Simultaneous removal of malachite green and hexavalent chromium by *Cunninghamella elegans* biofilm in a semi-continuous system

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Abstract

The present study was conducted to evaluate the potential of the fungus *Cunninghamella elegans* for simultaneous decolourisation of a triphenylmethane dye malachite green (MG) and hexavalent chromium [Cr(VI)] in the same media. This fungus can degrade MG through its reduction into leucomalachite green and then demethylation followed by oxidative cleavage. Along with MG degradation, *C. elegans* biofilm could effectively and repeatedly remove Cr(VI) from the liquid cultures even in the presence of high concentrations (40 g L^{-1}) of NaCl and various other metal ions. *C. elegans* biofilm was also found to adsorb different dyes (reactive black-5, acid orange 7, direct red 81 and brilliant blue G) concurrently with Cr(VI). Based on its potential for simultaneous removal of dyes and Cr(VI) as well as reusability, *C. elegans* biofilm is envisaged as an efficient bioresource to devise strategies for treatment of wastewaters loaded with multiple pollutants.

Keywords: Immobilization; fungus; dye decolorisation; textile wastewater; adsorption

1. Introduction

Dyes are a common constituent of wastewaters originating from various industrial processes. Malachite green (MG) is a triphenylmethane cationic dye which is used in textile, leather, medical, food and paper industries in addition to its use as a biocide to control protozoan and fungal infections in fish farming (Culp and Beland, 1996; Srivastava et al., 2004). However, discharge of MG-loaded wastewaters into the environment reduces light penetration in the water bodies and affects the living organisms present owing to the carcinogenic, mutagenic and teratogenic properties of MG and its metabolites (Culp and Beland, 1996; Srivastava et al., 2004; Donya et al., 2012). For example, MG is toxic to mammalian cells and has been shown to cause cancer in different organs including liver and thyroid of experimental animals (Rao, 1995; Srivastava et al., 2004; Donya et al., 2012). Leucomalachite green, which is a major metabolite arising from the reduction of malachite green, is also of particular concern owing to its toxicity, mutagenicity and its relatively higher lipophilicity, which result in it being retained in fish muscle and fat (Bilandzic et al., 2012). Despite the fact that MG has been banned in some countries it is still being used in others owing to its low cost, ready availability and high efficacy. In addition to dyes, wastewaters originating from different industries, including textile and leather, have also been found to contain considerable amounts of different salts and metal ions (Tuzen et al., 2008; Ngah and Hanafiah, 2008). The latter are present either from the use of metal complex dyes or metal-containing salts as mordant for better fixation of dyes. Among the metal ions, hexavalent chromium [Cr(VI)] is a common pollutant which co-exists with dyes in the wastewaters originating from textile and leather industries (Desai et al., 2009). It is not only the second most common inorganic contaminant of ground water and hazardous waste sites but also listed by the United States Environmental Protection Agency among the 17 chemicals for posing the greatest threat to human health (Horton et al., 2006; Cheung and Gu, 2007; Quintelas et al., 2008). In addition to disruption of biochemical and physiological functions in bio-systems owing to its strong oxidizing nature, high solubility in water and rapid permeability, it has also been reported to harbor mutagenic, carcinogenic and teratogenic properties (McLean and Beveridge, 2001; Ilias et al., 2011). Hence, the co-existence of Cr(VI) and synthetic dyes, including malachite green, in wastewaters is a matter of serious concern and there is a need to find effective, innovative and economic treatment technologies to eliminate them or minimize their quantity in the environment.

Exploitation of microorganisms for bioremediation of contaminated environments has attracted attention as a cost-effective and environmentally friendly approach. Several

researchers have isolated and characterized various bacterial and fungal strains for removal and detoxification of chromium in soil and water resources (Prigione et al., 2008; Dhal et al., 2010; Ilias et al., 2011; Essahale et al., 2012; Maqbool et al., 2015). Similarly, a number of bacterial strains belonging to different genera have been isolated and characterized for decolourisation of MG (Li et al., 2009; Kalyani et al., 2012). The potential for decolourisation and degradation of this dye has also been reported in various fungi including *Phanerochaete chrysosporium, Cyathus bulleri, Cyathus stercoreus, Cyathus striatus*, and *Penicillium ochrochloron* (Vasdev et al., 1995; Jadhav and Govindwar, 2006; Shedbalkar and Jadhav, 2011; Jasinska et al., 2012). The non-lignolytic fungus *Cunninghamella elegans* is well known for its ability to transform a broad range of xenobiotics (Murphy, 2015) and the inactivated biomass of the fungus is an effective biosorbent (Tigini et al., 2010). Cha et al. (2001) observed the formation of leucomalachite green, *N*-demethylated and *N*-oxidized metabolites upon incubation of *C. elegans* with the MG. Microsomal fractions also catalysed the production of leucomalachite green and *N*-demethylated metabolites, and the biotransformation was inhibited by 1-aminobenzotriazole, metyrapone and SKF 525-A, thus it was reasoned that the reduction and *N*-demethylation reactions were catalysed by cytochrome P450. Kim et al. (2010) purified a cytochrome c, CeCyt, from the mitochrondria of *C. elegans*, that catalysed the decolourisation of malachite green and suggested that the protein functions to reduce malachite green under conditions of oxidative stress.

Whilst there are some reports on the simultaneous removal of different dyes and Cr(VI) from synthetic textile wastewaters by using some multifunctional bacterial strains (Desai et al., 2009; Mahmood et al., 2013; Anwar et al., 2014; Maqbool et al., 2016), to the best of our knowledge, simultaneous microbial removal of MG and Cr(VI) has not yet been the focus of any study. Moreover, there is no report regarding the application of fungal strains for such simultaneous removal of dyes and metal ions. In this context, the present study has been conducted for simultaneous removal of MG and Cr(VI) by using *C. elegans*. Biofilms of this fungus have already been reported to demonstrate improved biotransformation of drugs and xenobiotics compared with suspended cells (Amadio et al., 2013; Mitra et al., 2013; Quinn et al., 2015). The aim of this study is to extend the possible application of the fungal biofilm to the bioremediation of dye/metal contaminated wastewater.

2. Materials and Methods

2.1 Dyes

Malachite green (technical grade) was acquired from BDH (Poole, UK), reactive black-5 and direct red-81 were purchased from Santa Cruz Biotechnology (Dallas, TX, USA), and acid orange-7 (≥ 85 %) and brilliant blue G were obtained from Sigma Aldrich (Arklow, Ireland).

2.2. Cultivation of *C. elegans* biofilm and planktonic cells

Cunninghamella elegans DSM 1908 was grown on sabouraud glucose agar for 120 h at 28 °C. Inoculum was prepared by homogenizing one plate of agar and mycelia in 100 mL of 0.8% autoclaved saline. The planktonic cell cultures were grown in 250 mL Erlenmeyer flasks containing 45 mL of sterilised sabouraud dextrose broth and 5 mL of *C. elegans* homogenate. For cultivating biofilms the method described by Amadio et al. (2013) was followed. For biofilm cultivation, stainless steel compression springs (1.2 mm, T316 wire, Shannon Coiled Springs, Ireland) were placed at the bottom of 250 mL Erlenmeyer flasks containing sterilized sabouraud dextrose broth (49 mL). The springs were kept completely in contact with the inner walls of the flasks for optimum biofilm growth. Each flask was inoculated with *C. elegans* homogenate (1 mL) and incubated for 72 h with rotary agitation (150 rpm) at 28 °C.

2.3. Decolourisation of MG by *C. elegans* biofilm and planktonic cells

After 72 h of biofilm growth, the medium in the flasks was replaced with 50 mL sterile MG aqueous solution (80 μM or 29 mg L^{-1}, unless stated) and incubated with shaking (150 rpm) at 28 °C, alongside an un-inoculated control. When the MG was degraded and both the supernatant and biofilm had been decolourized, fresh MG was added to the flasks. For estimation of decolourisation by planktonic cultures, the cells were harvested by centrifuging (3500 rpm for 15 min) and the biomass was re-suspended in 50 mL of the aqueous MG solution and incubated as before; for the biofilm cultures, the supernatant was decanted and replaced. The supernatants (1.5 mL) and biomass (200 mg) of biofilm and planktonic cells were collected aseptically at regular intervals. Malachite green decolourisation in the supernatant was determined spectrophotometrically as previously described (Jasinska et al., 2012) by measuring the change in absorbance at 617 nm (λ_{max}). The biomass was immersed in 1 mL of methanol and shaken vigorously for 30 s. The methanol extract was then used to determine malachite green decolourisation (Nanodrop 1000). To recycle biofilms, the supernatants were decanted directly and fresh aqueous dye solution was added. Planktonic cells were harvested by centrifuging and the biomass re-suspended in 50 mL of fresh dye.

The biofilms and the planktonic cells were both rejuvenated by replacing the supernatants with 50 mL of fresh sabouraud dextrose broth and incubated for up to 16 h.

2.3.1. Effect of pH on MG decolourisation by *C. elegans* biofilm

To determine the effect of pH on MG decolourisation, biofilm was cultivated as described and incubated for 48 h with dye dissolved in water (50 mL). The supernatant was decanted and replaced by 50 mL of 20 mM phosphate buffer (pH 3, 5-7) or 2-(*N*-morpholino) ethanesulfonic acid (pH 4) containing malachite green; the pH experiments were conducted with the same biofilm, starting with pH 7 and ending at pH 3.

2.3.2. Metabolite identification

The degradation products of malachite green were extracted from the biomass by incubating it with 50 mL of ethyl acetate for 3 hours. The organic layer was evaporated to dryness, the residue redissolved in 1 mL ethyl acetate and analysed by gas chromatography-mass spectrometry (GC-MS) using a method similar to that described by Du et al. (2011). Samples (1 μL) of the extract were injected in the splitless mode onto a HP5MS column (30 m × 0.25 mm × 0.25 μm). The oven temperature held at 120 °C for 2 min and then increased to 300 °C at 10 °C min^{-1}. The metabolites were identified by retention time and mass spectra.

2.4. Simultaneous removal of dye and Cr(VI) by *C. elegans* biofilm and planktonic cells

C. elegans biofilm and planktonic cells were prepared as described in section 2.1 and incubated under the standard conditions with either MG (80μM) or Cr(VI) (20 mg L^{-1}) only, or a combination of both dye and metal. Decolourisation was monitored spectrophotometrically and Cr(VI) removal was assessed following the diphenyl carbazide (DPC) method described by Maqbool et al. (2016). In order to test the reusability of the biofilms for MG decolourisation and/or Cr(VI) removal, the aqueous solutions of dye and/or metal were continuously replaced with the fresh solutions after >95% of the pollutants were eliminated from the supernatant. The biofilms were rejuvenated after every three cycles of decolourisation by replacing the supernatants with 50 mL of fresh sabouraud dextrose broth and incubating for up to 16 h under shaking (150 rpm) at 28ºC.

To evaluate the ability of *C. elegans* biofilms to decolourise other dyes (reactive black-5, acid orange-7, direct red-81 & brilliant blue G) concurrently with Cr(VI) removal, triplicate biofilms were separately incubated with aqueous solutions containing 20 mg L^{-1} of Cr(VI) and 50 mg L^{-1} one of the selected dyes. Triplicate un-inoculated controls were also

incubated for each treatment. Decolourization of the dyes in the supernatant was monitored spectrophotometrically at 597 nm (reactive black-5), 485 nm (acid orange-7), 540 nm (direct red-81) and 595 nm (brilliant blue G).

2.3.1. Impact of initial Cr(VI) concentration on removal efficiency

Triplicate biofilms were incubated with aqueous solutions of MG (80µM) and varying concentrations (20 mg L^{-1}, 40 mg L^{-1}, 60 mg L^{-1}, 80 mg L^{-1}, 100 mg L^{-1}, 150 mg L^{-1}) of Cr(VI). Triplicate un-inoculated flasks for each treatment were also incubated as controls. The decrease of both pollutants in the supernatants was measured as described previously. The re-usability of biofilms following rejuvenations at varying initial Cr(VI) concentrations was also evaluated.

2.4.2. Impact of NaCl and metal ions on simultaneous removal of MG and Cr(VI) by *C. elegans* biofilm

Biofilms were incubated with aqueous solutions containing MG (80µM) and Cr(VI) (20 mg L^{-1}) plus varying concentrations (up to 100 g L^{-1}) of NaCl. The presence of 20 mg L^{-1} various metal ions (Ag^{+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Ni^{2+}, Ba^{2+}, Fe^{3+}) on simultaneous removal of both pollutants was similarly investigated. Triplicate un-inoculated flasks for each treatment were also incubated as controls.

2.5 Statistical analysis

The results are presented as means ± standard deviation. The means were compared using Least Significance Difference (LSD) test after the analysis of variance (ANOVA) at $p \leq 0.01$ using the software R (3.4.1).

3. Results

3.1. MG decolourisation by *C. elegans*

MG was decolourised by *C. elegans* biofilm and planktonic cultures. Over the first 6 h incubation in the biofilm culture, the colour in the supernatant had decreased by approximately 60 % and, after 24 h incubation, almost a complete (> 95%) removal of colour was observed in the supernatant (data not shown). Upon the second addition of dye to the flasks the supernatant and biomass were monitored spectrophotometrically at different time points (Fig 1). The dye was removed from the supernatant within 15 min by absorption to the biomass (Fig 1A); the colour in the biomass dissipated more slowly (Fig 1 B and C). The

biomass of both the biofilm and planktonic cultures gave almost a similar pattern of colour removal from the supernatant and absorbance over the incubation period. Thus, rapid initial decolourisation of the supernatant through biosorption was followed by a slower biodegradation of the MG dye.

In order to study the impact of decreasing pH on decolourisation of MG by *C. elegans* biofilm, the decolourisation experiments were carried out at pH from 7 to 3. The cultures incubated at pH values from 4 to 7 were found to decolourise more than 95% of the initially added MG in the supernatants within the first 24 hours. However, only 80% decolourisation of the supernatant was observed in the same period with cultures at pH 3 (Supplemental Information). Furthermore, the time for complete decolourisation (i.e. supernatant and biomass) of MG by the cultures increased as pH was lowered.

3.2. Assessment of biodegradation of MG by *C. elegans*

The biomass from biofilm cultures incubated with 80 μM malachite green was extracted with ethyl acetate and the extractable metabolites were analyzed by GC-MS. The GC-MS analysis revealed the presence of leucomalachite green, *N*-demethylated metabolites, 4-(dimethylamino) benzophenone and aminobenzophenone (Table 1). The presence of these metabolites suggests a stepwise demethylation followed by oxidative cleavage as previously suggested by Cha et al. (2001). Interestingly, upon subsequent dye addition to the biofilm, no metabolites were detectable by GC-MS after 24 h incubation, indicating complete biodegradation.

3.3. Semi-continuous biofilm-catalyzed simultaneous removal of MG and Cr(VI)

3.3.1. Simultaneous removal of MG and Cr(VI) by *C. elegans* biofilm and planktonic cells

C. elegans biofilm and planktonic cultures were tested for their potential not only to remove MG and Cr(VI) individually but also for simultaneous removal of MG and Cr(VI) in the same solution. The data are summarized in Table 2 and show that MG removal was comparable in planktonic and biofilm cultures whether in the absence or presence of Cr (VI), with approx. 80 % decolourisation within 16 h and complete degradation within 22-26 h. Planktonic cultures were more effective at Cr(VI) removal than biofilm, with 83 % removed in 16 h compared to 71 %, and a shorter time required for complete removal (22 h compared with 24 h). However, whereas the efficiency of biofilm was not significantly impacted with

the combination of dye and metal, the removal of Cr (VI) in planktonic cultures after 16 h decreased noticeably compared with the cultures incubated with the metal only.

One of the main potential advantages of employing the biofilm is the ease of re-usability, which was demonstrated in these experiments, showing that complete (> 95 %) removal of dye and metal was possible for at least 19 repeated additions (Table 2). Planktonic cultures are more difficult to recycle, as a centrifugation (or filtration) step is necessary, and the suspended cells have previously shown to cease functioning after approx. three cycles (Amadio et al., 2013).

3.3.2. Impact of initial Cr(VI) concentration on biofilm efficiency

Varying initial concentrations of Cr(VI) had an impact on the simultaneous removal of MG and Cr(VI) by the *C. elegans* biofilm (Figure 2). After 16 h incubation, over 90% of the initially added MG was decolourized in the solutions containing Cr (VI) concentrations up to 60 mg L^{-1}; however, higher concentrations of the metal resulted in a decrease in decolourisation ability. The total amount of Cr (VI) removed within the same period in these experiments increased from 0.91 mg, when an initial concentration of 20 mg L^{-1} was used, up to 1.95 mg when the initial Cr (VI) concentration was 60 mg L^{-1}. At higher concentrations the removal progressively declines. Notably, increasing the initial concentrations of Cr(VI) also resulted in an increase in the time required for complete (>95%) simultaneous removal of Cr(VI) and MG, and a decrease in number of cycles of complete simultaneous removal of both the pollutants (Table 3).

3.3.3. Effect of NaCl and other metals on biofilm efficiency

Simultaneous removal of MG and Cr(VI) by the *C. elegans* biofilm was not substantially affected by NaCl concentrations of 20 g L^{-1} (Fig 3 and Table 3); however, at higher concentrations the removal efficiency after 16 h, the time required for complete removal and the number of cycles of complete dye/metal removal were all affected. Fig 4 shows the effect of a selection of metal ions (2 mM) on the simultaneous removal of dye and Cr(VI). Most of the metals tested inhibited the removal of both pollutants to some degree, although complete (>95 %) removal was still achieved within 40 h in all experiments.

3.4. Simultaneous removal of Cr(VI) and other dyes by *C. elegans* biofilm

The ability of *C. elegans* biofilm for simultaneous removal of Cr(VI) and other dyes (Reactive Black 5, Acid Orange 7, Direct Red 81 and Brilliant Blue G) was also examined.

This biofilm showed a good potential for parallel removal of Cr(VI) and different dyes from the culture supernatant (Table 4). After 40 h incubation, a complete (>95%) removal of Cr(VI) was observed along with at least 85 % simultaneous removal of the initially added dye. The dyes were biosorbed by the biofilms, but, unlike MG, the biomass was not completely decolourized, even after 120 hours incubation.

4. Discussion

Environmental pollution due to synthetic textile dyes is one of the leading contributors in degradation of natural resource. This negative impact of synthetic dyes is intensified when these dyes loaded effluents are also accompanied by the presence of different pollutants. Hexavalent chromium [Cr(VI)] is one of such pollutants which has often been found to co-exist as a contaminant with synthetic dyes in textile and tanneries effluents. Hence, there is need to devise the strategies for concurrent removal of such co-existing pollutants and the present study was conducted to evaluate the potential of *C. elegans* biofilm for simultaneous removal of a synthetic dye, malachite green (MG), and Cr(VI) in a semi-continuous system.

The decolourisation of MG in planktonic and biofilm cultures occurred following a similar pattern, with the dye rapidly adsorbed by the biomass, followed by a slower biodegradation step, resulting in complete removal of 80 µM dye in 24 h. This pattern of decolourisation with initial biosorption followed by degradation has also been observed in other studies that focused on fungal biodegradation of MG (Jadhav and Govindwar, 2006; Jasinska et al., 2012). It was observed here that the time taken for dye decolourisation by *C. elegans* biofilm upon initial addition of MG was longer compared to the subsequent rounds, in contrast to planktonic cultures. One possible reason for this difference is that in the biofilm there are specific genes required for decolourisation that are induced upon dye addition, but in planktonic cells the genes are already expressed. Transcriptomic and proteomic analyses of other fungi demonstrate that expression of genes can vary between planktonic and biofilm cultures (Gutierrez-Correa et al., 2012).

In general biofilms are stable and active over long periods (Halan et al., 2012) and *C. elegans* biofilms have been shown to be conveniently reused for biotransformations (Amadio et al., 2013; Quinn et al., 2015), thus they have potential for application in continuous or semi-continuous processes. The *C. elegans* biofilm can decolourize MG over a range of acidic pH, which is an added advantage; however, at pH 3 decolourisation ability is compromised. This is comparable with the decolourisation activity of some other fungal

297 strain including *Penicillium ochrochloron*, which is completely inhibited at pH 3 (Shedbalkar
298 and Jadhav, 2011).
299 Leucomalachite green, demethylated leucomalachite green, 4-(dimethylamino)
300 benzophenone and aminobenzophenone were observed as intermediate metabolites during
301 initial decolourisation of MG by *C. elegans* biofilm. Cha et al. (2001) identified mono-, di-,
302 and tri-demethylated derivatives of malachite green and leucomalachite green after
303 decolourisation by suspended *C. elegans* ATCC 36112. The other metabolites detected in the
304 present study had not previously been identified from the fungus, but are known
305 intermediates in the biodegradation of malachite green in other microorganisms. For
306 example, 4-(dimethylamino) benzophenone and 4-aminobenzophenone were observed during
307 degradation of MG by *Micrococcus* sp. strain BD15 (Du et al., 2013). 4-(Dimethylamino)
308 benzophenone has also been detected during decolourisation of MG by *Shewanella*
309 *decolourationis* NTOU1 under anaerobic conditions (Chen et al., 2010).
310 *C. elegans* biofilm as well as planktonic cultures were shown here for the first time to
311 simultaneously remove MG and Cr(VI) from contaminated water. Although bacterial
312 cultures have been reported to concurrently remove Cr(VI) and different azo-dyes (Maqbool
313 et al., 2016; Anwar et al., 2014; Mahmood et al., 2013), to the best of our knowledge, there is
314 no report of the simultaneous removal of MG and Cr(VI) by any single microbial strain.
315 Furthermore, the biofilm can tolerate the presence of Cr(VI) up to 60 mg L^{-1}, and up to 40 g
316 L^{-1} NaCl, but is sensitive to higher concentrations of both, resulting in longer times for
317 complete removal of dye/metal and a reduction in the number of times the biofilm can be re-
318 used. Simultaneous removal of dye/metal by the biofilm is possible even in the presence of
319 metal ions, such as silver and copper, albeit at a slower rate.
320 There are numerous reports of immobilized fungi applied to the decolourisation of
321 dye-contaminated water (Couto, 2009), but only a handful of these concern MG, and none
322 that also involve Cr(VI) removal. Barapatre et al. (2017) reported MG decolourisation in
323 *Aspergillus flavus* and demonstrated that immobilization on a number of inert materials, such
324 as polyurethane foam and clay brick, resulted in improved decolourisation compared with
325 suspended culture. However, no experiments to investigate the recycling of the immobilized
326 fungus were done. In the present study, *C. elegans* was immobilized as a biofilm, which
327 enabled repeated use (at least 19 cycles of dye/metal removal), which is attractive for
328 bioremediation applications. Furthermore, a screen of other dyes demonstrated that the
329 biofilm could biosorb these also, thus expanding its potential for remediation of dye-
330 contaminated water.

Conclusion

Based on the findings of this study, it can be concluded *C. elegans* biofilm might serve as potential bioresource to devise the strategies for simultaneous removal of Cr(VI) and MG even in the presence of NaCl and metal ions that are characteristically present in real textile and tanneries effluents. Confirmation of the re-usability of this biofilm is an important feature for its potential use in wastewater treatment processes, which require continuous operation.

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Conflict of interest

None

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466

Figure legends

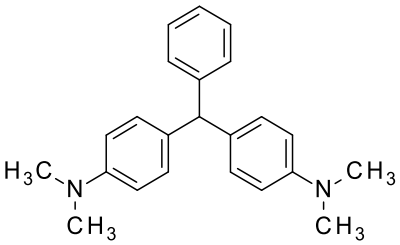
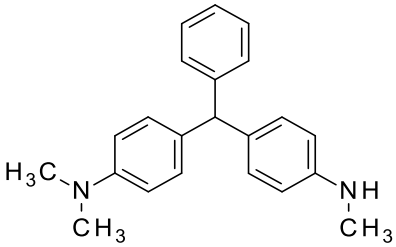
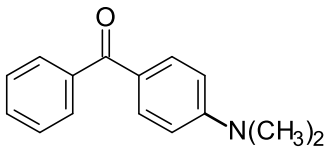
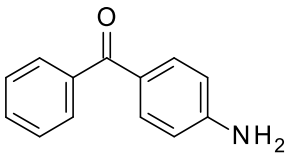
Fig 1. The decolourisation malachite green in *C. elegans*. (**A**) Absorbance (617 nm) of supernatants (S/N) and biomasss (BM) after 15 min incubation with the fungus. Error bars represent standard deviation n=2. (**B**) Absorbance spectra of methanolic extracts of planktonic biomass. (**C**) Absorbance spectra of methanolic extracts of biofilm biomass. The slightly lower absorbance in biofilm reflects the effectiveness of the extraction method using methanol

Fig 2. The effect of initial Cr (VI) concentration on the simultaneous removal of MG and Cr (VI) after 16 h incubation with the fungus. The means of MG removal compared using Least Significance Difference (LSD) test after the analysis of variance (ANOVA) at $p \leq 0.01$ (LSD value=17.43). The mean values labelled by the same letter(s) are not significantly different.

Fig 3. The effect of NaCl concentration on simultaneous removal of MG and Cr (VI). The means of MG removal and Cr(VI) removal compared using Least Significance Difference (LSD) test after the analysis of variance (ANOVA) at $p \leq 0.01$ (LSD value for MG removal=13.04, LSD value for Cr(VI) removal=17.42). The mean values within either response (MG removal or Cr(VI) removal) labelled by the same letter(s) are not significantly different.

Fig 4. The effect of metal ions on the removal of MG (**A**) and Cr (VI) (**B**) by *C. elegans*. The means of MG removal and Cr(VI) removal at varying time intervals compared using Least Significance Difference (LSD) test after the analysis of variance (ANOVA) at $p \leq 0.01$ (LSD value for MG removal after 8 h= 4.57, LSD value for MG removal after 24 h= 5.73, LSD value for Cr(VI) removal after 8 h= 5.01). The mean values within either response (MG removal or Cr(VI) removal) at a specific time labelled by the same letter(s) are not significantly different. The unlabelled mean values for MG removal over 40 h and Cr(VI) removal over 24 hours were found statistically non-significantly different among themselves.

494 Table 1. GC-MS data for the metabolites of malachite green incubated with *Cunninghamella*
495 *elegans* biofilm

Intermediate products	Molecular structure	T_R (min)	*m/z* of M^+
Leucomalachite green		14.65	330
Desmethyl Leucomalachite green		14.29	316
4-(Dimethylamino) benzophenone		15.39	225
4-Aminobenzophenone		14.16	197

496

497

498 Table 2. Removal of Cr(VI) and malachite green (MG) by the suspended cells (planktonic)
499 and biofilm of *Cunninghamella elegans.* > 95 % Decrease in dye/metal is considered
500 complete removal.

Culture condition		**Cr (VI) Removal**	**MG Removal**	**Simultaneous removal**	
				Cr (VI)	**MG**
Planktonic	% Removal after 16 hours	82.9±5.9	81.6±4.4	74.3±3.4	79.5±6.1
	Time for complete removal (h)	22	22	24	24
Biofilm	% Removal after 16 hours	71.4±3.5	82.5±6.2	69.2±2.9	80.5±4.6
	Time for complete removal (h)	24	22	26	26
	No. of cycles of complete removal	24	19	23	20

501

502

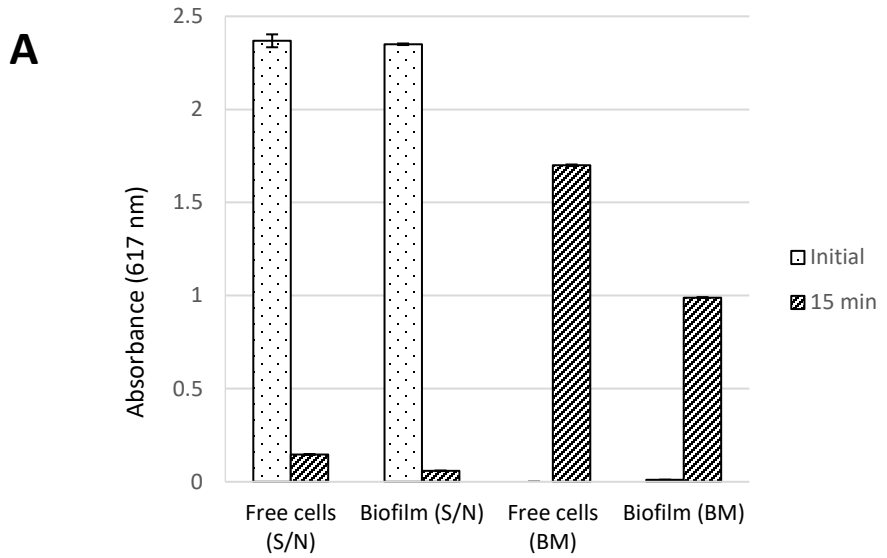
Table 3. Effect of NaCl and initial Cr(VI) concentrations on simultaneous removal of Cr(VI) and malachite green (MG) by *Cunninghamella elegans* biofilm. > 95 % Decrease in dye/metal is considered complete removal.

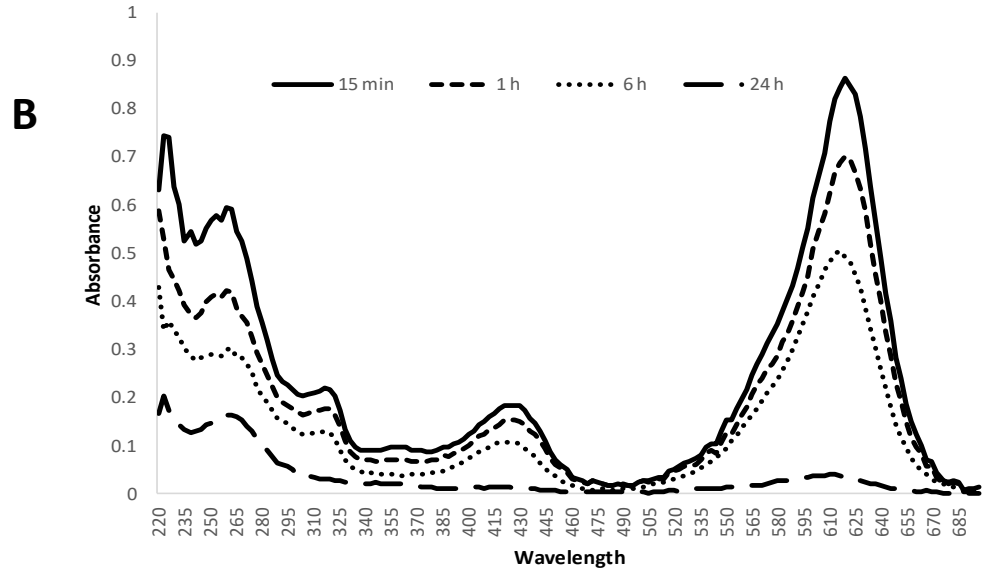
NaCl concentration	**No NaCl**		**20 g L^{-1}**		**40 g L^{-1}**		**60 g L^{-1}**		**80 g L^{-1}**		**100 g L^{-1}**	
	MG	**Cr(VI)**	**MG**	**Cr(VI)**	**MG**	**Cr(VI)**	**MG**	**Cr(VI)**	**MG**	**Cr(VI)**	**MG**	**Cr(VI)**
Time for complete removal (h)	24	24	24	24	28	32	32	54	96	144	>144	>144
No. of cycles of complete removal	>10	>10	>10	>10	6	4	3	2	1	1	0	0
Initial Cr(VI) concentration	**20 mg L^{-1}**		**40 mg L^{-1}**		**60 mg L^{-1}**		**80 mg L^{-1}**		**100 mg L^{-1}**		**150 mg L^{-1}**	
	MG	**Cr(VI)**	**MG**	**Cr(VI)**	**MG**	**Cr(VI)**	**MG**	**Cr(VI)**	**MG**	**Cr(VI)**	**MG**	**Cr(VI)**
Time for complete removal (h)	24	24	24	32	28	52	48	72	76	>144	>144	>144
No. of cycles of complete removal	>10	>10	6	5	2	2	1	1	1	0	0	0

Table 4. Simultaneous removal of Cr(VI) and various dyes by *Cunninghamella elegans* biofilm.

	Colour Removal (%)		**Cr(VI) Removal (%)**	
	24 h	**40 h**	**24 h**	**40 h**
Reactive Black 5	38.6 ± 3.6	90.7 ± 3.1	85.3 ± 4.5	98.6 ± 2.1
Acid Orange 7	51.9 ± 3.1	92.6 ± 3.9	82.1 ± 3.4	96.6 ± 2.6
Direct Red 81	72.7 ± 1.4	96.3 ± 2.6	89.1 ± 2.4	96.7 ± 3.1
Brilliant Blue G	58.9 ± 2.3	86.6 ± 3.9	90.1 ± 1.9	96.6 ± 2.8

Fig 1.


A
2.5
2
1.5
1
0.5
0
Absorbance (617 nm)
Initial
15 min
Free cells (S/N)
Biofilm (S/N)
Free cells (BM)
Biofilm (BM)


B
15 min
1 h
6 h
24 h
Absorbance
Wavelength


C
15 min
1 h
6 h
24 h
Absorbance
Wavelength

Fig 2.

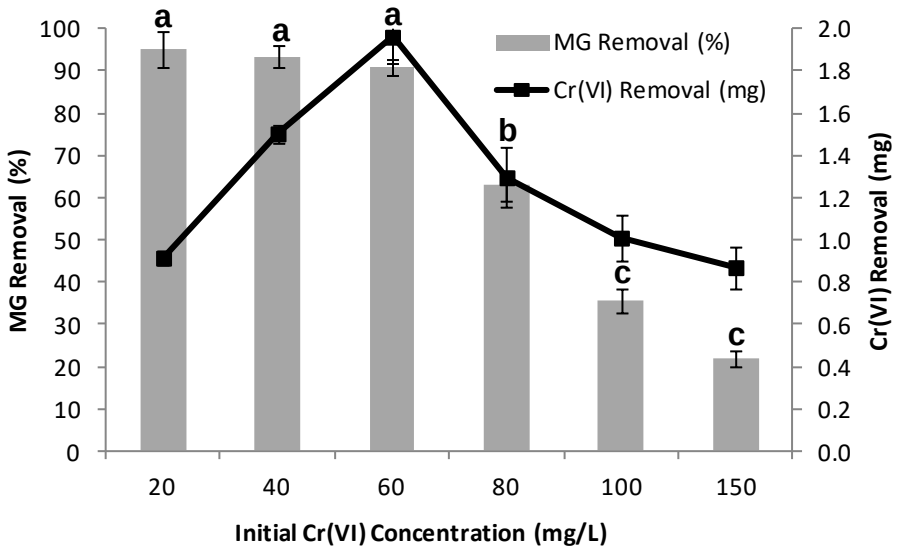

a
a
a
b
c
c
MG Removal (%)
Cr(VI) Removal (mg)
MG Removal (%)
Cr(VI) Removal (mg)
100
90
80
70
60
50
40
30
20
10
0
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
20
40
60
80
100
150
Initial Cr(VI) Concentration (mg/L)

Fig 3

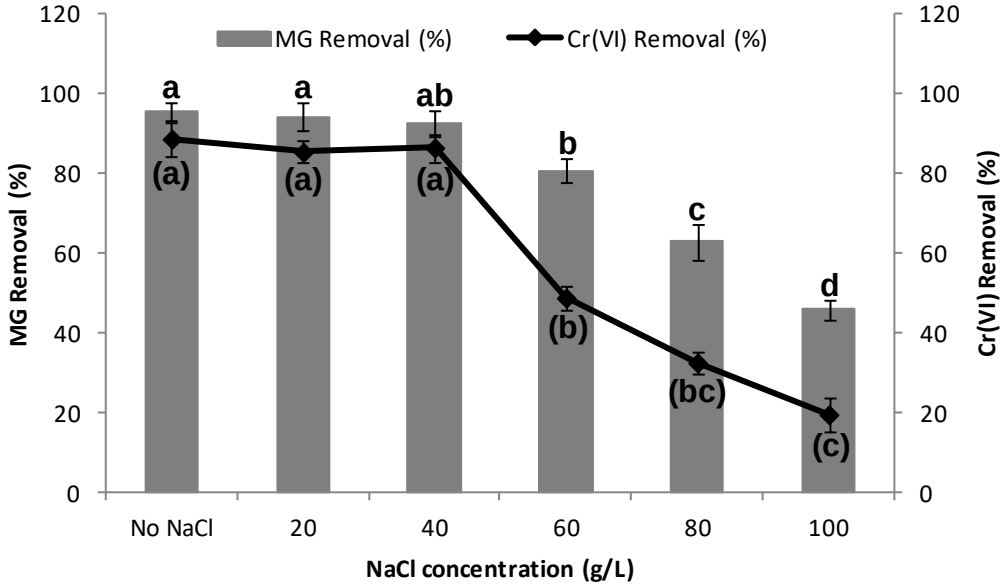
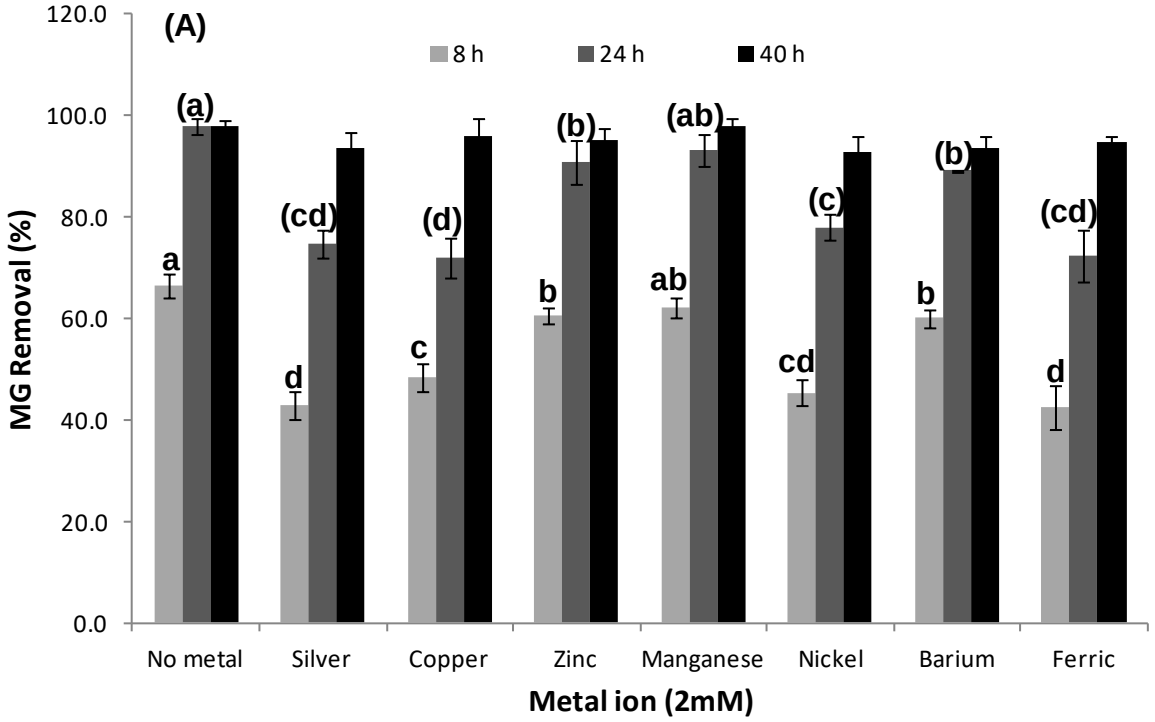

MG Removal (%)
Cr(VI) Removal (%)
MG Removal (%)
Cr(VI) Removal (%)
NaCl concentration (g/L)
No NaCl
20
40
60
80
100
0
20
40
60
80
100
120
a
a
ab
b
c
d
(a)
(a)
(a)
(b)
(bc)
(c)

Fig 4


(A)
8 h
24 h
40 h
MG Removal (%)
120.0
100.0
80.0
60.0
40.0
20.0
0.0
(a)
a
(cd)
d
(d)
c
(b)
b
(ab)
ab
(c)
cd
(b)
b
(cd)
d
No metal
Silver
Copper
Zinc
Manganese
Nickel
Barium
Ferric
Metal ion (2mM)

w