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Potential of plant polyphenol oxidases in the decolorization and removal of textile and non-textile dyes

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Abstract

In this study an effort has been made to use plant polyphenol oxidases; potato (*Solanum tuberosum*) and brinjal (*Solanum melongena*), for the treatment of various important dyes used in textile and other industries. The ammonium sulphate fractionated enzyme preparations were used to treat a number of dyes under various experimental conditions. Majority of the treated dyes were maximally decolorized at pH 3.0. Some of the dyes were quickly decolorized whereas others were marginally decolorized. The initial first hour was sufficient for the maximum decolorization of dyes. The rate of decolorization was quite slow on long treatment of dyes. Enhancement in the dye decolorization was noticed on increasing the concentration of enzymes. The complex mixtures of dyes were treated with both preparations of polyphenol oxidases in the buffers of varying pH values. Potato polyphenol oxidase was significantly more effective in decolorizing the dyes to higher extent as compared to the enzyme obtained from brinjal polyphenol oxidase. Decolorization of dyes and their mixtures, followed by the formation of an insoluble precipitate, which could be easily removed simply by centrifugation.

Key words: decolorization; textile dyes; polyphenol oxidases; potato; brinjal; wastewater treatment; removal of dyes

Introduction

There are more than 100000 commercially available dyes with over 7×10^5 t of dyestuff produced annually (Zollinger, 1987; Robinson *et al*., 2001). Due to their chemical structure, most of the dyes are resistant to fading on exposure to light, water and many chemicals (O'Neill *et al*., 1999). There are many structural varieties of dyes that fall into cationic, anionic or nonionic type. Anionic dyes are the direct, acid and reactive dyes (Mishra and Tripathy, 1993). Reactive dyes are the only colorants designed to bond covalently with the fabrics. Reactive dyes contain chromophoric groups such as azo, anthraquinone, triarylmethane etc.,and reactive groups e.g., vinyl sulfone, chlorotriazine, trichloropyrimidine etc., that form covalent bonds with the fiber (Sumathi and Manju, 2001). Azo reactive dyes, the largest class of water-soluble synthetic dyes with the greatest variety of colors and structures are generally resistant to aerobic biodegradation and are not amenable to conventional biological wastewater treatment (Moran *et al*., 1997; Wilmott *et al*., 1998). Possible reason for their non-biodegradability is the lack of requisite enzymes in the conventional biological treatment plants. Anaerobic transformation of azo dyes begins with reductive fission of the azo linkage, resulting in the formation and accumulation of colorless aromatic amines, which can be highly toxic and even carcinogenic (Van der Zee *et al*., 2001). Azo dyes are generally non-degradable by bacteria under aerobic conditions (Abadulla *et al*., 2000).

Conventional chemical and physical methods of dye decolorization/removal are actually outdated due to some unresolved problems (Robinson *et al*., 2001; Lin *et al*., 2003). Biodegradation appears to be a promising technology but unfortunately the analysis of contaminated soil and water has shown that these toxic pollutants persist even in the presence of microorganisms that are completely capable of mineralizing the pollutants. Often the environment of the microorganisms is not optimal for rapid degradation. There is a need to find alternative treatments that are effective in removing dyes from large volumes of effluents and are low in cost (Duran and Esposito, 2000).

Recent studies indicate that an enzymatic approach has attracted much interest in the removal of phenolic pollutants from aqueous solutions as an alternative strategy to the conventional chemical, physical as well as microbial treatments, which pose some serious limitations (Duran and Esposito, 2000; Husain and Jan, 2000; Bhunia *et al*., 2001). Oxidoreductive enzymes such as peroxidases and polyphenol oxidases are participating in the degradation/removal of aromatic pollutants from various contaminated sites. These enzymes can act on a broad range of substrates and they can also catalyze the degradation or removal of organic pollutants present in very low concentration at the contaminated sites (Husain and Jan, 2000). In view of the potential of enzymes in treating the phenolic compounds, several microbial and plant peroxidases and polyphenol oxidases have been considered for the treatment of dyes but none of them has

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been exploited at the large scale due to low enzymatic activity in biological materials and their high cost of purification (Nagai *et al*., 2002; Souza *et al*., 2002; Verma and Madamwar, 2002). Plant peroxidases are catalyzing decolorization of wide spectrum of dyes but they require expensive H_2O_2 as a co-substrate (Bhunia *et al*., 2001; Shaffiqu *et al*., 2002).

Hence an attempt has been done to use systematically partially purified potato and brinjal polyphenol oxidases (PPOs) for the treatment of various textile and other industrially important dyes. Ammonium sulfate fractionated partially purified enzymes were used in this study. The selected dyes were treated with soluble potato and brinjal PPO under various experimental conditions to optimize the conditions for the decolorization of industrially important dyes. Complex mixtures of dyes were also treated with these enzymes.

1 Materials and methods

1.1 Materials

Coomassie Brilliant Blue G-250, Coomassie Brilliant Blue R-250 and PAGE Blue 83 were purchased from Sigma Chemicals Co., USA. Carmine, Naphthalene Black, Methylene Blue, Violet 6 B and 1,2-Naphthaquinone 4-sulphonic acid and Tropaeolin were the products of BDH Ltd., Poole, England. Evans Blue was purchased from Eastman Organic Chemicals, USA. Reactive textile dyes supplied as a free gift by Atul Pvt. Ltd, Gujarat, India. Potato and the brinjal were purchased from the local vegetable market. All other chemicals and reagents employed were of analytical grade and were used without any further purification.

1.2 Extraction of polyphenol oxidases

Potato or brinjal (200 g) were homogenized with 400 ml of pre-cooled 50 mmol/L sodium citrate buffer, pH 4.0 in the presence of benzoic acid (1.8 mg/L). Benzoic acid was used to stop the enzymatic browning during enzyme extraction. The mixture was immediately filtered through the four layers of cheesecloth. The extract was then centrifuged at 3000 g for 10 min at 4°C in a Remi Cooling Centrifuge C-24. The supernatant was then subjected to 0–60% ammonium sulfate fractionation by overnight continuous stirring in cold. The precipitated proteins were then collected by centrifugation at 1000×*g* for 20 min at 4°C and the pellets obtained were then redissolved in 25 ml of 50 mmol/L sodium citrate buffer, pH 4.0. The dissolved proteins were dialyzed against the assay buffer containing benzoic acid 1.8 mg/L (Khan *et al*., 2005).

1.3 Effect of pH on the decolorization of dyes

Eight textile reactive dyes (Reactive Blue 4, Reactive Blue 160, Reactive Blue 171, Reactive Red 11, Reactive Red 120, Reactive Orange 4, Reactive Orange 86 and Reactive Yellow 84) were selected for this study. Each dye was incubated with soluble potato and brinjal PPO, 1.5 EU/ml in the buffers of varying pH values 3.0–5.0 at 37°C for 1 h. The molarity of each buffer was 50 mmol/L. Dye decolorization by PPO was monitored at the specific wavelength. The decolorization percentage was calculated by taking untreated dye solution as control of each buffer (100%).

1.4 Effect of PPO concentration on the decolorization of dyes

The eight textile reactive dyes as the above were decolorized with increasing concentrations of potato and brinjal PPO. Each dye solution was incubated with increasing concentration of potato and brinjal PPO (0.45–1.35 EU/ml) in 50 mmol/L sodium citrate buffer, pH 3.0 at 37°C for 1 h. Dye decolorization was monitored at their respective wavelength maxima. The decolorization rate was calculated by taking untreated dye solution as control (100%).

1.5 Effect of time on the decolorization of dyes

The independent dye solution was incubated with PPO 1.5 EU/ml in 50 mmol/L sodium citrate buffer, pH 3.0 at 37°C and different time. The disappearance of dye color by PPO treatment was recorded at each specific wavelength. The percent decolorization was calculated by taking untreated dye solution as control (100%).

1.6 Decolorization of non-textile dyes

Nine non-textile dyes like Coomassie Brilliant Blue G 250, Coomassie Brilliant Blue R 250, Evans Blue, PAGE Blue 83, Bromophenol Blue, 1,2-Naphthaquinone-4-sulphonic acid, Carmine, Naphthalene Black, Methyl Violet and Tropaeolin were selected for this study. Dye solutions were prepared in the concentration range of 50–100 mg/L in distilled water (Akhtar *et al*., 2005a). All dyes were independently treated with potato and brinjal PPO 1.5 EU/ml in 50 mmol/L sodium citrate buffer, pH 3.0 for 1 h at 37°C. The treated samples were first centrifuged before monitoring decrease in absorbance at a particular wavelength of a dye. Untreated dyes were considered for the calculation of percent decolorization.

1.7 Treatment of mixture of dyes

Mixtures of equal volume four independent dyes were prepared. These mixtures incubated with soluble potato and brinjal PPO 1.5 EU/ml in 50 mmol/L sodium citrate buffer, pH 3.0 for 1 h at 37°C. Dye decolorization by PPO was monitored at the specific wavelength of the mixture. The decolorization rate was calculated by taking untreated dye mixture as control (100%).

1.8 Absorption spectra of Reactive Blue 160 and a mixture of dyes

Reactive Blue 160 solution was prepared in 50 mmol/L sodium citrate buffer, pH 3.0. Dye was independently treated with potato and brinjal PPO 1.5 EU/ml of reaction mixture for 1 h at 37°C. After 1 h the insoluble precipitate was removed by centrifugation and absorbance spectrum was recorded at various wavelengths by using a spectrophotometer Cintra 10e, Australia.

The mixture of four dyes (Reactive Red 120, Reactive Red 11, Reactive Orange 4 and Reactive Blue 160) was prepared by taking each dye in equal proportions. This mixture was treated with potato and brinjal PPO 1.5 EU/ml of reaction volume for 1 h at 37°C. After treatment the insoluble precipitate was removed by centrifugation and the absorbance spectrum was recorded in UV-visible regions of the spectrum using a spectrophotometer Cintra 10e, Australia.

1.9 Calculation of dye decolorization rate

To compare various experiments, the decolorization rate was calculated for each dye or mixture of dyes assay. This parameter is defined as:

$$r = \frac{A_u - A_t}{A_u} \times 100$$

where, r is decolorization rate, A_u is absorbance of the untreated dye, A_t is absorbance after treatment.

1.10 Assay of the PPO activity

The method of Vieira and Fatibello-Filho (1998) was used with some slight modifications to assay the PPO activity. The reaction was initiated by adding a definite quantity of enzyme to a reaction mixture containing 1.0 ml of 20 mmol/L catechol in a total volume of 3.0 ml with 50 mmol/L sodium citrate buffer, pH 4.0. The reaction mixture was incubated at 37°C for 1.5–3.0 min. Reddish colored complex formed due to oxidation of catechol was measured at 420 nm using a spectrophotometer Cintra 10e. There was a linear rise in the formation of colored complex up to 3.0 min.

One unit of PPO activity is defined as the amount of enzyme protein that catalyzes the formation of 1.0 μmol/min of colored complex after the oxidation of catechol at 420 nm.

1.11 Protein assays

The protein concentration was determined by the procedure of Lowry *et al.* (1951). Bovine serum albumin was used as standard.

2 Results and discussion

The potato and brinjal extract showed 96 and 148 EU PPO/g of the intact vegetable, respectively. These enzyme preparations were used for dye decolorization studies. In this work, for the first time we successfully demonstrated the decolorization and removal of textile and other industrially important dyes by using plant polyphenol oxidases. The plant products such as potato and brinjal have been selected as the source of PPO because of their low cost and easy availability. These dyes were treated with potato and brinjal PPO under various experimental conditions to optimize the conditions for dyes decolorization.

2.1 Effect of pH on the decolorization of reactive textile dyes

Table 1 summarizes the effect of pH on the decolorization of textile reactive dyes by soluble potato and brinjal PPO. Most of the tested dyes were decolorized maximally at pH 3.0. The decolorization rate was significantly high in the buffers of lower pH, it was continuously decreased on increasing the pH of the buffers. Potato PPO was much more effective in decolorizing the dyes in all the investigated buffers of varying pH values than the brinjal PPO. There was no decolorization at pH 5.0 in the case of brinjal PPO treated dyes. There were several earlier reports regarding the maximum decolorization of dyes by various plant peroxidases (Bhunia *et al*., 2001; Akhtar *et al*., 2005a; Mohan *et al*., 2005) and microbial polyphenol oxidases (Nyanhongo *et al*., 2002; Unyayar *et al*., 2005) in the buffers of acidic pH values.

2.2 Effect of PPO concentration on the decolorization of dyes

Eight textile reactive dyes were treated with increasing concentrations of potato and brinjal (Table 2). The rate of dye decolorization was continuously enhanced with increasing the amount of both types of PPOs. However, the rate of dye decolorization was marginally increased with potato PPO 0.9 EU/ml while the rate of decolorization was continuously increased with brinjal PPO 1.35 EU/ml. These observations suggested that the potato PPO was much more effective in decolorization of reactive dyes compared to brinjal PPO even at lower concentrations of the enzyme. The efficiency of plant PPOs was significantly higher as compared to the fungal enzymes used for the decolorization of dyes. An enzymatic dye decolorization study showed that a maximum of 19% Orange G was removed by laccase at 15 EU/ml whereas lignin peroxidase

Table 1 Effect of pH on the PPO mediated decolorization of reactive textile dyes

Dye	λ_{max} (nm)	Dye decolorization (%)					
		Potato PPO (pH 3.0)	Brinjal PPO (pH 3.0)	Potato PPO (pH 4.0)	Brinjal PPO (pH 4.0)	Potato PPO (pH 5.0)	Brinjal PPO (pH 5.0)
Reactive Blue 4	600	80	31	83	23	53	0
Reactive Blue 160	616	96	43	82	26	47	0
Reactive Blue 171	607	97	50	91	21	53	0
Reactive Orange 4	489	78	30	75	0	29	0
Reactive Orange 86	413	78	23	62	0	29	0
Reactive Red 11	545	85	36	66	5	18	0
Reactive Red 120	511	91	45	87	36	70	0
Reactive Yellow 84	414	91	42	88	11	33	0

Each dye was incubated with potato and brinjal PPO 1.5 EU/ml in the buffers of varying pH values 3.0–5.0 at 37°C for 1 h. Each value represents the mean for three-independent experiments performed in duplicate, with average standard deviation <5%.

Table 2 Effect of PPO concentration on the decolorization of reactive dyes

Dye	Dye decolorization (%)					
	Brinjal (1.25 EU/ml)	Potato (1.25 EU/ml)	Brinjal (2.5 EU/ml)	Potato (2.5 EU/ml)	Brinjal (3.75 EU/ml)	Potato (3.75 EU/ml)
Reactive Blue 160	8	44	20	84	37	85
Reactive Blue 171	20	74	43	85	56	85
Reactive Red 11	18	62	29	70	37	79
Reactive Orange 4	8	29	15	40	21	52
Reactive Yellow 84	13	47	21	78	33	85
Reactive Orange 86	7	42	10	60	19	62
Reactive Blue 4	8	49	21	75	28	77
Reactive Red 120	28	77	41	83	57	83

Each dye solution was incubated with increasing concentration of potato and brinjal PPO (0.45–1.35 EU/ml) in 50 mmol/L sodium citrate buffer, pH 3.0 at 37°C for 1 h. Each value represents the mean for three-independent experiments performed in duplicate, with average standard deviation <5%.

Table 3 Effect of time on the PPO mediated decolorization of reactive dyes

Dye	Dye decolorization (%)							
	Incubation time of 30 min		Incubation time of 60 min		Incubation time of 90 min		Incubation time of 120 min	
	Brinjal	Potato	Brinjal	Potato	Brinjal	Potato	Brinjal	Potato
Reactive Blue 160	39	93	45	94	49	94	52	94
Reactive Blue 171	40	84	46	90	50	96	56	96
Reactive Red 11	30	79	37	82	40	83	43	85
Reactive Red 120	35	87	46	90	46	93	49	94

Each dye solution was incubated with potato and brinjal PPO1.5 EU/ml in 50 mmol/L sodium citrate buffer, pH 3.0 at 37°C for varying times. Each value represents the mean for three-independent experiments performed in duplicate, with average standard deviation <5%.

(LiP) and manganese dependent peroxidase at the same concentration decolorized 13.5% and 10.8% of Orange G, respectively. A maximum decolorization of 12.0% and 15.0% for Congo Red and Amido Black 10B, respectively, was recorded by laccase (Selvam *et al*., 2003).

2.3 Effect of time on the PPO catalyzed decolorization of reactive dyes

Decolorization of textile reactive dyes with potato and brinjal PPO was examined by varying the time of incubation (Table 3). The decolorization of dyes was increased with time up to 1 h. However, the rate of dye decolorization was quite slow after 1 h which may be probably due to products inhibition. This observation suggested that initial first hour was significant for dyes decolorization. These results further showed that potato PPO was more effective in decolorization of high percentage of color as compared to brinjal PPO. This was probably due to higher affinity of potato PPO for the dyes as compared to brinjal PPO. These results were in agreement with earlier published work of HRP and BGP catalyzed decolorization of textile dyes (Akhtar *et al*., 2005a; Mohan *et al*., 2005).

2.4 Decolorization of non-textile dyes by PPO

Table 4 illustrates the effect of potato and brinjal PPO on the decolorization of other industrially important dyes. Removal of color from all tested dyes by potato PPO was significantly higher as compared to brinjal PPO. Six of nine dyes were decolorized by more than 60% with potato 1.5 EU/ml of reaction volume. However, the decolorization of dyes by the same amount of brinjal PPO was marginally lower than potato PPO. 1,2-Naphthaquinone-4-sulphonic acid, Methylene Blue and Tropaeolin were hardly decolorized as compared to other tested dyes by both PPOs.

Table 4 Decolorization of other industrially important dyes by PPO

Dye	λ_{max}	Dye decolorization (%)	
		Potato PPO	Brinjal PPO
PAGE Blue 83	557	70	43
Coomassie Brilliant Blue G 250	556	87	59
Coomassie Brilliant Blue R 250	585	60	20
Methylene Blue	664	35	20
Naphthaquinone-4-sulphonic acid	400	25	13
Carmine	518	60	43
Naphthalene Black 12 B	617	63	38
Tropaeolin	446	30	18
Evans Blue	607	70	42

Each dye was treated with potato and brinjal PPO 1.5 EU/ml in sodium citrate buffer, pH 3.0 for 1 h at 37°C. Each value represents the mean for three-independent experiments performed in duplicate, with average standard deviation <5%.

2.5 Treatment of mixture of dyes by PPO

To monitor the feasibility of treating industrial effluents that may contain the complex mixture of dyes. We prepared various complex mixtures of textile Reactive dyes by mixing four dyes in equal proportions. Table 5 shows the effect of potato and brinjal PPO on the decolorization of complex mixture of dyes. Potato PPO preparation was significantly effective in decolorizing the higher percentage of color from all dye mixtures than the brinjal PPO at pH 3.0, 4.0 and 5.0 (Table 5). The results further demonstrated that decolorization of dyes even in the complex mixtures was maximum at pH 3.0. This result is comparable to the decolorization of dyes by same enzymes in their independent form (Table 1).

2.6 UV-visible spectra of Reactive Blue 160 and a mixture of dyes

The treatment of textile dyes or their mixtures resulted in the formation of insoluble precipitate due to

Table 5 Decolorization of mixture of four reactive dyes by PPO

Composition of the mixtures	λ_{max} (nm)	Dye decolorization (%)					
		Potato PPO (pH 3.0)	Brinjal PPO (pH 3.0)	Potato PPO (pH 4.0)	Brinjal PPO (pH 4.0)	Potato PPO (pH 5.0)	Brinjal PPO (pH 5.0)
Reactive Blue 160 + Reactive Blue 171 + Reactive Yellow 84 + Reactive Red 11	551	97	16	59	0	16	0
Reactive Red 120 + Reactive Orange 86 + Reactive Orange 4 + Reactive Blue 4	510	87	44	62	17	39	0
Reactive Blue 160 + Reactive Orange 86 + Reactive Yellow 84 + Reactive Orange 4	400	99	18	63	0	38	0
Reactive Blue 4 + Reactive Blue 160 + Reactive Red 120 + Reactive Red 11	544	98	21	72	2	36	0
Reactive Blue 160 + Reactive. Red 11 + Reactive Orange 86 + Reactive Orange 4	513	87	12	46	0	15	0
Reactive Red 120 + Reactive Red 11 + Reactive Orange 4 + Reactive Blue 160	515	90	20	62	1	31	0

The mixtures were incubated with soluble potato and brinjal PPO 1.5 EU/ml in 50 mmol/L sodium citrate buffer, pH 3.0 for 1 h at 37°C. Each value represents the mean for three-independent experiments performed in duplicate, with average standard deviation <5%.

quinones-derivative formation, which mediates the aggregation of aromatic pollutants. Several earlier reports about the treatment of phenols, aromatic amines and dyes with peroxidases and polyphenol oxidases are available (Husain and Jan, 2000; Bhunia *et al*., 2001; Akhtar *et al*., 2005a). The treatment of phenols with such type of enzymes resulted in the formation of large insoluble aggregate, which could be easily removed by simple centrifugation, sedimentation or filtration from the reaction mixture (Wada *et al*., 1995; Akhtar *et al*., 2005a). The decolorization and removal of Reactive Blue 160 with the help of UV-visible absorbance spectroscopy are shown in Fig.1. After treatment, a remarkable diminution in absorbance peaks in UV and visible regions was observed. These results suggested that decrease in absorbance peaks of dyes was due to removal of dyes from polluted water in the form of insoluble products. Fig.2 demonstrates the removal of dyes from the mixture after the treatment with potato and brinjal PPO. There was a clear demarcation in the decolorization of mixture of dyes by same amount of PPO from two different sources; a spectrum of potato PPO treated dye mixture was significantly different as compared to brinjal PPO.

Several investigators have earlier shown that the aromatic compounds were treated by various methods either they were degraded or get precipitated by the action of peroxidases/polyphenol oxidases (Tatsumi *et al*., 1996; Mielgo *et al*., 2001; Duran and Esposito, 2000; Husain and Jan, 2000; Mohan *et al*., 2005). The absorption spectra for the treated dyes exhibited decreased absorbance at

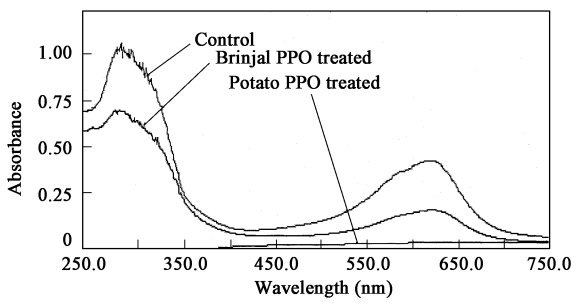

Fig. 1 Absorption spectra of Reactive Blue 160 before and after treatment.

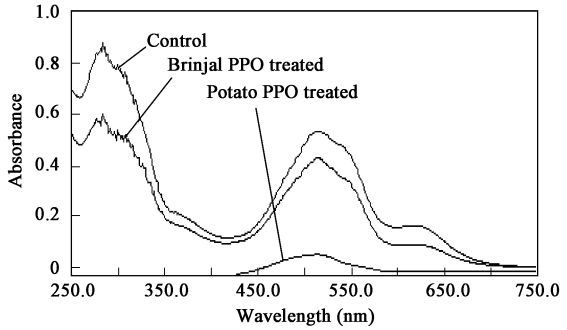

Fig. 2 Absorption spectra of mixture of reactive dyes before and after treatment.

various wavelengths in their spectra regions as compared to the untreated dyes (Bhunia *et al*., 2001; Mielgo *et al*., 2001; Gouvea *et al*., 2000; Coute *et al*., 2002). There was a disadvantage of using peroxidases for wastewater treatment, as these enzymes required quite expensive hydrogen peroxide as co-substrate for catalyzing reaction. It made decolorization process more expensive and dependent (Bhunia *et al*., 2001; Shaffiqu *et al*., 2002; Akhtar *et al*., 2005a). Moreover, the use of inexpensive, stable and reusable polyphenol oxidases can overcome the difficulty of using H_2O_2 because these enzymes could catalyze reactions in the presence of molecular O_2. The use of laccases and peroxidases in the decolorization of a number of recalcitrant textile and non-textile dyes need redox mediators for effective treatment of wastewaters (Bourbonnais and Paice, 1990; Soares *et al*., 2001; Claus *et al*., 2002; Akhtar *et al*., 2005b). However, the use of such type of expensive compounds further enhanced the cost of treatment and it also added pollutants to the environment. In order to avoid the use of such compounds, polyphenol oxidases could be better alternatives to the laccases and peroxidases because they catalyze the decolorization of dyes at lower pH by means of formation of insoluble dye-product, precipitate.

3 Conclusions

The ammonium sulfate precipitated proteins of potato and brinjal have very high PPO activity. These PPOs have

great potential in the decolorization of textile and non-textile dyes. Moreover, these polyphenol oxidases required no redox mediator and were quite capable of decolorizing and removing the dyes from the polluted wastewaters without requiring any redox mediator. Plant polyphenol oxidases decolorized dyes via insoluble precipitate formation, which could be easily removed by the process of simple filtration, sedimentation or centrifugation. The removal of insoluble precipitate resulted in the loss of aromatic compounds from wastewater. Potato polyphenol oxidases were more effective in decolorization of independent dye or mixture of dyes over the brinjal polyphenol oxidases. The use of partially purified enzyme may be extendable to the effluents coming out of industries and mixtures of dyes present in wastewaters. These plant polyphenol oxidases in their immobilized form may be applied at the large-scale treatment of wide spectrum of structural dyes present in the industrial effluents. This as well as the scale up of enzymatic processes is the subject of further study.

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