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Decolourization of bromophenol blue and bromocresol green by laccase enzyme from *Brassica oleraceae*

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Abstract

Industrialization has led to a serious threat to water bodies and causing harmful impacts on living organisms. Industries discharge untreated waste water directly into the environment causing environmental pollution. Biotechnologists across the world are trying to develop innovative processes to check global pollution. However, the quantity of various pollutants discharged in the water bodies is increasing tremendously. One of the harmful pollutants is the synthetic dyes from textile industries. They are toxic and carcinogenic and are hazardous to human health. The present research work focused on solving this problem cost-effectively to some extent. Laccase (E.C.1.10.3.2) is a significant and ubiquitous enzyme which plays avital role in degradation of synthetic dyes. In the current study, laccase enzyme from Brassica oleraceae has been isolated. The substrate used for laccase wasguaiacol. The enzyme activity was found to be 0.83U/ml. The kinetic parameters were studied and optimum conditions recorded were as follows: pH: 4, temperature: 100°C, incubation time: 30 minutes. The crude laccase enzyme was subjected to purification steps that involved ammonium sulphate salt precipitation followed by dialysis and anion-exchange chromatography using DEAE cellulose. The decolorization of synthetic dyes by laccase was seen after incubation of 72hrs. Complete degradation was accomplished in eight days.

Keywords: Biodegradation, laccase, bromophenol blue, bromocresol green, guaiacol, *Brassica oleraceae*.

Introduction

The untreated water which contains toxic synthetic dyes from textile industries is discharged directly into the water bodies. This water is unfit for agricultural land and is harmful to crop plants. Textile industries use both natural and synthetic dyes[1]. Natural dyes like turmeric, red sandalwood, etc. are biodegradable but synthetic dyeslike azo dyes, acidic dyes, etc. are non-biodegradable and pose a threat when disposed into water bodies[2]. They cause water pollution and contaminate water resources causing threat to aquatic life. These dyes are xenobiotic, toxic and carcinogenic and have serious effects on man. The synthetic dyes are used in textile industries to make fabric look colorful but they are hazardous to the environment[3].

Laccase (E.C.1.10.3.2) is an oxidoreductase enzyme and belongs to the blue multicopper oxidases. It participates in the degradation of polymers and ring cleavage of aromatic compounds[4]. These enzymes have gained application in textile, pulp and paper and food industry. In recent years it has been used as a bioremediation agent to clean up herbicides and pesticides in the soil. It also can oxidize and degrade the phenolic, non-phenolic and synthetic dyes[5].

It is broadly distributed among bacteria, fungi, plants and insects. It is extensively studied in fungi[6,7]. Very few workon laccase has been reported in plants. The present study deals with the extraction of laccase enzyme from the plant *Brassica oleracea* and its decolorization effect on synthetic dye. This investigation reported decolorization of bromophenol blue by up to 63% and bromocresol green dye was decolorized up to 44%.

The objective of this study was to assess the screening of laccase from plants and its extraction, isolation and partial purification from *Brassica oleracea,* enzyme assay and kinetic studies of laccase and to determine dye degradation using bromophenol blue and bromocresol green[8-11].

Materials and methods

Buffer extraction: 10g pulp of German turnip was weighed and homogenized in 100ml of 0.1M phosphate-citrate buffer (pH=5) and then centrifuged at 10,000rpm for 15 minutes at 4°C. The supernatant was used as crude enzyme extract[12,13].

Enzyme assay: Various substrates were used for laccase such as syringaldazine, guaiacol, DMP (2,6-dimethoxyphenol), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), etc. In the current investigation, guaiacol was used as the substrate. Enzymatic assay was carried out by adding 3.0ml 100mM phosphate-citrate buffer (pH=5), 1.0ml 10mM guaiacoland 1.0ml of the crude enzyme.

A blank was also prepared by adding 3.0ml buffer, 1.0ml guaiacol, and 1.0ml of distilled water.

The tubes were incubated at room temperature for 10minutes and absorbance was read at 470nm in UV- Visible spectrophotometer[14].

The enzyme activity was expressed in the international unit. The laccase activity was determined by using the formula[15]:

$$\text{Laccase enzyme activity} = \frac{\text{A X 4 X Vt X D. F}}{\text{E X Vs}}$$

Where, A = absorbance at 470nm, 4 = derived unit definition and principle, V_t= final volume of reaction mixture, D.F = dilution factor, E = Extinction co-efficient of guaiacol (0.6740µm cm^{-1}), V_s= sample volume.

The protein was estimated by Bradford's method[16]: Enzyme Kinetics: For pH studies phosphate-citrate buffer (0.1M) of pH ranging from 4,5,6,7 and 8 were used for the enzyme assay and the tubes were incubated at room temperature for 30 minutes and absorbance was read at 470nm in UV-Visible spectrophotometer[14].

For temperature studies, 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 viz., 4°C, 21°C, 26°C, 40°C, 100ºC for 30 minutes and absorbance was read at 470nm in UV - Visible spectrophotometer.

Similarly for substrate evaluation, the enzyme assay was performed using different volume of guaiacol and then 0.1M phosphate-citrate buffer was added and incubated at room temperature for 30 minutes and absorbance was read at 470nm in 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 in a beaker by 80% saturation and placed on a magnetic stirrer. Ammonium sulphate salt was added little by little with continuous stirring. The beaker was then placed at 4°C overnight and the sample was centrifuged at 10,000rpm for 15 minutes. Supernatant was discarded and the pellet was dissolved in 10mM tris-HCl[15,18].

Dialysis: The dialysis bag was activated by boiling the bag in 100ml of water for 20 minutes. Sodium bicarbonate (2%) was added in small amounts to the boiling water. After 10 minutes the bag was then transferred to fresh 100mlboiling 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 containing water and incubated at 4°C overnight. After overnight incubation, the beaker was placed on a magnetic stirrer for 2 hrs. The water was changed after every half an hour[17].

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 and 175mM). The gel was cast, washed and calibrated with the elution buffer and the sample was loaded. The eluted samples were subjected to enzyme assay[18].

Dye degradation: Two synthetic dyes were chosen for the present investigation namely, bromophenol blue and bromocresol green. Stock solutions (1mg ml^{-1}) of both dyes were prepared by dissolving 0.1g of dye in 10ml of solvent. A stock solution of 0.25ml bromophenol blue was added to 99.75mL of distilled water to get 25ppm concentration and bromocresol green dye was also prepared likewise. The dye solution was incubated with the enzyme sample for dye decolorization[19,20].

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

$$\%\text{ of decolorization} = \frac{\text{Initialdecolorization} - \text{Final decolorization}}{\text{Initialdecolorization}} x\ 100$$

Results and discussion

The protein was estimated by Bradford assay and the protein concentration in the sample was found to be 84.8µg/ml. In this present work an effort was made to degrade synthetic dyes with promising results. The laccase enzyme isolated from Brassica oleraceae has shown effective degradation of bromophenol blue by upto 64% and bromocresol green was degraded by 44%. The decolorization of synthetic dyes were seen after incubation of 72hrs. Complete degradation was accomplished in eight days. This method is the cost effective and environment-friendly.

Table-1: Enzyme activity.

Sample	Buffer(ml)	Substrate (ml)	Incubate at room temp. for 10min	Enzyme(ml)	Abs @ 470nm	Enzyme activity(U/ml)
Blank	3.0	1.0		-	0.0	0.0
Turnip	3.0	1.0		1.0	0.029	0.83

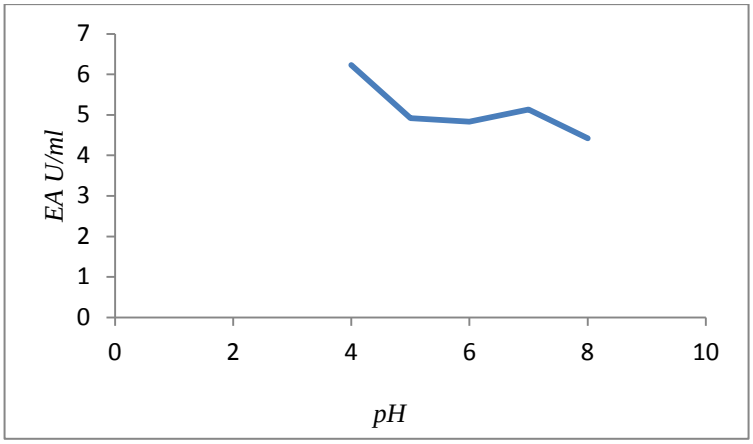

Figure-1: The optimum pH of the laccase enzyme in *Brassica oleraceae* was found to be pH 4.

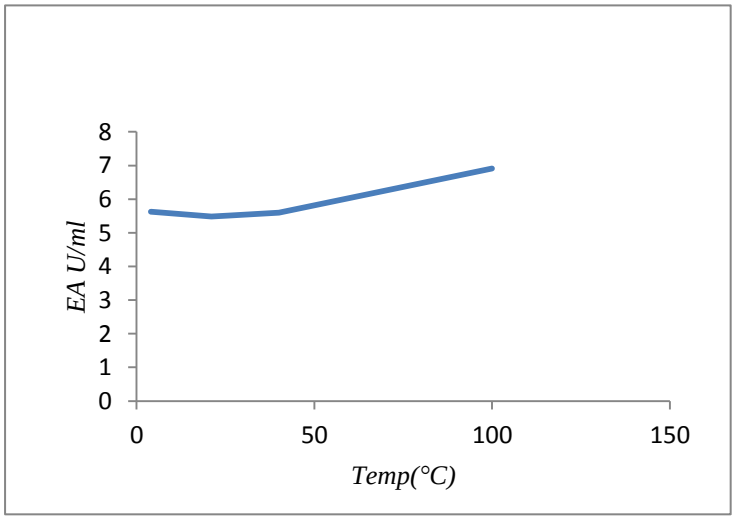

Figure-2: The optimum temperature of was found to be 100°C. Hence this laccase is found to be thermo stable.

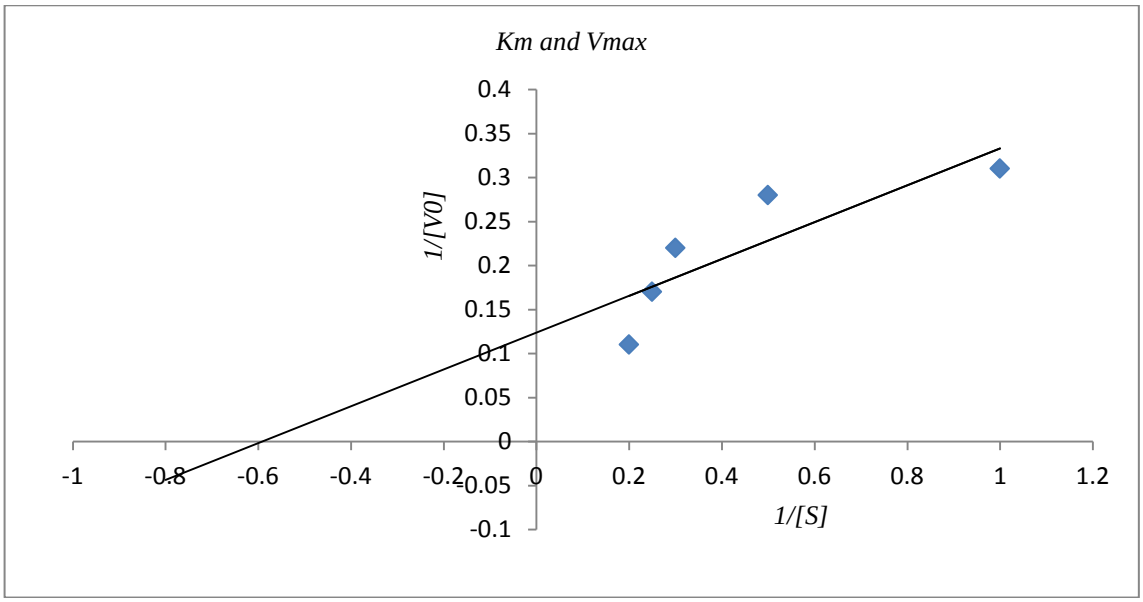

Figure-3: K_m was found to be 66mM and V_{max} was 8µmol/min.

Table-2: Activity of enzyme in different purification steps.

Activity of enzyme	Ammonium Sulphate ppt.	Dialysis	Ion exchange
Enzyme activity (U/ml)	1.36U/ml	1.09U/ml	0.771U/ml

Table-3: Dye degradation.

Dye degradation	Crude (%)	Pure (%)
Bromophenol blue	58.9	63.7
Bromocresol green	41.5	44.0

Figure-4: Degradation of bromophenol green by 44%.

Figure-5: Degradation of bromophenol blue up to 64%.

Conclusion

The synthetic dyes from the textile industry when discharged into the environment, pollutes the water bodies and is harmful to the aquatic life and also humans. They are not degraded by microbes. Many approaches like chemical degradation, precipitation, etc. have been done but are found to be ineffective. Their limitations are, high running cost, time-consuming, and disposal of sludge during the process. An effort has been made in this present work to degrade the synthetic dyes by isolating laccase enzyme from *B.oleraceae* and has shown promising results. Effective degradation of bromophenol blue by up to 64% was recorded and bromocresol green was degraded by 44%. The present method is cost-effective and environmental friendly, an alternative for dye degradation of the effluent from textile industries. Hence further investigation in this area is required for commercial application.

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