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ORIGINAL ARTICLE

BIODEGRADATION OF AZO DYE USING PEROXIDASE ENZYME EXTRACTED FROM MOSSES & CHARACTERIZATION OF DEGRADED PRODUCT

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Abstract:

Peroxidase enzyme from mosses was extracted and purified by using 70% ammonium sulphate precipitation. In burette, it was found that crude sample of Rhincostagella contains more proteins as compared to Poheli and Plagiominium. The maximum absorbance of enzyme was noted at 470 nm, which confirms the isolated enzyme is peroxidises. Guaiacol showed maximum reactivity in staining activity. Optimum temperature and pH were found to be 30oC and 7oC respectively. It was observed that all metals have inhibitory effect on enzyme activity except ZnSO4 and cystein. Beta mercaptoethanol showed maximum inhibition while ystein shows least inhibition while ZnSO4 and cystein showed Inducers effect at 1mM concentration of enzyme. As the substrate concentration increases the specific activity of enzyme also increased. The decolourization of malachite green with peroxidases was recorded up to 70 to 85 %. The degradation was monitored by UV-Visible spectrophotometer, GC-MS.

KEY WORDS:
Mosses, Peroxidases enzyme, Purification, Reactivity, Dye decolourization etc.

.INTRODUCTION

Different types of pollutants are discharged in the surroundings from large number of industries and factories, especially in the form of dyes and dye intermediates. Dyestuffs are chemically inorganic pollutants and comprise traces of heavy metals which are toxic, hazardous to the environment as they are non-degradable and tedious to recycle (Mohamed et al., 2012). Dyes that adds bright colour to our life have always been fascinating to us. The majority of the colour effluents consisting of dyes are released into the environment by various industries. Some industries include textile, pulp and paper, dyestuff and dye manufacture, leather industries etc. Staining pollution in the environment has become a major problem (Kalyani, 2007).

Large numbers of chemically different dyes are used in various industrial applications. A significant proportion of these dyes are introduced into the environment in the form of wastewater, spoiling the environment (Jadhav et al., 2007). A major class of synthetic dyes includes azo, anthroquinone and triphenylmethane dyes (Parshetti et al., 2006). Azo dyes represent a major group of dyes mostly used in industry, which are causing environmental concern because of their color, biorecalcitrance, potential toxicity to animals and human (Parshetti et al., 2007). Biotransformation of azo dyes leads to formation of aromatic amines. Industries generate a number of harmful pollutants which are discharged into the surrounding environment without treatment. Many biological agents can be used to mitigate the pollution problem.

The enzymatic approach has attracted much interest in the decolorization and degradation of textile and other industrially important dyes from wastewater (Hussain et al., 2000; Duran et al., 2000). Bryophytes grow on soil, rocks, tree trunks, branches, leaves, buildings, old monuments etc. and in

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wetlands (Sahu et al., 2007). Hence treatment of such effluents with bryophytes for degradation of dyes is simple, eco friendly, natural, time and efforts saving method. And this method has no known drawbacks. Further bryophytes have many advantages over the conventional chemical, physical as well as microbial treatment methods which pose some serious limitations (Duran et al., 2000).

MATERIAL AND METHODS

Mosses Sample used are Plagiominiumcuspidatum, Rhincostagelladivaricatifolia, Poheliagedeana. Bryophyte samples were collected from Ranikhet and Mukteshwar of Kumoon hills (Western Himalaya, India).

Extraction of Peroxidases from mosses:

Extraction of peroxidases was carried out by following Putter (1974) method:

Preparation of crude extract, 30g of fresh sample was homogenized with 0.1M phosphate buffer (pH 7.4) in the ratio 1:1 (w/v) (TanX, L. P. Y. 1988). Filter the extract, this filtrate was centrifuged and supernatant was used as a crude peroxidase enzyme source for further experimentation. Purification of the Peroxidases from Crude sample:

The supernatant was saturated with 70% solid ammonium sulphate [(NH4)2SO4]. The precipitated enzyme peroxidases, was separated by centrifugation at 10,000 rpm for 15 min. The pellet obtained was dissolved in 0.1 M phosphate buffer (pH 7.4) and dialyzed at 4°C for overnight. The dialyzed solution was used as purified enzyme. Peroxidases activity was determined by measuring an increase in absorbance at 470 nm, with guaiacol as a substrate.

Protein Quantification:

The protein content was measured by Biuret method using BSA as the standard procedure. Biuret reagent was prepared and distributed in all test tubes. The control contains only 3 ml biuret reagent and 0.5 ml phosphate buffer, while test samples contain 3 ml of biuret reagent and 5 ml enzyme. Both the samples were incubated at room temperature for half hour analysed on spectrophotometer at 520 nm. Standards were prepared and concentrations of crude, purified enzyme samples were calculated.

Substrate Specificity:

Substrate specificity of purified enzyme was determined by using different substrates such as catechol, L-DOPA, and guaiacol.

Protein partial characterization:

Protein identification was done by using polyacrylamide gel electrophoresis (PAGE). Two gels were run simultaneously. Equal amounts of enzyme sample applied to both the gels. One gel was used for activity staining with substrate guaiacol while other gel is used for CBB. After native PAGE, the gel was stained with CBB for 2 to 3 hours. Marker is run along with samples.

Activity Staining:

After native PAGE, peroxidases activity was visualized with guaiacol as substrate. The gel was equilibrated for 15 min at RT in 0.1 M phosphate buffer (pH 7.4) and incubated for 30 min in guaiacol under vigorous aeration and 2 to 3 drops of hydrogen peroxide (H2O2) were added to start the reaction. White band indicated sites of peroxidases activity.

Effect of different Parameters on Enzyme activity:

The parameters taken into consideration were temperature, pH, effect of Metals, effect of Inhibitors (Inc. Concentration of substrate), effect of increasing concentrations of dye.

Effect of inhibitors on Peroxidases:

Peroxidases activity were measured by using ten different inhibitors (citric acid -192.12 (C6H8O7, EDTA), ascorbic acid (Vit C -176.13), sodium chloride (NaCl -58.44), zinc sulphate (ZnSO4), Beta –mercaptoethanol (C2H6O5, -78.13) , barium chloride (BaCl2), manganese chloride (MnCl2 - 197.90), L- dopa and sodium azide at two concentrations 1mM and 10 mM with guaiacol as the substrate.

Inhibition by increasing concentration of dye on Peroxidases:

The solution of guaiacol was prepared in phosphate buffer (pH 7). A set of five test tubes containing 0.1 ml to 0.5 ml of dye solution were prepared to these test tubes 1.9 ml to 1.5 ml phosphate buffer were added respectively to make final volume 2 ml. About 0.5 ml of enzyme was added to these test

tubes one by one and activity were calculated at optimum pH and temperature.

Effect of concentration variability of dye on Peroxidases activity:

The increasing concentration of the dye were analysed by two methods, in first method enzyme was used once and in second method the repeated use of the enzyme was made to calculate its efficiency. In first method ten test tubes containing 1 to 10 ml of dye were prepared; 0.5 ml of enzyme extract was added to each test tube for initial reading. A test sample was left for decolourization, after decolourization time was noted; final values were recorded at 470 nm for specific activity calculations. In the second method 1 ml of dye (22 mg/20 ml) and 0.5 ml enzyme extract was taken in a test tube and initial values were recorded at 470 nm. After decolourisation absorbances were noted, one more ml of dye was added. The procedure was repeated till 10 ml dye is used and every time values were taken and specific activity was calculated. Small decrease in specific activity was observed.

Action of Peroxidases on textile dyes:

Action of enzyme on dyes, malachite green was checked spectro-photometrically. Two samples were prepared; one control contains 1.5 ml dye solution (conc. 2mg/100ml) and 0.5 ml phosphate buffer (pH 7.4). Second test sample contains 1.5 ml methyl red and 0.5 ml crude extract. The disappearances of colour by peroxidases were monitored at predetermined λmax of the respective dye solution. The absorbance at 470 nm were recorded every 5 sec decrease in values (OD) indicated the degradation of the dye. The percent decolourizations were calculated by taking the maximum absorbance of each untreated dye solution as control (100%).

Gas Chromatography Mass Spectroscopy (GC-MS):

GC-MS analysis of metabolites were carried out using a Shimadza 2010 MS engine, equipped with integrated gas chromatography with a HPI column (60m long, 0.25mm id, non polar). Helium was used as a carrier gas at a flow rate of 1ml/min. the injector temperature was maintained at 28°C with oven conditions as: 80°C kept constant for two min.- increased up to 200°C with 10°C/min.- raised up to 28°C with 280°C/min rate. The compounds were identified on the basis of mass spectra using quadrapole detector and the NIST library.

RESULT AND DISSCUTIONS

Protein estimation using Biuret method:

Three samples of crude enzyme showed the highest protein concentration, further enzyme with 50% ammonium sulphate precipitation, then enzyme with 70% ammonium sulphate precipitation (Crude > P (50%) > P (70%))

Meaning protein concentrations decrease after purification and purity of crude enzyme sample increases (P (70%) > P (50%) > P (Crude))

Comparative study:

Crude sample of Rhincostagella contains more proteins as compare to Pohelia and Plagiominium i.e. enzyme concentration in three sample are Rhincostagella>Plagiominium>Pohelia

Sample	*Plagioniumcuspidatum*	*Rhincostagelladivaricatifolia*	*Poheliagedean*
	Spcific activity	Spcific activity	Specific activity
Protein crude	0.657	1.476	1.819
Protein purified	3.121	2.207	2.494

Table 1. Extraction and purification of peroxidise enzyme from three different samples

These values showed that the extraction and purification of crude enzyme were carried out successfully.

Comparative Study:

Crude sample of Plagioniumcuspidatum was purified by 4.75 times sample of Pohelia was purified by 2.40 times, while Sample of Rhincostagella was purified by 1.37 times.

Substrate specificity:

It showed maximum affinity with guaiacol, followed by L-DOPA, and catechol. Meaning isolated enzyme is peroxidases and showed substrate affinity in table 2.

Substrate	*Plagioniumcuspidatum*		*Rhincostagelladivaricatifolia*		*Poheliagedean*	
	Enzyme activity	**Specific enzyme activity.**	**Enzyme activity**	**Specific enzyme activity.**	**Enzyme activity**	**Specific enzyme activity.**
Catechol	0.018	0.038	0.027	0.094	0.026	0.134
Guaiacol	**0.709**	**2.997**	**0.235**	**0.824**	**0.406**	**2.092**
L – Dopa	0.056	0.118	0.128	0.449	0.123	0.634

Table 2. (Substrate specificity of Peroxidase enzyme).

Molecular Weight determination and CBB staining:

Purified enzyme produced a single band on gel which shows that isolated enzyme is monomer. All enzymes showed bands with markers, indicating their weight is near to 44KDa, confirming that samples run are peroxidases. First well contained marker, Second well contained sample from plagionium, third well contained sample of Rhincostagella while sample from Pohelia is present in fourth or last. (Figure 1: CBB staining)

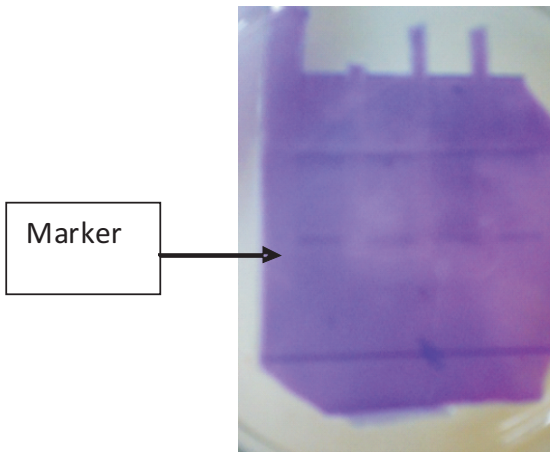


Figure 1. CBB staining

Activity Staining:

The bands obtained by electrophoresis are then treated with different substrate in activity staining. It showed maximum reactivity with guaiacol (figure 1) in comparison to other substrates. (Figure 2: Activity staining) Samples from Plagionium, Rhincostagella, Pohelia are present in first, Second and third well respectively.

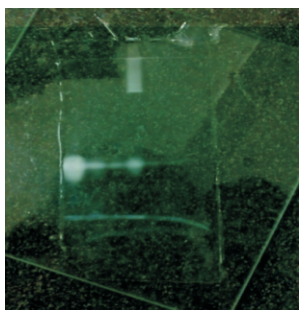


Figure 2. Activity staining

Spectroscopic properties:

When Absorbance was taken to the different at different wavelengths, it was found that the maximum absorbance of enzyme from all the three samples was observed at 470 nm. This observation confirmed the enzyme preparation to be homogeneous.

Effect of temperature on peroxidases:

Optimum temperature:

The purified enzyme shows an optimum temperature of 300C i.e. maximum specific activity of peroxidase was seen at 300C.which increase from 20 to 30 and then decreases after 300C and becomes very less at 700C.

Optimum pH:

During testing the effect of pH on enzyme activity an optimum pH of 7 was found for plagionium and pohelia, which increases from pH 5 to 7 and then decreases from 7 to 9. Least activity is seen at pH 9, while 7.4 was found to be optimum for pohelia.

Effect of increasing substrate concentration:

When the substrate concentration (guaiacol) of enzyme was increased from 0.1 to 0.5, it was observed that specific activity of enzyme was also increased as the substrate concentration increased.

Effect of various metals on enzyme activity:

1) Effect of lower metal concentration on enzyme activity:

All the metals showed absorbance less than control meaning that nearly all metals have inhibitory effect on enzyme activity except ZnSO4. Beta mercaptoethanol showed maximum inhibition while ZnSO4 showed inducers effect at 1mM concentration of enzyme.

2) Effect of higher metal concentration on enzyme activity (10mM):

After using higher concentration of metals, all metals showed inhibitory effect. Betamercaptoetanol showed maximum inhibition, while MnCl2 showed minimum inhibition as comparison with other metals. While BaCl2 and cystein showed nearly same inhibitory effect.

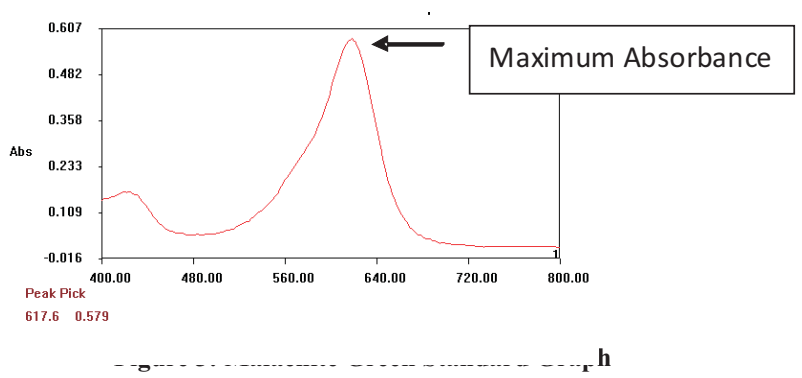
Calculations for IC50:

By using Na – Azide

When increasing concentration of Na-Azide is used, 0.005 concentration does not showed inhibition on enzyme activity. Maximum inhibitions were seen at 0.03 concentration of Na – Azide, while 0.04 and 0.1 concentration showed increased enzyme activity. 50% inhibition was found at 0.02 concentrations.

UV-Visible Spectra of methyl red and malachite green treated with peroxidase:

The observed standard line showed the UV spectra of malachite green before and after the treatment with peroxidase. Malachite green showed maximum absorbance at 590 nm however, there was a remarkable decrease in absorbance peaks after the treatment with enzymes. The complete decolourisation of the dye took 48 to 72 hrs. Peroxidase enzyme caused 100% decolourization of malachite green. In the present study aggregation of dyes were not observed. This indicated the removal of dyes was due to degradation and not due to precipitation (Figure 3 to 6).



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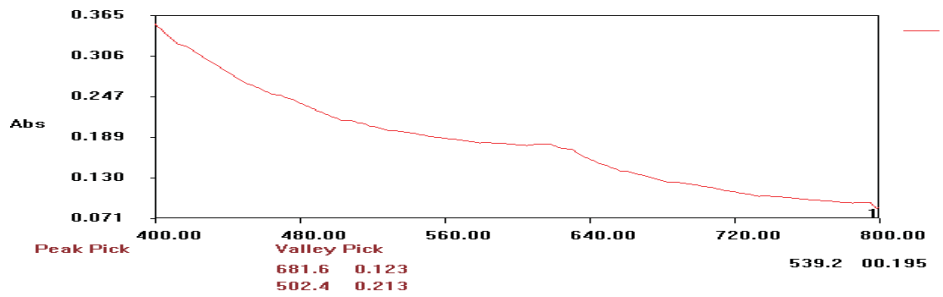

Figure 4: Degraded product of malachite green by peroxidases from r*hincostagella*

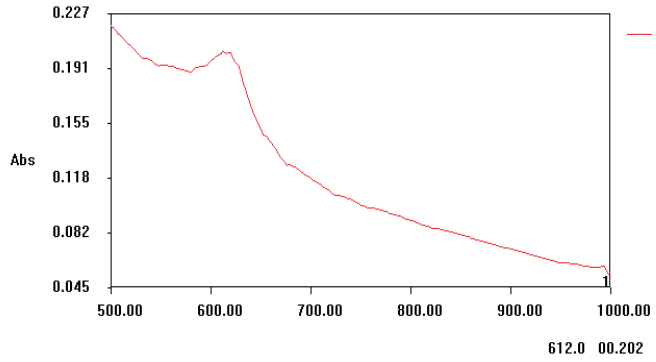

Figure 5: Degraded product of malachite green by peroxidases from *plagionium*

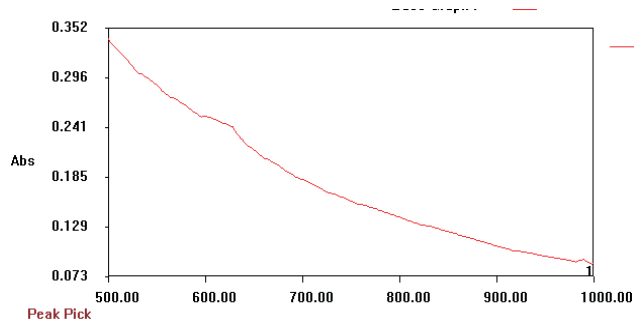

Figure 6: Degraded product of malachite green by peroxidases from *pohelia*

Decolorization of Textile Dyes by and peroxides:

When dye degradation capacity of peroxidases, was observed, the time required for dye decolorization ranged from 48 to 72 hrs for decolourisation. Percentage decolorization was calculated and is shown in table 3

Sample	% Decolourization.	Hours required.
Plagionium	80 to 85	72
Rhincostagella	75 to 80	72
Pohelia.	70 to 80	72

Table 3 - Percentage Decolorization of 2mg/100ml concentrated dye

Effect of Degraded sample on seed germination:

The effect of degraded dye on seed germination was demonstrated by using the paper towel method of seed germination. In this method different type of seeds, at least each 10 in number were soaked in dye solutions (negative control), clean water (positive control) and degraded dye solution (sample). Seeds were allowed to soak for at least for 6-10 hours. After soaking seeds were wrapped in wet filter paper leaving a distance of 2-3 cm between them. The filter papers, with seeds, were given the proper moisture after proper time intervals. The seeds germinated in three to five days. After the seeds germinated the frequency of germination from all the solutions were measured.

Seed used	Plagionium	Rhincostagella	Pohelia.	Positive Control (Water)	Negative control (conc. Dye)
Cereals	13/20	15/20	17/20	19/20	5/20
pulses	14/20	18/20	18/20	17/20	2/20
Grams	17/20	15/20	16/20	19/20	3/20

Table 4: Effect of dyes and degraded sample on seed germination

Result of GC-MS:

The samples were prepared in ethyl acetate, and then GC-MS and HPLC analysis of samples were carried out. The standard curves were obtained. The curve of GC showed different peaks with different retention time. MS showed different m/z ratio for all samples which shows that different compounds are present in different sample (Figure 7 to 9)

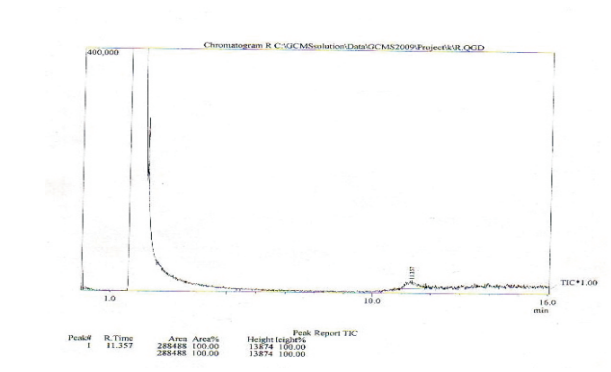

Figure 7: GC-MS analysis of r*hincostagella* sample

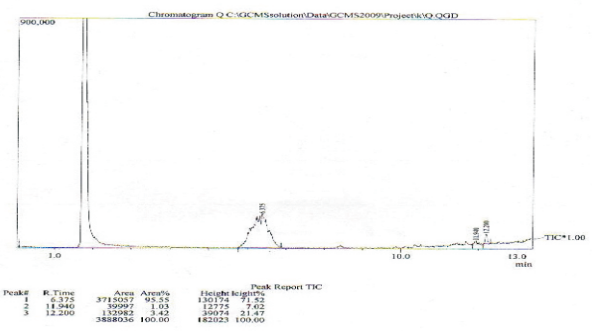

Figure 8: GC-MS analysis of *plagionium* sample

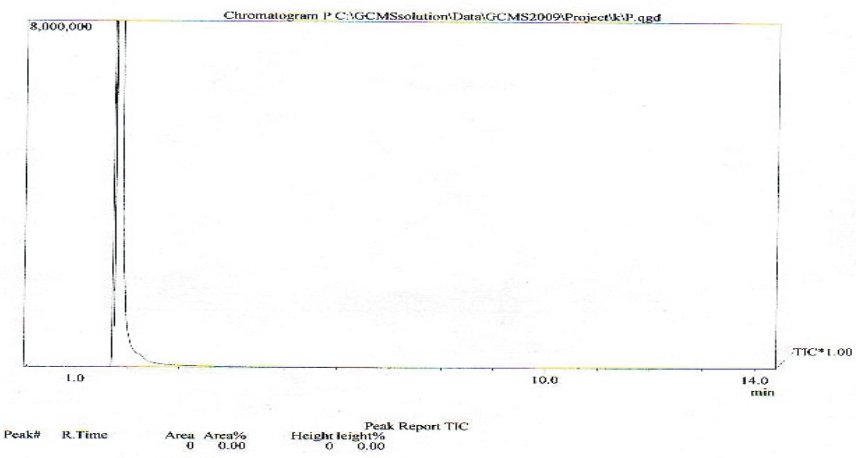

Figure 9: GC-MS analysis of *pohelia* sample

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