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Research article

Biofilm formation of filamentous fungi *Coriolopsis* sp. on simple muslin cloth to enhance removal of triphenylmethane dyes

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ABSTRACT

The isolate *Coriolopsis* sp. (1c3) was cultured on muslin cloth to induce formation of filamentous biofilm. The biofilm and the free-mycelium forms (control) were then used to treat two triphenylmethane dyes; Cotton Blue (CB) and Crystal Violet (CV). The biofilm comprised primarily of a compact mass of mycelium while sparse mycelium network was detected in free-mycelium forms. Results revealed significant decolourization activities by filamentous biofilm of 1c3 for CB (79.6%) and CV (85.1%), compared to free-mycelium forms (72.6 and 58.3%, for CB and CV, respectively). Biodegradation occurred in both biofilm and free-mycelium forms. FTIR spectra revealed that biofilm formation (stacking of mycelium), did not have severe implications to the number and types of functional groups available for dye biosorption. The findings here suggested that formation of biofilm in 1c3 was induced effectively on muslin cloth, leading to enhanced decolourization activities. This technology is simple, feasible and can be adopted and further improved to obtain biofilm to enhance their dye removal efficiency in aqueous solutions.

1. Introduction

Triphenylmethane dyes are colourants used in the many leather and textile industries (An et al., 2002), resulting in the generation and release of almost 20% of unspent dyes in their wastewaters (Sankar et al., 1999). These dyes comprise of complex aromatic structures that are resistant to degradation, resulting in their gradual accumulation in the environment. Their persistence in the environment and their resistance to degradation, give rise to their mutagenic and carcinogenic properties that are highly toxic to other living organisms and the environment. The conventional approach to mitigate dye problems are through the use of various physico-chemical techniques based on adsorption, precipitation, flocculation, redox processes, and electrochemical treatments (Chen and Ting, 2017). These processes were however, expensive and generates large amount of toxic sludge (Saratale et al., 2009). These processes were also unable to degrade synthetic dyes; hence the complete removal of dyes is often not achieved. As an alternative, bioremediation is sought.

Bioremediation by fungi has been demonstrated to be effective for the removal of triphenylmethane dyes. Bioremediation is typically achieved with the use of white-rot fungi *Phanerochaete chrysosporium* (Radha et al., 2005; Hu et al., 2016) and the multi-coloured *Trametes versicolor* (Casas et al., 2009). In recent years, lesser known fungi have also shown dye removal potential, which include *Irpex lacteus* (Kalpana et al., 2012), *Fusarium solani* (Chaudhry et al., 2013), *Coriolopsis* sp. and *Penicillium simplicissimum* (Chen and Ting, 2015a, 2015b). Fungi are highly-preferred as they are easily cultured and they produce a range of enzymes useful for dye degradation (Shedbalkar et al., 2008; Hofrichter et al., 2010). They naturally secrete enzymes that are capable of degrading the harmful dye molecules into less or non-toxic simpler forms (Trovaslet et al., 2007). In this study, isolate *Coriolopsis* sp. (1c3) was selected as this isolate has been reported to demonstrate efficient decolourization of Crystal Violet (CV; 100 mg l^{-1}), Methyl Violet (MV; 100 mg l^{-1}), Cotton Blue (CB; 50 mg l^{-1}), and Malachite Green (MG; 100 mg l^{-1}), with 94, 97, 91 and 52% of decolourization efficiency, within 7, 7, 1 and 9 day(s), respectively (Chen and Ting, 2015a).

In recent years, fungi have been identified to have the potential to form biofilm. This is primarily attributed to their natural affinity to attach to structures. Biofilm formation in filamentous fungi has been "induced" by providing solid surface or structure to support

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growth (Villena and Gutiérrez-Correa, 2007). It has been reported that the formation of biofilm in fungi mimics the biofilm formation in bacteria. In biofilm forms, fungi benefits from better growth and enhanced production of extracellular enzymes and bioactive compounds (Villena and Gutierrez-Correa, 2006; Gutierrez-Correa et al., 2012), as well as better amenability for process control and up-scaling for industrial applications (Villena and Gutiérrez-Correa, 2007; Gutierrez-Correa et al., 2012).

In this study, it is hypothesized that biofilm in fungi can be artificially-induced by providing a thin surface layer for the fungi to form a dense network of mycelium. This is achieved through the use of a simple, inexpensive approach of using muslin cloth. Muslin cloth has perforated surface, which enables the uptake of nutrients to support fungal growth as well as enabling the formation of biofilm. The perforated surface also allows removal of dye molecules as the biofilm has surface contact with the dye solutions. This study therefore embarks to investigate the possibility of adopting muslin cloth to induce biofilm formation of isolate *Coriolopsis* sp. (1c3). The structure of biofilm formed (via Scanning Electron Microscopy and Fourier Transformed Infrared Spectroscopy) and their decolourization and possible biodegradation activities were examined and compared against free-mycelium forms.

2. Materials and methods

2.1. Establishing fungal culture for free-mycelium and biofilm forms

The fungal isolate *Coriolopsis* sp. 1c3 (accession number KM403574) was isolated from the Empty Fruit Bunch compost of oil palm by Chen and Ting (2015a). This isolate was cultured on Potato Dextrose Agar (PDA, Merck®) for 7 days at room temperature (24 ± 2 °C). The free-mycelium inoculum was prepared by inoculating five mycelial plugs into 40 mL of Potato Dextrose Broth (PDB, Difco®) in 250 mL flasks. The culture was incubated for 10 days at room temperature (24 ± 2 °C) to give rise to free-mycelium cultures of 1c3. After incubation, the fungal biomass was filtered through filter paper (90 mm diameter Whatman® No. 1) and washed three times with autoclaved distilled water. The biomass obtained was weighed to 1 g portions and used for subsequent dye decolourization test.

To initiate biofilm cultures of 1c3, muslin cloth (Calbiochem®) was used to provide a thin surface layer required for formation of filamentous biofilm. The muslin cloth was first cut into a circle (7.5 cm diameter) with four long strips (11 cm in length, 2 cm in width). The muslin cloth was then suspended in 250 mL beaker containing 40 mL of Potato Dextrose Broth (PDB, Difco®), to allow the circular piece to gently touch the surface of the broth in the beakers. This set-up was sterilized by autoclaving. Five mycelial plugs were then inoculated to the suspended muslin cloth. The cultures were incubated as standing cultures for 10 days at room temperature (24 ± 2 °C). After incubation, the muslin cloth (along with the fungal biomass on it) was removed from the beakers, washed 3 times with sterile distilled water, and separated into portions of 1 g biomass (on muslin cloth) for subsequent decolourization test.

2.2. Dye decolourization test

Dye solutions were first prepared using powdered Cotton Blue (Sigma-Aldrich®) and Crystal Violet (Merck) dye to concentrations of 50 mg L^{-1} and 100 mg L^{-1}, respectively (Chen and Ting, 2015a, 2015b). The dye solutions were adjusted to pH 5, and 100 mL of the dye solution was then dispensed into 200 mL beakers. To each beaker, 1 g of free-cell biomass was added and gently swirled. For biofilm cultures, the muslin cloth (from section 2.1) was transferred to beakers containing dye solutions, with the muslin cloth placed gently on the surface of the dye solution (similar to section 2.1). Several control sets were also prepared. They include sets of beakers containing only dye solutions (without fungal free-cell or biofilm), and beakers of dye solutions with muslin cloth only (no biofilm). At every 24-h interval for the next 14 days, 3 mL of the dye solutions were pipetted and dispensed into centrifuge tubes. Centrifugation was then performed at 24 ± 2 °C, 10 000 rpm for 10 min (Microfuge® 22R Centrifuge, Beckman Coulter). The resulting supernatant was then collected and transferred into a 24-well microplate (Jet Biofil®) and the absorbance read at 599 and 590 nm for Cotton Blue and Crystal Violet, respectively, using a microplate reader (TECAN®, Infinite M 200 plate reader) (Chen and Ting, 2015a,2015b). The absorbance values were recorded and the decolourization efficiency (DE, %) (Parshetti et al., 2006) calculated as in Equation (1).

$$DE(\%) = \frac{\text{Initial Absorbance} - \text{Absorbance after treatment}}{\text{Initial Absorbance}} \text{x} 100\% \quad (1)$$

2.3. Ultraviolet–visible (UV–vis) spectral analysis to detect biodegradation potential

Dye solutions were also sampled for UV–visible spectral analysis to detect the occurrence of biodegradation. Positive biodegradation is concluded when observable change in peaks between control and treated dye samples were detected (Kalpana et al., 2012). The samples (2 mL) were collected from the dye solutions at the beginning and the end of the experiment, centrifuged (10,000 rpm, 10 min) and the resulting supernatant read between the wavelengths of 300–800 nm, using the microplate reader (TECAN®, Infinite M 200 plate reader) (Chen and Ting, 2015a, 2015b). Spectra peaks for dyes with and without treatment was plotted and compared.

2.4. Scanning electron microscopy

Scanning Electron Microscopy (SEM) was performed to analyze and compare the morphological structure of free-mycelium and biofilm forms of 1c3. Firstly, samples were fixed by immersing the collected biomass in 2.5% glutaraldehyde solution and kept overnight at 4 °C. The samples were then removed from the glutaraldehyde solution and washed thrice with 15 mL of 0.1 M phosphate buffer (pH 7.4) (15 min each washing). Samples were then subjected to dehydration by immersing in a series of ethanol solutions, beginning with 30% ethanol for 10 min, followed by 50, 70, 80, 90, 95 and 100% ethanol. The absolute ethanol (100% ethanol) was repeated twice at 15 min each cycle. Once dehydrated, samples were further placed into a desiccator for 3 days to accelerate the drying process. The samples were then sputter-coated with gold (QUORUM, Q15ORS) and examined using a Scanning Electron Microscope (HITACHI, S-3400 N).

2.5. FTIR-analysis of biofilm and free-mycelium forms

FTIR analysis was performed to characterize the functional groups present on the cell wall of biofilm and free-mycelium forms of 1c3. The fungal biomass from section 2.1 was oven-dried and ground to powder form. Single-reflection attenuated total reflection spectra (within 4000–400 cm^{-1}) were obtained using the FTIR spectrometer (Varian 640-IR) conducted in ambient temperature. Data were collected within the mid-infrared region from 4000 to