Desalination and Water Treatment
www.deswater.com

doi: 10.5004/dwt.2018.22933

122 (2018) 293–297
August

Strain improvement of *Pleurotus citrinopileatus* MTCC 1796 for enhanced production of laccase enzymes and its environmental application

Velayutham Karthikeyan, Vasuraj Surya Praba, Madhava Anil Kumar, Manickam Periyaraman Premkumar, Subramanian Sivanesan*

Department of Applied Science and Technology, Alagappa College of Technology, Anna University, Chennai, emails: siva@annauniv.edu, sivanesh1963@gmail.com (S. Sivanesan)

Received 28 March 2018; Accepted 6 August 2018

ABSTRACT

The present study aims to improve a fungal strain; *Pleurotus citrinopileatus* MTCC 1796 to produce laccase by inducing mutation through ultraviolet (UV) light irradiation. The laccase from the wild and mutant strains was partial purified and it was found that the mutant cells of the strain produced laccase with higher activity (16.32 U/mL) while, the wild type produced laccase with an activity of 9.75 U/mL. The decolorization efficiency of the laccase from the wild and mutant strains was tested against Basic Blue 7 (BB7), belonging to the triarylmethane class of dyes. The decolorization of BB7 was high in the case of reactions catalyzed by the laccase from the mutant cells. The optimization of the BB7 decolorizing factors such as incubating temperature and pH for the varying BB7 concentration and time was studied. UV–Vis spectrophotometric and Fourier transform infrared spectroscopic analyses of BB7 decolorization and degradation by the laccase from the mutant cells was analyzed. Conclusively, the toxicity tests using plant seeds were done to understand the toxicity of the BB7 and the products of laccase catalyzed reactions were done.

Keywords: Laccase; Ultraviolet irradiation; *Pleurotus citrinopileatus* MTCC 1796; Mutant; Wild type; Basic Blue 7

1. Introduction

The pervasive use of textile dyes has resulted in the escalated water pollution leading to the loss of the aesthetic values of the water bodies because of the colored effluents [1,2]. These colored synthetic dyeing effluents contain a diversified group of dyes and other auxiliary compounds causing serious deterioration of water quality [3–7]. Among the distinct classes of dyes, the azo (N=N) type of dye has stable structure because of highly substituted aromatic rings and is largely produced and consumed globally which is strongly recalcitrant due to its substituted ring structures [8–10].

The treatment techniques for abating the dyeing wastewater such as physico-chemical, biological and advanced oxidation methods are employed [11]. The biological wastewater treatment is considered to be the most viable technology to treat the dyeing effluents as they overcome the demerits such as low efficiency, production of hazardous by-products and high cost associated the physico-chemical and advanced oxidation treatment methods [11,12]. Laccase shows significant biotechnological potential in various industrial applications, including polymer synthesis, beverage stabilization, anticancer drug development, dye bleaching and sulfate pulping and the reduction of environmental pollution generated by waste effluents [13,14]. Biodegradation using laccase from fungal strains [15–19] makes the degradation process more meritorious [20–23]. However, industrial applications of laccases are usually delayed by low laccase yield, long fermentation period,

* Corresponding author.

Presented at the 3rd International Conference on Recent Advancements in Chemical, Environmental and Energy Engineering, 15–16 February, Chennai, India, 2018.

as well as poor enzyme stability [24]. In addition to enzyme source viz., microorganism and substrate, other influences should be considered to develop efficient enzymatic bioprocesses and to gratify the commercial needs.

Microorganisms are used to induce mutation by the random mutagenesis for better distinction. Mutagens such as UV irradiation, gamma irradiation, ethidium bromide (EtBr) and ethyl methyl sulfonate (EMS) are employed for inducing mutation [24–28]. In the recent years, random mutagenesis studies were conducted to improve the productivity of the enzymes. The present study aims to mutate a fungal strain *Pleurotus citrinopileatus* MTCC 1796 to enhance their laccase production and utilize the laccase enzyme to decolorize and degrade the BB7 dye. The degradation phenomena of BB7 can be studied using the analytical tools such as UV–Vis spectrophotometry and Fourier transform infrared (FT-IR) spectroscopy. The toxicity of the BB7 and the degraded products of the BB7 can be studied using plant toxicity tests.

2. Materials and methods

2.1. Chemicals, fungal culture and instruments

The BB7 (C.I. Basic Blue 7; λ_{max}, 615 nm; $C_{33}H_{40}ClN_3$; molecular weight, **514.14**) was purchased from Colourtex Industries, India. Syringaldazine was from Sigma-Aldrich, Bangalore (India). All the chemicals were of analytical grade and no further purification was required. The fungal strain *Pleurotus citrinopileatus* MTCC 1796 was procured from the Microbial Type Culture Collection Centre and GenBank (MTCC), Institute of Microbial Technology, Chandigarh, India. The strain was grown on potato dextrose agar (PDA) medium and the growth conditions were maintained at 25°C for 7 d. The BB7 concentrations were measured using the absorbance recorded on UV-Vis spectrophotometer (UV-1800, Shimadzu, Japan) and FT-IR spectra were recorded using 237B Infrared spectrometer (PerkinElmer, United States).

2.2. UV irradiation for strain improvement and harvesting crude laccase

To carry out UV mutagenesis, 10 mL of the actively grown cells of *P. citrinopileatus* MTCC 1796 were taken in petri dish and were exposed to UV light (290 nm) for 5 min. The distance between the petri dish and the lamp was 42 cm. The surviving cells after UV irradiation are known to be the mutant type and the mutants were sub-cultured further on PDA plates and incubated at 25°C for 7 d. The strains (wild and mutant) were individually grown on 250 mL Erlenmeyer flasks containing 50 mL of potato dextrose broth and incubated at 25°C for 5 d at 150 rpm. The cultures were centrifuged individually; the supernatants from individual flasks were designated as crude laccase from wild type and crude laccase from mutants. Laccase activity was estimated using syringaldazine as substrate and its oxidation was measured spectrophotometrically at 530 nm [29,30]. One unit of enzyme activity is defined as the change in absorbance units per minute per mg of protein. The enzyme assays were carried out at room temperature, where blank had all the reagents except the enzyme.

2.3. BB7 decolorization using laccase from wild type and mutants

The mutated sample was serially diluted to obtain 10^{-5} dilution. From that 0.1 mL was inoculated into PDA medium containing BB7 and incubated at the optimum temperature for 24 h. The colonies formed were counted and each colony was inoculated into a 10 mL of broth containing 100 mg/L of BB7. For control, the wild organism was inoculated separately into the broth of the same composition. The percentage decolorization was estimated after 12 h and the colonies that showed values more than the wild strains were considered as positive mutants. Furthermore, best mutants were finally isolated based on their decolorization efficiencies and the isolated mutants were further sub-cultured in the media containing BB7.

The BB7 decolorization was performed using the crude laccase produced from the wild type and mutants of *P. citrinopileatus*, for this purpose 10 µL of crude laccase wild type and mutant cells were introduced with 100 mg/L BB7 at shaking conditions. At the end of the reaction, sample solutions withdrawn at regular time intervals were centrifuged at 7,000 rpm and the BB7 and biomass concentration was estimated using the cell-free supernatant immediately after decolorization. The cell free solution was extracted for the products of the BB7 degradation catalyzed by the laccase and the extraction was done using ethyl acetate and the products were concentrated by simple evaporation. BB7 decolorization by the crude laccase of wild type and mutants was performed by individually incubating 10 µL of crude laccase varying the BB7 concentration (100–500 mg/L), pH (4.0, 6.0 and 8.0), temperature (28°C, 37°C and 45°C) and time (60, 90, 120, 150 and 180 min).

2.4. Tests for toxicity

The toxicity level of the BB7 and the products of laccase catalyzed degradation were monitored using *Vigna radiata, Cicer arietinum* and *Pisum sativum*. The seeds were supplemented with equal volumes of water, 100 mg/L BB7 and degraded BB7 separately. The tests were carried out in the dark condition and the plumule and radicle length was recorded and compared against the control sets (supplemented with distilled water) at the same time [31].

3. Results and discussion

3.1. UV irradiation for inducing mutagenesis and laccase production

The percentage of survival values for the UV mutagenesis of *P. citrinopileatus* MTCC 1796 is shown in Table 1. The dosage of the UV mutagenesis was expressed in terms of UV

Table 1
Dose–response values for UV mutagenesis

No.	Time of exposure (min)	Survival (%)
1	0	100
2	1.0	73
3	2.0	59
4	3.0	31
5	4.0	15
6	5.0	3

irradiation and the time range between 1 and 5 min to identify the mutation effects and survival capacity of *P. citrinopileatus* MTCC 1796. The survival of *P. citrinopileatus* MTCC 1796 got decreased with increase in UV light exposure, time interval and this may be attributed to the reason that the UV light has caused aberrations in the DNA, distortion in the rigidity of the cell membrane and the cellular proteins [24]. After a prolonged exposure, the strain was unable to survive because of uncontrolled DNA repair. The cells which were able to survive up to 5 min of UV exposure and produce laccase are known as mutants and the control cells were termed as wild type. The survival of the mutated cells was tested by inoculating the cells in the potato dextrose broth incubated at 25°C for 5 d at 150 rpm. Both the cells were tested for the ability to produce laccase and it was evident that the laccase production was high (16.32 U/mL) when in case of the mutant cells of *P. citrinopileatus* when compared with the wild type (9.75 U/mL). The increased yield in laccase production is evidence that the strain efficiency has been improvised by exposing the cells to UV radiation.

3.2. BB7 decolorization studies

The effect of initial BB7 concentration on the percentage decolorization by the crude laccase was studied and the results are shown in Fig. 1. The BB7 decolorization by the crude laccase of wild type and mutants was performed by individually incubating 10 µL of crude laccase varying the BB7 concentration (100–500 mg/L), pH (4.0, 6.0 and 8.0), temperature (28°C, 37°C and 45°C) and time (60, 90, 120, 150 and 180 min). Increasing the initial BB7 concentration, the percentage of decolorization was decreased. Wild type laccase requires 34–42 h for the complete degradation of BB7 at higher concentrations (100–500 mg/L), whereas the mutant laccase required less than 26 h. The ability of the mutant laccase indicates that the industrial dye effluent can treat effectively.

Initial pH was varied from 4.0 to 8.0 for wild and mutated laccase to analyze the decolorization ability and the results obtained demonstrated that the percentage decolorization was practically the same in the pH range 5.0–7.0 for the mutant type. The mutant laccase survived significantly under extreme pH (3.0 and 4.0) conditions, but the wild type was found to survive under all the tested pH conditions but the growth was insignificant.

The effect of temperature on BB7 dye biodegradation by wild type and mutant laccase was analyzed at different temperature in the range of 28°C–45°C. The temperature range between 30°C and 40°C for mutant laccase was decolorized completely at the similar time interval and the growth was significantly high. The mutant laccase showed good resistance to the temperature and the pH variations 30°C–40°C and 3–5, respectively.

UV–Vis spectrophotometric analysis was done to understand the effect of laccase catalysis on the decolorization of BB7. The spectral scan of the initial BB7 and decolorized solutions were recorded and it was observed that the spectrum of BB7 had a maximum absorbance at 615 nm whereas, in case of the decolorized solution, the absorbance maxima shifted to a lower wavelength as shown in Fig. 2. The aforementioned observation confirms that the laccase from mutant cells of *P. citrinopileatus* had degraded and decolorized BB7 [32].

3.3. FT-IR spectroscopic examination of the BB7 degradation

The functional groups on the initial BB7 compound and the products from the laccase from the mutant cells of *P. citrinopileatus* were compared using FT-IR spectroscopic analysis. FT-IR absorption spectra of BB7 had peak at 1,052 cm^{-1} for the strong C–O stretch and 1,137 cm^{-1} for the presence of the aliphatic ether. The peak at 843 cm^{-1} describes the moderate appearance of the C–Cl stretch of the halo compound. The presence of phenol was indicated by the O–H bend at 1,384 cm^{-1} and peak at 1,503 cm^{-1} describes the strong appearance of the N–O stretch of the nitro compound. The C=O stretch of amide was observed at 1,621 cm^{-1} and C ≡ C stretch of alkynes at 2,171 cm^{-1} inferring the chemical complexity of the BB7 as shown in Fig. 3(a).

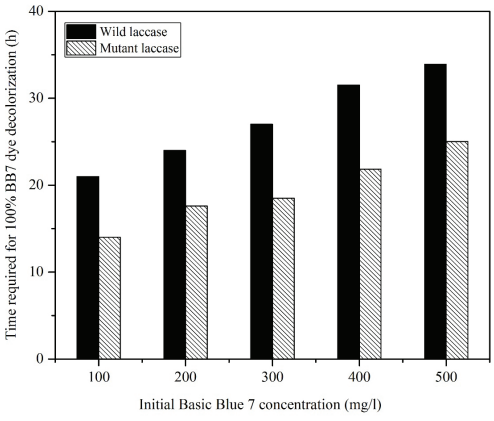

Fig. 1. Effect of initial dye concentration on time required for 100% BB7 decolorization by crude laccase of wild type and mutant.

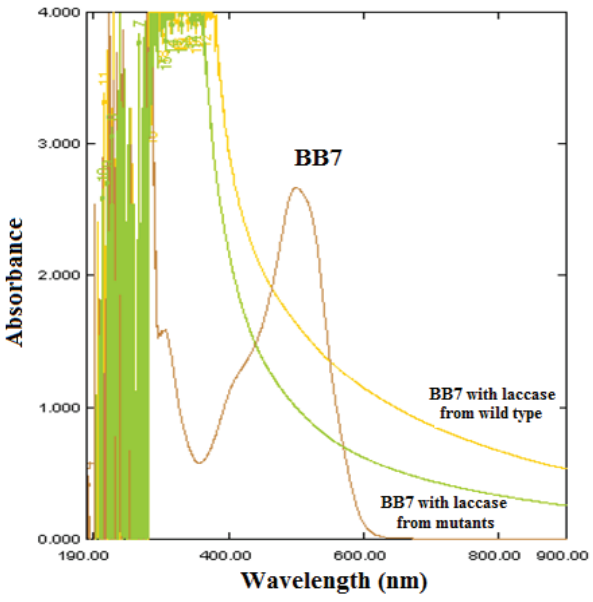

Fig. 2. UV–Vis spectra of initial and decolorized BB7 solution.

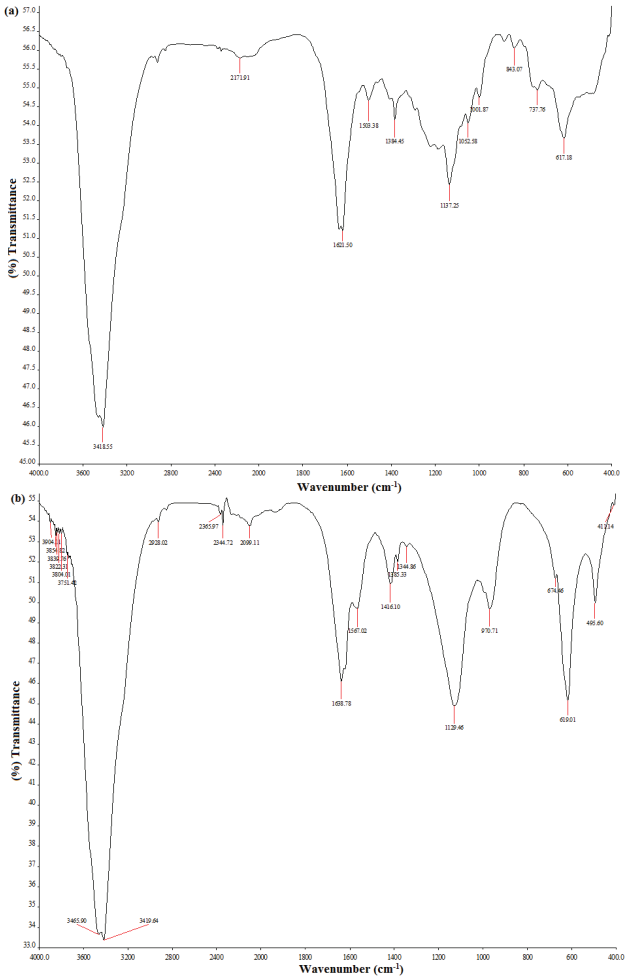

Fig. 3. FT-IR spectra of (a) BB7 and (b) degraded products.

FT-IR spectra of products of the BB7 degradation from the laccase from the mutant cells of *P. citrinopileatus* had considerable shifts in the peak intensity. 3,465 and 3,419 cm^{-1} indicates the presence of O–H stretch of alcohols, while the peak at 2,928 cm^{-1} refers to the N–H stretch of amines. The peak at 1,416 cm^{-1} describes the strong appearance of the C–HO bend of aldehydes and C–O bending of aliphatic ether was observed at 1,129 cm^{-1}. The deformation of the BB7 structure at out-of-plane twist stretching was evident by the peaks at 970 cm^{-1} for the C=C alkene groups as shown in Fig. 3(b).

3.4. Outcome of plant toxicity studies

The relative growth sensitivity of *Vigna radiata*, *Cypripedium arietinum* and *Pisum sativum* seeds toward the BB7 and the products of BB7 degradation from the laccase from the mutant cells of *P. citrinopileatus* were assessed. It was found that the germination of seeds was severely affected by the presence of BB7 describing the toxicity of the BB7 as shown in Table 2. The seeds when irrigated with the products of the BB7 degradation by laccase from the mutant cells of *P. citrinopileatus* did not express inhibitory effect on the seeds which explains the decline in the toxicity of degraded products.

Table 2
Toxicity tests of the BB7 and products of biodegradation

No.	Solution	GP (%)	Length (cm)	
			Plumule	Radicle
Vigna radiata				
1	Control	100	9.17 ± 0.048	5.16 ± 0.093
	BB7	45	3.4 ± 0.021	3.2 ± 0.081
	Products	84	4.1 ± 0.013	4.5 ± 0.064
Cicer arietinum				
2	Control	100	8.7 ± 0.032	8.6 ± 0.039
	BB7	65	5.1 ± 0.043	4.8 ± 0.045
	Products	81	5.7 ± 0.027	7.0 ± 0.063
Pisum sativum				
3	Control	100	5.1± 0.019	6.8± 0.021
	BB7	58	3.8 ± 0.089	5.3 ± 0.073
	Products	65	4.8 ± 0.033	6.2 ± 0.058

4. Conclusions

The mutant strain of *P. citrinopileatus* showed an enhanced laccase production and it can be effectively degraded to the BB7 compared with wild laccase. The BB7 was decolorized using laccase from the mutants of *P. citrinopileatus* and the BB7 decolorization was due to the biodegradation which was confirmed by the spectral analyses and the factors influencing decolorization were optimized. Mutagenesis using UV irradiation was found to be effective. The phytotoxicity results suggested that degradation products of the BB7 are non-toxic to the seeds.

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