REMEDIATION OF NAVY BLUE RX DYE USING BIOLOGICAL AND ADVANCED OXIDATION PROCESS

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ABSTRACT

Synthetic dyes used in the textile industry are causing worldwide concern due to their harmful impact on the environment. In this study, microorganisms capable of decolourizing Navy Blue RX dye were isolated from textile effluent samples and identified using 16S rRNA sequencing. The effects of temperature, pH, initial dye concentrations and mechanical agitation on the dye decolourization ability of individual organisms and their consortia were examined. For improved storage, stability and utility, the organisms were inoculated and incubated with solid substrates - rice flour and compost, and dried to obtain a powdered formulation. Treatment with only a microbial formulation requires a longer time and does not result in complete dye remediation. Therefore, advanced oxidation process, such as the Fenton process and ozonation, was combined with microbial treatment. The effect of different treatment methods and their combinations was analysed by checking the percentage of decolourization using a UV spectrophotometer. Analysis of control and treated samples by gas chromatography-mass spectrometry confirmed the degradation of the parent dye. According to this study, ozonation combined with microbial treatment results in about 98 % decolourization of Navy Blue RX dye.

Keywords: *Navy Blue RX, microbial treatment, Fenton process, ozonation*

INTRODUCTION

Effluents discharged from the textile industry contain a large amount of unbound synthetic dyes which, when discharged into water bodies, have a harmful effect on human health and the environment. About 8,000 synthetic dyes are listed in the Colour index under 40,000 trade names. The global dyes and pigments market is expected to reach USD 49.1 billion by 2027 [1]. About 70 million tons of synthetic dyes for the textile industries are produced worldwide annually. Almost 10 % of the dyes are released into the environment as process wastewater [2]. Prolonged exposure to synthetic dyes can cause skin irritation, cancer, diarrhoea, vomiting, headaches and cognitive impairment. Untreated synthetic dyes from textile effluents cause water pollution that adversely affects aquatic life and ecosystems causing a decline in biodiversity and soil pollution that affects the microbial population and plant growth [3]. Navy Blue RX is a reactive dye that can react with fibres to form

a covalent bond. Reactive dyes are those dyes used for cotton dyeing and printing in the textile industry due to the wide range of colours they provide, high fastness, easy application and low cost. It is crucial to detoxify synthetic dyes before they are released into water sources. Existing physical and chemical dye treatment methods require high costs and strict reaction conditions [4]. Biological treatment methods are relatively inexpensive and involve the use of microorganisms such as *Rhizobium radiobacter*, *Aeromonas sp*., *Pseudomonas sp.*, *Halomonas sp.*, *Neocardia sp.*, *Proteus mirabilis* [5 - 10], etc. for dye remediation. Known microbial dye degraders need to be converted into a form that can be easily transported, stored and used even by an unskilled person. Therefore, in this study, microorganisms capable of dye remediation were isolated, inoculated and incubated on a solid substrate such as rice flour and compost that can facilitate transportation and application. In addition to microbial treatment, advanced oxidation processes such as ozonation and Fenton process were applied. Advanced oxidation processes (AOPs) are those groups of technologies that lead to the formation of hydroxyl or sulphate radical, which further results in degradation of complex dye structures. The Fenton reaction is the oxidation process that uses hydrogen peroxide (H_2O_2) as an oxidant and iron (Fe) as a catalyst in the presence of an acidic (H^+) medium. Fenton oxidation process begins with the formation of a free hydroxyl radical. Hydroxyl radicals belong to the most active oxidants and can react 10^6 - 10^{12} times faster than ozone, depending on the substrate to be degraded [11]. In the case of ozonation, the mechanism of formation of hydroxyl radical is based on the decomposition of ozone in a chain of reactions initiated by hydroxyl ions [12].

MATERIALS AND METHODS

Materials

Navy Blue RX dye was procured from Jain Colour and Chemical Co. (an industrial supplier of textile dyes). Effluent samples were obtained from five different textile industries in Maharashtra, India, which mainly use reactive dyes for dyeing cotton fabric. Nutrient agar and nutrient broth were procured from HiMedia. Rice flour (RF) and compost (CT) were used in a 1:1 ratio to prepare powdered formulation. Ferrous sulphate and hydrogen peroxide for the Fenton reaction come from Loba Chemicals. Kent ozone generator was used for ozonation (product specification: voltage - single phase 220 - 240 V AC, 50 - 60 Hz, rated power dissipation - 13 W). A UV spectrophotometer (Equiptronics EQ-825A, microprocessor-based UV spectrophotometer) was used to check absorption maxima (λ_{max}) and percentage of decolourization. Separating funnel, solvent system (0.48 g of cetrimide in 100 ml isoamyl alcohol) and Perkin Elmer Clarus 600C gas chromatograph/mass spectrometer were used for gas chromatography-mass spectrometry (GC-MS) analysis.

Screening of dye decolourizers and identification using 16s rRNA sequencing

Effluent samples obtained from textile industry were serially diluted eight times using sterile saline. Dilutions of 10^{-6}, $10^{-7}$and 10^{-8} were plated on nutrient agar (NA) containing 0.1 % Navy Blue RX dye. Organisms were isolated and inoculated into nutrient broth (NB) containing 0.05 % Navy Blue RX dye. In order to determine the wavelength at which the percentage of decolorization has to be calculated, the absorption maxima of Navy Blue RX dye were determined. The percentage of decolourization was determined after four days using a UV spectrophotometer and the following equation:

$$\text{Percentage of decolourization} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \cdot 100$$

where: absorbance of control is the absorbance reading for 0.05 % dye in nutrient broth without treatment and absorbance of test is the absorbance reading for 0.05 % treated dye solution at 605 nm (λ_{max} for Navy Blue RX dye is 605 nm).

Three isolates (A1, O3 and A2) which gave the maximum percentage of decolourization were sent for microbial identification by 16S rRNA sequencing to Barcode Biosciences, Bangalore. The following isolated organisms were identified: A1 - *Neobacillus niacini,* O3 - *Enterobacter hormaechei* and A2 - *Enterobacter ludwigii.*

Effect of temperature, pH and initial dye concentration on the decolourization of Navy Blue RX dye

Effect of temperature – The percentage of decolourization of 0.05 % Navy Blue RX dye was calculated after incubation with *Neobacillus niacini*, *Enterobacter hormaechei* and *Enterobacter ludwigii* for four days at different temperature conditions (0, 27, 37 and 50 °C).

Effect of pH - 0.05 % Navy Blue RX dye was adjusted to different pH values (2, 5, 9 and 12) and inoculated with *Neobacillus niacini*, *Enterobacter hormaechei* and *Enterobacter ludwigii*. The percentage of decolourization was calculated after four days of incubation at 37 °C.

Initial dye concentration - Navy Blue RX solution was prepared at different concentrations (0.25, 0.5, 1 and 2 mg/ml) and incubated with *Neobacillus niacini*, *Enterobacter hormaechei* and *Enterobacter ludwigii*. The percentage of decolourization was calculated after four days of incubation at 37 °C.

Effect of consortia on decolourization of Navy Blue RX dye

The effect of individual isolates and their combinations on the percentage of decolourization of Navy Blue RX dye was analysed. 0.05 % Navy Blue RX dye in NB was inoculated with *Neobacillus niacini* (A1), *Enterobacter hormaechei* (O3)*, Enterobacter ludwigii* (A2) and its combinations (A1 + O3, O3 + A2, A1 + A2 and A1 + O3 + A2), and incubated at 37 °C.

Preparation of microbial formulation

To prepare the microbial formulation, 2 g of powdered rice flour and compost respectively was placed in a glass Petri plate and autoclaved. Since the consortium of three organisms (*Neobacillus niacini* (A1) + *Enterobacter hormaechei* (O3) + *Enterobacter ludwigii* (A2)) gave a higher percentage of decolourization in a shorter time period of two days, 2 ml of each overnight grown culture of *Neobacillus niacini*, *Enterobacter hormaechei* and *Enterobacter ludwigii* in NB was inoculated in a sterile Petri plate containing rice flour and compost as a solid substrate in 1:1 ratio and dried for five days at 40 ˚C to obtain a powdered formulation.

Fenton process

25 ml of 0.05 % Navy Blue RX dye solutions were adjusted to pH 2, 3, 4, 5, 6 and 7 using 0.1N NaOH and 0.1N H_2SO_4. 10 ml of 2.5M ferrous sulphate was added to dye solution at different pH, followed by addition of 5 ml of 30 % w/v hydrogen peroxide. After the effervescence had ceased, more hydrogen peroxide was added to check for decolourization (total volume of hydrogen peroxide added was 20 ml). The reaction was carried out at room temperature (27 °C) in presence of light. The ferric sludge formed during this process was filtered, dried and weighed.

Ozonation

100 ml of 0.025, 0.05, 0.1 and 0.2 % Navy Blue RX dye solution was subjected to ozonation using a Kent ozone generator. The experiment was carried out in a 250 ml conical flask at room temperature and pH 7.5 in presence of visible light, and the ozone output was 200 mg/hr. During the ozonation process, samples were collected at 15-minute intervals, and the percentage of decolourization was calculated using a UV spectrophotometer.

Combined effect of microbial formulation and ozonation on Navy Blue RX dye

2 g of powdered microbial formulation from the Petri plate was added under sterile conditions to a 250 ml conical flask containing 100 ml of 0.05 % Navy Blue RX dye solution. The control flask (dye solution) and test flask (dye solution with powdered formulation) were incubated in a shaker at room temperature. The percentage of decolourization was measured after 24 and 48 hours. The supernatant obtained after 48 hours of treatment with the powdered microbial formulation was then subjected to ozonation at pH 7.5 in presence of visible light, at room temperature, until it became colourless (about 98 % decolourization when checked with a UV spectrophotometer).

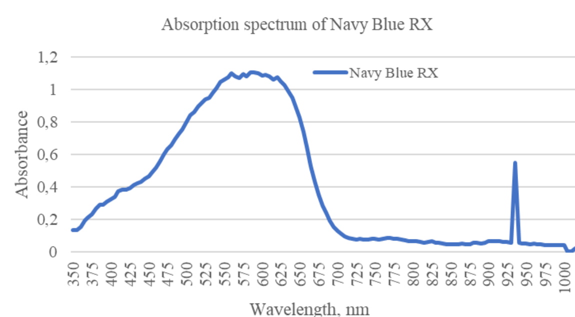
Solvent extraction and GC-MS analysis

For solvent extraction, several solvents and their combinations from research articles were tested. After standardization, it was found that 0.48 g of cetrimide in 100 ml of isoamyl alcohol could extract the dye and its products from water. Therefore, control and treated water samples were extracted in solvent (iso-amyl alcohol with cetrimide) using a separating funnel. 50 ml of sample and 25 ml of solvent were added to a separating funnel, mixed by shaking and left to stand overnight. The upper layer was collected in clean test tubes and used for GC-MS analysis. Analysis was carried out on a Perkin Elmer Clarus 600C GC-MS instrument with a single quadrupole mass analyser in electron impact (EI) mode. A GsBP-5MS column was used for separation (30 m x 0.25 mm x 0.25 µm). 1µL of sample was injected with a split ratio of 20:1 at an injection temperature of 275 °C. The oven temperature was initially set at 40 °C and then increased to 280 °C at a rate of 10 °C/min with a final hold time of 4 min. The GC-MS interface temperature was set at 280 °C and the MS source temperature was set at 250 °C. Peaks were identified using National Institute of Standards and Technology (NIST) library.

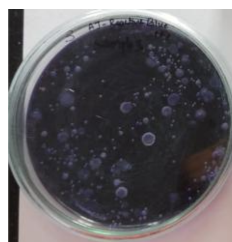
RESULTS

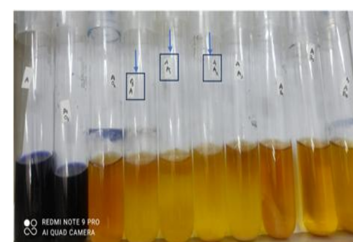
Screening of Navy Blue RX dye decolourizers and identification using 16s rRNA sequencing

The absorption spectrum of Navy Blue RX dye was studied using a UV spectrophotometer and absorption maxima was found at 605 nm (Figure 1a). Figure 1b shows organisms from an effluent sample spread on NA with 0.1 % Navy Blue RX dye. Figure 1c shows nine isolated organisms inoculated into NB, showing decolourization after four days. Based on the reports of sequencing and phylogenetic analysis of the three isolated organisms that gave the highest percentage of decolourization, they were determined to be *Neobacillus niacini* (A1), *Enterobacter hormaechei* (O3) and *Enterobacter ludwigii* (A2).



a)



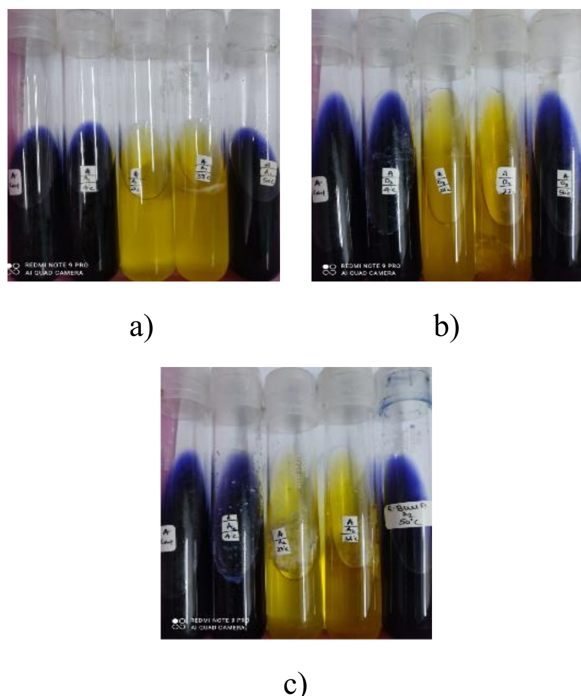


b) c)

Figure 1. a) Absorption spectrum of Navy Blue RX dye, b) spread plate method with organisms from effluent sample on NA containing 0.1 % Navy Blue RX dye, c) decolourization of Navy Blue RX dye in NB by 9 isolates from effluent sample obtained from spread plate (from left to right: control and test tubes inoculated with isolates)

Effect of temperature, pH and initial dye concentration on the decolourization of Navy Blue RX dye

Temperature - Figures 2a, 2b and 2c show the decolourization of 0.05 % dye by *Neobacillus niacini*, *Enterobacter hormaechei* and *Enterobacter ludwigii* at 27 and 37 °C after four days. In test tubes incubated at 4 and 50 °C, no significant decolourization was achieved

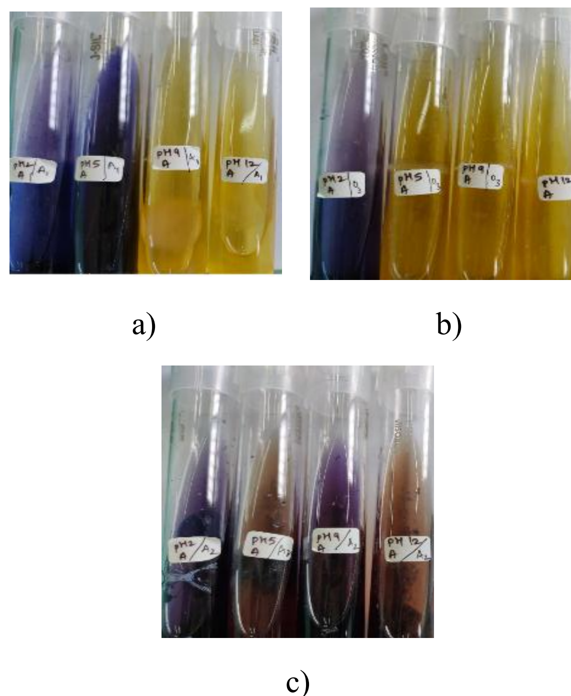


a) b)

c)

Figure 2. Effect of temperature on the decolourization of Navy Blue RX dye by: a) *Neobacillus niacini*, b) *Enterobacter hormaechei*, c) *Enterobacter ludwigii* (from left to right: control tube, 4, 27, 37 and 50 °C)

pH - 0.05 % Navy Blue RX solution was adjusted to different pH 2, 5, 9 and 12. After inoculation and incubation of the isolated organisms for four days, higher percentage of decolourization was achieved at pH 9 and 12 for *Neobacillus niacini* (Figure 3a), at pH 5, 9 and 12 for *Enterobacter hormaechei* (Figure 3b), and low decolourization was achieved using *Enterobacter ludwigii* (Figure 3c) at pH 5, 9 and 12. None of the organisms showed decolourization at pH 2.



a) b)

c)

Figure 3. Effect of pH on decolourization by: a) *Neobacillus niacini*, b) *Enterobacter hormaechei*, c) *Enterobacter ludwigii* (from left to right: 0.05 % Navy Blue RX dye at pH 2, 5, 9 and 12)

Initial dye concentration - *Neobacillus niacini* could decolourize up to 0.2 % of Navy Blue RX dye when incubated for four days in nutrient broth (Figure 4a). *Enterobacter hormaechei* can decolourize up to 0.1 % (Figure 4b) and *Enterobacter ludwigii* up to 0.05 % of Navy Blue RX dye (Figure 4c).

Effect of consortia

The percentage of decolourization of the isolated organisms and their consortia was studied after 24, 48, 72 and 96 hours. About 80 % decolourization was achieved in a shorter period of time (48 hours) when all three organisms were used in a consortia compared to decolourization achieved by individual organisms (Figure 5). Individual isolates can result in about 80 % dye decolourization in 96 hours, however the same results can be achieved by consortia in 48 hours.

a) b)

c)

Figure 4. Effect of initial dye concentration on decolourization of Navy Blue RX dye by: a) *Neobacillus niacini*, b) *Enterobacter hormaechei*, c) *Enterobacter ludwigii* (from left to right: control and test tubes with initial dye concentrations of 0.025, 0.05, 0.1 and 0.2 %)

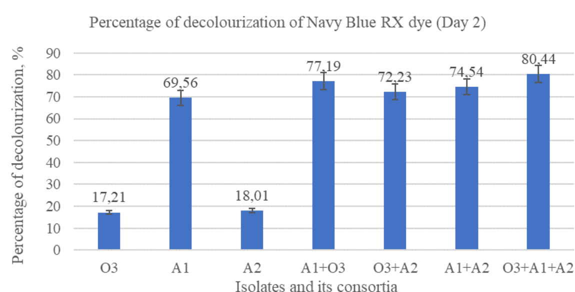
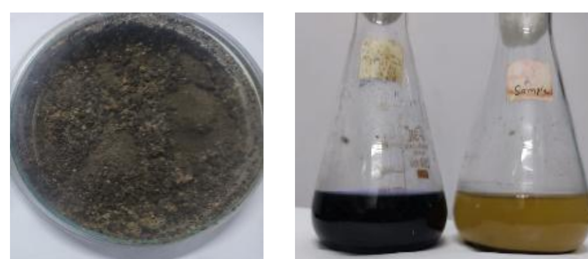


Figure 5. Percentage of decolourization of isolates and their consortia

Preparation of microbial formulation

2g of powdered formulation with consortia of *Neobacillus niacini*, *Enterobacter hormaechei* and *Enterobacter ludwigii* (Figure 6a) was inoculated in 100 ml of 0.05 % dye solution and incubated at room temperature in a shaker which resulted in decolourization after 48 hours (Figure 6b).



a) b)

Figure 6. a) Dried powdered formulation of RF + CT with consortia of *Neobacillus niacini*, *Enterobacter hormaechei* and *Enterobacter ludwigii*, b) effect of powdered formulation on 100 ml of 0.05 % Navy Blue RX dye solution (control and test sample)

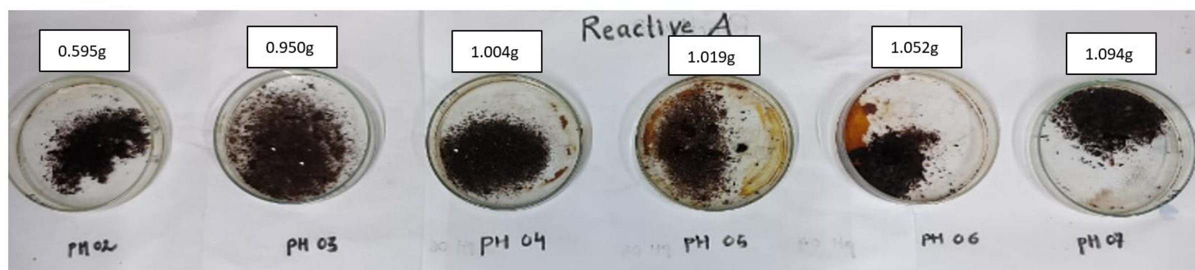
Fenton process

The Fenton reaction did not result in significant dye decolourization at pH ranging from 2 to 7 (Figure 7a). It resulted in the formation of ferric sludge that settled at the bottom of the beaker. The ferric sludge was separated, dried and weighed. With the increase in the pH of the dye, the amount of ferric sludge formed also increased (Figure 7b).

Ozonation

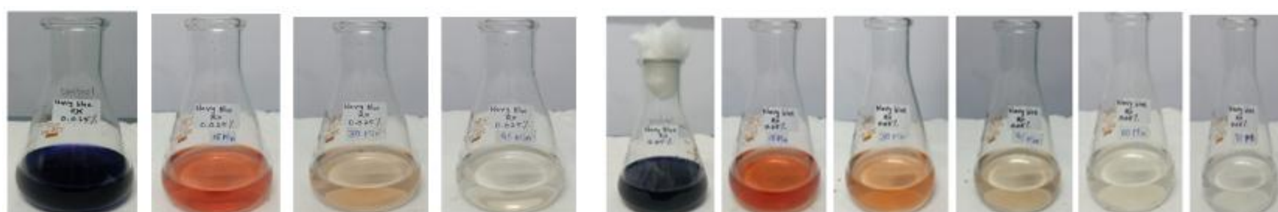
100 ml of 0.025, 0.05, 0.1 and 0.2 % Navy Blue RX dye solution was subjected to ozonation. The time required for almost complete decolourization of 0.025, 0.05, 0.1 and 0.2 % Navy Blue RX was 45 min, 75 min, 135 min and 255 min, respectively (Figures 8a, 8b, 8c, 8d and 8e). Therefore, the time required for decolourization is directly proportional to the initial dye concentration.

a)

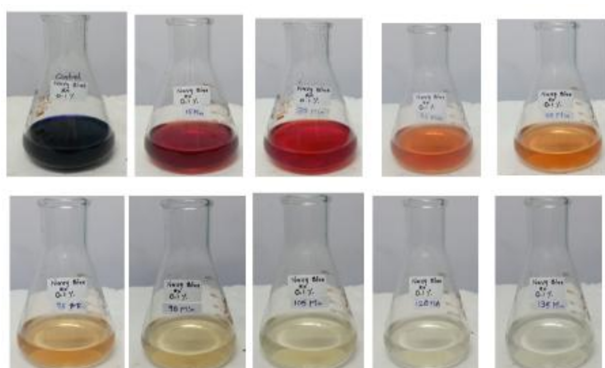


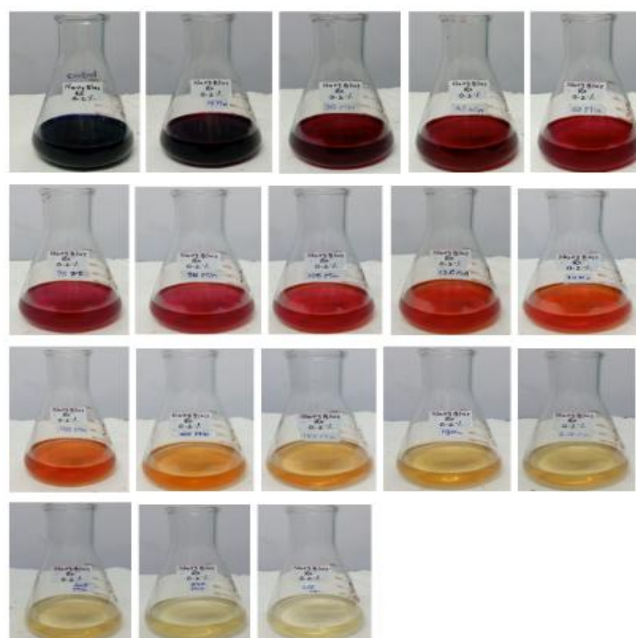
b)

Figure 7. a) Fenton reaction on Navy Blue RX dye at different pH (from left to right: control sample, pH - 2 to 7), b) ferric sludge and its weight in grams from Fenton reaction on Navy Blue RX dye (from left to right: pH - 2 to 7)



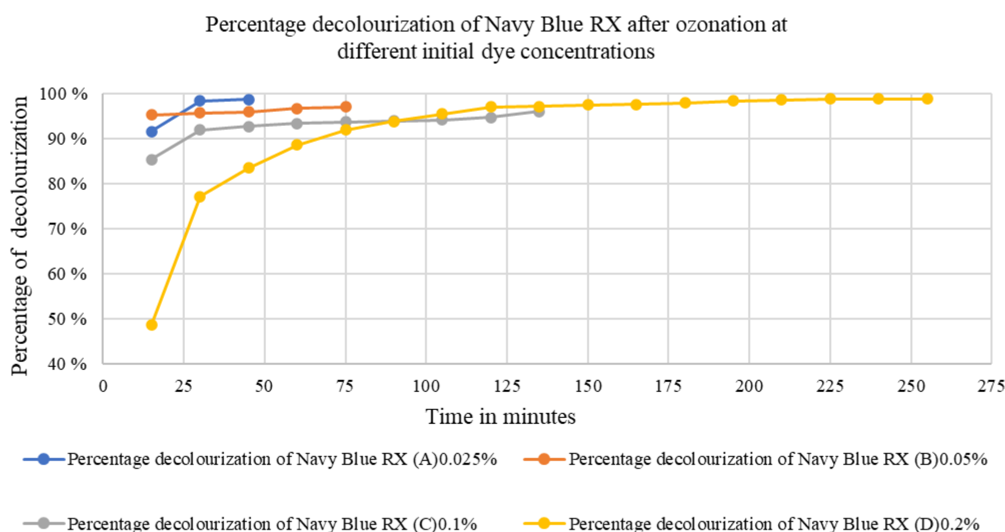
a) b)





c) d)

Figure 8. Effect of ozonation at 15-minutes intervals on the decolourization of 0.025 % (a), 0.05 % (b), 0.1 % (c) and 0.2 % (d) Navy Blue RX dye solution



e)

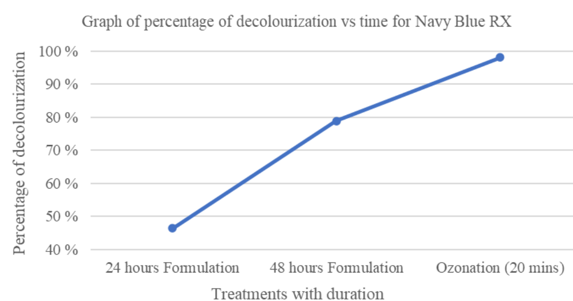
Figure 8 (continued). e) graph for percentage decolorization of Navy Blue RX after ozonation at different initial dye concentrations

Combined effect of microbial formulation and ozonation on Navy Blue RX dye

Unlike the Fenton reaction, treatment with microbial formulation and ozonation gave a good percentage of decolourization. Therefore, a combined method of initial treatment with a microbial formulation for two days followed by ozonation was tested for the treatment of 0.05 % Navy Blue RX dye solution. The results show that 98.07 % of dye decolourization can be achieved with the combined method (Figures 9b, 9c and 9d).

a) b) c)

Figure 9. a) control sample - 0.05 % Navy Blue RX dye solution, b) dye solution treated with microbial formulation for two days, c) dye solution treated with a microbial formulation for two days followed by 20 min of ozonation



d)

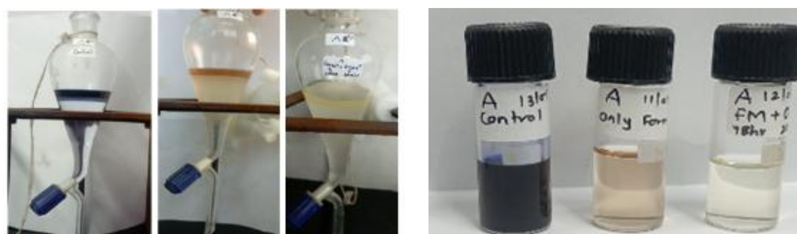
Figure 9 (continued). d) graphical representation of the percentage decolourization of Navy Blue RX dye after 24 and 48 h of treatment with microbial formulation followed by 20 min of ozonation

Earlier results for ozonation of 0.05 % dye solution showed that 75 min was required for decolourization of ~ 98 %, which has now been reduced to 20 min. Therefore, the time required for ozonation can be reduced by pre-treating the dye solution with microbial formulation.

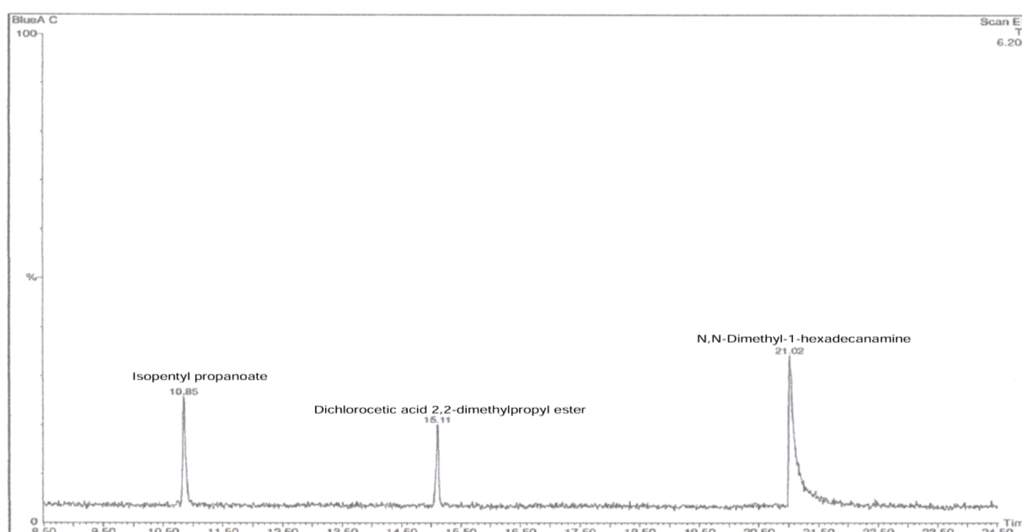
Solvent extraction and GC-MS analysis

Control and treated water samples were extracted for GC-MS analysis using solvent extraction (Figure 10a). The different retention

times of the peaks obtained for control (Figure 10b) as compared to the treated samples indicate the conversion of the parent dye into other metabolites. The decrease in the area under curve (AUC) of the peaks in the microbially treated sample (Figure 10c) compared to the control sample indicates a decrease in the dye concentration, while the increase in the AUC of the peaks in the ozonated samples (Figure 10d) indicates the formation of products after dye degradation. Compound names obtained from the MS NIST library are shown on the chromatograms.



a)



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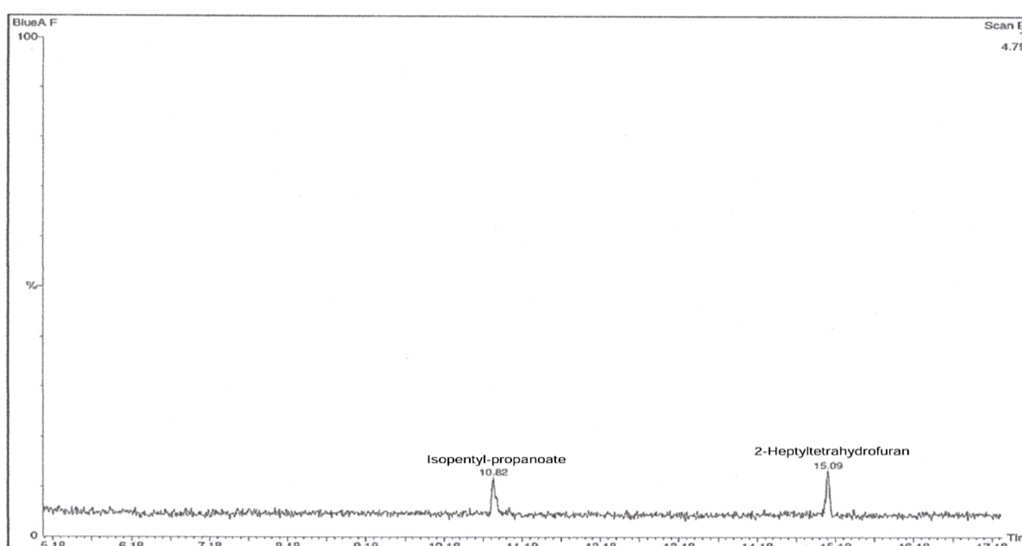
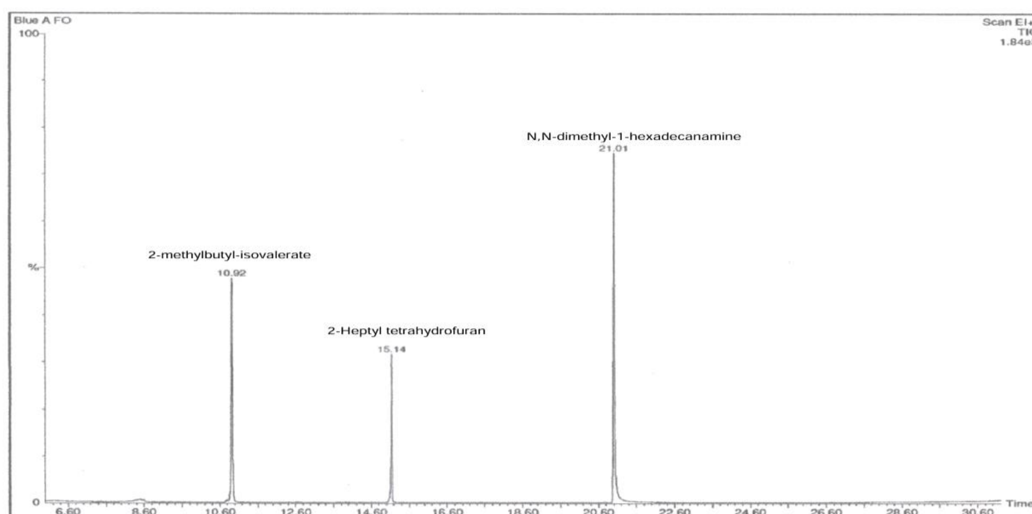


c)

Figure 10. a) Solvent extraction in a separating funnel and samples collected for GCMS, b) chromatogram for Navy Blue RX dye control sample, c) chromatogram for Navy Blue RX dye solution treated with microbial formulation for two days



d)

Figure 10 (continued). d) chromatogram for Navy Blue RX dye solution treated with a microbial formulation for two days followed by 20 min of ozonation

CONCLUSION

A number of organisms capable of remediating textile dyes can be isolated from textile effluent water, which, if converted into a usable formulation, can be a potential biological agent for wastewater treatment. Treatment of 0.05 % Navy Blue RX solution with a consortium of *Neobacillus niacini*, *Enterobacter hormaechei* and *Enterobacter ludwigii* showed a higher percentage of decolourization compared to treatment with individual organisms. Powdered formulation of consortium facilitated longer shelf life and easy storage of the dye remediation organisms. The Fenton reaction had limited application in the treatment of Navy Blue RX dye due to the formation of ferric sludge. Ozonation resulted in complete dye decolourization, but required more time compared to the combined method of microbial treatment and ozonation. GC-MS results show that, in addition to decolourization, the treatment resulted in a decrease in dye concentration and its degradation into other metabolites. Further research need to be conducted on metabolites formed by dye degradation and its degradation pathways. This combination method can be extrapolated to verify its effect in treating other reactive dyes.

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