Final Report of the Minor Research Project

"Production and partial characterization of peroxidase enzyme from the general plant body and callus cultures of *Piper nigrum* and its application in bioremediation."

1. Introduction

Our ecosystem is getting polluted due to the dumping of large amount of waste ranging from sewage to nuclear waste thereby posing serious problems hindering the survival of mankind. Though many physical and chemical methods are used for waste degradation, ecologists are still in search of new degradation methods since the former methods can lead to the addition of more dangerous substances to the environment.

Crystal violet or gentian violet (also known as methyl violet 10B or hexamethyl pararosaniline chloride) is a triarylmethane dye used as a histological stain and has antibacterial, antifungal, antihelminthic, antitrypanosomal, antiangiogenic, and antitumor properties and antitumor properties.. It is still being extensively used in human and veterinary medicine as a biological stain and as a textile dye in textile industry. (Mani & Bharagava, 2016). In spite of its many uses, it has been reported as a biohazardous recalcitrant dye molecule that pose toxic effects in environment.

Though different physico-chemical methods such as adsorption, coagulation and ion-pair extraction, activated carbon (Mohanty et al., 2006) etc. are reported for the removal of the dye, these methods are insufficient for the complete removal and also produce large quantity of sludge containing secondary pollutants (Ghasemi *et al*., 2011).. Like any other dye, in the case of crystal violet also biological degradation methods are proved to be better due to low cost of the process, its environmental friendliness, the production of less secondary sludge and completely mineralized end products which are

nontoxic (Selim et al., 2015). Studies are going on around the world on the use of enzymes in biodegradation and biotransformation of aromatic compounds which are the most deleterious compounds in waste water.

Monoxygenases, dioxygenases, laccases and peroxidases are the most important enzymes used in the field of bioremediation. Most important among these is peroxidase, an oxidoreductase enzyme seen in most of bacteria, fungi and higher plants and is the major enzyme involved in the detoxification of toxic organic compounds through oxidative coupling.

One of the latest reports on biodegradation of dyes is by a newly isolated bioluminescent bacterium *Photobacterium leiognathi* strain where the degradation was done using laccase enzyme. (Sutar *et al*., 2019). Some studies are based on the use of *in situ* plants including the ornamental plants for phytoremediation of dyes (Chandanshive *et al*., 2018). Scientists are even concentrating on recombinant production of the horseradish peroxidase enzyme in *Escherichia coli* due to its increased demand in the industry. (Gundinger & Spadiut, 2017). Polymerization of phenol using free and immobilized horseradish peroxidase has also been reported. (Pradeep *et al.*, 2012). Though biological methods can possibly lead to complete degradation of the dye molecules to carbon dioxide and water, these methods also have certain limitations. Therefore, there is an urgent need to develop such eco-friendly and cost-effective biological treatment methods, which can effectively remove the dye from industrial wastewaters for the safety of environment, as well as human and animal health along with cost effectiveness.

The current study focuses on the use of locally available potential source of peroxidase, *Piper nigrum* for dye degradation studies. Protocol standardized for callus production from the plant for more enzyme production and thereby better degradation.

2. Materials and Methods

Piper nigrum is considered as king of spices and is a plant having various medicinal properties and very high antioxidant property. (Damanhouri, 2014).

A large number of plants were screened for peroxidase activity and *Piper nigrum* (Piperaceae) was selected owing to high peroxidase enzyme activity. Fresh leaves of the plant were taken from an unpolluted village near Kattakkada, Thiruvananthapuram district of Kerala.

2.1. Enzyme activity studies.

Partial purification of enzyme.

Fresh mature leaves were homogenized with distilled water in a mortar with a pestle for 5–10 minutes at 30 ± 2°C. The extract was filtered and centrifuged at 14,000 X g for 10 minutes. The enzyme was precipitated from the crude extract with 50–75% ammonium sulfate at 4 ± 1 °C. The salt was added very slowly and stirring was done for 1-3 hours and the precipitated enzyme was centrifuged at 14,000 X g for 10 minutes. The precipitate was dissolved in 0.05 M acetate buffer (pH 4.5) and dialyzed overnight against the same buffer. Ultra filtration of the sample was done in Amicon ultrafiltration tubes having a NMWCO (nominal molecular weight cut off) of 10 kD. The enzyme was dialyzed against deionized water for 16 hours. This partially purified enzyme was used for enzyme assay and dye degradation studies.

Peroxidase assay procedure using ABTS as substrate (Smith *et al*., 1990): Peroxidase activity towards ABTS was measured by monitoring the absorbance increase at 405 nm in mixture containing 0.36 mM ABTS and 5 mM H_2O_2 in 0.05 M acetate buffer, pH 5.0 ($\varepsilon 405 = 36.8\ mM^{-1}\ cm^{-1}$) at 26±2°C. The enzyme international activity unit (U) was calculated as the number of μmols of ABTS free radicals formed per minute under standard conditions (ε 405-36.8 $mM^{-1}\ cm^{-1}$).

Enzyme Activity was calculated using the formula, $V \times (\delta E/\delta t)/ \varepsilon \times d \times v$ U/ml

V - assay volume

$(\delta E/\delta t)$- increase in absorbance/min

ε - extinction coefficient

d - path length (1cm)

v - volume of sample

Specific Activity = Enzyme activity in units/mg of protein

Protein Estimation was done by Lowrys' method (Lowry *et al.*, 1951) and specific activity of the enzyme was calculated as unit activity per milligram of protein. In all the above, one unit of peroxidase activity is represented as IU (International unit) or U and is the amount of enzyme catalyzing the oxidation of 1 μmole of the substrate in 1 min. Specific activity in all the experiments are represented in international unit (i.e., units/mg of protein) as that is the most used unit than the SI unit, katal/kg for better comparison with reported values.

pH and temperature studies for enzyme activity

pH optimum was determined by incubating the enzymes for 20 minutes in appropriate buffer (pH 3 to 6–acetate buffer , pH 6 to 9–phosphate buffer). Stability was also determined at different pH (4.0, 5.0 and 6.0) in the corresponding buffer for definite periods of time. To find out the optimum temperature, the enzymes were subjected to different temperatures ranging from 20–90 ºC at an interval of 10 degree rise of temperature. The enzyme samples were quickly cooled on ice and the activity was studied. In all these experiments, the substrate used was ABTS and each experiment was repeated thrice.

2.2. Initiation and maintenance of callus cultures.

Leaves and tender stem segments were excised from the plants. Explants were washed well under running tap water for 30 min. and were treated with 10% (v/v) labolene (a neutral detergent, Qualigens, India) for 10 min. and washed thoroughly with sterile water. The surface sterilization was done with 0.1% (w/v) aqueous mercuric chloride for 5 min. and finally washed six to seven times with sterile double-distilled water.

Leaf segments with midrib (7 X 10 mm) and stem segments (1.0–1.5 cm) were placed on to basal Murashige and Skoog's medium (MS)(1962) supplemented with 100 mg l^{-1} myo-inositol, 3% (w/v) sucrose and 0.8% (w/v) agar. Hormones used were kinetin (6-Furfuryl amino purine), BAP (Benzyl amino purine), 2,4-D (2,4-Dichlorophenoxyacetic acid), NAA (Naphthalene acetic acid), IBA (Indole-3-butyric acid) alone and in different combinations. pH of the medium was adjusted to 5.8 before adding agar and 20 ml of the media were dispensed in test tubes of 150 X 25 mm size and autoclaving was done at 121 °C for 15 min.. After inoculation, cultures were incubated at 25 ± 2 ^{0}C and 70–90% humidity under cool white fluorescent light with a total irradiance of 36 μmol m^{-2} s^{-1} for a photo period of 12/12 h. Further subculturings for callus proliferation as well as shoot multiplication were done onto 70 ml media in 250 ml conical flasks (Borosil, India). Each treatment consisted of 5 explants and was replicated 5 times. Once established, the callus cultures were maintained by the subsequent subculturing.

Use of PVP (Polyvinyl pyrolidone) in culture medium:

0.5–2% (w/v) (PVP) was provided in the medium in addition to the growth regulators to remove the browning of callus due to phenolics.

2.3. Dye degradation studies

Crude enzyme extract was used for the degradation studies at the optimum pH and temperature. Different concentrations of dye ranging from 5 mg/l to 30 mg/l were studied. The spectrum of the dye was scanned by a UV spectrophotometer in the range of 290-690 nm. The percentage of degradation was calculated from the difference between the initial and final λ_{max} value (585) of the mixture (dye+ enzyme).

Analysis of degradation – TLC and HPLC

The dye samples after and before treatment with the enzyme were analyzed by TLC using silica gel, developed with the sol vent system, Hexane

and Ethyl acetate (4:6 V/V). Degradation was confirmed by HPLC analysis using an isocratic mobile phase consisting of of 80:20 acetonitrile with acetate buffer (pH 4.5)

3. Results and Discussion.

3.1. Enzyme activity.

Partially purified enzyme extract from the leaves showed very good enzyme activity of 120 U/g of fresh leaves and specific activity shown was 39 U/mg. After ammonium sulfate precipitation, the specific activity was increased to 120 U/mg. Ultrafiltration further removed particles having small mass, which was not completely removed during the previous step, this process also concentrated the sample thereby increasing the specific activity to 235 units/mg.

Studies on optimum pH and Temperature

Optimum pH of the pepper peroxidase is in the range of 4.5-7 and the stability was better in the higher range of pH (6-7).

Optimum temperature for the catalytic activity was found to be 35-50 °C and it showed good thermal stability lower temperature (35°C). Similar pH and temperature optima are reported in some other plant peroxidases too. (Wang *et al*.., 2019).

3.2. Initiation and maintenance of callus cultures.

- **Table 1-Combined effect of 2, 4-D and BA on callus induction**

Medium	Concentration of hormones (mg/l)		Nature of response	Intensity of callus
	2,4-D	BA		
MS	2.0	0.01	No response	–
	2.0	0.1	Yellow friable callus	+
	2.0	0.5	Green friable callus	+ +
	2.0	1.0	Green friable callus	+ +
	2.0	1.5	Greenish brown friable	+ ++
	2.0	2.0	Brown compact callus	+
	2.0	2.5	Explants got dried	–
	1.0	0.1	Yellow compact callus	+
	1.0	0.3	Yellow compact callus	+ +
	1.0	0.5	Yellow compact callus	+ + +
	1.0	1.0	White compact callus	+ +
	1.0	1.5	White compact callus	+
	1.0	2.0	White compact callus	+

+ + + Good callusing

+ + Moderate callusing

+ Meagre callusing

– No callusing.

Though callus initiation occurred from the explants of *Piper nigrum* within 10 days (Figure 1), further proliferation was very slow. Also, along with proliferation, it resulted in browning of callus due to the formation of phenolics. Phenolics were extruded into the medium from the *Piper nigrum* callus after a period of about 20 days (Figure 2). But, to the surprise, the extruded phenolics showed tremendous enzyme activity. There are reports that peroxidase level increases with different stress situations like drought stress (Phimchan, 2014). Tissue culture conditions also creates a stress situation to the plant callus so that it induces production of more enzyme in the system. But, some of the cultures without browning showed the presence of some fungus that hindered further proliferation too. Endophytic fungus in *Piper nigrum* has been reported that are supposed to have improved the growth, yield and piperine content in the plant. (Anith *et al.,* 2018). There are only a few reports on tissue culture and callus regeneration of Piper nigrum owing to this phenolic exudates except some reports on *in vitro* propagation by nodal explants (Padhan, B., 2015; Khan *et al*., 2017) and a report on callus regeneration; but using comparatively high hormone concentrations (Wankhade, 2014). Indirect regeneration through leaf explants have been reported in another species of this genus, *Piper longum* (Basak *et al.,* 2014). Different hormone combinations used for callus initiation and proliferation are shown in the table (1). Optimum green callus formation was achieved when 2 mg/l 2,4-D and 1.5 mg/l BA was used in the MS medium.

When the medium was provided with 1% (w/v) Polyvinyl pyrolidone (PVP) (optimum concetration) in addition to the growth regulators, the phenolic formation was found to be reduced. Newly regenerated callus showed white compact callus but the peroxidase enzyme activity of the callus was extremely low. Most of the phenolic compounds are well known substrates of peroxidases and hence a suppression of the same will naturally lead to reduction in enzyme production.

Fig. 1

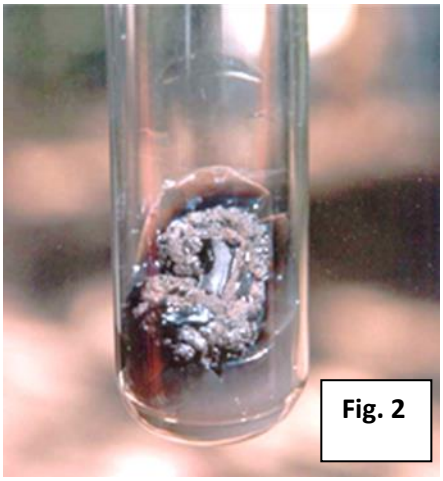


Fig. 2

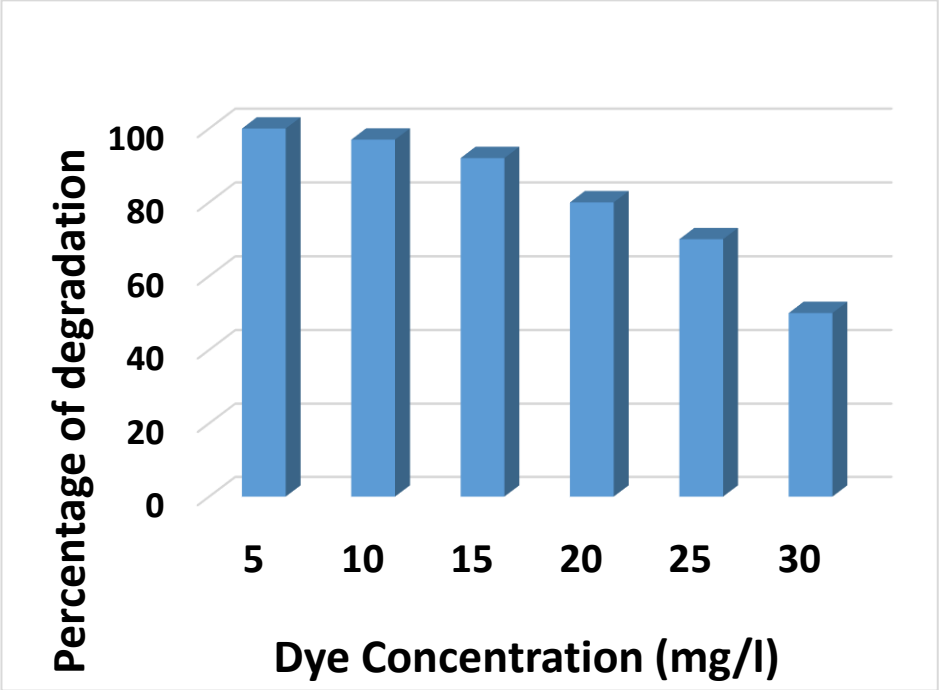
Though there are no reports regarding pepper callus peroxidase on dye degradation, in vitro cultures of *Piper nigrum* have been reported to be effective against toxic metabolite-producing pathogenic microbes like *Escherichia coli*, *Pseudomonas aeruginosa* etc. (Ahmad *et al*., 2018)

3.3. Dye degradation studies

Different concentrations of dye ranging from 5 mg/l to 30 mg/l were studied. Percentage of degradation was different at different dye concentrations. Almost complete degradation was observed up to a dye concentration of 7.5 mg/l. (Figure 3)

Enzymatic degradation of dyes from different effluents has been reported by different peroxidase enzymes. (Chiong *et al*., 2016). But, the plants studied, ie., soybean and *Luffa acutangula* and also the most common commercial source plant, horse radish are not abundant in the Kerala zone and hence a common local plant, *Piper nigrum* was selected for the study and it was found to be a very good alternative to the above mentioned plants. There are also reports on using horseradish peroxidase immobilized by copolymerization into cross-linked polyacrylamide gel for the degradation and detoxification of an azo dye, methyl orange. (Gobinath et al., 2019). But, most of these are costly methods.

Figure 3- Degradation of different concentrations of dye



Conclusion

In the current study, a very common local plant, *Piper nigrum* which is a very good source of peroxidase enzyme has been used for enzyme activity studies, partial purification, partial characterization and its use in dye degradation studies also done pertaining to the commonly used dye, Crystal violet. A cost effective protocol has also been established for the callus proliferation which shows tremendous enzyme production along with phenolic extrusion. Dye degradation can be made cost effective by immobilizing the enzyme producing callus cultures in different cost effective media and making it reusable (Rani& Abraham, 2016). Further studies are started pertaining to the work.

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