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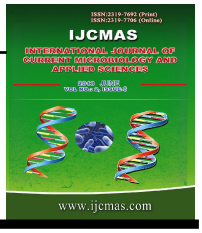

Original Research Article

Ecofriendly approach of textile dye effluent decolorization by using microbial source

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A B S T R A C T

Dye molecules play an important role in textile industries due to its color. Management of water pollution is one of the major challenges for environment. About 2% of these dyes are directly discharged as aqueous effluent and 10% are lost during textile coloration process .Several combinations of treatment methods have been developed in order to effectively process textile waste water, decolourization is one of the method. The microorganisms were isolated out from the textile effluent for decolourization. And various biochemical assays were performed for the characterization of microorganisms. Dyes were taken at different concentration and organisms were inoculated for estimating the degradation efficiency and the desired microorganism was screened. The genome of the effective microorganism against textile effluent was identified. Moreover, protein pattern of microorganism were studied while degrading the textile effluent.

Keywords

Textile industries; Effluent; De-colorization; Treatment methods.

Introduction

The advancement of science and technology, the different dye stuffs having diverse chemical structures and characteristics are being developed to obtain bright colors and shades as well as for resistance against natural decay, including biodegradation. Textile manufactures select the dyes based on several factors with the material to be dyed and fashion as major factors. Rapid industrialization and urbanization causes an environmental pollution and releases several xenobiotic compounds into the environment. Many of them have been listed as priority pollutants by the United States Environment Protection Agency due to their toxicity and persistence in nature. Textile industries are the largest industrial consumers of water (Marcucci *et al.,* 2001).They use water and chemicals for processing (AEPA,1998).The water after usage is left into water bodies without any treatment. The main reason for dye loss is the incomplete exhaustion of dyes on to the fiber (Willmott, 1997) . The presence of very low concentration of dyes in effluent is highly visible (Nigam *et al.,* 2000). Some of the chemicals used in textile industries include enzymes, detergents, dyes, acids, sodas and salts (Aslam *et al*., 2004). Dyes are more stable. Due to the stability in waste water they are

not totally degraded by conventional treatments (Leisinger *et al.,* 1981). Moreover textile waste water quality is variable with time and may include many types of dyes, detergents, sulphide compounds , solvents ,heavy metals and inorganic salts ,their amount depends on the the kind of process that generates effluent (Lopez *et al.,*1999). The decolorization is a challenge for textile industry as well as for waste water system, it can be overcome by using micro organisms (Balan, 1999; Balan and Monterio, 2000).Moreover decolorization and degradation can also toxify the effluent effectively without leaving any residues. Bacteria and fungi along with their products such as enzymes (Whitely and Lee, 2006) and exopolymeric substances aid in bioremediation (Liao *et al.,* 2001).

The present investigation was to isolate and identify the effective strains from the textile dye effluent collected from Kumarapalayam, Erode. To achieve decolorization and detoxification of dye containing textile mill effluent varying in their pH, chemical and dye composition. Efficiency of the whole cultures in removal of color and toxicity, reduction in COD is to be studied. To perform the protein profiling of effective strain by using SDS PAGE.

Materials and Methods

Collection of samples

The sample was collected from the textile industries during the discharge of effluent in Erode district by using water bag. The sample was immediately covered tightly and preserved at room temperature for further analysis.

Analysis of textile dye effluent

Estimation of total solids in effluent sample

Dry silica crucible was measured and recorded as W_i to which 100 ml of unfiltered is transferred and evaporated in hot air oven at 105°C for 1 hour. The sample was cooled in a desiccators and weight was recorded as W_f. The experiment was repeat until constant weight was obtained. Total solids of the sample was calculated as,

$$Total\ solids\ (mg/l) = \frac{Wi - Wf}{Sample\ volume\ (ml) \times 1000}$$

W_i =initial weight of the crucible
W_f =final weight of the crucible

Analysis of total dissolved solids in effluent sample

Dry silica crucible was measured and recorded as to W_i which 100 ml of unfiltered sample was transferred and evaporated in hot oven at 180ºC for 1 hour. The sample was cooled in a desiccators and weight was recorded as W_f. The experiment was repeated until constant weight was obtained. Total dissolved solids of the sample was calculated as,

$$Total\ solids\ (mg/l) = \frac{Wi - Wf}{Sample\ volume\ (ml) \times 1000}$$

W_i =initial weight of the crucible
W_f =final weight of the crucible

Estimation of total suspended solids

Total suspended solids can be obtained from subtracting the total dissolved solids from total solids.

Total suspended solids = total solids - total dissolved solids

Estimation of biological oxygen demand

50 ml of effluent sample was added to one liter of 8 mg/l of oxygen containing water sample. BOD bottle was rinsed clearly with water and pH was neutralized by using acid or alkali. Two BOD bottles was filled with effluent sample and to one bottle 2 ml of manganese sulphate and 2 ml of alkali iodine azide solution was added. The bottle was observed for brown colored precipitate and it was allowed to settle at halfway .Two to three drops of sulphuric acid was added to the bottle. 50 ml of acidified sample was titrated against sodium thiosulphate and observed for pale yellow color. Starch indicator was added to the sample and again titrated up to the disappearance of blue color. The remaining bottle was incubated at 20-27ºC for 3-5 days. The final oxygen concentration was estimated after 5 days of incubation. Dissolved oxygen of the effluent sample was calculated by formula,

$$2mg/l = \frac{\text{Titrant value x 0.025 x 8 x 100}}{\textit{Volume of sample}}$$

0.025-normality of the titrant
8-molecular weight of oxygen
BOD of the effluent was calculated by the formula: BOD = D1-D2

Estimation of chemical oxygen demand

50 ml of effluent sample was taken in one conical flask and 50 ml of distilled water in another conical flask, to that 5 ml of potassium dichromate solution was added and it was incubated for 1 hour at 100ºC in water bath. Cool it for 10 minutes. Mix 5 ml of potassium iodide solution and 10 ml of sulphuric acid. Titrate the effluent with 0.1 M sodium thiosulfate and amount of thiosulfate used was observed. Add 1 ml of starch solution, the color turns blue. Again titrate with sodium thiosulfate till the complete disappearance of blue color. Volume of sodium thiosulfate used was noted,

$$\text{COD (mg/l)} = \frac{8\times C\times V_B - V_A}{V_S}$$

C = Concentration of titrant (mM/l)
V_A= Volume (ml) of titrant used for control
V_B = Volume (ml) of titrant used for water sample
V_S = Volume (ml) of water sample taken

Isolation and identification of bacterial strains

Serial dilution

The effluent sample was added to 9 ml of sterile water, it will give 1:10 or 10^{-1} dilution of original sample, (i.e) the original sample has been diluted to $1/10^{th}$. Similarly 1:100,1:1000 1:10,000 and so dilution of the original sample was prepared. Finally one ml aliquot of all dilution was added to a sterile Petri dish to which are added 9 ml of sterile, cool, molten nutrient agar medium. The dishes were incubated at suitable temperature. Within few days colonies of colonies of each kind of bacteria were grown in Petri dish**.**

Quadrant streaking

A loop full of the culture was obtained and aseptically transferred to the Petri plate containing media. The inoculums were streaked into the first quadrant and then the loop was shown in flame to sterilize it. Using the sterile loop it was streaked from quadrant one to quadrant two. It is

streaked several times through quadrant one to pick up some organism on the loop. The same general procedure was repeated for quadrant three and four respectively. The loop was shown in flame between each quadrant. The plates were inverted and incubated for 24-48 hours at room temperature.

Gram staining

A thin smear of the bacterium was prepared on the slide. To the smear crystal violet solution was applied for 30 seconds. The slide was then rinsed in clean water and iodine solution was applied for 30 seconds. This in turn was rinsed off. All cells would be deeply stained and appear blue-purple if the slide was examined. Then 70% ethyl alcohol was applied. This usually takes 20 seconds to one minute. Microscopic examination of the slide will reveal the gram positive bacteria retain the violet iodine combination, whereas gram negative loose the blue-purple color after alcohol wash, and will be of original color. The species which retain the stain are called gram positive, whereas those which yield the strain to alcohol were called gram negative.

Biochemical identifcation of unknown bacteria

Catalase test

One of the TSA slants inoculated for stock working cultures was used. After incubation a few drops of 3 % H_2O_2 were placed on the slant ,vigorous bubble `production on either indicates a positive reaction. If there are no bubbles, the test is negative.

Starch hydrolysis

A loop full of bacteria was streaked line on the plate containing starch agar medium and incubated for 24 to 48 hours. After incubation results were obtained by pouring iodine over the streak line. Iodine solution reacts to produce a blue color. Where the starch was hydrolyzed the medium was uncolored (clear). This indicates a positive test. The results were read within a few minutes**.**

Urea hydrolysis

Inoculate the urea broth medium with bacterial culture. Incubate the culture at 37ºC for 48 hours. The phenol red indicator will turn to pink due to alkaline nature of the medium because of ammonia production . Otherwise, the indicator will remain yellow at acidic range of pH. Then it shows no urease production by the given microorganism.

Indole test

Prepare tryptone broth and inoculate it with bacterial culture. Incubate the culture at 37ºC for 48 hours. Add 0.5 ml of Kovac's reagent after the bacterial growth. After 2 minutes a red color band appears at the junction of medium and reagent as in the case of *E.coli.*

Methyl red

Prepare MR VP broth tubes , one for bacteria and one as control. Inoculate the broth with bacterial culture and incubate it for 48 hours. Add 5 drops of methyl red indicator into one tube of each bacterium , if it turns red color it shows positive result. If it remains yellow it shows negative result.

VP test

Prepare MR VP broth tubes, one for

bacteria and one as control. Inoculate the broth with bacterial culture and incubate for 48 hours. Add ten drops of VP I reagent and two to three drops of VP II reagents. If it appears pink red color it shows positive result, if it remains faint brown color it shows negative result.

Citrate test

Prepare Simmons's citrate agar slants. Inoculate the bacterium by stabbing to the base of the slant. Thereafter, streak the surface. Incubate the tube at 37ºC for 48 hours. Blue color indicates the citrate utilization , unutilized citrate indicates no color change.

Triple Sugar Test

Three different broth were taken sucrose, lactose, glucose along with beef extract, peptone and pH indicator phenol red for acid detection. Durham tube was inverted in each tube. Culture was inoculate and incubate it at 37ºC for 24 hours. Purple or red color indicates no fermentation ,yellow color indicates acid production only and yellow color with gas accumulation in Durham tube indicates acid and gas production.

Screening of bacterial isolates against effluent

Dye at different concentrations was taken (3 ml, 5 ml, 7 ml, and 10 ml). Decolorization ability of bacterial isolates was then analyzed for effluent in minimal broth medium under static condition for 7 days. Freshly prepared inoculums of selected bacteria in nutrient broth were taken and used in these experiments. Samples of treated dye containing medium were taken into centrifuge tubes. These samples were centrifuged under 8500 rpm for 10 minutes. The residual amounts of each dye in medium were monitored through Shimadzu UV/VIS spectrophotometer at its maximum absorbance.

SDS PAGE

The gel plate was fixed with appropriate spacer on the gel plate. Resolving gel buffer solution was prepared and adds TEMED and APS. The solution was poured and it was allowed for polymerization. Stacking gel was poured on the top of separating gel. The bottom spacer was removed after 20 minutes and gel was fixed in slab gel unit. Then the sample mixture and the standard protein were loaded then it was subjected to electrophoresis**.**

Results and Discussion

Physiochemical parameters of effluent sample

The effluent sample collected from the dyeing industry with smell and temperature of 30ºC. The pH of the effluent was alkaline (8.8). Total solids in the effluent was 89.33 mg/l and total dissolved solids (TDS) was 144.26 mg/l and total suspended solid (TSS) was 54.93 mg/l (Table.1).

The physiochemical parameter of the effluent sample collected from the dyeing industry was analyzed .The alkaline pH of textile effluent was associated with the process of bleaching and it is extremely undesirable in water ecology. Both chemically and biologically mediated adsorption/reduction of dyes were mediated with the decreasing pH level under redox-mediating compounds . Decrease in pH of the effluent down the stream significantly improved bacterial count and thereby associated remediation.

Isolation and identification of bacterial strains

Serial dilution

The effluent sample were serially diluted and plated for the growth of bacteria and it was incubated for 48 hours at 37°C.

Isolation of pure culture

A loop full of the mixed culture was obtained and aseptically transferred to the Petri plate containing media. The inoculums were streaked for the pure culture of bacteria. The plates were inverted and incubated for 24-48 hours at room temperature.

Table.1 Physiochemical analysis of effluent sample

S.No	Parameters	Results
1	pH	8.8
2	Temperature (ºC)	30
3	Smell	Disagreeable
4	Total Dissolved Solids (mg/l)	144.26
5	Total Solids (mg/l)	89.33
6	Total suspended Solids (mg/l)	54.93
7	Biological Oxygen Demand (mg/l)	250
8	Chemical Oxygen Demand (mg/l)	390

Gram staining

Three organism selected for decolorization were stained using gram staining technique and viewed through microscope for gram positive or gram negative. The shape of the bacteria was also analyzed through microscope. The result of gram staining whether it is gram positive or gram negative was identified through the stain color.

The positive result in Catalase, Citrate, Oxidase and negative result in Indole, MR VP test and starch test confirmed that the unknown organism 1 was *Pseudomonas spp.* The positive result in Starch, citrate, glucose and negative result in Oxidase, Urease confirmed that the unknown organism 2 was *Bacillus spp*. The Positive result in Catalase, Citrate, Oxidase and negative result in nitrate, Starch, Indole, Methyl red and VP confirmed that the unknown organism 3 was *Alcaligenes spp* (Table.2).

Table.2 Biochemical identification of Bacteria

Biochemical test	Organism		
	1	2	3
Catalase	+	-	+
Indole	-	-	-
Methyl red	-	-	-
VP	-	+	-
Citrate	+	+	+
Starch	-	+	-
Glucose	+	+	-
Lactose	-	-	-
Sucrose	-	-	-
Shape	Rod	Rod	Rod
Gram Positive /Negative	-	+	-

Decolorization

Decolorization assay was performed to check the dye effluent decolorization by

bacterial strains, in which the bacterial colonies from pure culture were transferred.

Effect of different concentrations of dye on decolorization

The efficiency of dye decolorization by the bacterial isolate was carried out at different concentration of dye 3ml, 5ml, 7ml and 10ml. The Decolorization of dye by bacterial strain was found to be higher in 3 ml concentration when comparing to other concentrations.

The time taken for the decolorization of the dye increases as the concentration increase. The results of the present study are in accordance with Ajay Kumar Pandey *et al.* (2012). They stated that with increase in dye concentration the dye decolourizing efficiency of the bacterial strain decreases.

Statistical analysis

The results of degradation of dye using *Psuedomonas* spp ,*Bacillus* spp *and Alcaligenes* spp at different dye concentration for different time period was carried out using ANOVA (Table 3,4 ,5 & 6).

The critical difference value for dye degradation time by *Psuedomonas spp, Bacillus spp and Alcaligenes* spp. at various concentrations was calculated. It was found that the CD value was higher at increasing dye concentration.

The Bacteria isolated from dye effluent sample was treated against dye at various concentrations. The degradation rate was higher in *Bacillus spp.* Oren *et al*.(1992) and Ventosa *et al.*(1998) reported that the rate of decolourization may be due to the high metabolic diversity being seen in the halophiles due to there extremophilic nature.

Table.3 Decolorization percentage of effluent by different bacterial strains at varying dye concentration in first day of interval

Bacteria	Dye Concentration	Time Interval	Wavelength (500 nm)		
			Avg±S.D	SEM	CD
Pseudomonas spp	3ml	1st day	0.72±0.007	0.03	0.13
Bacillus spp			0.76±0.008	0.03	0.13
Alcaligenes spp			0.833±0.009	0.03	0.13
Pseudomonas spp	5ml	1st day	1.24±0.01	0.01	0.06
Bacillus spp			1.25±0.008	0.01	0.06
Alcaligenes spp			1.42±0.012	0.01	0.06
Pseudomonas spp	7ml	1st day	1.08±0.005	0.04	0.17
Bacillus spp			1.198±0.011	0.04	0.17
Alcaligenes spp			2.256±0.103	0.04	0.17
Pseudomonas spp	10 ml	1st day	2.1±0.060	0.06	0.24
Bacillus spp			2.293±0.021	0.06	0.24
Alcaligenes spp			2.56±00.151	0.06	0.24

SEM-Standard Error Mean;**CD**- Critical Difference

Table.4 Decolorization percentage of effluent by different bacterial strains at varying dye concentration in fifth day of interval

Bacteria	Dye Concentration	Time Interval	Wavelength (500 nm)		
			Avg±S.D	SEM	CD
Psuedomonas spp	3ml	5^{th} day	0.26±0.001	0.01	0.07
Bacillus spp			0.340±0.008	0.01	0.07
Alcaligenes spp			0.716±0.03	0.01	0.07
Psuedomonas spp	5ml	5^{th} day	1.24±0.012	0.05	0.2
Bacillus spp			1.34±0.136	0.05	0.2
Alcaligenes spp			1.42±0.014	0.05	0.2
Psuedomonas spp	7ml	5^{th}day	1.3±0.022	0.01	0.06
Bacillus spp			1.38±0.027	0.01	0.06
Alcaligenes spp			1.85±0.012	0.01	0.06
Psuedomonas spp	10 ml	5^{th} day	1.91±0.005	0.05	0.2
Bacillus spp			2.179±0.10	0.05	0.2
Alcaligenes spp			2.311±0.10	0.05	0.2

SEM-Standard Error Mean,**CD**- Critical Difference

Table.5 Decolorization percentage of effluent by different bacterial strains at varying dye concentration in tenth day of interval

Bacteria	Dye Concentration	Time Interval	Wavelength (500 nm)		
			Avg±S.D	SEM	CD
Psuedomonas spp	3ml	10^{th} day	0.24±0.016	0.01	0.04
Bacillus spp			0.248±0.01	0.01	0.04
Alcaligenes spp			0.64±0.022	0.01	0.04
Psuedomonas spp	5ml	10^{th} day	1.12±0.009	0.02	0.09
Bacillus spp			1.186±0.05	0.02	0.09
Alcaligenes spp			1.535±0.01	0.02	0.09
Psuedomonas spp	7ml	10^{th}day	0.64±0.022	0.01	0.03
Bacillus spp			1.23±0.012	0.01	0.03
Alcaligenes spp			1.325±0.008	0.01	0.03
Psuedomonas spp	10 ml	10^{th} day	0.65±0.007	0.2	0.9
Bacillus spp			1.32±0.02	0.2	0.9
Alcaligenes spp			1.26±0.003	0.2	0.9

SEM-Standard Error Mean,**CD**- Critical Difference

Table.6 Decolorization percentage of effluent by different bacterial strains at varying dye concentration in fifteen days of interval

Bacteria	Dye Concentration	Time Interval	Wavelength (500 nm)		
			Avg±S.D	SEC	CD
Psuedomonas spp *Bacillus* spp *Alcaligenes* spp	3ml	15th day	0.22±0.01 0.24±0.013 0.403±0.016	0.02 0.02 0.02	0.09 0.09 0.09
Psuedomonas spp *Bacillus* spp *Alcaligenes* spp	5ml	15th day	0.0167±0.01 0.82±0.01 1.23±0.02	0.03 0.03 0.03	0.13 0.13 0.13
Psuedomonas spp *Bacillus* spp *Alcaligenes* spp	7ml	15th day	0.51±0.053 0.81±0.01 1.12±0.11	0.05 0.05 0.05	0.2 0.2 0.2
Psuedomonas spp *Bacillus* spp *Alcaligenes* spp	10 ml	15th day	1.17±0.01 1.2±0.01 2.13±0.1	0.04 0.04 0.04	0.1 0.1 0.1

SEM-Standard Error Mean,**CD**- Critical Difference

At the fifth day of interval the absorbance value found to be reduced than first day due to the degradation of dye by the organism. The degradation rate was observed to be slow in the increased dye concentration. The time taken for the Decolorization of the dye increases as the concentration increases. The results of the present study is in accordance with Ajay Kumar Pandey *et al.,* (2012).

At the tenth day of interval the degradation rate was high, this results in the less absorbance value than first day and fifth day interval of time. The enzyme rate released by the organism for the degradation was higher. The results of Saratale *et al.,* (2009) showed that the isolate showed complete Decolorization, this could be due to a greater production of enzymes.

The bacterial isolated from dye effluent were reported to be used against dye and its product for degradation. The organism *Pseudomonas* spp and *Bacillus* spp can grow in alkaline environment of dye and they gave similar decolorization results. This study is in accordance with Chang *et al.,* (2011).They suggested that the *Bacillus* spp. grows very well in the pH range of 5–9 and *Pseudomonas* spp. exhibited best decolorization at pH 7.0 with constant decolorization rate up to pH 9.5. *Alcaligenes* spp also found to degrade dye effluent and its product but in low rate than other two organisms. *Alcaligenes* spp can able to grow in alkaline environment.

SDS page

SDS-PAGE was carried out with 8% separating gel and 4% stacking gel. SDS PAGE was done after the treatment of dye effluent samples by the isolated organisms. The protein concentration was found to be increased from each day. It was observed from the protein bands.

Lane 4 was loaded with fifth day , Lane 3 was loaded with tenth day degraded sample and Lane 2 was loaded with fifteenth day degraded sample .The result showed that protein concentration was high in the lane 2 than lane 3 and 4. This is due to release of protein by the organism for degradation of the dye in the effluent. The analysis of protein in the sample was shown in the figure 4.5.1.

The textile effluent collected from the Kumarapalayam area has lagre amount of chemicals. The method of textile dye degradation is a ecofriendly and cost effective method in which bacterial isolates are used for dye degrading. In this study,a decolorizing bacterial strains were isolated from the effluent. *Pseudomonas* spp showed higher decolorizing activity through a degradation mechanism rather than other two strains. With high degradative and decolorizing activity against dyes it can be used by the textile industries. The experimental results showed that the biological method of treatment is effective alternative method for removing the color from the effluent. The pattern profiling of effective strain was carried out. The degradation method is useful to remove the dye from the effluent. The present invention indicates that microbial decolourization could be a viable means in ridding dye waste water. Hence the treated water can be reused in the dying industries.

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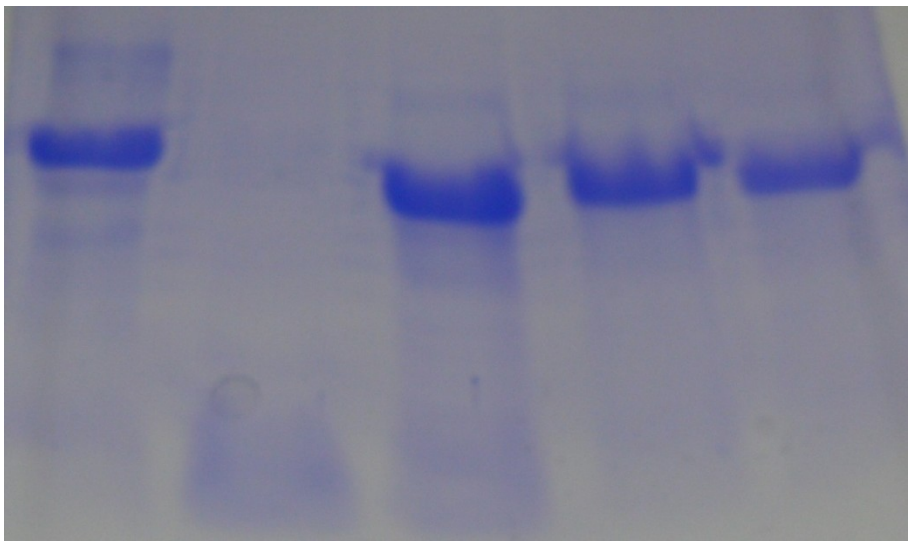
Figure.1 SDS PAGE (8%) Showing different Protein bands of *Pseudomonas* spp.



Lane 1: Marker
Lane 2: *Pseudomonas spp* dye degraded sample (fifteenth day)
Lane 3: *Pseudomonas spp* dye degraded sample (tenth day)
Lane 4: *Pseudomonas spp* dye degraded sample (fifth day)

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