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Original Research Article

Decolourization of Azo dyes in a two-stage process using novel isolate and advanced oxidation with Hydrogen peroxide / HRP system

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A B S T R A C T

Azo dyes such as Reactive Black 5 and Disperse Red 343, commonly used in the textile industries have been investigated for their complete decolourization and degradation through a two-stage treatment process. The first stage of treatment involved decolourization of the Reactive Black 5 and Disperse Red 343 using novel bacterial isolates (*Paenibacillus lautus SK21* and *Bacillus subtilis SK48* respectively) under standardized conditions of pH 7, 37^{o}C / static condition with the dye concentration of 100 mg l^{-1}. Reactive Black 5 was decolourized to 24 % in 72 hours while that of Disperse Red 343 to 88 % in 42 hours in minimal media. In the second stage of treatment, the decolourized dye was subjected to Advanced Oxidation Process with Hydrogen Peroxide and Horse Radish Peroxide system in which Reactive Black 5 was decolourized to 91 % and Disperse Red 343 was decolourized to 99% within 6 hours under sunlight. The spectrophotometric and chromatographic characteristics of the dyes after stage I and stage II revealed sufficient evidences of having undergone molecular re-arrangement with simultaneous loss of the native color.

Introduction

Azo dyes, which are aromatic compounds with one or more –NQN– groups, constitute the largest class of synthetic dyes used in commercial applications (Anjali *et al*., 2007). These dyes are widely used in a number of industries, such as textile dyeing, food, cosmetics, paper printing, with the textile industry as the largest consumer. All dyes do not bind to the fabric; depending on the class of the dye, its loss in wastewaters could vary from 2% for basic dyes to as high as 50% for reactive dyes, leading to severe contamination of surface and ground waters in the vicinity of dyeing industries (Ganesh *et al*., 1994; O'Neill *et al*., 1999). Many dyes are visible in water at concentrations as low as 1 mg l^{-1}. Textile processing wastewaters with dye contents in the range of 10–200 mg l^{-1} are highly colored. Some of the dyes and their degradation products are carcinogenic in

nature (Levine, 1991). A conventional biological wastewater treatment process is not very efficient in treating a dye wastewater due to the low biodegradability of dyes. It is usually treated by physical or chemical-treatment processes, but these procedures have inherent drawbacks as they generate a significant amount of the sludge or cause secondary pollution due to the formation of hazardous byproducts (Khehra *et al*., 2005). These constraints have led to the consideration two stage treatment process involving advanced oxidation processes (AOP) and biological methods as attractive options for the treatment of dye-containing wastewaters. The ability of microorganisms to carry out dye decolourization has recently received much attention. Advanced oxidation is one of the potential alternatives to decolourize and to reduce recalcitrant wastewater loads from textile dyeing and finishing effluents. This process implies generation and subsequent reaction of hydroxyl radicals, which are the most powerful oxidizing species after fluorine (Legrini *et al*., 1993). AOP methods do not produce solid waste. However, both AOP and membrane filtration methods are energy and cost intensive. The main objective of the present study is to decolourize and degrade azo dyes in a two stage treatment process using bacterial isolate and advanced oxidation process involving H_2O_2 and horse radish peroxidase (HRP). Azo dye Reactive Black 5 (C.I.) and Disperse Red 343 (C.I.) was selected as the model azo dye for the characterization of the breakdown process during decolourization involving both biological and advanced oxidation process.

Materials and Methods

Commercial Dyes and Chemicals

The dyes that were used in the study were procured from United Bleachers (UBL) Pvt. Ltd, Mettupalayam, Tamil Nadu, India. All other chemicals and reagents were of Analytical grade (Himedia, Mumbai, India).

Spectral analysis of the Dye

Reactive Black 5 and Disperse Red 343 were initially studied for absorption spectrum in a UV-Vis Spectrophotometer (Schimadzu UV-Vis 1800, Japan) from 200nm to 900nm (Muhammad *et al*., 2007; Safia *et al*., 2005).

Chosen Bacterial Culture

The novel isolates were tested and standardized earlier was chosen for the present study (Senthil, 2011).

Culture Maintenance and Media

The bacterial strain (Stock Culture) was maintained routinely on Nutrient Agar containing (g l^{-1}): NaCl 5.0, bacteriological peptone 10.0, Yeast Extract 2.0, Beef Extract 1.0 and Agar Agar 15.0, and stored at 4°C until use (Jadhav *et al*., 2010). The organism from the stock culture was used for the decolourization studies after pre-culturing in Minimal broth (Cappuccino and Sherman, 2004).

Decolourization in Minimal media

The dyes were subjected to decolourization experiments under Minimal media following the optimal conditions as described earlier (Senthil, 2011).

Identification of the novel isolate

The chromosomal DNA of the strains with the best decolourization potential was

isolated according to the procedure described earlier (Rainey, 1996). A partial DNA sequence for 16S rRNA gene was amplified by using 5'- ATG GAT CCG GGG GTT TGA TCC TGG CTC AGG-3' (forward primer) and 5'-TAT CTG CAG TGG TGT GAC GGG GGG TGG-3' (reverse primer) (Jing *et al*., 2004; Senthil *et al*., 2010). The nucleotide sequence analysis of the sequence was done at Blast-n site at NCBI server (http://www.ncbi.nlm.nih.gov/ BLAST). The alignment of the sequence was done by using CLUSTALW program V1.82 at European bioinformatics site (http://www.ebi.ac.uk/clustalw). The sequence was refined manually after crosschecking with the raw data to remove ambiguities and submitted to the NCBI.

Analysis of the Decolourized Product

UV-Vis Spectral Analysis

The samples were collected before and after the decolourization processes and filtered through 0.2 µm membrane filters. The filtrate was then scanned in the UV-Vis Spectrophotometer (Schimadzu, Japan) within the range of 200 – 900nm. Appropriate blank was also subjected to the scanning process. The band width was set to 1 nm during the scanning program. The absorbance was noted at the respective characteristic peak area (λ_{max}) for the interpretation of results (Sagarika *et al*., 2006).

TLC Analysis

The decolourized supernatant processed for the UV-Vis spectrophotometric analysis was evaporated in a watch glass and resuspended in methanol. The methanolic suspension was then concentrated by allowing evaporation and used as sample for separation of component(s) by thin layer chromatography. The decolourized supernatant sample was resolved on a 20 x 20 cm sized pre-coated TLC silica gel 60 F (Merck, Mumbai, India). Organic solvent mixture of methanol: propanol: water: ammonia used in the ratio 3:6:1:4 was used as the eluent. After elution, the separated bands were observed under UV illumination.

Advanced oxidation process with hydrogen peroxide and horse radish peroxidase

The microbial mediated decolourized dye was subjected to advanced oxidation process using hydrogen peroxide (H_2O_2) and horse radish peroxidase (HRP) system.

Extraction of horse radish peroxidase enzyme from the horse radish

Around 500 gm of washed and cleaned horse radish was cut into small pieces. The pieces were minced thoroughly to extract the juice with 250 ml of distilled water. The juice was strained using a muslin cloth and then filtered through a fine nylon filter. The filtrate was then centrifuged at 6000 rpm for 10 minutes to obtain a clear supernatant. This supernatant was used as a crude source of the peroxidase enzyme in all the experiments.

Second Stage decolourization of the microbial decolourized dye by advanced oxidation process

The microbe-free supernatant was subjected to the second stage of decolourization through advanced oxidation process using the mixture of decolourized supernatant, hydrogen

peroxide and horse radish peroxidase in the ratio of 8:1:1 and kept under sunlight for 24 hours time period. The mixture was centrifuged at 6000 rpm for 10 minutes and subjected to UV-Vis spectrophotometric analysis (200 – 900 nm) and TLC.

Analysis of decolourized dye products after advanced oxidation process by TLC

The second – stage decolourized supernatant processed for the UV-Vis spectrophotometric analysis was evaporated in a watch glass and resuspended in methanol. The methanolic suspension was then concentrated by allowing evaporation and used as sample for separation of component(s) by thin layer chromatography as described earlier.

Results and Discussion

Spectral Analysis

Reactive Black 5 and Disperse Red 343 were analyzed for the absorption maxima by UV-Vis spectrophotometry and found to be at 587 nm and 522 nm respectively (Fig. 1 & 2).

Decolourization of Reactive Black 5 using novel isolate *Paenibacillus lautus SK21*

The isolate *Paenibacillus lautus SK21* was able to decolourize Reactive black 5 to 21 % in 42 hours under optimal conditions such as pH 7, 37^{o}C, static condition and with the dye concentration of 100 mg l^{-1} in minimal media (Fig.3). (Selection of novel isolate and optimization of parameters were performed earlier and data are not shown).

Decolourization of Disperse Red 343 using novel isolate *Bacillus subtlis SK48*

The isolate *Bacillus subtilis SK 48* was able to decolourize the dye significantly to 88 % in 42 hours under optimal conditions such as pH 7, 37^{o}C, static conditions and with the dye concentration of 100 mg l^{-1} in minimal media (Fig. 4).

Analysis of decolourization by UV-Vis Spectrophotometry

Reactive Black 5 was decolourized by the novel isolate SK21 in such a way that its absorption peaks at 587 and 484 nm (Fig.1) in the visible range disappeared. At the same time, the absorption peak in the UV range also diminished and many new peaks appeared. Similarly, Disperse Red 343 was also decolourized by the SK48 with the disappearance of absorption peaks at 522 and 545 nm (in Fig. 2) in the visible range. In the case of Disperse red 343, there were no new peaks seen in the spectrum (both visible and UV range) after decolourization treatment.

Analysis of decolourized dye products by TLC

Reactive Black 5 that was decolourized by the novel isolate was resolved in TLC (Fig. 5 & 6). The TLC when observed under the UV light at 365 nm showed 2 bands with distinct Rf namely 0.58 and 0.69 in comparison to the native dye (0.73). Similarly, TLC of Disperse Red 343 when observed under the UV light at 365 nm showed 5 bands with distinct Rf values (0.26, 0.32, 0.46, 0.59 and 0.70).

Fig.1 UV- Vis spectrophotometric analysis of the (a) native dye – Reactive Black 5, (b) decolourized and (c) AOP-treated dyes.

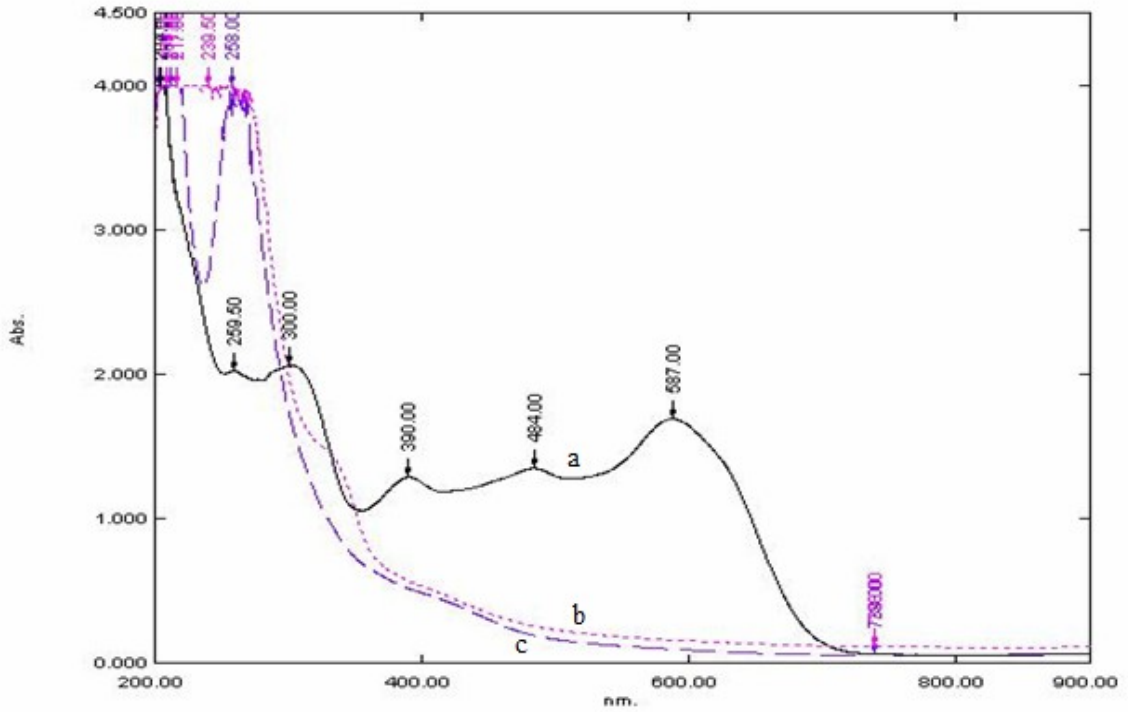


Fig.2 UV- Vis spectrophotometric analysis of the (a) native dye – Disperse Red 343, (b) decolourized and (c) AOP-treated dyes

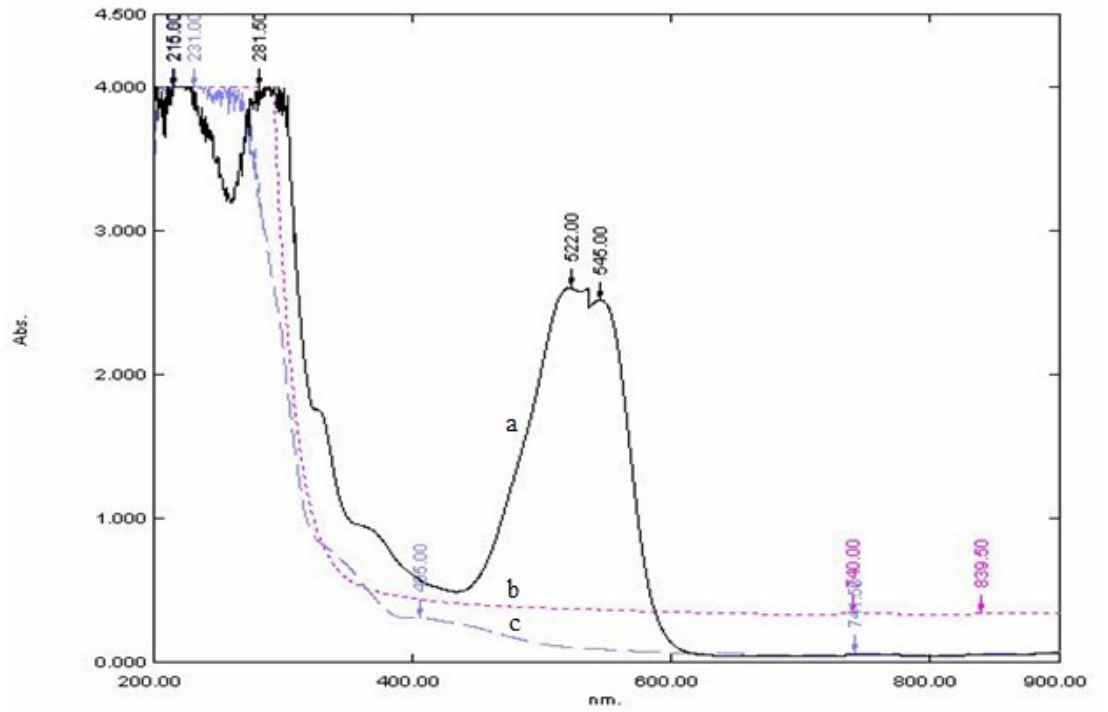


Fig.5 Thin layer chromatogram of Reactive black 5 under UV light (365 nm) (a) with native dye, (b) after biological treatment and (c) after AOP

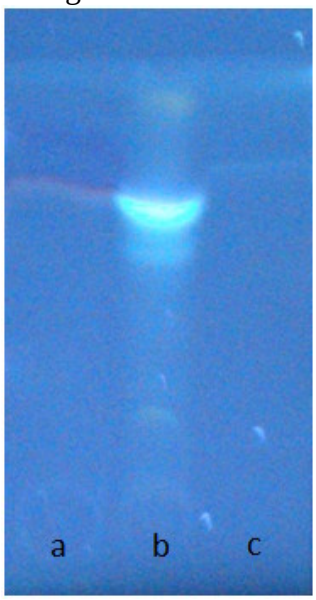
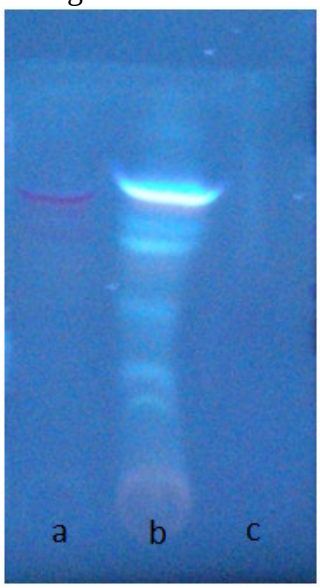


Fig.6 Thin layer chromatogram of Disperse Red 343 under UV light (365 nm) (a) with native dye, (b) after biological treatment and (c) after AOP



Advanced oxidation process with hydrogen peroxide and horse radish peroxidase

The biologically decolourized supernatant of the dye Reactive 5 and Disperse red 343 was decolourized to 91 % and to 99% within 6 hours under sunlight.

Identification of decolourized dye products after advanced oxidation process by TLC

The AOP-decolourized solution when subjected to TLC did not produce any visible band under the UV illumination (Fig.5 & 6).

Knowledge of biological decolourization of textile dyes is still inadequate especially with reference to azo group of dyes. Reactive Black 5 is widely used in textile dying and is reported to be toxic too (Sagarika *et al*., 2006). There has not been usually nonspecific and bacterial decolourization is generally faster. Research on bacterial strains that are able to decolourize azo dyes under aerobic and anaerobic conditions have been extensively reported (Rafii *et al*., 1990; Kudlich *et al*.,1997; Suzuki *et al*., 2001; Blumel *et al*., 2002; Olukanni *et al*., 2006; Dos Santos *et al*., 2007; Vijaykumar *et al*., the key enzyme responsible for this reductive azo-dye degradation in bacterial species (Franciscon *et al*., 2009). Dye degradation was performed under microaerophilic conditions until no residual color was observed. The degradation products of the dye were characterized using UV–Vis, and TLC techniques (Kalyani *et al*., 2008). The results in the foregoing sections suggest that decolourization of Reactive Black 5 and Disperse Red 343 occurs during microaerophilic reaction set-in by the isolate obtained from the acclimatized dumping ground of the sludge within the textile industry (Senthil *et al*., 2011). When both the dyes subjected to bacterial decolourizaiton process, the spectral analysis revealed complete shift in the absorption maxima from its native form. This suggests that the dye has undergone structural modification during the decolourization process. This is possibly due to the reductive cleavage of the azo bond, resulting in the formation of colorless aromatic amines (Khalid *et al*., 2008). To date, very few reports are available on the intermediates or the products of biodegradation of azo dyes (Chen *et al*., 2008). To further substantiate the findings, the TLC chromatograms of any exclusive report on to 88% only in static conditions indicating microaerophilic nature of the process involving the novel bacterial culture. The bacterial reduction of the azo bond is

2007; Hsueh and Chen, 2008; Lin and Leu, 2008). However, Reactive Black 5 demonstrated very poor decolourization amounting to 21 %. This is possibly due to its structural difference and complexity. Zimmerman *et al*., 1982 reported similar observation while investigating the degradability of difference structures of azo dyes. Azo Reductase is thought to be the media extracted by the organic phase before and after biological decolourization under UV light showed that the decolourized sample had additional bands which might have originated from the dye metabolites. Comparison of the samples before and after decolourization also showed the disappearance of the dye band in decolourized media indicating the complete decolourization involving molecular rearrangement in the dye. Although biological degradation methods are one of the most economic processes for wastewater treatment, they are often ineffective to degrade molecules of refractive nature, like those present in textile industry waste waters. Also, the survival of anaerobic biomass in the presence of high concentration of azo dyes is a difficult task. Therefore, for the treatment of this type of waste water, Advanced Oxidation Process involving H_2O_2 and HRP system has been proposed. In the present study, treatment of the microbe-mediated decolourized dye has been further subjected to advanced oxidation process as the second stage of treatment to achieve near to 100% decolourization to obtain a solution as clear as water. Before and after biological treatment of both the dyes under UV-Vis

spectral analysis, revealed existence of absorption maxima at different areas of the visible region. However, these peaks disappeared remarkably and remained flat after the advanced oxidation treatment. This is an evidence of complete molecular re-arrangement of the native dye. Thin Layer Chromatography of the AOP-dye products did not reveal any bands suggesting that the oxidized product of the dye to have either undergone modification or did not resolve satisfactorily to be visualized. However, the dye has undergone a total and radical color change to become like that of water. Toxicity of the decolourized water is yet to be analyzed to evaluate the microbial and AOP-treated dye to be let into the environment or water bodies. The results of this study will form the basis for development of cost effective and robust indigenous process for treatment of textile waste water.

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