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BIODEGRADATION OF BROMOPHENOL BLUE AND BROMOCRESOL GREEN BY LACCASE ENZYME FROM MALUS DOMESTICA.

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ABSTRACT The rapid increase in industrialization and urbanization across the globe has increased the harmful contaminants into our ecosystem. One such is the synthetic dyes from textile industries. The synthetic dyes from these industries contaminate the water bodies causing water pollution and is a major environmental hazard. The Laccase (E.C.1.10.3.2) is a potent enzyme that catalyzes the degradation of synthetic dyes. It is extensively studied in microorganisms. Not much is explored from plant sources. In our current study, we have isolated laccase from Malus domestica (fruit pulp of apple). The substrate used for laccase was guaiacol. The maximum enzyme activity was found to be 2.67U/ml. The kinetic parameters were studied and optimum conditions were pH - 7, temp was 26°C, time- 30 min. The crude enzyme was subjected to a series of purification steps that included ammonium sulphate precipitation followed by dialysis and the dialyzed sample was subjected to anion exchange chromatography using DEAE cellulose. The decolorization of dye was seen after incubation of 72hrs. Complete degradation was accomplished in 8 days.

KEYWORDS : biodegradation, laccase, synthetic dyes, Malus domestica.

Currently across the globe, the textile industries uses over 10,000 different dyes and in specific 7×10^5 tons of synthetic dyes are annually produced worldwide. These synthetic dyes make the fabric look attractive but they are hazardous to the environment. These synthetic dyes are washed away as effluents into the water bodies. The wastewater from textile plants is presumed to be the most recalcitrant pollutant in the environment. Also, the increased demand for textile products is proportional to the use of synthetic dyes and both together contribute to water pollution as the synthetic dyes are not degraded. And this dye wastewater is one of the substantial sources for severe pollution in current times (Farah Maria Drumond Chequer et al., 2013) Synthetic dyes are xenobiotic, toxic and carcinogenic and have serious effects on man. The present research is focused on biodegradation of synthetic dyes using laccase enzyme from the apple fruit.

Laccase (E.C.1.10.32) is an oxidoreductase enzyme. It belongs to the blue multi-copper oxidases, which can degrade the synthetic dyes (Shraddha et al., 2011). It is broadly distributed among bacteria, fungi, plants and insects. It is extensively studied in fungi. Very few works on laccase has been reported in plants (Vinit Kumar et al., 2013). In the current piece of work, laccase enzyme is isolated from the fruit pulp of the plant *Malus domestica* (apple) and dye decolorization studies were carried out. The dye decolorization studies showed 60% dye decolorization of bromophenol blue and 44% in bromocresol green.

OBJECTIVES

- Screening of laccase enzyme from different plant source.
- Extraction, isolation and partial purification of the laccase enzyme from *Malus domestica.*
- Enzyme assay and kinetic studies.
- Dye degradation studies using bromophenol blue and bromocresol green.

MATERIALS AND METHODS

Buffer Extraction

10g of the fruit pulp of *Malus domestica* was weighed and homogenized in 100ml of 0.1M phosphate-citrate buffer (pH=5). It was centrifuged at 10000 rpm for 15 minutes at 4°C. The supernatant was used as crude enzyme sample. (Benjamin, 1997)

Enzyme Assay

Many substrates are used for laccase such as syringaldazine, guaiacol, DMP, ABTS, etc. Guaiacol was used as the substrate for laccase in our investigation. Enzymatic assay was carried out by adding 3.0ml of 100mM phosphate-citrate buffer (pH = 5), 1.0ml of 10mM guaiacol and 1.0ml of the crude enzyme and a blank was prepared by adding 3.0ml of buffer, 1.0ml of guaiacol and 1.0ml distilled water. The tubes were incubated at room temperature for 10 minutes and absorbance was read at 470nm using UV-Visible spectrophotometer. (Jadhav et al., 2009) Enzyme activity was expressed in the international unit. The laccase activity was determined by using the formula:

$$\text{Enzyme activity} = \frac{A \times 4 \times V_t \times D.F}{E \times V_s}$$

Where, A = absorbance at 450nm, 4 = derived from unit definition and principle, V_t = final volume of reaction mixture, D.F = Dilution factor, E = extinction co-efficient of guaiacol (0.6740µm/cm), V_s = sample volume.

Bradford Assay

Protein in the sample was estimated by Bradford assay. Coomassie Brilliant Blue G-250 was dissolved in 50ml of 95% ethanol. The resulting solution was diluted to a final volume of 1 litre (Marion.M.Bradford, 1976). The standard protein used was BSA (1mg/ml).

Enzyme Kinetics

Effect of pH: Phosphate-citrate buffer (0.1M) of pH ranging from 4,5,6,7 and 8 was used and the enzymatic assay was carried out and tubes were incubated at room temperature for 30 minutes and absorbance was read at 470nm using UV-Visible spectrophotometer.

Effect of Temperature: The enzymatic assay was carried out by using 0.1M phosphate-citrate buffer, 100mM guaiacol and crude enzyme source, the tubes were incubated at varied temperature 4°C, room temperature(21°C), 26°C, 40°C, boiling water bath(100ºC) for 30 minutes and absorbance was read at 470nm using UV- Visible spectrophotometer.

Effect of Substrate: The enzymatic assay was done using the different volume of guaiacol and then buffer was added and incubated at room temperature for 30 minutes and absorbance was read at 470nm using UV-Visible spectrophotometer.

Partial purification of laccase:

Ammonium Sulphate Precipitation: Crude enzyme was filtered through Whatman filter paper No.1 and the total volume measured was 100ml. The crude enzyme was precipitated by 80% saturation. The beaker containing the sample was placed on a magnetic stirrer and ammonium sulphate salt was added little by little with continuous stirring. Once the total salt was added the beaker placed at 4°C

overnight. It was then centrifuged at 10000rpm for 15 minutes. Supernatant was discarded and the pellet was dissolved in 10mM Tris-HCl. (Distasioe et al., 1976)

Dialysis: The dialysis bag was activated by boiling the bag in 100ml water for 20 minutes. Then to that water 2% sodium bicarbonate was added little by little for 10 minutes, the bag was then transferred to fresh 100ml boiling water for 10 more minutes. Then the bag was cooled at room temperature. The partially purified enzyme was poured in the activated dialyzed bag and was tied tightly; this bag was placed in a beaker of water and incubated at 4°C overnight. After overnight incubation, the beakers were placed on the magnetic stirrer for 2 hours, every half an hour the water was changed.

Purification

The dialyzed sample was further subjected to ion-exchange chromatography. The resin used was DEAE-Cellulose-52. Elution buffer was prepared using NaCl and Tris-HCl with different concentration (50mM, 75mM, 100mM, 125mM,150mM, 175mM), the gel was cast, washed and calibrated with the elution buffer and the sample was loaded and different elution was collected (volume = 2ml) and checked for enzyme activity.

Dye Degradation

Two synthetic dyes were chosen bromophenol blue and bromocresol green. Stock solutions (1mg/ml) of both dyes were prepared. Stock was prepared by dissolving 0.1g of dye in 10ml of solvent. 0.25ml stock and 99.75ml of distilled water was added to get 25ppm concentration. The dye solution was incubated with enzyme sample for dye decolorization.

The λ_{max} of bromophenol blue is 590nm and the λ_{max} of bromocresol green is 423nm. The absorbance after incubation period was read in UV-Visible spectrophotometer.

$$\% \text{ Decolorization} = \frac{\text{initial decolorization} - \text{final decolorization}}{\text{initial decolorization}} \times 100$$

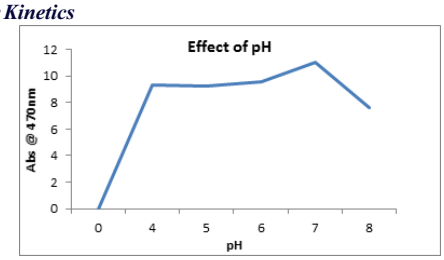
RESULTS

Table 1
Enzyme activity

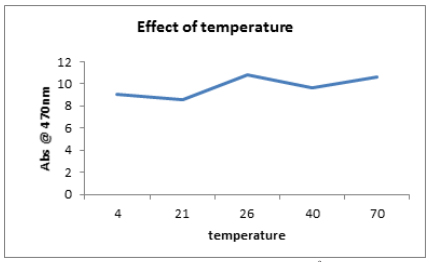
Sample	Buffer (ml)	Substrate (ml)		Enzyme (ml)	Abs @ 470nm	Enzyme activity (U/ml)
Blank	3.0	1.0	Incubate at room temp. for 10 mins	-	0.0	0.0
Apple	3.0	1.0		1.0	0.090	**2.67**

The protein was estimated by Bradford assay and the protein concentration in the sample was found to be **1080µg.**

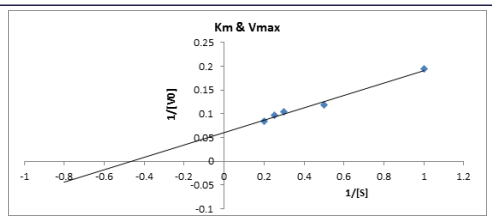
Enzyme Kinetics



The maximum enzyme activity was seen at pH 7 which was 11.06 U/ml and the least enzyme activity was seen at pH 5 which was 9.22U/ml.



The maximum enzyme activity was seen at 26°C = 10.8U/ml and the least enzyme activity was seen at 4°C = 9.02U/ml.



K_m=2.22mM; V_{max}= 16µmol/min

Partial purification

The enzyme activity of the crude enzyme which was partially purified by ammonium sulphate precipitation and was found to be **2.9 U/ml.**

Dialysis

The dialyzed sample was recovered and the enzyme assay was carried out. The enzyme activity was **2.96U/ml.**

Purification

Table 2: Ion-Exchange Chromatography

Elution no.	Vol of Enzyme (ml)	Vol of substrate (ml)	Vol of Buffer (ml)		Abs at 450nm	Enzyme activity (U/ml)
T_1	0.0	0.5	1.5	Incubate at room temp. for 10 mins	0.0	0.00
T_2	0.5	0.5	1.5		0.007	0.207
T_3	0.5	0.5	1.5		0.017	0.504
T_4	0.5	0.5	1.5		0.009	0.267
T_5	0.5	0.5	1.5		0.100	0.296
T_6	0.5	0.5	1.5		0.026	**0.771**
T_7	0.5	0.5	1.5		0.09	0.029

The dialyzed sample was subjected to anion exchange chromatography and the maximum enzymatic activity was found to be **0.771U/ml** in the eluted sample.

Dye Degradation:

Table 4: Dye Degradation Percentage

	Crude (%)	Ion exchange (%)
Bromophenol blue	59.3	65.4
Bromocresol green	43.7	47.4

Effective degradation of bromophenol blue by upto 65% was recorded from laccase purified by *Malus domestica*.

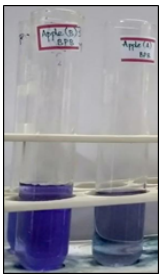


Figure1: Degradation of Bromophenol Blue

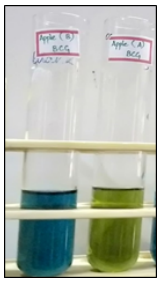


Figure2: Degradation of Bromocresol Green

CONCLUSION

The synthetic dye from the textile industry pollutes the water bodies and is harmful to the aquatic life and even humans. They are not degraded by microbes and hence an effort is made in the present work to degrade the synthetic dyes by isolating laccase enzyme from *M. domestica* and has shown promising results, hence further investigation in this area is required for commercial application.

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