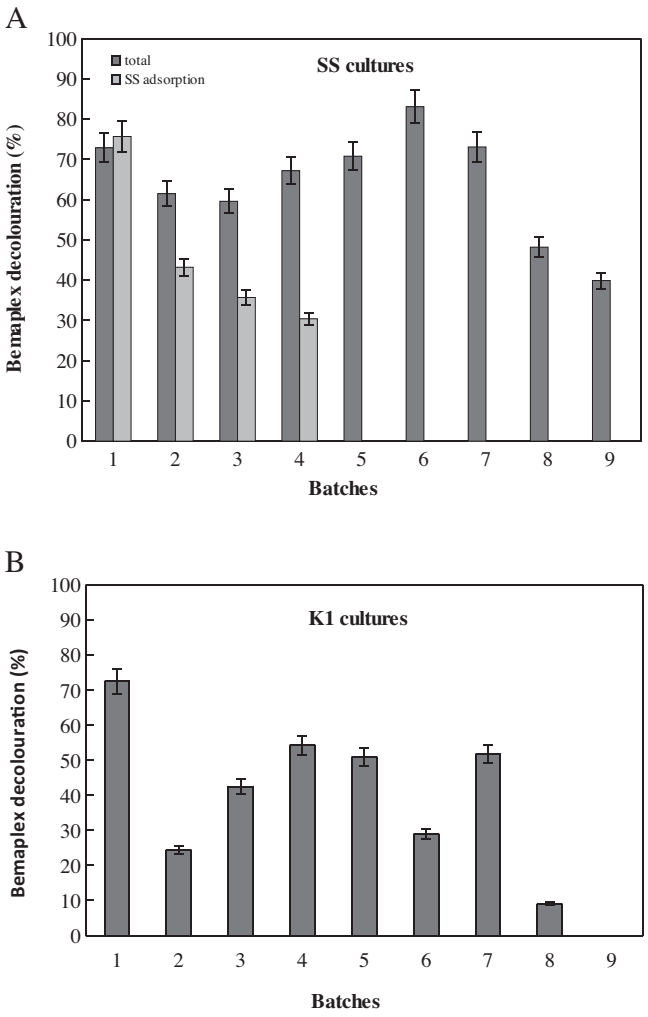

Fig. 3. Decolouration of the metal-complex dyes Bemaplex Navy M-T in nine successive batches by semi-solid-state cultures of *T. pubescens* grown on (A) sunflower-seed shells and (B) carriers Kaldnes™ K1. Each batch consisted of fresh medium plus dye (150 mg/L) and lasted 24 h except for the last two batches which consisted only of an aqueous solution of the dye (150 mg/L) and lasted 96 h.

of around 0.6 g/L. As for K1 cultures, glucose steeply decreased from day 3 to 6 and from here onwards it was maintained at residual levels of around 0.4 g/L.

3.2. Laccase production

Laccase enzymes were the only enzymes detected in the culture broth of both cultivations. They were produced after glucose consumption (Fig 2A), i.e. during the secondary metabolism. As shown in Fig. 2B SS cultures led to much higher laccase activities than K1 ones. Thus, SS cultures exhibited activities higher than 10,000 U/L from day 12 onwards whereas K1 cultures showed activities around 3000 U/L for the same cultivation days (Fig. 2B). The stimulation of laccase activity by lignin-based supports has already been reported by different researchers [10,15,20].

3.3. Dye decolouration

3.3.1. Decolouration of Bemaplex Navy

In Fig. 3A the decolouration of Bemaplex Navy (150 mg/L) by SS cultures of *T. pubescens* is presented. In the first four batches the decolouration was due to two phenomena: adsorption onto support (i.e. SS) and fungal action (biosorption and biodegradation by laccase enzymes) and from the 5th batch onwards decolouration was only due to fungal action because the support was saturated in dye. The last two batches (8th and 9th), consisting only of an aqueous solution of the dye (150 mg/L, final concentration in the flasks), were also decolourised to a significant extent. Thus, a decolouration percentage around 50 and 40% was attained for the 8th and 9th batches, respectively, in 96 h.

As for K1 cultures of *T. pubescens* (Fig. 3B) the dye was not adsorbed onto the support (i.e. K1 carriers), so the decolouration was only due to fungal action. Decolouration percentages higher than 40% were attained except for the 2nd and 6th batches. Surprisingly, contrary to the SS cultures, the batches containing only dye (8th and 9th) were not decolourised or hardly decolourised by K1 cultures.

3.3.2. Decolouration of Bezaktiv Blue

The dye Bezaktiv Blue showed less resistance to degradation by *T. pubescens* cultures than the dye Bemaplex Navy. Thus, as shown in Fig. 4A total dye decolouration was achieved in the 7th batch by SS cultures of *T. pubescens*. As in Bemaplex decolouration, in the first four batches the decolouration was due to two phenomena: adsorption onto the support (i.e. SS) and fungal action and from the 5th batch onwards decolouration was only due to fungal action. The last two batches (8th and 9th), which consisted only of an aqueous solution of the dye (150 mg/L, final concentration in the flasks), also showed significant decolouration. Thus, a

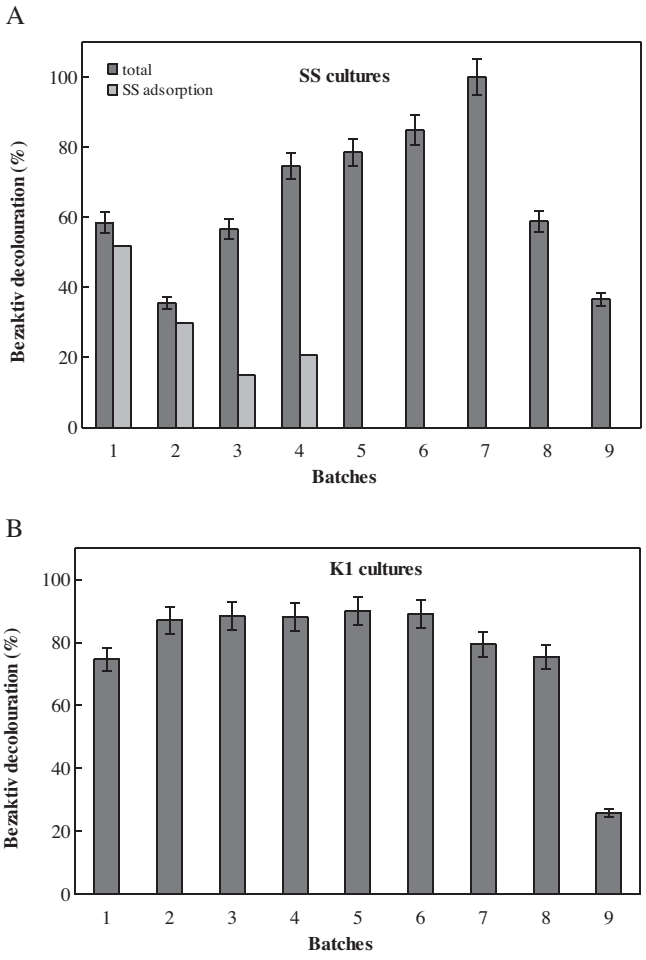

Fig. 4. Decolouration of the metal-complex dyes Bezaktiv Blue BA in nine successive batches by semi-solid-state cultures of *T. pubescens* grown on (A) sunflower-seed shells and (B) carriers Kaldnes™ K1. Each batch consisted of fresh medium plus dye (150 mg/L) and lasted 24 h except for the last two batches which consisted only of an aqueous solution of the dye (150 mg/L) and lasted 96 h.

decolouration percentage around 59 and 37% was attained in the 8th and 9th batches, respectively, in 96 h (Fig. 4A).

As for the K1 cultures, high decolouration percentages were attained in all the batches (between 74 and 90%) except for the last one (Fig. 4B). Surprisingly, these decolouration percentages are higher than that obtained by SS cultures. This is likely due to differences in the isoenzymatic complex secreted by *T. pubescens* when grown under different conditions. This shows that dye affinity is different for different isoenzymatic complexes, underlining the influence of the support on the efficiency of each particular process. Recently, Kumar et al. [8] studied the laccase production and textile effluent decolouration by the white-rot fungus *Coriolus versicolor* immobilised on different supports under SSF conditions and found that the characteristics of the supporting material played an important role in both decolouration and laccase activity. Amongst the different supports tested, they found that the K carriers led to the highest laccase production (2600 U/L on the 14th cultivation day) and effluent decolouration (73% on the 12th cultivation day.)

Dye adsorption onto the mycelium of heat-killed controls was observed with the naked eye. However, in living cultures the dyes were adsorbed onto the fungal mycelium (biosorption) and subsequently the dyed mycelium was bleached along cultivation. This was likely due to both extracellular enzymes (i.e. laccases) and intracellular or mycelia-bound enzymes [21,23]. Pazarlioglu et al. [13] also found that biodegradation was the main mechanism of direct azo dye decolouration in living cultures of free and immobilised cultures of the white-rot fungus *Phanerochaete chrysosporium*.

The dyes were not adsorbed onto K1 carriers. However, a considerable amount of dye was adsorbed onto SS and at the end of the 4th batch they were saturated in dye. Therefore, this approach would create the additional problem of the dyed-SS disposal. A possible solution for their disposal would be to use them for the production of laccase enzymes [18]. Another alternative would be to burn the dyed SS to generate power.

It is worth mention that *T. pubescens* was able to decolourise the dye solutions without the addition of nutrients, buffer or redox mediators to a significant extent. This indicates the high degrading ability of this fungus, which was also reported by other authors [1,4]. So in view of these encouraging results, studies on dye decolouration by this fungus under more realistic conditions will be pursued at my laboratory.

4. Conclusions

Semi-solid-state cultures of the white-rot fungus *T. pubescens* have been shown to be very effective to decolourise textile metal-complex dyes in successive batches, even with neither nutrient addition nor pH adjustment. The inert support K1 seemed more suitable for dye decolouration since the dye was not adsorbed onto it. In addition, the support integrity was maintained along fermentation allowing its recovery and re-utilisation. This would make the cost of the overall process more favourable. The utilisation of lignocellulosic supports in dye decolouration by white-rot fungi could be advantageous in countries with a huge amount of lignocellulosic wastes.

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