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

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Abstract

Peroxidases are oxidoreductase enzymes with significant potential for the bioremediation of synthetic dyes from industrial wastewater. This study investigates the production, partial characterization, and dye degradation efficiency of peroxidase enzyme extracted from the leaves and callus cultures of *Piper nigrum* L. (black pepper), a locally abundant plant in Kerala, India. The enzyme was partially purified using ammonium sulfate precipitation (50–75%), dialysis, and ultrafiltration (10 kDa NMWCO), resulting in a specific activity of 235 U/mg. The partially purified peroxidase exhibited optimal activity at pH 4.5–7.0 and temperature range of 35–50°C, with good thermal stability at 35°C. For enhanced enzyme production, callus cultures were initiated from leaf and stem explants on Murashige and Skoog (MS) medium supplemented with various concentrations of 2,4-D and BA. The optimal callus induction (green friable callus) was achieved with 2.0 mg/L 2,4-D and 1.5 mg/L BA. However, callus proliferation was accompanied by phenolic exudation and browning, which surprisingly showed tremendous peroxidase activity. Supplementation with 1% (w/v) polyvinyl pyrrolidone (PVP) reduced browning but simultaneously suppressed enzyme activity. The crude enzyme extract was effectively employed for the degradation of crystal violet (gentian violet), a recalcitrant triarylmethane dye. Almost complete degradation was achieved at dye concentrations up to 7.5 mg/L under optimal conditions. Degradation was confirmed by UV-Vis spectrophotometry (λmax 585 nm), TLC, and HPLC analysis. The findings demonstrate that *Piper nigrum* leaf peroxidase is a cost-effective, eco-friendly alternative to commercial horseradish peroxidase for dye bioremediation. Furthermore, callus cultures showing phenolic-associated enzyme activity present a promising avenue for developing reusable immobilized biocatalyst systems for industrial wastewater treatment.

Index Terms:Bioremediation, callus culture, dye degradation, peroxidase, *Piper nigrum*, phenolic exudation, plant tissue culture, triarylmethane dye.

I. INTRODUCTION

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. It is still being extensively used in human and veterinary medicine as a biological stain and as a textile dye in the textile industry [1]. In spite of its many uses, it has been reported as a biohazardous recalcitrant dye molecule that poses toxic effects in the environment.

Though different physico-chemical methods such as adsorption, coagulation, ion-pair extraction, and activated carbon treatment [2,3] are reported for the removal of the dye, these methods are insufficient for complete removal and also produce large quantities of sludge containing secondary pollutants. Like any other dye, in the case of crystal violet also, biological degradation methods are proved to be better due to the low cost of the process, environmental friendliness, production of less secondary sludge, and completely mineralized non-toxic end products [4].

Biodegradation of dyes using microbes [5], plant peroxidases [6], recombinant production of horseradish peroxidase enzyme in *Escherichia coli* [7], etc. shows its high demand in the industry. Polymerization of phenol using free and immobilized horseradish peroxidase has also been reported [8]. Though biological methods can possibly lead to complete degradation of dye molecules into carbon dioxide and water, these methods also have certain limitations. Therefore, there is an urgent need to develop eco-friendly and cost-effective biological treatment methods that can effectively remove dyes from industrial wastewater for environmental safety as well as human and animal health. Plant peroxidases are preferred over microbial sources due to broad substrate specificity and stability. *Piper nigrum* L. is reported to contain high peroxidase levels [9], but callus-derived enzyme for dye degradation remains unexplored.

The current study focuses on the use of a locally available potential source of peroxidase, *Piper nigrum*, for dye degradation studies. A protocol was standardized for callus production from the plant for enhanced enzyme production and thereby better degradation efficiency.

II. MATERIALS AND METHODS

Fresh leaves of the plant were collected from an unpolluted village near Kattakkada, Thiruvananthapuram district of Kerala.

Enzyme Activity Studies

Partial Purification of Enzyme

Fresh mature leaves were homogenized with distilled water using a mortar and pestle for 5–10 minutes at 30 ± 2°C. The extract was filtered and centrifuged at 14,000 × 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 slowly with continuous stirring for 1–3 hours and the precipitated enzyme was centrifuged at 14,000 × g for 10 minutes. The precipitate was dissolved in 0.05 M acetate buffer (pH 4.5) and dialyzed overnight against the same buffer.

Ultrafiltration of the sample was carried out using Amicon ultrafiltration tubes having a nominal molecular weight cut-off (NMWCO) of 10 kD. The enzyme was further dialyzed against deionized water for 16 hours. The partially purified enzyme thus obtained was used for enzyme assay and dye degradation studies.

Peroxidase Assay Procedure Using ABTS as Substrate

Peroxidase activity towards ABTS was measured by monitoring the increase in absorbance at 405 nm in a reaction mixture containing 0.36 mM ABTS and 5 mM H_2O_2 in 0.05 M acetate buffer (pH 5.0) at 26 ± 2°C [10]. The enzyme international activity unit (U) was calculated as the number of µmoles of ABTS free radicals formed per minute under standard conditions.

Enzyme activity was calculated using the formula:

$U/ml = V \times (\delta E/\delta t) / \varepsilon \times d \times v$

Where:

VVV = assay volume

(δE/δt)(\delta E/\delta t)(δE/δt) = increase in absorbance/min

ε\varepsilonε = extinction coefficient

ddd = path length (1 cm)

vvv = volume of sample

Specific Activity = Enzyme activity in units/mg of protein.

Protein estimation was carried out by Lowry's method [11], and specific activity of the enzyme was calculated as unit activity per milligram of protein. One unit of peroxidase activity is represented as IU (International Unit) or U and is defined as the amount of enzyme catalyzing the oxidation of 1 µmole of substrate in 1 min. Specific activity in all experiments was represented in international units (units/mg protein) for easier comparison with reported values.

pH and Temperature Studies for Enzyme Activity

The optimum pH was determined by incubating the enzymes for 20 minutes in appropriate buffers (pH 3–6: acetate buffer; pH 6–9: phosphate buffer). Stability was also determined at different pH values (4.0, 5.0, and 6.0) in the corresponding buffer for definite periods of time.

To determine the optimum temperature, the enzyme samples were subjected to temperatures ranging from 20–90°C at intervals of 10°C. The samples were rapidly cooled on ice and enzyme activity was measured. In all experiments, ABTS was used as the substrate and each experiment was repeated five times.

Initiation and Maintenance of Callus Cultures

Leaves and tender stem segments were excised from the plants. Explants were washed thoroughly under running tap water for 30 min and treated with 10% (v/v) labolene (Qualigens, India) for 10 min, followed by washing with sterile water. Surface sterilization was carried out using 0.1% (w/v) aqueous mercuric chloride for 5 min, followed by six to seven washes with sterile double-distilled water.

Leaf segments with midrib (7 × 10 mm) and stem segments (1.0–1.5 cm) were inoculated onto basal Murashige and Skoog's (MS) medium [12] supplemented with 100 mg l^{-1} myo-inositol, 3% (w/v) sucrose, and 0.8% (w/v) agar. Hormones used included kinetin (6-furfuryl aminopurine), BAP (benzyl aminopurine), 2,4-D (2,4-dichlorophenoxyacetic acid), NAA (naphthalene acetic acid), and IBA (indole-3-butyric acid), individually and in various combinations.

The pH of the medium was adjusted to 5.8 before adding agar. Twenty milliliters of medium were dispensed into test tubes (150 × 25 mm) and autoclaved at 121°C for 15 min. After inoculation, cultures were incubated at 25 ± 2°C and 70–90% humidity under cool white fluorescent light with a total irradiance of 36 µmol m^{-2} s^{-1} and a photoperiod of 12/12 h. Further subculturing for callus proliferation and shoot multiplication was carried out using 70 ml medium in 250 ml conical flasks (Borosil, India). Each treatment consisted of five explants replicated five times. Once established, the callus cultures were maintained through regular subculturing.

Use of PVP in Culture Medium

Polyvinyl pyrrolidone (PVP) at concentrations ranging from 0.5–2% (w/v) was added to the medium along with growth regulators to reduce browning of callus caused by phenolic exudation.

Dye Degradation Studies

Crude enzyme extract was used for dye degradation studies at optimum pH and temperature conditions. Different concentrations of dye ranging from 5 mg/l to 30 mg/l were studied. The absorption spectrum of the dye was scanned using a UV spectrophotometer in the range of 290–690 nm. Percentage degradation was calculated from the difference between the initial and final λmax value (585 nm) of the dye-enzyme mixture.

Analysis of Degradation – TLC and HPLC

The dye samples before and after enzymatic treatment were analyzed by thin-layer chromatography (TLC) using silica gel plates developed with Hexane:Ethyl acetate (4:6 v/v) solvent system. Degradation was further confirmed by HPLC analysis using an isocratic mobile phase consisting of 80:20 acetonitrile and acetate buffer (pH 4.5).

III. RESULTS AND DISCUSSION

Enzyme Activity

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

Studies on Optimum pH and Temperature

The optimum pH of pepper peroxidase was found to be in the range of 4.5–7, and the stability was better in the higher pH range of 6–7.

The optimum temperature for catalytic activity was found to be 35–50 °C, and good thermal stability was observed at lower temperature (35 °C). Huang et al. [20] also reported good thermal stability for *Brassica chinensis* peroxidase.

Initiation and Maintenance of Callus Cultures

Table 1. Effect of Different Hormone Combinations on Callus Induction in *Piper nigrum*

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

Though callus initiation occurred from the explants of *Piper nigrum* within 10 days (Figure 1), further proliferation was very slow. Along with proliferation, browning of callus due to the formation of phenolics was observed. Phenolics were extruded into the medium from the *Piper nigrum* callus after about 20 days (Figure 2). Surprisingly, the extruded phenolics showed tremendous enzyme activity.

There are reports that peroxidase levels increase under stress conditions such as drought stress [15]. Tissue culture conditions also create stress situations in plant callus, thereby inducing enhanced enzyme production. Some cultures without browning showed the presence of fungi that hindered further proliferation. Endophytic fungi in *Piper nigrum* have been reported to improve growth, yield, and piperine content in the plant [2].

Only a few reports are available on tissue culture and callus regeneration of *Piper nigrum* due to phenolic exudation, except reports on in vitro propagation through nodal explants [10], [14] and a report on callus regeneration using comparatively higher hormone concentrations [19]. Indirect regeneration through leaf explants has also been reported in another species of this genus, *Piper longum* [3].

Different hormone combinations used for callus initiation and proliferation are shown in Table 1. Optimum green callus formation was achieved in MS medium supplemented with 2 mg/l 2,4-D and 1.5 mg/l BA.

When the medium was supplemented with 1% (w/v) Polyvinyl pyrrolidone (PVP), phenolic formation was considerably reduced. Newly regenerated callus showed white compact morphology, but the peroxidase enzyme activity of the callus was extremely low. Most phenolic compounds are known substrates of peroxidases, and therefore suppression of phenolic production naturally reduced enzyme synthesis

Figure 1: Callus initiation occurred from the explants of *Piper nigrum*

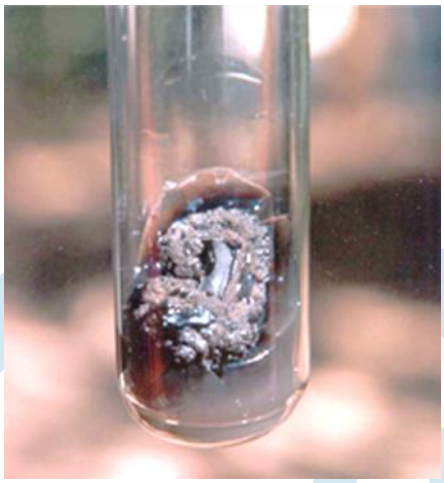

Figure 2: Phenol exudation into the medium of *Piper* callus

Though there are no reports regarding pepper callus peroxidase in dye degradation, in vitro cultures of *Piper nigrum* have been reported to be effective against toxic metabolite-producing pathogenic microbes such as *Escherichia coli* and *Pseudomonas aeruginosa* [1].

Dye Degradation Studies

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

Enzymatic degradation of dyes from different effluents using plant peroxidases has been previously reported [5]. However, the plants commonly studied, such as soybean, *Luffa acutangula*, and horseradish, are not abundantly available in Kerala. Hence, the locally available plant *Piper nigrum* was selected for the present study and was found to be a good alternative source of peroxidase.

There are also reports on the use of horseradish peroxidase immobilized by copolymerization into cross-linked polyacrylamide gel for degradation and detoxification of azo dyes such as methyl orange [8]. However, most of these methods are costly. Therefore, extensive research is being carried out to identify alternative source plants for commercial horseradish peroxidase.

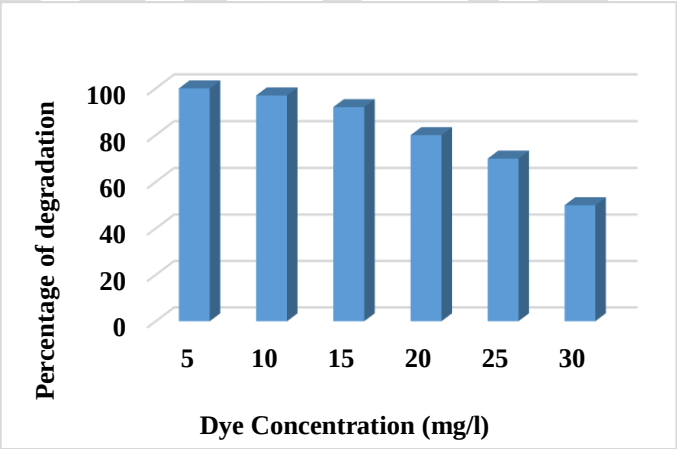

Figure 3 Degradation of different concentrations of dye

IV. 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 media making it reusable.

V. ACKNOWLEDGEMENT

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