Journal of Environmental Chemical Engineering 5 (2017) 222–231

Contents lists available at ScienceDirect

Journal of Environmental Chemical Engineering

journal homepage: www.elsevier.com/locate/jece

Trichoderma asperellum laccase mediated crystal violet degradation–Optimization of experimental conditions and characterization

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ARTICLE INFO

Article history:
Received 30 September 2016
Received in revised form 27 November 2016
Accepted 29 November 2016
Available online 30 November 2016

Keywords:
Crystal violet
Dye degradation
Laccase
Genotoxicity
Trichoderma asperellum

ABSTRACT

Biodegradation of crystal violet (CV) by *Trichoderma asperellum* laccase was herein reported. The process parameters viz., enzyme concentration, dye concentration, pH and incubation time were experimentally optimized for the maximum dye degradation. The degradation of CV was physico-chemically characterized by UV–vis spectroscopy, FTIR, HPTLC, HPLC and GC–MS analyses. In UV–vis spectroscopy, a major reduction in characteristic peak from 588 nm to 575 nm has suggested the disappearance of triphenyl methane chromophore. From the FT-IR spectra, the formation of new peaks at 3707 cm^{-1} (—OH stretching) and 1726 cm^{-1} (C=O stretching) indicates the successful transformation of the crystal violet. Similarly, the existence of a prominent peak ($R_f - 0.39$) in HPTLC and a significant reduction in peak intensity of crystal violet had suggested the laccase mediated dye degradation. GC–MS analysis of crystal violet degraded metabolites were identified as Leucocrystal violet, Olivetol dimethyl ether (1,3-Dimethoxy-5-pentylbenzene) and 1,3,5-trimethoxybenzene by a hydride ion (H-) transfer followed by oxidative methylation. The genotoxicity studies by *Allium cepa* assay has shown that the degraded intermediates of CV are minimally toxic than the original dye. Owing to the above-discussed significance, *T.asperellum* laccase may be considered for the development of more environmental friendly treatment processes.

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1. Introduction

Synthetic dyes play an extensive role in the textile, dying and printing industry, which in turn, consumes a large amount of water (up to 150 l of water to dye 1 kg of cotton) and generates huge quantity of polluted effluents. It is estimated that 8×10^5 tons of synthetic dyes are produced annually and nearly 15% of the untreated dyes are directly discharged into water streams, which pose a serious threat to the environment [1]. Triphenylmethane dyes are the major group of bright coloured cationic dyes used in various dying processes. These cationic dyes are recalcitrant and contain mutagenic substances owing to the presence of a delocalized electron in its aromatic ring structure and they are highly toxic over anionic dyes [2]. These dyes cause serious health issues by navigating into the entire food chain and leading to biomagnifications [3,4]. Though conventional physicochemical methods are available for the removal of ionic dyes from textile wastewater such as adsorption, ozonation, precipitation and electrochemical methods [5], they have only limited applicability and are commonly associated with economic unfeasibility and accumulation of concentrated quantities of sludge, which further leads to secondary toxicity [6,7].

Biological methods using microbes have been continuously researched for the decolorization and degradation of textile dyes since they are cost-effective and eco-friendly. It was reported that Sol-Gel immobilized *Pseudomonas* sp. had produced more than seven times higher amounts of extracellular enzymes which results in the efficient biodegradation of azo dyes [8]. Unlike bacteria, White-rot fungi are known for strong bioremediation capacity due to its extracellular lignolytic enzyme assembly where it can be able to degrade a range of xenobiotic compounds hence,

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http://dx.doi.org/10.1016/j.jece.2016.11.044
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researchers paid more attention on fungi in recent years [9]. Currently, biological degradation of dyes by fungi or their enzyme systems is of great interest due to its specificity, low energy cost, ease of control and low impacts on environment [10]. This treatment procedure results in the complete or partial conversion of toxic dyes into safe (non-toxic) end products, which combats the issue of generation of secondary toxic pollutants. For dye decolorization, most of the studies have employed only a single fungal strain and recently fungal co-culture technology has emerged as an efficient method of dye decolorization. The lignocellulolytic fungi *Pleurotus florida* (PF) and *Rhizoctonia solani* (RS) has resulted in highest dye decolorization (98.54%) at 2% (w/v) of synthetic brilliant green carpet industry dye as compared to monocultures [11].

Ligninolytic enzymes, such as lignin peroxidase (LiP; EC 1.11.1.14), manganese peroxidase (MnP; EC 1.11.1.13) and laccase (*p*-diphenol: dioxygen oxidoreductase; EC 1.10.3.2) can be applied in dye degradation [12]. Decolorization of synthetic dyes by the lignin peroxidase (LiP) and manganese peroxidase (MnP) are highly sensitive to the acidic pH and further requires the addition of H_2O_2 [13]. These, laccase-based treatment deserves much attention because it catalyzes the oxidation of an array of substrates by the reduction of molecular O_2 to water and it can be further extended by the addition of redox mediators. Since it produce molecular O_2 and water as the only-by product, laccase-based catalytic reactions are considered as green-chemistry approach and have been increasingly used in bioremediation processes [14].

The experimental factors viz., dye concentration, enzyme concentration, pH, temperature and incubation time has been used to determine the efficacy of the enzymatic dye degradation process. Optimization of these factors provides a commercially viable evaluation of operational conditions for dye degradation as well as the identification of plausible synergic or antagonistic interactions which exist between them. Herein, the optimization of experimental variables such as enzyme concentration, dye concentration, pH and incubation time have been reported for the laccase-mediated crystal violet degradation. The results of the dye degradation process and its intermediates were further confirmed by UV–vis spectroscopy, FTIR, HPTLC, HPLC and GC–MS analyses. The toxicity of enzyme degraded crystal violet by-products were also assessed by the genotoxicity studies. This is the first report of *Trichoderma asperellum* laccase mediated dye degradation using the response surface methodology.

2. Materials and methods

2.1. Microorganism and culture conditions

The surface grown mycelium of wood rotting fungi *Trichoderma asperellum* BPLMBT1 (Accession number KC243781.1) used in the study was collected from the Western Ghats, South India (latitude: 10.1667°N, longitude: 77.0667°E). The inoculum was prepared as described by [15] with slight modifications. The spores were harvested after 6 days of incubation on 2% (w/v) malt extract agar slants without disturbing the mycelial growth using a sterile Camel hairbrush. The spore concentration was adjusted to 10^5 spores/mL and used as inoculum for further studies.

2.2. Enzyme assays and protein determination

The cell free supernatant of *T. asperellum* obtained from C–limited medium was served as the enzyme source. Lignin peroxidase activity was measured by the oxidation of dye azure B in presence of H_2O_2 at 651 nm [16]. Manganese peroxidase activity was assayed at 610 nm by using phenol red and H_2O_2 [17]. One unit of enzyme activity was defined as a change in absorbance (units per milligrams of protein per minute). The laccase activity (EC 1.10.3.2) was determined by measuring the oxidation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) at 436 nm ($\varepsilon_{436} = 36{,}000\ M^{-1}\ cm^{-1}$). Briefly, the reaction mixture contained 0.9 ml of 1 mM ABTS in 0.1 M sodium acetate buffer (pH 4.5) and 0.1 ml of enzyme. The enzyme unit were expressed as international units (IU) where one unit of laccase activity was defined as the amount of enzyme that oxidized 1 μM ABTS per min [18].

2.3. Laccase production and purification

Laccase production was performed on the C-limited medium at 27 ± 2 °C under agitation (125 r/min) for 6 days [19]. The purification of laccase was adapted from the protocol as described by Sadhasivam et al. [15] with modifications. For the enzyme purification, 0.1 M sodium acetate buffer of pH 4.5 was used and the enzyme purification steps were carried out at 4 °C, unless otherwise specified. To obtain the crude enzyme filtrate, 6 days old liquid medium was centrifuged at 6000 rpm for 10 min and the clear supernatant was collected. The supernatant fractionated using 66% pre-chilled acetone (v/v) was kept at −20 °C for 6 h and then centrifuged at 5,600 x *g* for 10 min. The as-obtained pellet was resuspended in 0.1 M sodium acetate buffer (pH 4.5) and dialyzed against the same buffer. The partially purified enzyme was obtained by loading the dialysate onto a pre-equilibrated Sephadex G-100 column (45 × 2.5 cm) and subsequently eluted with 0.1 M NaCl in buffer. The fractions showing laccase activity were pooled, concentrated by freeze-drying and stored at −4 °C. The freeze-dried sample was loaded onto a DEAE-Sepharose FF anion exchange column chromatography equilibrated with sodium acetate buffer and washed with the same buffer until the A_{280} reading was less than 0.02. A linear gradient of NaCl (0–0.5 M) prepared in buffer was used to elute the bound proteins. The active fractions showing laccase activity were pooled, dialyzed (membrane molecular weight cut-off 12,000 Da) overnight against the same buffer and concentrated using Amicon ultra spin concentrator column (Millipore) with a molecular cut-off of 10 kDa. At each step, the protein content [20] and laccase activity [18] were determined.

2.4. Dye degradation experiment

A recalcitrant triphenylmethane dye, crystal violet was purchased from Himedia and used without any further purification (Mumbai, India). The dye degradation experiments were carried out by the desired concentrations of the dye as obtained from the dilution of stock solution (1000 ppm) and stored at room temperature in dark condition until further use. The dye degradation experiment was performed in a 5.0 ml reaction mixture consists of 50 ppm of dye solution in 0.1 M sodium acetate buffer (pH 4) and 0.5 U ml^{-1} of laccase was incubated for 60 min at room temperature under static conditions. All the experiments were performed in triplicates and a heat inactivated enzyme was served as control. The residual dye concentration was measured spectrophotometrically (Shimadzu UV 1800) at 580 nm (λ_{max}) and the% dye degradation was calculated by the below given formula:

$$D = \frac{(A_{ini} - A_{obs})}{A_{ini}} \times 100$$

where *D* is the degradation of crystal violet (%), A_{ini} is the initial absorption at time zero and A_{obs} is the absorption at determined

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