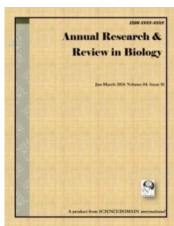

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Azo Dye Decolourisation using Crude Peroxidase from *Armoracia rusticana* Hairy Roots

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Authors' contributions

This work was carried out in collaboration between both authors. Author MA performed the experimental work, statistical analysis, interpretation and wrote the draft of the manuscript. Author UM designed the experiments, arranged the literature review and interpreted results for the manuscript. Both authors read and approved of the manuscript.

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ABSTRACT

Aim: The objective of the present work was to assess the ability of crude peroxidase from hairy roots of *Armoracia rusticana* (horseradish) to decolourise two model azo dyes: The mono-azo dye Methyl Orange (MO) and the di-azo dye Bismarck Brown (BB).
Study Design: The enzymatic decolourisation of aqueous solutions of the model dyes was performed by varying reaction parameters like pH of reaction mixture, crude enzyme concentration, dye concentration and hydrogen peroxide concentration; at ambient temperature (25±2ºC). The emphasis was on using a crude extract that showed high enzyme activity, despite being obtained by minimum processing, and virtually no

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purification.
Methodology: Hairy root cultures of *A. rusticana* were used as the source of crude peroxidase. The hairy root tissue was macerated using phosphate buffer (pH 5.8) and centrifuged. The clear supernatant was used as crude enzyme. The decolourisation of the dyes was assessed using spectrophotometric method. Appropriate controls to account for bleaching due to hydrogen peroxide were maintained. Decolourisation of MO and BB by the Fenton reaction was used as a positive control for decolourisation.
Results: The crude peroxidase extract can bring about up to 73% and 32% decolourisation of MO and BB respectively within a reaction time of two hours (2h).
Conclusion: The outcome of the present work can find application in the enzymatic treatment of wastewater containing azo dyes. This oxidative decolourisation method appears to be better suited to the decolourisation of mono-azo dyes.

Keywords: Hairy roots; peroxidase; azo dyes; decolourisation.

ABBREVIATIONS

HRP: Horseradish Peroxidase; MO: Methyl Orange; BB: Bismarck Brown.

1. INTRODUCTION

Azo dyes form the largest faction of synthetic dyes that are utilized in dyeing of textile, leather, paper and food as well as in the manufacture of coloured cosmetic products [1,2]. The high tinctorial strength and stability of these dyes have contributed to the wide-spread use of these dyes. However, these properties of azo dyes also make them recalcitrant [3] and potentially toxic pollutants [4].

The presence of dyestuffs in effluents can cause serious problems. Effluents containing azo dyes can be a threat to water bodies that they contact. The high tinctorial strength of the azo dyes severely hampers the absorption of incident sunlight by photosynthetic organisms in the water body [1]. The role of primary producers in any food chain is critical [5]. The delicate balance of the ecosystem of receiving water body is thus adversely affected. Low photosynthetic rates also lead to lower levels of dissolved oxygen. Thus the oxidative degradation of organic material in the water body is hindered. The partial reductive degradation of some azo dyes can lead to the formation of potentially carcinogenic amines [6]. Anoxic conditions and the accumulation of organic matter eventually deteriorate the water body completely. Hence, the decolourisation of effluents before they reach water bodies is necessary.

The decolourisation of dyestuffs in effluent can be achieved by many physico-chemical methods. Many of these are efficient in the removal of colour but may suffer from drawbacks that limit their application. Ozonation for example, is an efficient method for decolourisation, but the running cost is very high [2]. The Fenton and Fenton like processes are efficient and cost effective. However, the need for adding Fe^{2+} and Fe^{3+} during the reaction can be a disadvantage [2].

In recent times, the development of alternative methods to decolourise effluents, is gaining importance since they are considered less aggressive towards the environment. The use of enzymes for decolourisation is an imminent method for the decolourisation of effluents.

Since enzymes are biological in origin, the use of enzymes for wastewater treatment is considered environmentally more acceptable [7].

Oxidative enzymes like peroxidases [8,9] and laccases [6,10] have been investigated for their ability to decolourise azo dyes. The commercial source of the enzyme horseradish peroxidase (HRP) is the roots of the plant *Armoracia rusticana* or horseradish. Studies have shown that hairy roots can produce significantly higher amounts of root associated macromolecules as compared to normal roots [11].

In the present study, the ability of crude peroxidase from the hairy roots of *A. rusticana* to decolourise solutions of two model azo dyes was assessed. Methyl Orange (mono-azo dye) and Bismarck Brown (di-azo dye) were used as model dyes. Various reaction parameters like pH of reaction mixture, crude enzyme concentration, dye concentration and hydrogen peroxide concentration were optimised at ambient temperatures for a reaction time of 2h. The aim of the study was to develop an alternative method to decolourise dyestuff containing effluents, using a minimally processed crude enzyme to obtain appreciable decolourisation in a relatively short reaction time.

2. MATERIALS AND METHODS

2.1 Establishment of Hairy Root Cultures

Plantlets of *Armoracia rusticana* were grown on modified MS medium [12] supplemented with 3% sucrose and 0.5ppm Indole 3-Acetic Acid (IAA) for 5-6 weeks. The cultures were initiated using node, petiole and leaf as explants. The temperature was maintained at 25±2ºC. The plantlets were given 8 hour photoperiod (photon flux density 40.5 μmol $m^{-2}s^{-1}$) using cool white fluorescent tube lights.

The bacterial cultures of *Agrobacterium rhizogenes* ATCC # 39207 were obtained from ATCC (USA). The bacterial culture was maintained on Yeast extract and Mannitol (YEM) medium [13]. A culture of *A. rhizogenes* ATCC# 39207 that was 48 hour old was used as inoculum. The plantlets were inoculated using Scalpel method [14]. The inoculated plantlets were transferred to modified MS medium devoid of phytohormones until the emergence of adventitious or hairy roots at the site of inoculation. The adventitious roots were excised and cultured separately in modified MS medium at 25ºC in the dark for 2 weeks. After sufficient growth of the adventitious roots was seen, the actively growing root tips were excised and cultured on phytohormone free modified MS medium with 250ppm cefotaxime [15] to disinfect and free the roots from *A. rhizogenes*. This was done periodically to ensure complete disinfection of the roots.

2.2 Chemicals used

The dyes used for the study were Methyl Orange (MO) and Bismarck Brown R (BB) representing mono-azo and di-azo dyes respectively. MO was obtained from S.D. Fine Chemicals (Boisar), India; while BB was obtained from Burgoyne Burbridges and Co. (India), Mumbai. Stock solutions of 1mg mL^{-1} of both dyes were prepared in distilled water. Different volumes of these stock solutions were utilized as required in the study. Guaiacol (2-methoxy phenol) was purchased from Sisco Research Laboratories, India. Hydrogen peroxide was purchased from Qualigens Fine Chemicals, Navi Mumbai, India. Chemicals for buffer

preparation, like KH_2PO_4, KOH, Citric acid, Trisodium citrate were purchased from S.D. Fine Chemicals (Boisar), India; while Tris-HCl was purchased from Loba Chemie, Mumbai.

2.3 Preparation of Extract

Approximately 0.2g of hairy root tissue was macerated in a pre-chilled mortar and pestle, with 2.0mL of phosphate buffer (0.1mol L^{-1}, pH 5.8). The suspension was centrifuged at 3000 rpm for 10 min (805g at 25±2ºC). The supernatant was used as the source of crude peroxidase.

2.4 Activity Assay of Crude Peroxidase

The activity of peroxidase was estimated spectrophotometrically using a JASCO V-530 Spectrophotometer utilizing the method described by [16]. In the reaction, the coloured product tetraguaiacol is estimated at 470 nm (λmax). The calculation of crude peroxidase activity (U/mL) of the extract towards guaiacol has been shown in Equation 1.

The reaction mixture (3.0mL) contained 0.1mol L^{-1} phosphate buffer (pH 6.0), 0.02mL crude enzyme (dilution factor 10) and guaiacol and hydrogen peroxide at final concentrations of 5 mmol L^{-1} and 1mmol L^{-1} respectively.

Equation 1:

$$U/mL = (\Delta OD/min) \times (RmV/CeV) \times (df/ \varepsilon_{470})$$

Where,

ΔOD/min = increase in absorbance per minute (min^{-1})
RmV = reaction mixture volume (mL)
df = dilution factor
ε_{470} = molar absorptivity of tetraguaiacol at 470nm (mL μmol^{-1} cm^{-1})
CeV = crude enzyme volume (mL)

2.5 Decolourisation Studies

Decolourisation was assessed by measuring initial and final absorbance using JASCO V-530 spectrophotometer. The reaction volume was 3.0mL, the duration of observation was 2 hours and all the decolourisation experiments were performed at ambient temperature (25±2ºC). Positive controls (reported in section 4. Discussion) were prepared for MO (20 ppm) and BB (30ppm) using HCl to obtain pH 3.0, Ferrous ammonium sulphate (0.70mmol L^{-1}) and H_2O_2 (500ppm) was used in the modified Fenton process [17]. All samples were centrifuged at 2500 rpm for 10 min (559 g at 25±2ºC) before reading absorbance at 460nm and 400 nm for MO and BB respectively. The percentage of decolourisation was calculated using the formula given below in Equation 2 [18]:

Equation 2:

$$\% \text{ decolourisation} = [(Ai-Af) \times 100] / Ai$$

Where,

Ai = Initial absorbance
Af = Final absorbance

2.6 Optimisation of pH of Reaction Mixture

The pH was varied in the range 3 to 9 at intervals of 1 pH unit. Citrate buffers (0.1mol L^{-1}) were used to obtain pH 3 to 5, phosphate buffers (0.1mol L^{-1}) for pH 6-8 and Tris-HCl buffer (0.1mol L^{-1}) was used to obtain pH 9. The reaction mixtures contained 0.02mL of crude peroxidase (guaiacol activity 5.26±0.25U/mL). The concentrations of dyes were 20 ppm and 30ppm for MO and BB respectively. The concentration of hydrogen peroxide was maintained at 2 mmol L^{-1}. Since the colour of MO is affected by pH, the absorbance of MO containing reaction mixtures was read at the respective λ_{max} for each pH. The absorbance of reaction mixtures containing BB was read at 400 nm for all pH since pH did not affect the λ_{max} of BB.

2.7 Optimisation of Amount of Enzyme

Different volumes of enzyme suspension in the range (0.025mL to 0.200mL) of crude peroxidase enzyme (guaiacol activity 3.47±0.22U/mL) were used for these experiments. The pH was maintained at pH 4.0 for MO and pH 3.0 for BB. The concentrations of dyes in the reaction mixture were 20ppm and 30ppm for MO and BB respectively. The concentration of hydrogen peroxide was maintained at 2mmol L^{-1}.

2.8 Optimisation of Initial Dye Concentration

The initial concentration of MO and BB were varied in the range 10ppm to 60ppm. The pH was maintained at 4.0 and 3.0 for MO and BB respectively. The concentration of hydrogen peroxide was maintained at 2mmol L^{-1}. The volumes of crude enzyme used were 0.05mL and 0.075mL (guaiacol activity 3.47±0.22U/mL) for MO and BB respectively.

2.9 Optimisation of Hydrogen Peroxide Concentration

The concentration of hydrogen peroxide was varied in the range 1 mmol L^{-1} to 10mmol L^{-1}. The pH was maintained at pH 4.0 and pH 3.0 for MO and BB respectively. The concentrations of dyes were 20ppm and 30ppm for MO and BB respectively. The volumes of crude enzyme used were 0.05mL and 0.075mL (guaiacol activity 3.47±0.22U/mL) for MO and BB respectively.

2.10 Statistical Analysis of Data

The reported values are mean± SD (*n*=3). All the statistical analysis of the data obtained from the studies described above, were performed using SPSS version 19. The results of the analysis were obtained for *P*=.05. In cases where ANOVA has been performed, multiple comparisons were made using Duncan's Multiple Range Test (DMRT). Unless stated otherwise, the 'MO' series has been assigned groups represented by the letters (A>B>C…) and the 'BB' series has been assigned groups represented by the letters (P>Q>R ...). The means (within a single series, i.e. MO or BB) that have been assigned the same letter(s) are not significantly different from each other at *P*=.05.

3. RESULTS

A comparison between the hairy roots and normal roots is shown in Fig. 1. The hairy roots (3.47U/mL) have nearly 6 times as much activity as the normal roots (0.59U/mL). The mean activities of the normal roots and transformed roots have been assigned different letters, indicating that they are significantly different from each other (*P*<.001.).

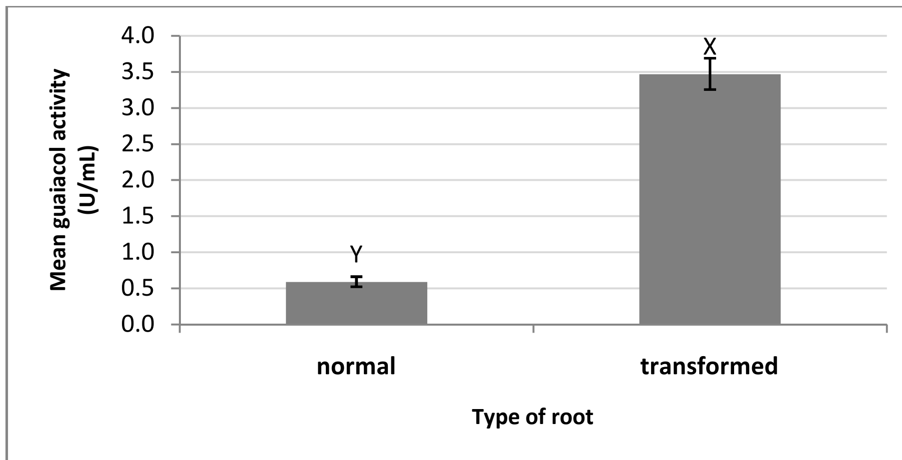

Fig. 1. Crude peroxidase activity from normal and transformed roots

As seen in Fig. 2, the highest decolourisation of MO (50.73%) and BB (38.64%) are obtained at pH 4.0 and pH 3.0 respectively. Hence the optimum pH values for the decolourisation of MO and BB are pH 4.0 and 3.0 respectively.

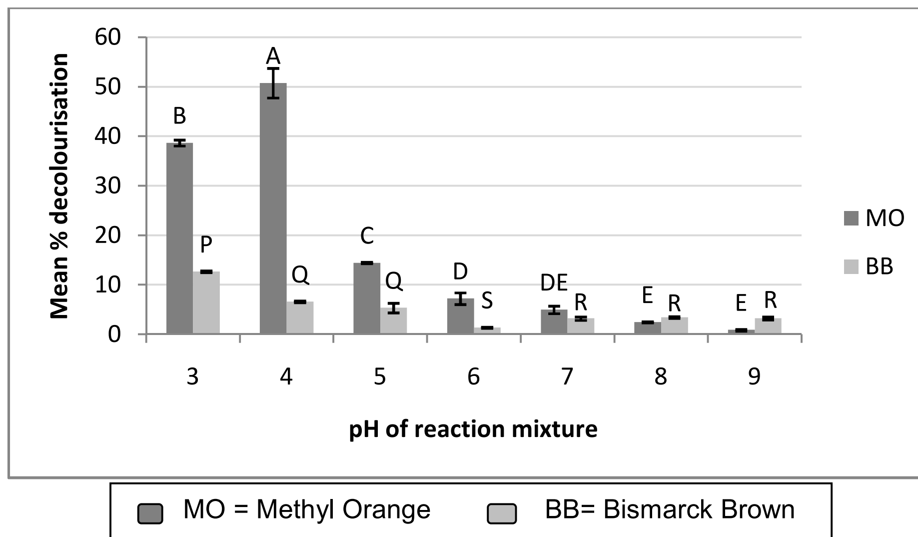

Fig. 2. Decolourisation of methyl orange and bismarck brown at different pH using crude hairy root peroxidase

Note: In a given series, means assigned the same letter(s) are not significantly different from each other P=.05

As seen in Fig. 3 maximum decolourisation of MO occurs when the enzyme concentration in the reaction mixture 0.09U/mL (73.00%). At higher concentrations of enzyme, the concentration of the substrate (MO and/or H_2O_2) starts becoming the limiting factor and consequently, lower activity of the crude enzyme is seen. Hence the decolourisation decreases at higher concentration of the enzyme. The decolourisation of BB is relatively high in the range 0.03 to 0.09U/mL, though the maximum decolourisation of BB occurs when the concentration of enzyme is 0.09U/mL (62.97%), beyond which the decolourisation decreases significantly. In case of both dyes, some amount of decolourisation is seen even in the absence of enzyme (0.00U/mL). This is due the bleaching action of H_2O_2 which is significantly lower than all the enzyme treated sets in case of MO. In the case of BB, the bleaching effect of H_2O_2 is comparable to that seen in the sets in which the decolourisation is limited by the availability of H_2O_2.

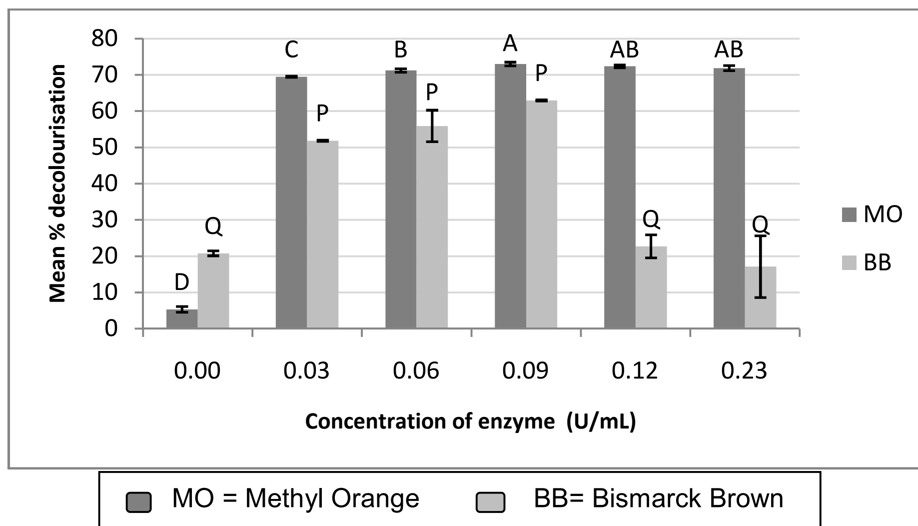

Fig. 3. Decolourisation of Methyl Orange and Bismarck Brown using different concentrations of crude hairy root peroxidise

Note: In a given series, means assigned the same letter(s) are not significantly different from each other P=.05

The decolourisation of different initial concentrations of MO and BB are shown in Fig. 4. The decolourisation of MO increases after 10ppm, and does not change significantly between 20 and 50ppm. Beyond 50ppm, the decolourisation decreases due to the saturation of the crude enzyme with the dye substrate. In the case of BB it appears that the crude enzyme becomes saturated at lower concentrations of the dye i.e. above 30ppm.

As seen in Fig. 5, the optimum decolourisation of MO occurs when the concentration of H_2O_2 is 1mmol L^{-1}. Beyond this concentration, the decolourisation decreases steadily. The activity of Peroxidase towards some substrates is known to be inhibited at high concentrations of H_2O_2 [19,20]. The decolourisation of BB increases with the concentration of H_2O_2 up to 4 mmol L^{-1}. Beyond this concentration, the decolourisation does not change significantly. This suggests that the H_2O_2 requirement of crude peroxidase for the decolourisation of BB is higher than that for decolourisation of MO.

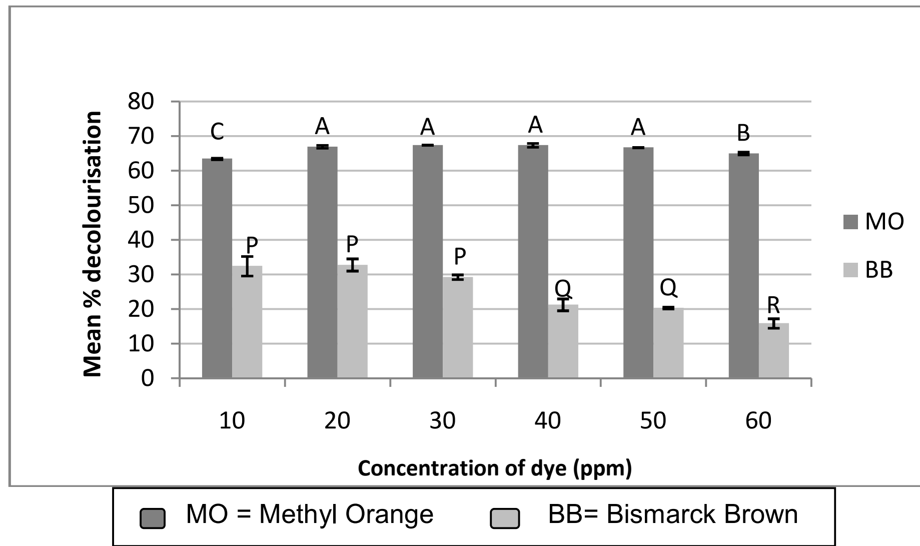

Fig. 4. Decolourisation of different concentrations of methyl orange and bismarck brown using crude hairy root peroxidase

Note: In a given series, means assigned the same letter(s) are not significantly different from each other P=.05

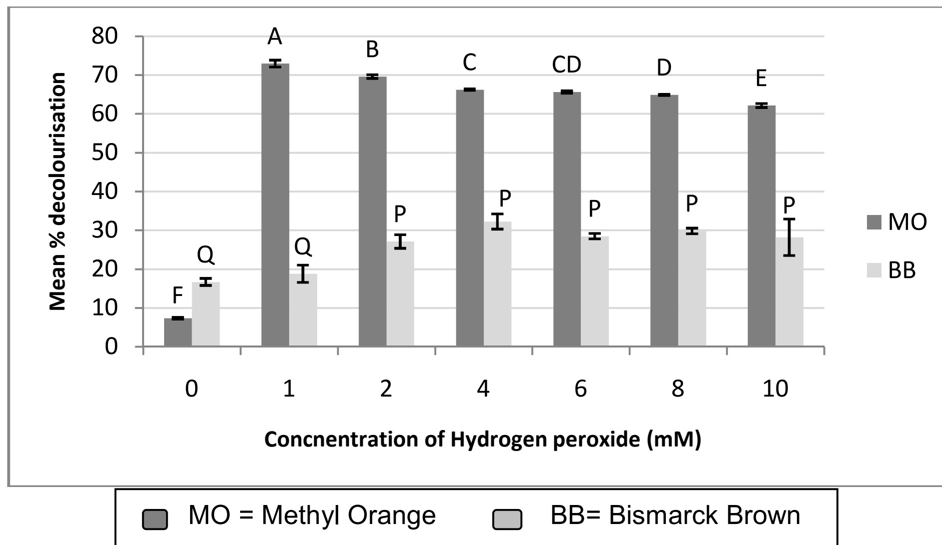

Fig. 5. Decolourisation of methyl orange and bismarck brown at different concentrations of Hydrogen peroxide

Note: In a given series, means assigned the same letter(s) are not significantly different from each other P=.05

4. DISCUSSION

The growth rate and metabolism of hairy roots is higher than that of normal roots. Hairy root cultures are ideal for large-scale synthesis of metabolites such as proteins; especially those

associated with root tissue [21]. In many cases, the quantity of the metabolite synthesized is comparable to that obtained from *in vivo* grown plantlets [22,23].

Stable and continuous production of root associated macromolecules like peroxidase, in the case of horseradish is possible using hairy root cultures. Hairy root cultures of Horseradish have been reported to produce significantly higher levels of horseradish peroxidase (HRP) than normal cultures [24]. Our experiments too have shown similar results (Fig. 1). An important advantage of hairy root cultures is that they are amenable to scale-up in column type and bubble type bioreactors. The treatment of these cultures with ions that affect permeability of the root cells can lead to enhanced secretion of peroxidase into the medium [11]. Previous studies with horseradish have shown that treatment of *in vitro* plantlets with 25mmol L^{-1} $CaCl_2$ can significantly increase secretion of peroxidase from roots into the medium while treatment of plantlets with 25mmol L^{-1} $Ca(NO_3)_2$ can significantly increase peroxidase content of horseradish roots [25]. Hence, it is likely that relatively simple modifications in the medium may considerably improve the production of peroxidase by hairy root cultures of horseradish.

Decolourisation of synthetic wastewater using various bacteria that produce enzymes that can degrade azo dyes has been reported [3]. An extensive list of dye degrading bacteria has been given in a review article by Forgacs and co-workers [26]. Cultures of white rot fungi like *Phanerochaete chrysosporium* can oxidatively decolourise azo dyes like Orange II and Congo red in 6-9 days [26]. Decolourisation of dyes like Crystal Violet (98%) and Malachite Green (96%) using mainly the adsorptive property of the biomass of cultures of the fungus *Fusarium solani* was obtained in 2 days [27]. Often, when cultures of living organisms are used to degrade a pollutant, the organisms need to adapt metabolically to the pollutant in order to break it down. This 'lag period' causes the duration of such degradation reactions to extend to 3-5 days. Most of the organisms capable of degrading pollutants can do so due to the production and/or secretion of suitable enzymes. When cell free extracts of crude enzymes are used to degrade pollutants, the need for acclimatisation is circumvented. Hence, the duration of reaction using enzymes is consequently shortened to few hours. In cases where the concentration of enzyme is very high or when purified enzyme is used, the reaction may take only a few minutes [1].

At concentrations of crude enzyme higher than 0.26 U/mL (Fig. 3), the decolourisation of both MO and BB decrease. In case of MO, the decrease in decolourisation is much smaller than with BB. It is likely that as the enzyme concentration increases, the availability of either MO or H_2O_2 limits the extent of decolourisation. The crude enzyme extract probably contains a mixture of different peroxidases, which show activity towards guaiacol, but do not necessarily react with the dyes. The mixture is likely to contain Ascorbate peroxidase with a considerably greater affinity for H_2O_2 (K_m = 3μM), which seems to quickly utilise the available H_2O_2 leaving the crude HRP (K_m = 0.27mM) [28] relatively little H_2O_2 for reaction with the dyes. This decreases the decolourisation of BB at higher concentrations of crude enzyme. This competition for H_2O_2 can be eliminated at higher concentrations of H_2O_2. As seen from the H_2O_2 variation graph (Fig. 5), the H_2O_2 requirement of crude HRP for BB decolourisation is relatively higher than that for MO. Hence, the concentration of H_2O_2 becomes the limiting factor in the decolourisation of BB.

It appears that the crude enzyme is capable of decolourising MO more readily than BB (Fig. 3). The decolourisation of MO is above 60% between 20 to 50 ppm. Above 50 ppm, the decolourisation of MO decreases by a small amount. However, in case of BB, the decolourisation decreases by a large amount beyond 30ppm (Fig. 4).

The presence of the azo linkage (-N=N-) in azo dyes is a major contributor to the stability of the dyes and consequently their recalcitrance as pollutants (Figs. 6a and 6b). The electron withdrawing nature of the linkage makes azo dyes less liable to oxidative degradation [8]. The decolourisation of MO and BB obtained using the Fenton reaction (positive control) was 96.5±0.29% and 26.5±2.76% respectively. The significantly lower decolourisation of BB obtained with the Fenton reaction shows that the number of azo linkages, present in a molecule can affect its readiness to be oxidised. BB is a diazo dye, containing two azo linkages (Fig. 6b). It is therefore likely to withstand oxidation by crude HRP from hairy roots. The effect of the presence of two azo linkages is also seen in the fact that the decolourisation (by oxidation) of BB requires higher concentration of H_2O_2 than MO and that the decolourisation of BB occurs optimally at lower concentrations than MO.

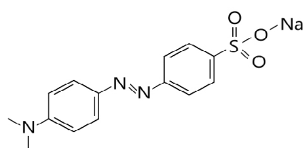

Fig. 6a. Methyl Orange (MO)

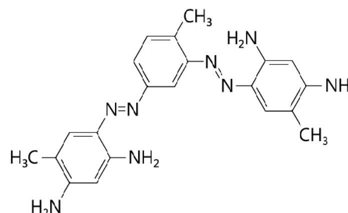

Fig. 6b. Bismarck Brown R (BB)

The optimum conditions for the decolourisation of MO are - pH 4.0, enzyme concentration 0.09U/mL, dye concentration 30ppm and H_2O_2 concentration 1mmol L^{-1}. For the decolourisation of BB, the optimum conditions are – pH 3.0, enzyme concentration 0.09U/mL, dye concentration 20ppm and H_2O_2 concentration 4mmol L^{-1}. The findings of the present study are significant in the enzymatic treatment of effluents containing dyestuffs. The enzyme used in the study is in a very crude form. The enzyme has been extracted using minimum processing and yet it retains appreciable activity at ambient temperatures. The cost of using pure enzymes for a large scale operation like treatment of industrial effluents is prohibitive. Crude enzymes that are easily obtained and show high activity present an important alternative to pure enzymes. Also the reaction time is relatively short with considerable decolourisation especially for MO. Modifications such as immobilising the crude enzyme on a suitable support may be made to improve the decolourisation method by increasing reusability of the crude enzyme.

5. CONCLUSION

The present study describes an enzymatic treatment method for the decolourisation of effluents containing azo dyes using crude peroxidase from horseradish hairy roots. Though the method may be better suited to the decolourisation of monoazo dyes (up to 73.00% decolourisation), the important advantages of the method include high activity of enzyme despite minimal processing, and short reaction time (2 hours) at ambient temperatures. Growing hairy root cultures of *Armoracia rusticana* on a large scale, for crude peroxidase mediated effluent treatment can provide an environmentally more acceptable alternative to existing effluent treatment methods.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Da Silva MR, De Sá LRV, Russo C, Scio E, Ferreria-Lietão VS. The use of HRP in decolorization of reactive dyes and toxicological evaluation of their products. Enzyme Research; 2010. Article ID 703824 Accessed 15 December 2013. DOI:10.4061/2010/703824.
   Available: http://www.hindawi.com/journals/er/2010/703824.
2. Abo-Farah SA. Comparative study of oxidation of some azo dyes by different Advanced Oxidation Processes, Fenton, Fenton-like, photo-Fenton and photo-Fenton-like. J Am Sci. 2010;6:128-42.
3. Franciscon E, Zille A, Dias GF, Ragaanin De Menezes C, Durrant L R, Cavaco-Paulo A. Biodegradation of textile azo dyes by a facultative *Staphylococcus arlettae* strain VN-11 using a sequential microaerophilic/aerobic process. Int Biodeter Biodegr. 2009;63:280-88.
4. Omar HH, Algal decolorization and degradation of monoazo and diazo dyes. Pak J Biol Sci. 2008;11(10):1310-16.
5. Odum EP. Fundamentals of Ecology. 3rd ed Philadelphia: W.B. Saunders Company; 1971.
6. Zille A, Górnacka B, Rehorek A, Cavaco-Paulo A. Degradation of azo dyes by *Trametes villosa* laccase over long periods of oxidative conditions. Appl Environ Microbiol. 2005;71(11):6711-18.
7. Adam W, Lazarus M, Saha-Moller C, Weichold O, Hoch U, Haring D Schreier P. Biotransformation with peroxidase. Adv Biochem Eng/Biot. 1999;63:73-107.
8. Maddhinni VL, Vurimindi HB, Yerramilli A. Degradation of azo dye with horseradish peroxidase (HRP). J Indian Inst Sci. 2006;86:507-14.
9. Ulson de Souza SMAG, Forgiarini E, Ulson de Souza AA, Toxicity of Textile Dyes and their Degradation by the Enzyme Horseradish Peroxidase (HRP), J. Hazard. Mater. 2007;147:1073-78.
10. Kandelbauer A, Maute O, Kessler RW, Erlacher A, Gübitz GM. Study of dye decolorization in an immobilized laccase enzyme-reactor using online spectroscopy. Biotechnol Bioeng. 2004;87(4):552-63. DOI:10.1002/bit.20162.
11. Rudrappa T, Neelwarne B, Kumar V, Lakshmanan V, Venkataramareddy SR, Aswathanarayana RG. Peroxidase production from hairy root cultures of red beet (*Beta vulgaris*). Electron J Biotechn. 2005;8:66-78.
12. Dodd JH, Roberts LW. Experiments in plant tissue culture, 2nd ed Cambridge: Cambridge University Press; 1985.
13. American Type Culture Collection (ATCC) material safety data sheet for *Agrobacterium rhizogenes* ATCC# 39207 and 15834; 2009.
14. Ooms G, Kamp A, Bunrell MM and Roberts J. Genetic modifications of potato development using Ri-DNA. Theor Appl Genet. 1985;70:440-46.

15. Mariya John KM, Joshi SD, Mandal AKA, Kumar SR and Kumar RR. *Agrobacterium rhizogenes*-mediated hairy root production in tea leaves [*Camellia sinensis* (L.) O. Kuntze] IJBT. 2009;8:430-34.
16. Kim YH, Yoo JY. Peroxidases production form carrot hairy root cell culture. Enzyme Microb Tech. 1996;18:531-53.
17. Habib MA, Ismail IMI, Mahmood AJ, Ullah MR. Decolourization and mineralization of brilliant golden yellow (BGY) by Fenton and photo-Fenton processes. Afr J Pure Appl Chem. 2012;6:153-58. DOI: 10.5897/AJPAC 10.024.
18. Singh AD, Sabaratnam V, Abdullah N, Sekaran M. Enzymes from spent mushroom substrate of *Pleurotus sajor-caju* for the decolourisation and detoxification of textile dyes. World J Microbiol Biotechnol. 2011;27:535-45.
19. Veitch NC. Horseradish peroxidase: A modern view of a classical enzyme. Phytochemistry. 2004;65:249-59. DOI: 10.1016/j.phytochem.2003.10.022.
20. Hollmann F, Arends I. Enzyme initiated radical polymerizations. Polymers. 2012;4:759-93. DOI: 10.3390/polym4010759.
21. Hu ZB, Du M. Hairy root and its application in plant genetic engineering. J Integr Plant Biol. 2006;48(2):121-27.
22. Giri A, Narasu MJ. Transgenic hairy roots: Recent trends and applications. Biotechnol Advances. 2000;18:1-22.
23. Guillon S, Tremouillaux–Guiller J, Patil PK, Rideau M, Gantet P. Harnessing the potential of hairy roots: dawn of a new era. Trends Biotechnol. 2006;24:403-09.
24. Uozumi N, Kato Y, Nakashimada Y, Kabayashi T. Excretion of peroxidase from horseradish hairy root in combination with ion supplementation. Appl Microbiol Biotechnol. 1992;37:560-65.
25. Ambatkar M, Mukundan U. Calcium salts enhance activity and azo dye decolourisation capacity of crude peroxidase from *Armoracia rusticana*. AJPS. 2014;5(2):212-18. DOI: 10.4236/ajps.2014.52028.
26. Forgacs E, Cserháti T, Oros G. Removal of synthetic dyes from wastewaters: A review. Environ Int. 2004;30:953-71.
27. Abedin MAR. Decolorization and Biodegradation of Crystal Violet and Malachite Green by *Fusarium solani* (Martius) Saccardo, A Comparative Study on Biosorption of Dyes by the Dead Fungal Biomass. Am-Euras J Bot. 2008;1(2):17-31.
28. Penel C, Castillo FJ. Oxidoreduction at the plasma membrane: Relation to growth and transport Crane FL, Morré DJ, Löw HE, editors. Boca Raton (USA): CRC Press. 1991;2.

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