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Bioremediation of textile dyeing industry effluent from small scale industries using a microbial consortium of *Bacillus* sp., *Escherichia coli*, and *Aspergillus niger*

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ABSTRACT

Dyes are the visible contaminants that are released from the textile industries. Decontamination of textile dye effluents using microbes is environmentally viable over chemical, physical and other mechanical methods. Bacteria, fungi, yeast, and algae have synergistic metabolic activities that alter the chromogen and degrade the absorbed dye color. This work was aimed at investigating the dye decolorization potential of a mixed microbial culture (MMC) obtained from different soil and sludge samples. A single dye (Direct Blue 53) was used for comparison studies. The MMC were incubated for 9 days in mineral salt medium with dye and the absorbance of its filtrate at 647 nm (Blue dye) and 308 nm (Industrial dye) was noted down for every 22 h. The color removal efficiency (CRE) by MMC were 47.04%, 46.77%, 45.21%, and 35.02% for soil of textile dyeing unit (DS), sludge from STP (SE) soil (SS) from drying bed of STP and sludge from membrane reactor of dyeing unit (DE) respectively. Further, the maximum CRE of 98.35% was recorded by microbial culture from drying bed soil of STP (SS), followed by microbial culture from STP sludge (SE) was 97.96%, textile dyeing unit soil (DS) of 96.99%, and sludge form membrane reactor of dyeing unit (DE) was 96%. *Bacillus* sp. isolated from eco-bio block was tested against the blue dye and gave color removal of about 89.25%. The study concluded that the microbe present in soil obtained from the dyeing unit is naturally acclimatized to the dye waste and hence shows highest dye removal efficiency.

1. INTRODUCTION

Textile industry is one of the largest industries in India providing employment for more than 35 million people in the entire country. Around 14% of the world's production of textile fibers and yarns were contributed from India. Textile industries accounts for 30% of the total exports and 14% of the total industrial production, thus playing an important role in deciding the national economy [1]. The textile industry is interdependent to the dyestuff sector. Nearly 70% of the dyestuff produced is consumed by the textile industry [2]. Different types of intermediate dyes were used in textile industries for fabric coloring such as azo dyes, direct dyes, disperse dyes, vat dyes, and reactive dyes. The color of the dye is due to the presence of the chromophore group, wherein the auxochrome group helps the dye in imparting the color on the fabric. In the process of dyeing, the dyestuff will get attached with the cloth in different ways such as (i) creates a covalent bond, (ii) form a complexes with metals or salts, and (iii) adhere onto the surface of the fabric by physical adsorption or by mechanical retention [3].

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The dyestuff used in the dyeing process is not completely taken-up by the material; about 10–15% of the total dye is lost or discharged as waste during the process. Every year 28,000 tons of dye wastes were discharged from the dyeing industry [4]. The waste discharge from textile dyeing industry contains toxic organic and inorganic compounds. Despite causing environmental pollution, some dyes and their N-substituted aromatic biotransformation products are toxic and/or carcinogenic. Release of dye contaminated water into fresh water bodies tends to reduce the penetration of light into it and thereby affecting the photosynthetic activities of aquatic flora. Further, it decreases the dissolved oxygen and increases the biological oxygen demand of the contaminated water bodies [5]. The contaminated water solvating the pollutants to the agricultural fields adversely affect the nutritional quality of the agricultural products, animal and human health causing skin itching, blindness, chemosis, and dermatitis [6].

Conventional effluent treatment methods consist of physiochemical processes such as chemical oxidation, precipitation, adsorption, electrolysis, and coagulation. However, the chemical stability of dyestuffs in the effluent and high operational cost makes the conventional treatment methods ineffective [7]. Thus, there is a need for the development of newer technologies that are efficient and environment friendly to reduce the dye contaminants to an acceptable level. Biological methods are

known to be environment friendly to treat the dye waste water [8,9]. It also operates at low cost and the end products formed are non-toxic in nature. These make the biological method the most effective method for the remediation process of dye effluent. Understanding the complex microbial community structure and their functional diversity adds significance for bio-augmentation and remediation of contaminated sites. However, the activity of microbes and their decolorization ability depends on the dye, other conditions such as temperature, nitrogen source, carbon source, and pH [10].

Bioremediation of textile dyes using microbes is drawing the attention of researchers in the recent past due to their decolorization potential. Microorganisms degrade dyes either by adsorption on microbial biomass or biodegradation by the cells or enzymes. Microbial adsorbents include bacteria, fungi and microalgae. In bio-adsorption process, the original dye structure is disrupted and often entirely decomposed [11]. Microbial dye degradation takes place in two steps: (i) Reductive cleavage of azo linkages resulting in colorless but potentially hazardous aromatic amines and (ii) degradation of aromatic amines [4,5]. The dye reduction is the rate limiting step in dye decolorization process. Dye decolorization occurs both under anaerobic, anoxic and aerobic conditions. Bacterial oxido-reductive enzymes are the key to degrade recalcitrant dye molecules. Most of the intracellular oxido-reductases will reacts while using cellular extracts [12-14]. Since, many dyes are polar in nature; the molecules will be permeable through the cell membrane. Besides, through electron transferring proteins, the bacteria form a link between their electron transport systems inside the cell and the extracellular dye [15]. This dynamic metabolism of bacteria enables them to utilize complex xenobiotic compounds as substrate and convert them into less complex metabolites. Recent studies have shown *Aeromonas hydrophila, Bacillus cereus, Bacillus subtilis, Lactobacillus* sp., *Proteus mirabilis*, and *Pseudomonas luteola* were marked as potential dyestuff bio-remediators [16-18].

The fungal oxidoreductases lignin peroxidase (LiP), manganese peroxidase (MnP), and laccases mineralize aromatic compounds and lignin, which are extracellular. Although extensive studies have been conducted on white-rot fungi for the mineralization of synthetic dyes, the long growth cycle, dependency on nutrient limitation and long hydraulic retention time for complete decolorization makes it more unreliable. Among the fungal species, *Aspergillus ochraceus, Bjerkandera adusta Neurospora crassa Phanerochaete chrysosporium, Pleurotus* sp., *Phlebia* sp., and *Trametes versicolor*, have gained much attention [19,20]. Gel entrapment and adsorption to a matrix enhanced the color removal efficiencies (CRE) of fungi, which is highly expensive [19]. Nevertheless, to single pure microbial culture, mixed microbial cultures (MMC) are effective in dye mineralization due to their co-operation for an enhanced effect. These different microbial groups would likely use different pathways and produce a versatile method that could be used for dye degradation [5]. In case of a microbial consortium, the individual microbial strain attacks the dye molecule at different positions or utilizes the metabolites produced by the co-existing strain for further decomposition [21]. Considerable research efforts have been taken in exploring MMC for textile dye treatment through bioprospecting biodiversity hotspots [22,23]. These studies do not address MMC from textile dye effluent contaminated soil and sludge. Hence, the present study demonstrated the dye mineralization potential of MMC isolated from textile dye effluent contaminated soil and sludge samples from the sewage treatment plant, Karunya Institute of Technology and Sciences (KITS), Coimbatore and textile industries, Tirupur, Tamil Nadu. The study explored the CRE of mixed microbial consortium comprising both fungi and bacteria.

2. MATERIALS AND METHODS

The dye effluent, soil, and sludge samples were collected from the sewage treatment plant at Karunya Ladies Hostel, Coimbatore and textile dyeing industry, Tirupur. The soil samples were collected in zip-lock bags from the surface to a depth of about 5–10 cm using a sterile spatula. The sludge samples were collected in sterile plastic containers. Onions with fungal molds are selected to isolate *Aspergillus niger*.

2.1. Microbial Strains, Culture Media, and Growth Conditions

Bacteria were isolated from the collected soil samples using nutrient agar (NA) medium. One gram of soil and sludge samples was serially diluted in 9 ml sterile phosphate buffered saline. The bacterial isolation was done by pour plate method. The plates were incubated at 30°C for 5 days and observed for the appearance of bacterial colonies. Well separated and morphologically distinct colonies were sub-cultured repeatedly to obtain pure cultures and maintained as agar slants at 4°C for further studies.

In this study, 2 ml of sludge (DE) sample collected from dyeing unit is used for isolating *A. niger* on Potato Dextrose Agar (PDA) plates. Once solidified, sludge sample dispensed with a sterile pipette onto the solidified media was spread plated using a flame sterilized L-shaped glass rod. The Petri plates were sealed using paraffin tape to avoid contamination and incubated for 5–7 days to obtain matured culture of *A. niger*. The cultured Petri plates were periodically examined for the growth. For isolating *A. niger* from onion, the surface spores were swapped with a cotton swap and spread onto PDA plates. The cultured plates were stored at room temperature (27–30°C) in the incubator for 3–5 day.

Similarly, 1 g of soil sample suspended in 10 ml of distilled water was streaked on eosin-methylene blue agar plates. The plates are incubated at 37°C for the growth of *Escherichia coli* colonies. Likewise, for isolating from sludge and dye effluent, 2 ml of samples were used.

2.2. Inoculum Preparation

The bacterial and fungal strains were inoculated into synthetic nutrient broth and incubated at 28 ± 2°C in shaking condition until the OD_{600} reached 0.8. Consequently, 2 mL of sludge and dye effluent, 2 g of soil from dyeing unit and from STP were transferred to the flask containing sterile nutrient medium and the cultures were inoculated. The culture flasks were then incubated at 28 ± 2°C for 24 h and used as the stock culture for further studies.

2.3. Assessment of Compatibility of the Bacterial Strains and Preparation of Consortia

To prepare MMC, the bacterial and fungal compatibility was assessed as follows: 1 mL (10^6 colony forming unit mL^{-1}) suspension of each bacterium was spotted onto NA plates. The spot to spot distance was 1 cm. The inoculated plates were then incubated at 28 ± 2°C in static condition for 72 h. If the growth of bacterial strains overlaps each other without inhibition, it is considered as compatible. The same compatibility assays was also performed for fungal culture versus bacterial cultures as described in Furuya *et al*. (1997) [24].

2.4. Acclimatization of MMC in Synthetic Dyes

Biodegradation abilities of MMC are increased by gradually introducing them to higher pollutants for treatment.

2.4.1. Sterile mineral salts medium (MSM)

1 g sodium chloride, 0.1 g calcium chloride, 0.3 g magnesium sulfate heptahydrate, 1 g potassium dihydrogen phosphate, 1 g disodium

hydrogen phosphate, and 3 g yeast extract were dissolved in 1000 ml of sterile distilled water (8 flasks with microbial culture inoculated and 2 control flasks without culture for each dye). Exactly 100 mL of MSM was aseptically added separately to each flask. The flasks are named from F1 to F10. F1 and F2 were used as control and added with blue dye and industrial dye separately. Flasks F3, F4, F5, and F6 are enriched with 50 mL (100 ppm concentration) of blue dye in each and four different stock microbial cultures was added separately in each flask. Wherein, F7, F8, F9, and F10 are added with 50 mL of industrial dye and four different microbial cultures were added separately. Initial absorbance was noted and further readings were taken after 24 h of inoculation.

2.5. Evaluation of Dye Decolorization by the Isolates as Mono and Mixed Cultures

Ten milliliters of 5-day old mixed cultures and respective mono cultures were inoculated into series of 250 mL Erlenmeyer's flask containing 100 mL of MSM added with filter sterilized 0.5 gL^{-1} of blue dye and industrial dye separately. The inoculated media were incubated on rotary shaker in 150 rpm speed at 28 ± 2°C for 10 days with the respective control (sterile medium amended with dye alone). The decolorization rate was measured by the percentage reduction of absorbance value. Original textile dye water is dark in color as it is a mixture of various single dyes. Percentage of decolorization was calculated using the formula;

$$\% \text{ of decolourization} = \frac{(Initial\ absorbance - Final\ absorbance)}{Initial\ absorbance} \times 100$$

Spectrophotometer reading was taken at every 22 h interval for all the 10 samples and the CRE was determined.

2.6. Changes in pH, Electrical Conductivity (EC) and Total Dissolved Solids (TDS)

EC, pH, and TDS (TS, TSS, and TDS) EC and TDS of the textile dye effluents after and before microbial amendments were determined using HACH EC-TDS meter where the electrode was directly dipped into the respective solutions to display result on a digital scale.

2.7. Statistical Analysis

The experiments were done in triplicates and the results were expressed as mean ± SD.

3. RESULTS AND DISCUSSION

Waste water contaminated sites harbor potential microbiome for remediating these environments. The present study envisages exploring the culturable non-fastidious microbial groups for decolorizing textile dye effluent.

3.1. Isolation of Fungal and Bacterial Cultures from Dye Unit Sludge, Soil and STPs

A. niger isolated from onion bulbs showed an identity of white to yellow mycelial culture surface and black conidia. On PDA, conidial heads are large (up to 15–20 µm in diameter), globose, becoming loosen, and well separated into loose columns with age. The color of the colonies changed from white to dark brown in 5–7 days of incubation period [Figure 1a]. Microscopic images showed smooth and colorless conidiophores and spores. *A. niger* changes dark to dark brown spores (conidia) from their heads which was further confirmed using a microscopic view [Figure 1b].

E. coli was isolated using selective media (EMB agar) from the sludge and soil samples. Eosin Y and methylene blue are used as pH indicators which form a dark purple precipitate and inhibited the growth of Gram-positive bacteria. In the study, sucrose and lactose act as fermentable nutrient sources which synthesize acids and form purple color. Microbes, while growing on the fermentation medium changed the color from dark purple to black. It was observed that *E. coli* showed a green metallic color. Some weak fermenters formed mucoid pink colonies. Generally the less-colored or colorless colonies showed that those microbes could not ferment any nutrient sources (lactose and sucrose) provided and it is not a fecal coliform [Figure 1c].

3.2. Performance of MMC in Blue Dye and Industrial Dye

The maximum peak was found at the wavelength of 647 nm for blue dye and 308.5 nm for the industrial dye water. The initial absorbance measurement was found to be 4.5453 AU for blue dye and 4.3599 AU for industrial dye. The results distinctly showed that the microbes from DS and SS samples could acclimatize with the synthetic blue dye and industrial dyes [Figures 2a and b). The microbial culture agglomerated into clusters of varied shapes and sizes and absorbed the dyestuff contaminant from the wastewater. This shows that the decolorization of the dye is due to adsorption on microbial biomass (biosorption).

Growth of bacterial cultures obtained from the sludge and dye effluents indicated that the lag phase lasted for 0–3 h. The logarithmic phase was attained at 10 h. During lag phase, the bacterial cells adapt themselves to the new medium accompanied by an increase in cell size with no changes in cell number. In log phase, the bacterial cells multiplied rapidly utilizing the medium provided. The curve was linear in this phase and the cells are enzymatically most active. The stationary phase was too long which prolonged up to 50 h beyond the stationary phase, as the nutrients drained, secondary metabolic products were accumulated. Hence, the growth is equals to the death rate and the cells gradually entered into the death phase. In the present study, the period of stationary phase is prolonged which might be the reason for acclimatization of microbes in the dye effluent and sludge samples [Figure 3]. Therefore, the microbial cultures entering the stationary phase and successively into the decline phase may result in subsequent reduction in color removal due to the inhibition of enzyme systems [25].

3.3. Microbial Acclimitization and Color Removal Efficiency (CRE)

Microbial dye decolorization predominantly depends on the adaptability of microbes and their reaction toward the dye molecule [26]. In the present study, the MMC exhibited a gradual increase in CRE percentage and it showed maximum removal on the 5th day [Figure 4]. Microbial culture enumerated from the dyeing bed soil showed a maximum CRE of 98.35%. The microbes from dyeing unit soil showed increased CRE because these naturally prevail in the extreme environmental conditions such as pH (slightly basic – above 7.5 or 8) and micronutrients (from the complex dye structures) whereas, the sludge samples from dyeing unit and STP have already passed through the treatment cycles which reduces the availability of the needed nutrients for microbial growth. Therefore, dyeing unit and STP sludges showed a reduced CRE compared to that of the microbes from soil sources.

The industrial dye effluent contains 30–40 individual disperse dyes being mixed together. This gives it a dark brown color. Disperse dyes are water insoluble and are mostly azobenzene or anthraquinone dyes. The azobenzene dyes contain two phenyl rings linked by a -N=N- bond and the anthraquinone dyes are polycyclic aromatic hydrocarbons. As

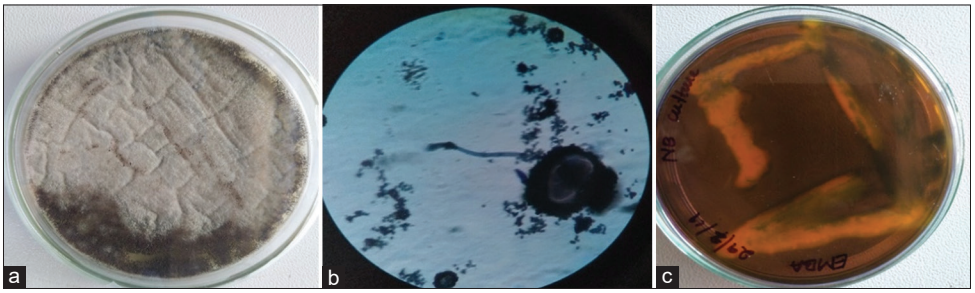

Figure 1: Microbes obtained from the sludge samples. (a) 7 days old matured black conidiophores of *Aspergillus niger*; (b) Microscopic image of *A. niger* showing long tail like hyphae and global shaped conidial heads; (c) Escherichia coli on eosin-methylene blue agar.

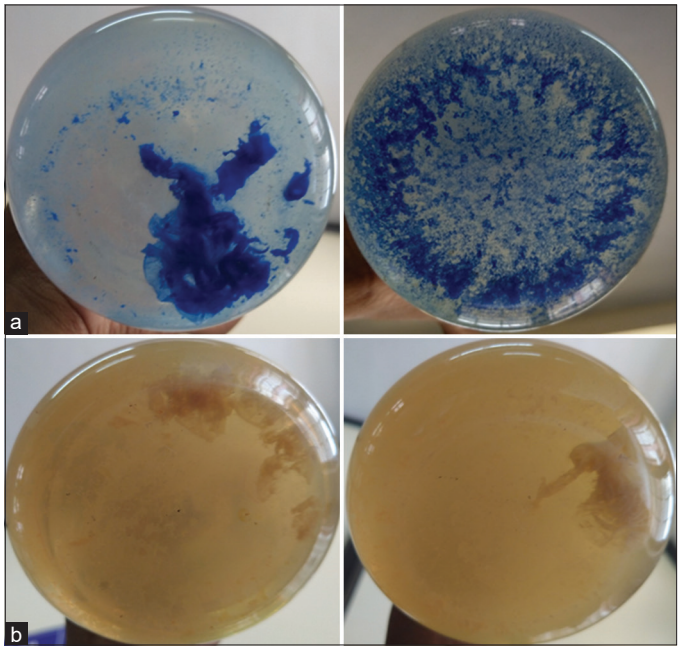

Figure 2: Acclimatization of microbial cultures in synthetic dyes. (a) Blue dye adsorbed by the mixed microbial culture from DS and SS samples (b) industrial dye adsorbed by the mixed microbial culture from DS and SS samples.

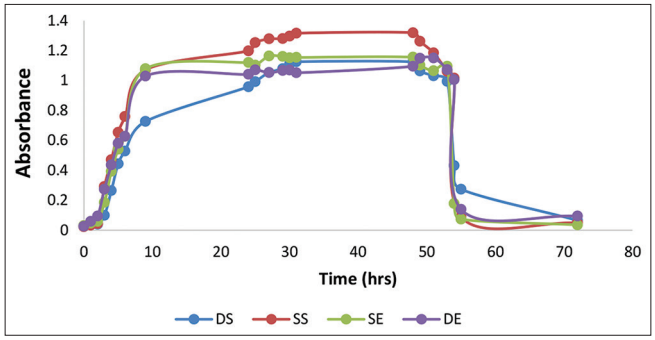

Figure 3: Growth pattern of MMC in DS, SS, SE, and DE samples at different time intervals.

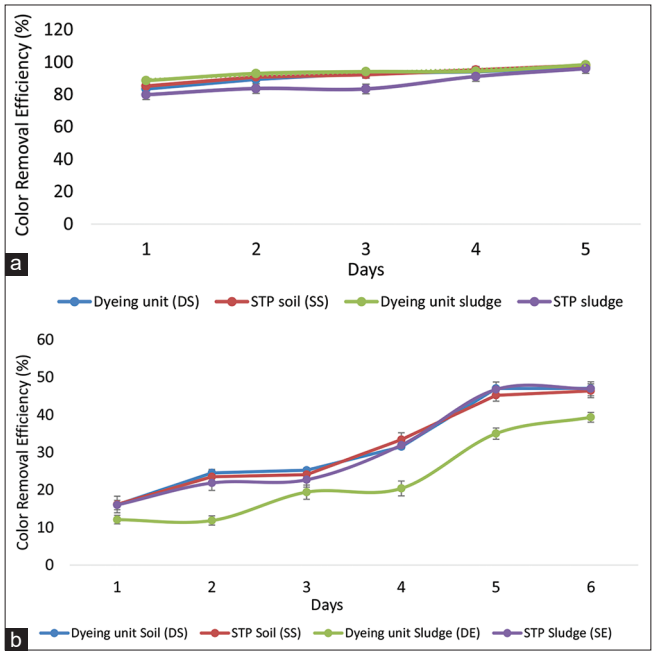

Figure 4: Color removal efficiency of mixed microbial culture (MMC) (a) blue dye (b) industrial dye.

disperse dyes are mostly azo dyes, the decolorization resulted would be due to the azoreductase mechanism. Similarly, MMC registered maximum color removal on the 6th days in the industrial dye. Microbial culture obtained from dyeing unit soil showed the maximum removal of 47.04%. This is because of the microorganisms prevailing in the dyeing unit soil is naturally acclimatized to the dye pollution. The microbes present in these sources utilize the dye stuff as carbon and nitrogen sources. The results are in accordance with Chen *et al*. [13] where microbial decolorizers were obtained from sludge samples. The mixed culture in this study has adapted to high dye concentrations as they are from the contaminated sites [27].

Bacillus was isolated from the available eco-bio block made of volcanic ash and dormant *Bacillus* strain. Nutrient broth media were used to culture *Bacillus* sp. A piece from the eco-bio block was taken and crushed into powder to be used as the inoculation sample. The media and the glasswares were autoclaved before use [Figure 4]. The maximum removal was recorded (89.25%) on the 5th days in flasks containing Blue dye. Flasks containing industrial dye and *Bacillus* culture samples showed only less removal of about 27.28%. This is because of the industrial dye exhibits a complex nature as it contains 30–40 dyes together [Figure 5]. Furthermore, significant reduction of EC and TDS in blue dye treated with *Bacillus* sp. was observed. Whereas, the culture samples with industrial dye water showed only less reduction. Industrial dye waste consisting of more than 30–40 individual dyes contributes to its complex nature, which hampers the decolorization potential of *Bacillus* sp. The removal of dye stuff from effluent is caused by the presence of the enzymes oxido-reductase

from *Bacillus sp.* This heme protein helps for the breakdown of lignin by oxidation. Peroxidases (LiP), catalyze phenolic substrates which results in radical formation using hydrogen peroxide as the electron donor. By combining MnP and LiP peroxidases, versatile peroxidases (VP), was produced. These VP can oxidize Mn^{2+} but phenolic and non-phenolic aromatic compounds called dyes. These peroxidases are produced by the series of reactions such as reductive cleavage.

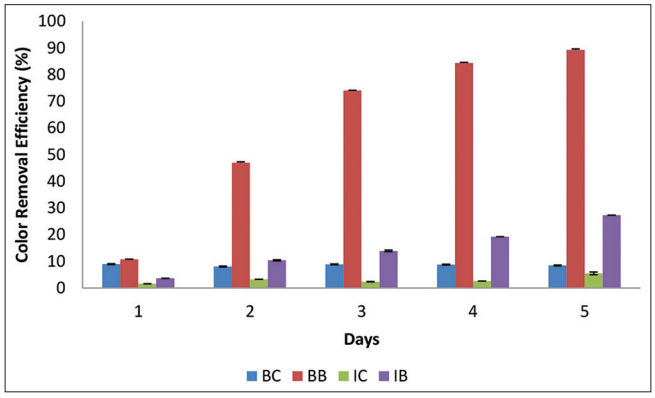

Figure 5: Color removal of different dye samples after 5 days of incubation by *Bacillus* sp.

Peroxidases utilizes the azo-reductase enzymes to cleave/break the N=N azo bond. The resulting product undergoes desulfonation and deamination which further metabolize the organic complex compounds to much simpler forms [Figures 6 and 7] [10].

E. coli is an efficient dye decolorizer and it possesses azo- reductase activity. The electron donor reduces the azo bond by Azoreductase. The azo- bond breakdown which require 2 moles of NADH to reduce 1 mole of a typical azo dye, into 2-aminobenzoic acid, and N, N-dimethyl-p phenylenediamine (Ping-Pong Mechanism) [28]. The enhancement of azoreductase during removal of azo dyes under stable condition was already reported [29]. The azoreductase enzyme synthesis some toxic amines while reacting with azodyes. Despite the fact that, azo dyes were degraded only in anaerobic conditions, this study reported the aerobic degradation of azo dyes. In general, anaerobic reduction is sensitive to molecular oxygen but aerobic reduction has not inhibited by molecular oxygen. Hence, aerobic enzymes are called as oxygen insensitive azoreductases. In the MMC, the fungus *A. niger* contributes to decolorization potential which occurs through deamination and dehydrogenation process [30]. The pathway is depicted in Figure 8. Similarly, excellent decolorization ability with high adsorption efficiency of 98% was observed with 100 mg L^{-1} acid dye [31]. The slow rate of decolorization in the industrial dye by the microbes can be related to higher molecular weights and the structural differences in the complex dyes [32].

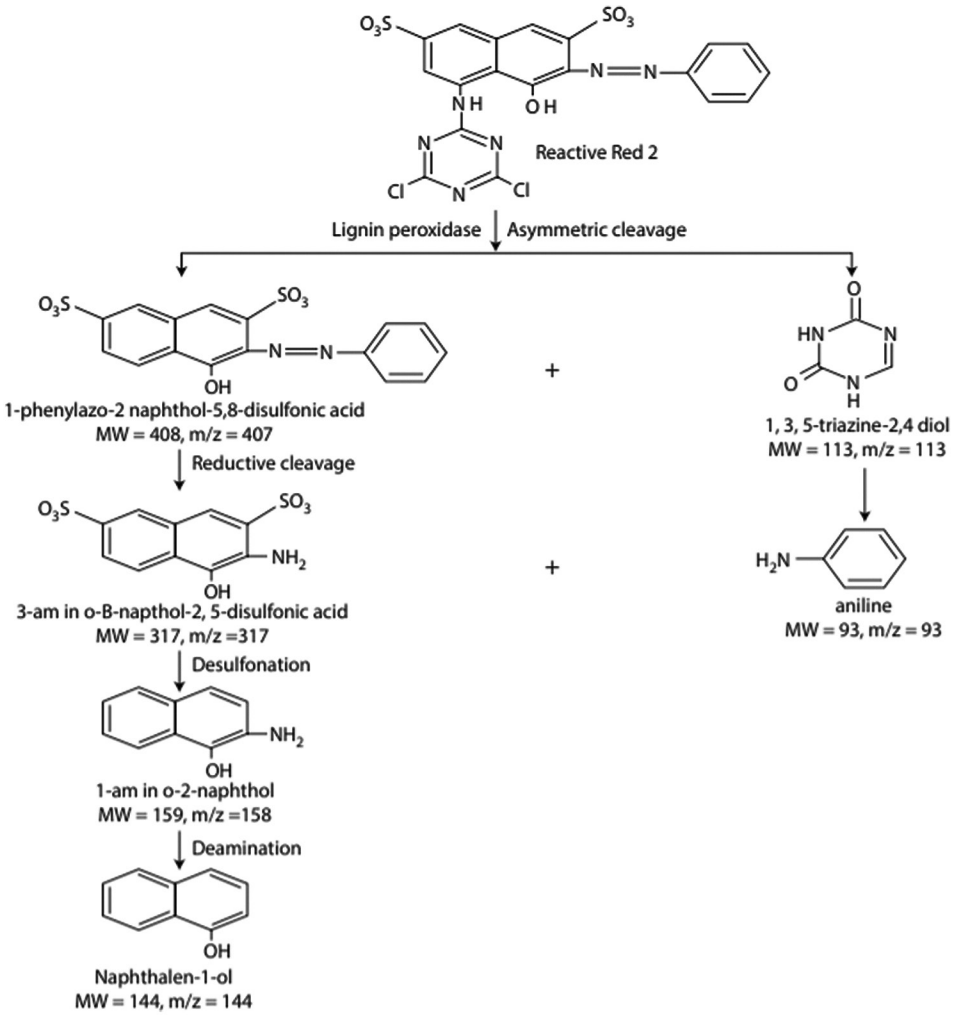

Figure 6: Biodegradation pathway of *Bacillus* sp. [33].

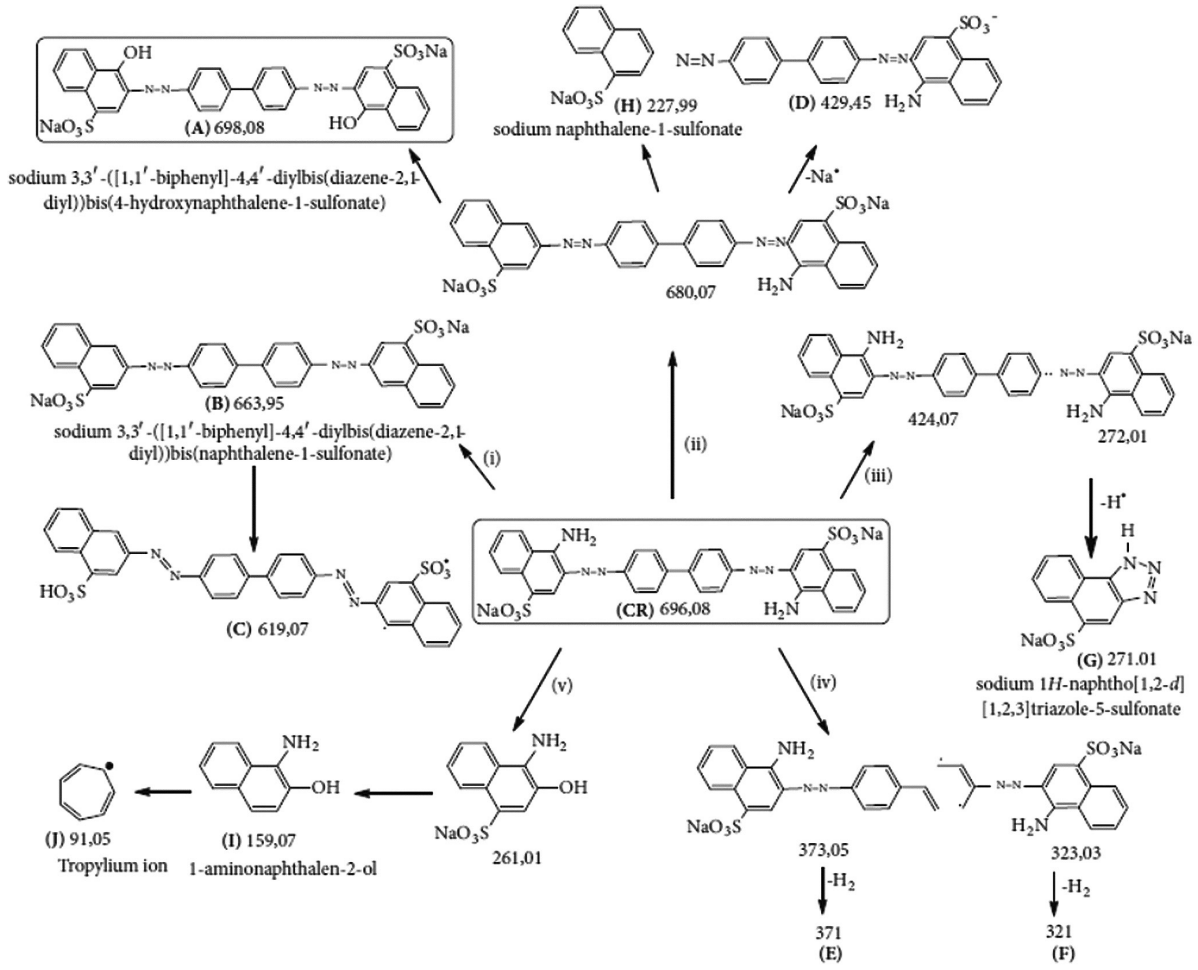

Figure 7: Biodegradation pathway of dye by *Aspergillus niger* [34].

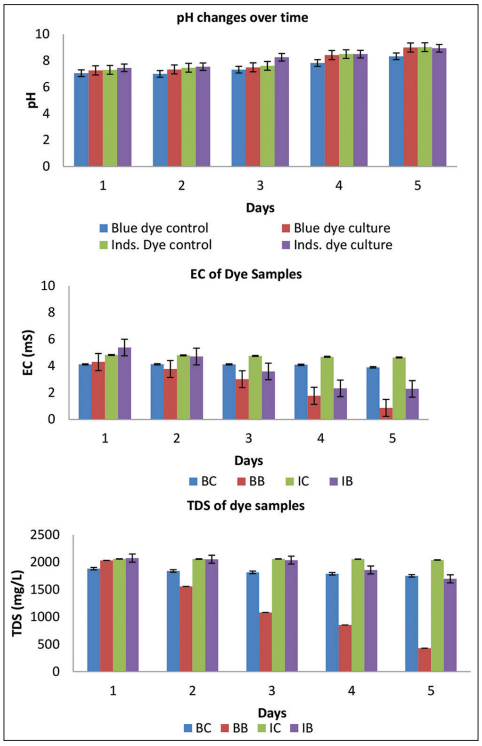

Figure 8: Changes in pH, EC, and TDS of dye samples by MMC. BC: Blue dye control, BB: Blue dye+MMC; IC: Industrial dye control, IB: Industrial dye+MMC.

The substantial removal of TDS and reduction of pH, EC levels shows that the toxic substances of the dye effluent are reduced significantly [Figure 8]. The increased decolorization rate with the MMC may be attributed to the synergism between the enzymatic systems and complex metabolic pathways present in the microbe [1,25].

4. CONCLUSION

The MMC comprising *Bacillus* sp., *E. coli,* and *A. niger* obtained from textile dye effluents and sludge samples valorized the textile dyes (blue dyes and industrial dyes) significantly. The CRE of blue dye is more than the industrial dye due to its complex nature. The synergistic enzyme action of the microbes in MMC increased the CRE. The microbial community from the dyeing unit soil is naturally acclimatized to the dye waste and hence showed highest color removal. From the results, it can be concluded that the different metabolic pathways of MMC involved in biodegradation process can be explored for further studies to address the serious threats of azodyes posed to agricultural lands in nearby textile dyeing units.

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7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects

9. PUBLISHER'S NOTE

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