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Original Research Paper

Decolourisation of Reactive Black 5 Dye by a White Rot Fungi *Trametes versicolor*

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ABSTRACT

Azo dyes represent the largest class of organic colourants listed in the Colour Index (60-70%) and makeup vast majority of the dyes discharged by textile industry. Due to high cost, low efficiency and inapplicability of several physico-chemical decolourisation techniques, biological processes such as the use of white-rot fungi provide an alternative to this problem. In the present work, ability of *Trametes versicolor* to decolourise an azo dye viz., Reactive Black 5 was evaluated under shaker conditions by statically grown culture using different sources of carbon and nitrogen. At the end of the 8 hours, Reactive Black 5 was decolourised to an extent of 25.12% in the medium containing glucose. The nitrogen source that supported the highest decolourisation (62.93%) of the dye at the end of 24 hours was yeast extract. Further optimization was carried out by the Orthogonal Array design for the optimization of the medium and to determine the effect of glucose, yeast extract and copper sulphate on the dye decolourisation. The data were analysed using Minitab software 15.

INTRODUCTION

The textile wastewater is rated as one of the most polluting among all the industrial sectors considering both volume and composition of the effluent. During dyeing processes about 10 to 40% of the dyes do not bind to their target substrates and, therefore, remain in the resulting wastewaters. It is estimated that 10 to 15 % of these dyes are lost in the effluent from industrial dyeing processes at concentrations of 10 to 15 ppm. The effluent is a complex and highly variable mixture of many polluting substances ranging from inorganic compounds and elements to polymers and organic products (Swamy 1998, Meenambal et al. 2006).

Globally, it has been estimated that 10% of the total dyestuff used, or about 7×10^5 tonnes per annum, are released into the environment (Shin et al. 2002, Asamudo et al. 2005). In general, the effluent is highly coloured with high biological oxygen demand (BOD) and chemical oxygen demand (COD), a high conductivity and alkalinity (Zille 2005). Azo dyes represent the largest class of organic colour ants listed in the Colour Index (60-70% of the total) and as their relative share among reactive, acid and direct dyes is even higher, it can be expected that they make up the vast majority of the dyes discharged by textile-processing industries (Zille 2005, van der Zee 2002).

Effluents derived from the textile and dyestuff activities can provoke serious environmental impact in the neighbouring receptor water bodies because of the presence of toxic reactive dyes, chlorolignin residues and dark colouration. As dyes are designed to be chemically and photolytically stable, they are highly persistent in natural environments. The degradation of dye molecules in the environment by microorganisms is likely to be slow which means that it is possible for high levels of dyes to persist and potentially accumulate. Furthermore, any degradation that occurs may produce

smaller molecules equally unfamiliar to the environment, such as amines, and which may also be toxic. The discharge of these waste residues into the environment eventually poison, damage or affect one or more species in the environment, with resultant changes in the ecological balance. The release of dyes may, therefore, present an ecotoxic hazard and introduce the potential danger of bioaccumulation that may eventually affect man by transport through food chains (Asamudo et al. 2005, Van der Zee 2002).

Removal of colour from the effluents is, thus, a major problem and implementation of tighter constraints on the discharges is forcing waste creators and managers to consider new options for the effluent treatment and disposal. These effluents can be treated by a number of physico-chemical processes. However, unfortunately such methods are of limited use due to constraints such as costs, general applicability and production of solid wastes. With economic constraints on pollution control processes, affordable and effective methods have become a necessity. Bioremediation using white-rot fungi can provide an alternative, cheap and cost effective method capable of degrading the hazardous dyes (Zille 2005, Murugesan & Kalaichelvan 2003).

Over the past decade, the white rot fungi have been extensively studied for their ability to degrade the complex plant polymer, lignin, and a broad spectrum of xenobiotic pollutants. Degradation is ascribed to non-specific oxidation by extracellular, lignin degrading enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP) and the laccases. The non-specificity of the ligninolytic enzymes allow for the degradation of a range of pollutants, without extensive acclimation. Since the enzymes are extracellular, large molecules such as dyes can be degraded without transport across the cell wall**,** which often limits bacterial degradation (Swamy 1998).

The aim of the present investigation was to optimize the method for decolourisation of an azo dye commonly used in the textile industry, i.e., Reactive Black-5 using *Trametes versicolor*, a white rot fungus.

MATARIALS AND METHODS

Reactive Black-5 was procured from Kiri Dyechem, Ahmedabad. All the media chemicals were AR grade purchased from Hi-Media Laboratories, Mumbai. *Trametes versicolor* NCIM-1086 was obtained from NCL, Pune and was maintained on Malt Extract Agar slants at 4°C with subculturing every 15 days.

Assessment of the decolourisation in the liquid medium: All the decolourisation studies were carried out using this common procedure. Ten discs of the fungal mycelia, grown on the malt extract agar at 28±1ºC for 7 days, were added to 100 mL of the synthetic basal medium **(**starch-20g, yeast extract-2.5g, KH_2PO_4-1g, Na_2HPO_4-0.05g, $MgSO_4.7H_2O$-0.5g, $CaCl_2$-0.01g, $FeSO_4.7H_2O$-0.01g, $MnSO_4.4H_2O$-0.001g, $ZnSO_4.7H_2O$-0.001g, $CuSO_4.5H_2O$-0.002g in 1L distilled water, pH 5.5) in 250 mL Erlenmeyer flasks. Dye was incorporated in each flask to a final concentration of 100 ppm after four days of growth of the fungus under static conditions. The flasks were then incubated under shaker conditions at 28±1°C. Decolourisation was measured at end of the stipulated time by removing the aliquots and centrifuging at 1200 rpm for 15 minutes. The clear supernatant obtained was used for determination of the rate of decolourisation spectrophotometrically by reading the absorbance at 597nm for Reactive Black-5. The rate of decolourisation was calculated as follows:

$(A_0\text{-}A_t/A_0) \times 100$

Where, A_0 = Initial O.D.

A_t = O.D. at time 't'

Optimization of the carbon and nitrogen sources for dye decolourisation: The effect of the different sources of carbon on dye decolourisation was studied at the end of 24 h by replacing the original carbon source from the medium using glucose, starch, glycerol, sucrose and the combination of starch and glucose (1:1). For carbon sources showing comparable results, decolourisation was studied at the end of 2, 4, 6, 8, 24 and 48 h. The effect of varying sources of nitrogen on dye decolourisation was studied at the end of 24 h after the optimization of the carbon sources by replacing yeast-extract (the original nitrogen source in the synthetic basal medium) with L-aspargine, peptone and ammonium sulphate. Control flasks without inoculum were also maintained.

Orthogonal array design for the maximization of the dye decolourisation: Taguchi's orthogonal array designs are widely used in screening experiments to identify the most important factors from a large number of factors. An Orthogonal array is usually represented by the following notation:

$L_a(b^c)$

Where, a = Number of the experimental runs or trials
b = Number of the levels of each factor
c = Number of columns in the array

The 'L' notation implies that the information is based on the Latin square arrangement of the factors.

The present investigation studied the effect of three factors viz., glucose as a source of carbon, yeast extract as a source of nitrogen and copper sulphate as an inducer of the enzyme (laccase) responsible for the dye decolourisation. The concentrations tested included concentrations one higher and one lower to the original concentration of the components initially present in the medium (Table 1). The experimental conditions were similar to those used for the previous experiments. The results obtained at the end of 2, 4, 6, 8 and 24 h were analysed using Minitab Software 15 (Revenkar & Lele 2006, Taguchi & Konishi 1987).

RESULTS AND DISCUSSION

Azo reactive dyes, the largest class of the water-soluble synthetic dyes with the greatest variety of colours and structures, are generally resistant to the aerobic biodegradation processes. However, biotreatment of the textile dyes by white rot fungi can prove to be a viable alternative to meet the environmental legislations and overcome the pollution problems caused by them (Yesilda & Ozcan 1998, Swamy 1998, Sumathi & Manju 2001). Hence, in the present investigation *Trametes versicolor* was evaluated for its ability to decolourise commonly used and one of the most polluting dyes viz., Reactive Black-5.

A combination of static conditions for the growth and shaker conditions for dye decolourisation was employed in course of the present study. The initial use of static conditions served to boost the production of lignolytic enzymes, while the shaker conditions employed later in the study helped in enhancing activity of the oxidative enzyme system due to an increase in mass and oxygen transfer between cells and the medium as shown by various workers (Machado & Matheus 2006, Anand & Vaidya 2008).

In the present investigation, the effect of carbon sources like glucose, sucrose, glycerol, starch

and a 1:1 combination of glucose and starch on decolourisation of Reactive Black-5 was studied at the end of 24 h. Glycerol and a combination of starch and glucose (1:1) proved to be highly inefficient in decolourising Reactive Black-5. Glycerol showed only 16.41 ± 0.45% decolourisation of Reactive Black-5, while a combination of starch and glucose (1:1) showed 23.97 ± 0.20% decolourisation. Starch was least efficient in removal of Reactive Black-5 showing a meagre 0.82 ± 0.50% decolourisation. Similar results showing inability of starch in decolourising Orange-2 dye by *Coriolus versicolor* have been demonstrated by Yesilda & Ozcan (1998). However, contradictory results by Revenkar & Lele (2006) showed 79% decolourisation of Amaranth by *Ganoderma* within 8 hours using starch as the sole source of carbon. In the present work both glucose and sucrose gave comparable results showing 62.93±0.31% and 60.30±0.33% decolourisation respectively at the end of 24 h, though the percent decolourisation increased up to about 83% by the end of 48 h. These results are in agreement with the results obtained by Vaidya & Konde (2008) who reported efficiency of glucose and sucrose in decolourisation of malachite green, a triphenylmethane dye.

Hence, these two sources were used in the further work where decolourisation was studied at the end of 2, 4, 6, 8, 24 and 48 h. When decolourisation was traced over a period of 24 and 48 h, glucose and sucrose showed comparable results for the decolourisation of the dye (Table 2). However, the initial rate of decolourisation was faster with glucose as compared to sucrose, especially during the first 4 h. The rate of decolourisation increased gradually with glucose as the sole source of carbon by the end of 8 h, though the major part of degradation occurred between the 8^{th} and 24^{th} hour. Reactive Black-5 though was degraded at a higher rate initially with both the sources of carbon, later on the rate of decolourisation slowed down considerably.

White rot fungi are capable of degrading the pollutants by the process of co-metabolism and glucose has been reported as the best carbon source for the fungal growth. Murugessan et al. (2003) reported that glucose concentration had a strong effect on the decolourisation of dyestuff. Fu & Viraraghavan (2001) reported that glucose is the best carbon source for decolourisation by the white rot fungi. However, the best glucose concentration giving the highest decolourisation rate was reported to be 5 g/L. In the present investigation glucose at 20 g/L was present in the medium which seemed to accelerate the initial rate of dye decolourisation. It is reported that laccase production is strongly dependent on the glucose concentration. In general, substrates that are efficiently and rapidly utilized by the organism result in high levels of laccase activity. The fungus has been found to utilize glucose and stabilize as soon as the glucose exhaustion occurs (Krishna Prasad et al. 2005). Hence, further work was carried out using glucose as the sole source of carbon.

It is conventionally accepted that C and/or N limitation triggers ligninolytic activity in white rot fungi and is required for the pollutant degradation. The requirement for N limitation is characteristic of secondary metabolism when metabolic activity is low. Murugesan & Kalaichelvan (2003) reported that degradation of Congo red by *Phanerochaete chrysosporium* was inhibited by a high concentration of nitrogen. Mineralization studies with several dyes have revealed that most of the dyes investigated were degraded only in a certain range of nitrogen concentration for *Phanerochaete chrysosporium* and *Trametes versicolor*. The synthetic medium used in the study contained yeast extract (2.5g/L) as a source of nitrogen presenting nitrogen limiting conditions. It was replaced by other sources of nitrogen to study their effect on dye decolourisation. The nitrogen sources evaluated included ammonium sulphate and three organic sources like yeast extract, peptone and aspargine.

Amongst the nitrogen sources tested yeast extract was found to be the best source of nitrogen showing 62.93 ± 0.31% of decolourisation at the end of 24 h. Peptone and aspargine reduced the

Table 1: Representation of $L_9(3^3)$ Orthogonal array for dye decolorisation.

Run	A Glucose	B Yeast Extract	C $CuS0_4$	A Glucose g/L	B Yeast Extract g/L	C $CuS0_4$ g/L
1	1	1	1	10	1.25	0.004
2	1	2	2	10	2.50	0.001
3	1	3	3	10	5.00	0.002
4	2	1	2	20	2.50	0.002
5	2	2	3	20	5.00	0.004
6	2	3	1	20	1.25	0.001
7	3	1	3	30	5.00	0.001
8	3	2	1	30	1.25	0.002
9	3	3	2	30	2.50	0.004

Table 2: % Rate of decolorisation of Reactive Black-5 with glucose and sucrose as the sole source of carbon by *T. versicolor*.

Hours	% Dye Decolorisation By *T. Versicolor*	
	Glucose	Sucrose
2	11.50±0.65	04.61±0.61
4	18.25±0.38	12.00±0.44
6	23.50±0.79	19.38±0.45
8	25.12±0.17	23.07±0.61
24	62.93±0.31	60.30±0.34
48	83.56±0.63	82.30±0.61

(± values indicate standard deviation)

Table 3: Orthogonal array for decolorisation of Reactive Black -5 by *T. versicolor* analysed by MINITAB 15.

Medium No.	Time required for decolorisation 2h	4h	6h	8h	24h
1	37.20±0.43	37.70±0.45	38.52±0.32	39.15±0.41	43.20±0.41
2	17.45±0.27	21.46±0.43	26.70±0.28	30.36±0.37	48.68±0.41
3	10.70±0.27	17.42±0.47	27.26±0.37	31.61±0.30	49.30±0.36
4	11.20±0.30	15.95±0.34	28.18±0.25	33.61±0.33	62.93±0.27
5	05.70±0.44	10.69±0.41	13.36±0.46	15.32±0.30	47.09±0.35
6	12.94±0.38	06.81±0.46	18.73±0.33	25.89±0.26	52.47±0.30
7	27.74±0.33	35.85±0.43	37.83±0.30	40.96±0.40	49.18±0.39
8	20.31±0.34	30.27±0.33	34.13±0.47	37.28±0.19	42.47±0.44
9	20.13±0.46	45.31±0.46	47.04±0.32	50.52±0.24	58.86±0.28

(± values indicate standard deviation)

Table 4: Response table for means for decolorisation of Reactive Black-5 by *T. versicolor*.

Level	A	B	C
1	47.06	51.77	46.05
2	54.14	46.05	56.82
3	50.17	53.54	48.50
Delta	7.08	7.49	10.78
Rank	3	2	1

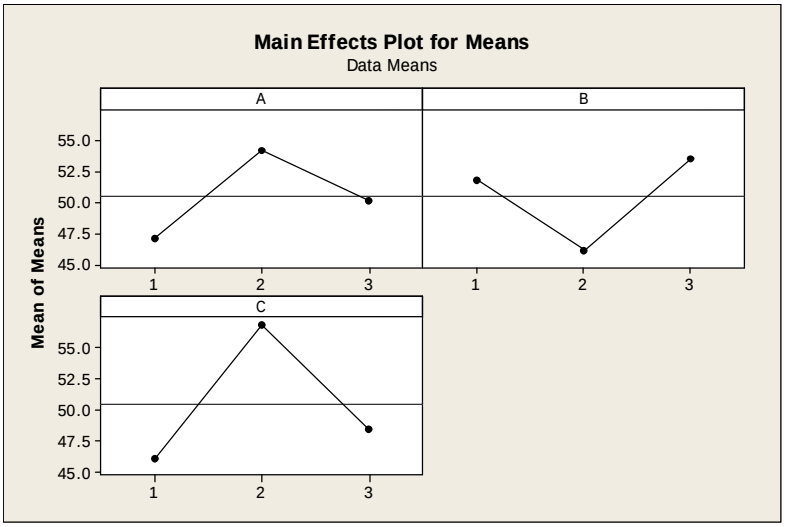

Fig. 1: Main effects plot for means for decolorisation of Reactive Black-5 by *T. versicolor*.

extent of dye decolourisation slightly as compared to yeast extract. Peptone showed 59.06 ± 0.54% decolourisation while aspargine showed 54.31 ± 0.33% decolourisation of Reactive Black-5. Ammonium sulphate showed the least effectiveness, decolourising only 42.59 ± 0.55% Reactive Black-5 at the end of 24 h. Revankar & Lele (2006) also showed ineffectiveness of the inorganic nitrogen sources like ammonium sulphate and urea in decolourisation of Amaranth showing only 24 and 18% dye decolourisation respectively. They showed efficiency of organic nitrogen sources in the dye decolourisation as compared to all the inorganic sources tested. They reported that yeast extract proved to be the best source of nitrogen with 75% decolourisation of Amaranth in 8 h, while only 61 and 51% decolourisation was achieved in 24 h with peptone and L-asparagine respectively. Similar results were obtained in the present study. Thus, glucose as a sole source of carbon and yeast extract as a sole source of nitrogen proved to be the most efficient in decolourisation of both the dyes. Hence, they were used in the further work for the optimization of the conditions for the dye decolourisation in the synthetic medium using Orthogonal array design.

Orthogonal array method facilitates analysis of the experimental data to establish the optimum conditions for the process, understanding the contribution of the individual factors and to evaluate the response under the optimal conditions. An orthogonal array (OA) is a matrix of numbers arranged in rows and columns. Each row represents the levels (or states) of the selected factors in a given experiment and each column represents a specific factor whose effects on the process performance (or product quality characteristics) can be studied. Orthogonal arrays have a balanced property which entails that every factor setting that occurs the same number of times for every setting of all the other factors considered in the experiment (Taguchi & Konishi 1987).

In OA L9, there are nine parameter-level combinations for each pair of columns, and each combination occurs once. Variables optimized in the present investigation were glucose, yeast extract and $CuSO_4$. Copper has been reported to be a strong inducer of laccase in the white rot fungi such as *Trametes versicolor* and *Phanerochaete chrysosporium* (Palmieri et al. 2000, Krishna Prasad et al. 2005). Thus, copper sulphate was chosen as one of the factors for the study due to its inducing effect

Table 5: Predicted (as anlysed by MINITAB 15) and observed values for decolorisation of the dyes at the end of 24 hours by *T. versicolor*.

Medium	Decolorisation of Reactive Black-5	
	Predicted values	Observed values
1	43.9656	43.20±0.41
2	49.0256	48.68±0.41
3	48.1889	49.30±0.36
4	61.8189	62.93±0.27
5	47.7756	47.09±0.35
6	52.8156	52.47±0.30
7	49.5256	49.18±0.39
8	41.3589	42.47±0.44
9	59.6256	58.86±0.28

on the laccase production which is responsible for the decolourisation of the dyes by *T. versicolor*. Glucose and yeast extract were the other two factors used in the study, as they have been found to be effective in enhancing the rate of decolourisation in the earlier steps.

The results analysed by MINITAB 15 software are shown in Table 3. 62.93 ± 0.27% decolourisation was observed at the end of 24 h in medium 4 containing 20g/L glucose, 1.25g/L yeast extract and 0.001 g/L $CuSO_4$. Table 4 represents the response table for means (nominal is best) obtained by analysing the data for the Orthogonal array. Rank and delta values help to determine the factors, which greatly affect the dye decolourisation. The order of the factors for Reactive Black-5 was $CuSO_4$ > yeast extract > glucose. This indicated that copper sulphate had a greater effect on the decolourisation of Reactive Black-5 in comparison with other factors. Revankar & Lele (2006), however, showed that yeast extract exerted a pronounced effect on the dye decolourisation of Amaranth. Amaranth is a monoazo dye while Reactive Black-5 is a diazo dye, presenting a more difficult structure to degrade. This might have attributed to the difference in order of factors responsible for their differing abilities in bringing about decolourisation by *T. versicolor*.

The optimum levels of each factor obtained by the statistical analysis are shown in Fig. 1. This represents the main effects plot for the system. A "main effect" is present when the different levels of a factor affect the response differently. A main effects plot graphs the response mean for each factor level connected by a line. When the line is horizontal (parallel to the X-axis), then there is no main effect present. Each level of the factor affects the response in the same way, and the response mean is the same across all the factor levels. When the line is not horizontal, then there is a main effect present. Different levels of the factor affect the response differently. The steeper the slope of the line, the greater the magnitude of the main effect. For each of the three variables at three different levels, one level increased the mean compared to the other level. This difference was the main effect, i.e., glucose at level 2, yeast extract at level 3 and $CuSO_4$ at level 2 for Reactive Black-5 (Table 4).

MINITAB 15 software also predicted the values for Amaranth decolourisation (100 ppm) after 24 h of incubation (Table 5). The values of decolourisation obtained on experimental verification, confirmed the findings. Thus, 20g/L of glucose, 5g/L yeast extract and 0.001 g/L $CuSO_4$ proved to be optimal concentrations for the decolourisation of Reactive Black-5. The present investigation showed the excellent performance of *T. versicolor* in the decolourisation of Reactive Black-5 enforcing its potential in environmental decontamination.

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