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Microbial Decolorization of Methyl Orange Dye by *Pseudomonas spp*. ETL-M

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Abstract The present study aimed at assessing the ability of *Pseudomonas spp*. to decolorize and degrade Methyl Orange dye. *Pseudomonas spp*. ETL-M could tolerate Methyl Orange dye upto 500 mg^{-1}. A bacterium identified as *Pseudomonas spp*. ETL-M was isolated from dye contaminated soil. This strain rapidly decolorized a methyl orange azo dye solution. Features of the decolorizing process related to biodegradation and biosorption were also studied. The dye was efficiently decolorized in static compared to shaked cultures. The bacterium exhibited a remarkable color removal capability over a wide range of dye concentration (50-200 mg/l), pH (6-10) and temperatures (30-40^{o}C). The *Pseudomonas spp*. ETL-M decolorized the repeated addition of methyl orange dye up to four cycles with variable decolorization rate (10-94%).

Keywords: *methyl orange, pseudomonas, temperature, biodegradation*

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1. Introduction

Rapid industrialization has necessitated the manufacture and use of different chemicals in day to day life [5,20]**.** The textile industry is one of them which extensively use synthetic chemicals as dyes. Wastewaters from textile industries pose a threat to the environment, as large amount of chemically different dyes are used. A significant proportion of these dyes enter the environment via wastewater [20]. Approximately 10,000 different dyes and pigments are used industrially and over 0.7 million tons of synthetic dyes are produced annually, worldwide [24]. Pollution due to textile industry effluent has increased during recent years. Moreover, it is very difficult to treat textile industry effluents because of their high BOD, COD, heat, color, pH and the presence of metal ions [4]. The textile finishing generates a large amount of waste water containing dyes and represents one of the largest causes of water pollution [6], as 10- 15% of dyes are lost in the effluent during the dyeing process. The traditional textile finishing industry consumes about 100 liters of water to process about 1 Kg of textile material. The new closed-loop technologies such as the reuse of microbial or enzymatic treatment of dyeing effluents could help reducing this enormous water pollution [1]. Azo dyes have been used increasingly in industries because of their ease and cost effectiveness in synthesis compared to natural dyes. However, most azo dyes are toxic, carcinogenic and mutagenic [22]. Azo bonds present in these compounds are resistant to breakdown, with the potential for the persistence and accumulation in the environment [29]. However, they can be degraded by bacteria under aerobic and anaerobic conditions [33]. Several physico-chemical techniques have been proposed for treatment of colored textile effluents. These include adsorption on different materials, oxidation and precipitation by Fenton's reagent, bleaching with chloride or ozone photo degradation or membrane filtration [25]. All these physical or chemical methods are very expensive and result in the production of large amounts of sludge, which creates the secondary level of land pollution. Therefore, economic and safe removal of the polluting dyes is still an important issue. Bioremediation through microorganisms has been identified as a cost effective and environment friendly alternative for disposal of textile effluent [9,23]. In recent years a number of studies have focused on some microorganisms capable of degrading and absorbing dyes from wastewater. A wide variety of microorganisms are reported to be capable of decolonization of dyes [2,3,7-8,11-15,18,26,31,34,18]. The present study deals with the isolation of textile dyes degrading bacterium from a dyes contaminated environment, its ability to degrade reactive dyes.

2. Materials & Methods

2.1. Screening of Decolorizers

Soil near to textile industrial outlet was used as source for enrichment and isolation of decolorizers. The screening medium (SM medium) contained: peptone, 10g; meat extract, 10g; NaCl, 5g; in 1 liter of distilled water with 0.5 g of Methyl Orange. Methyl Orange dye was sterilized by passing it through a 0.45- µm pore size filter,

while other components were sterilized at 121°C for 20 min. Ten grams of soil was then added to a 500-ml Erlenmeyer flask containing 100 ml of SM medium. The cultures were incubated at 32°C on a rotary shaker at 120 rpm. Next, the broth of the decolorized flask was transferred to fresh SM medium to screen the strain having color removing ability. The screening procedure in the liquid culture was conducted repeatedly until a decolorized culture occurred. A small amount of decolorized broth was then poured into an agar plate containing SM medium and it was incubated at 32°C. Colonies surrounded by decolorized zones were selected. Isolates were then tested for their color removal ability in a submerged culture and the best isolate was selected. Finally, identification of the isolate was done by Bergey's Manual of Determinative Bacteriology (2000) (Chen et al., 1999; and Syed et al., 2008).

2.2. Dyes

Methyl Orange (MO) was procured from local dye industry, Ankleshwar, Gujarat, India. Dye was checked for its color, solubility in water, ethanol, and absorption maximum. Stock solution of 6000 ppm was prepared by dissolving the dye in distilled water and was filter sterilized and kept at 4°C. Dye at different concentrations (50 ppm, 100 ppm, 150 ppm, 200 ppm ,400 ppm and 500 ppm) were used to study their effect on bacterial growth and adsorption after adding to the culture media.

2.3. Decolorization Experiments

All decolorization experiments were performed in three sets. The culture with OD 0.699 at 540 nm at concentration of 4% was inoculated in 250 ml Erlenmeyer flask containing 100ml Screening medium and incubated at 32°C for 24 h. After 24h of incubation, dye was added at concentration of 150 mg/l and 3 ml of the culture media was withdrawn at different time intervals. Aliquot was centrifuged at 6000 rpm for 10 minutes to separate the bacterial cell mass, clear supernatant was used to measure the decolorization at the absorbance maxima of the dye. Abiotic controls (without microorganism) were always included **(**Parshetti et al., 2006).

The percentage decolorization was calculated as follows-

$$\%\ Decolorization = \frac{Initial\ OD - Observed\ OD}{Initial\ DO} \times 100$$

2.4. Effect of Dye Concentration

The various concentrations of dye (50, 100 150, 250 and 400 mg/l) were added into the culture medium in order to examine the effect of initial dye on the decolorization in static conditions at various time intervals (21).

2.5. Effect of Temperature

The inoculated SM medium was incubated at various temperatures (10, 32, 37and 50°C) in static conditions for 48hrs. The effect of temperature on dye decolorization was checked spectrophotometrically after 48hrs [19].

2.6. Effect of pH of Culture Medium

The pH of the inoculated screening medium was adjusted to 2, 4, 6, 7, 8 and 10 with 1M HCL or 1M NaOH. The effect of pH on dye decolorization was checked spectrophotometrically after 48 hrs [19].

2.7. Decolorization at Static and Shaking Conditions

Decolorization ability of bacterial isolate was tested in shaking and static conditions at optimum pH (7.0) and temperature (32°C) using screening medium with 150 mg/l of Methyl orange. The supernatant was withdrawn at interval of 24 hrs for four days and was used for analysis of COD and decolorization. Decolorization was monitored by spectrophotometrically and chemical oxygen demand (COD) was determined according to standard method [30].

2.8. Effect of Glucose and Peptone on Dye Decolorization

To study the effect of carbon and nitrogen sources on decolorization of methyl orange, Mineral medium with trace element addition and varied concentration of glucose / peptone from 1-5% and 150 mg/l of dye was used [21].

2.9. Change in Absorption Spectra During Dye Decolorization

The change in peak in absorption spectrum reveals the dye adsorption or biodegradation during decolorization by isolate. Variation of UV- visible spectra of Azo dye solution at concentration of 150 mg/l methyl orange with *Pseudomonas spp.* ETL-M was checked at 0, 24 and 48 hrs intervals spectrophotometrically [10].

2.10. Assimilation of Dye

An attempt was carried to test the isolate ability to decolorize 150 mg/l methyl orange in mineral medium depleted from carbon or nitrogen or both. The decolorization was read spectrophotometrically after 48 hrs [19].

2.11. Fed Batch Decolorization of Methyl Orange by Isolate

The fed batch decolorization of methyl orange dye was also studied, in this study 150 mg/l dye was added into the 24hrs grown culture of bacterial isolate. After decolorization 150 mg/l dye added into the decolorized broth without supplement of additional nutrient. Dye was added continuously until culture does not lose decolorization ability. The dye concentration was determined by monitoring the absorbance of dye spectrophotometrically [30].

2.12. Decolorization of Azo Dyes in Consortium

Different Azo dyes viz. Methyl red, Methyl orange, Congo red at the final concentration of 150 mg/l was used in screening medium with isolate and dye reduction was checked spectrophotometrically for four days at 24 hrs intervals.

3. Results & Discussion

Industrial effluent is not stable and it varies often in a wide range depending upon the process practiced. South Asian countries are experiencing severe environmental problems due to rapid industrialization. This phenomenon

is very common where the polluting industries like textile dyeing, leather tanning, paper and pulp processing, sugar manufacturing, etc. thrive as clusters. Among these the Textile industries are large industrial consumers of waters as well as producers of wastewater. The effluent discharged by this industry leads to serious pollution of groundwater and soils and ultimately affects the livelihood of the poor [17]. The textile industry is one of the industries that generate a high volume of waste water. Strong color of the textile waste water is the most serious problem of the textile waste effluent. The disposal of these wastes into receiving waters causes damage to the environment. Dyes may significantly affect photosynthetic activity in aquatic life because of reduced light penetration and may also be toxic to some aquatic life due to the presence of aromatics, metals, chlorides, etc. Synthetic dyes are extensively used in the textile and printing industries. Azo dyes are the most important group of synthetic colorants. These are the largest class of dyes, and more than half of the annually produced dyes. Dye waste water from textile or dye stuff industry is one of the most difficult to treat because dyes have various synthetic origin and they contain complex aromatic molecular structures, which make them more stable and more difficult to be degraded. The removal of dyes from the textile waste effluent has been carried out by physical & chemical methods, such as flocculation, membrane filtration, electrochemical techniques, ozonation, coagulation and adsorption. In recent years, a number of studies have focused on some microorganisms which are able to biodegrade and biosorb dyes in waste waters. A wide variety of microorganisms capable of decolorizing a wide range of dyes include some bacteria, fungi and algae. The use of microorganisms for the removal of synthetic dyes from industrial effluents offers considerable advantages. The process is relatively inexpensive, it is simple method and the running costs are low and the end products of complete mineralization are not toxic. The present study was designed to test decolorization of azo dye by bacteria isolated from textile effluent drainage site. For this purpose soil near to textile effluent outlet was collected & enrichment and isolation for azo dye decolorizing bacteria was carried out in screening medium containing azo dye. Microorganism showing maximum decolorization in less time was selected & using Bergy`s Manual of Determinative Bacteriology (2000) was identified as *Pseudomonas spp.* The time course of methyl orange decolorization was studied at different initial concentrations (50 – 500 mg /L) in static cultures. Data in Figure 1 depict that at the lowest dye concentration (50- 200 mg/L) the dye was decolorized more than 84 % after four days incubation. Similar data were reported by Yan et al., 2004. As the dye concentration increased in the culture medium, a decline in color removal was attained. This might be attributed to the toxicity of dye to bacterial cells through the inhibition of metabolic activity, saturation of the cells with dye products, inactivation of transport system of the dye or the blockage of active sites of azoreductase enzymes by the dye molecules. Under given experimental conditions, 60% decolorization was attained upon using 500 mg /L of the dye after four days. Bacterial growth in presence of methyl orange was also studied using control without dye, which showed the dye has inhibitory effect on growth of bacteria as number of bacteria were decreased in presence of methyl orange as compare with control without dye (data not shown). The rate of chemical reaction is the direct function of temperature. Bacteria require optimum temperature for growth. Since dye decolorization is metabolic process hence shift in temperature from optimum results into decrease in dye decolorization as high temperature causes thermal inactivation of proteins & possibly of such cell structures such as membrane. The operating temperature of the incubation process varied between 10°C, 30°C, 37°C & 50°C, to study the effect of temperature on the decolorization process (Figure 2). At temperature below 37°C, due to slow growth of the bacteria, it took more days for decolorization and at temperatures above 37°C, the activity of *Pseudomonas spp.* and hence percentage of decolorization decreases. The variation in pH of the growth medium results in change in activity of bacteria & hence the bacterial growth rate as well as decolorization. Bacteria are active over certain range of pH. The optimum pH for the growth is the same for the dye decolorizing activity as it is mainly the metabolic process. In contrast with other decolorizing microbes like fungi with narrow pH range, *Pseudomonas spp.* cells proved to be of desirable characteristic, removing methyl orange color over a wide range of pH (6 – 10) with optimum at pH 8 (80 % dye decolorization). Large decrease in decolorization occurs at high acidic pH (2- 4) (Figure 3). This is an advantage of this bacterium for developing a practical bioprocess in treating dyeing mill effluents. In case of fungi increase in pH greater than 5.5 resulted in the fragmentation of mycelia pellets & below 5.5 there is no appreciable growth of fungi hence percentage of decolorization decreases. Hence the bacteria are preferred over fungi for dye decolorization. The pattern of methyl orange decolorization in static as well as in shaked cultures was elucidated in medium. Figure 4 shows that lower decolorization percentages were exhibited in shaked cultures compared to static ones. Maximal efficiency of methyl orange decolorization (86%) was achieved in four days incubated statically. These observations suggest that the decolorization performance of *Pseudomonas spp.* ETL-M was better in the presence of low oxygen content. The reason could be due to competition of abundant oxygen and the azo compounds for the reduced electron carriers under aerobic condition. Yan et al., 2004, also reported that to achieve an effective color removal, agitation and vigorous aeration should be avoided. The cell growth in shaking condition was higher than static condition but there was less decolorization (65%) with more COD removal (48 %) under shaking condition, while 86 % decolorization with less COD removal (25%) under static condition within four days (Data not shown). These findings are consisted with result shown by Guven Ozdemir et al., who suggested COD removal is more under shaking condition. Addition of a carbon source such as glucose at different concentrations has an effect on the percentage of decolorization (Data not shown). The concentration of glucose was varied from 1% to 5 % and it was found that the percentage of decolorization increases with the increase in concentration of glucose due to decrease in lag period. The percentage decolorization decreases with the increase in concentration of peptone up to maximum peptone concentration of 1% (75% dye decolorization) and after which there is decrease in percentage of decolorization. The decrease decolorization results from nitrate or nitrite, a reducing equivalent that cells generated from peptone consumption. These metabolites of nitrate/nitrite may

compete with the azo dye and result in less decolorization (Data not shown). In addition to a complex mixture of dyes, the textile mill effluents often contain heavy metals which generally affect the uptake and metabolism of azo dyes. Results obtained in the presence of different heavy metals are shown in Figure 5. Data indicates that the process of color removal is significantly inhibited by the presence of Mercuric chloride (11%) & Potassium dichromate (12%) especially during the initial period (1-2 days) of the incubation. Marginal inhibition in color uptake is noticed in the presence of Silver Nitrate, Zinc Sulphate & Cadmium Chloride. Hence the bacteria are able to tolerate the toxic effect of Silver Nitrate, Zinc Sulphate & Cadmium Chloride to achieve decolorization. Slow rates of color uptake in the presence of Chromium & Mercury may be related to heavy metal inhibition of enzymes and metabolic pathways. Similar data were reported by Sumathi et al., (2001) who studied effect of Cr^{+6} on *Aspergillus foetidus* in decolorization of procion dyes. Decolorization of the dye solution by bacteria could be due to adsorption to microbial cells or to biodegradation. In adsorption, examination of the absorption spectrum would reveal that all peaks decreased approximately in proportion to each other. If dye removal is attributed to biodegradation, either the major visible light absorbance peak would completely disappear or a new peak would appear. Dye adsorption would result in cell mats which are deeply colored because of adsorbed dyes, whereas those retaining their original colors are accompanied by the occurrence of biodegradation. Result displays the change of UV-visible spectra of Methyl orange, using the supernatant fluid of the culture at 0, 24, and 48 hrs (Data not shown).Decolorizing cultivation with *Pseudomonas spp.* The absorbance peak at 440nm disappears after cultivation. The fed batch decolorization study was carried out to check the ability of isolate for the decolorization of repeated added dye. The *Pseudomonas spp.* decolorized the repeated addition of methyl orange dye up to four cycles (each 24h) with variable decolorization rate (10 – 94%). In first cycle 93% decolorization occurred, 70% decolorization in second cycles & the percent decolorization goes on decreasing (up to 10% at 4^{th} cycle) as the number of cycle increases (Data not shown). Our isolate also has the ability to decolorize following azo dyes viz. Methyl orange, Methyl red, Congo red & Tartrazine in consortium. Figure 6 & Figure 7, depict that it can decolorize these mixed dye up to 75 % in four days.

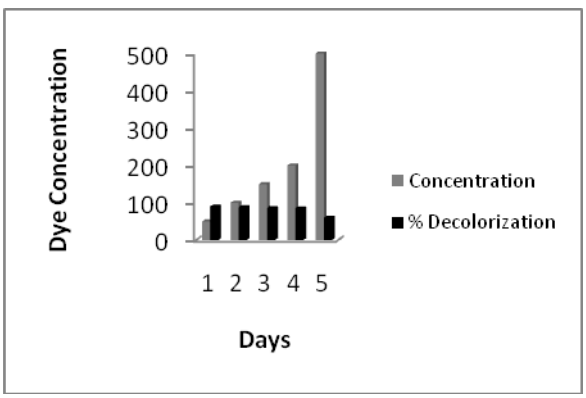

Figure 1. Effect of Dye concentration on decolorization performance of *Pseudomonas spp.* ETL-M.

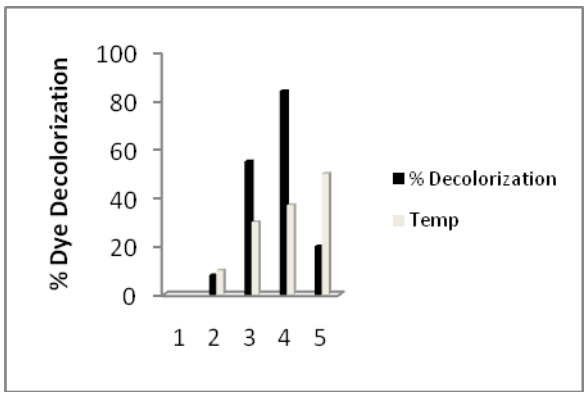

Figure 2. Effect of temperature on decolorization by *Pseudomonas spp.* ETL-M.

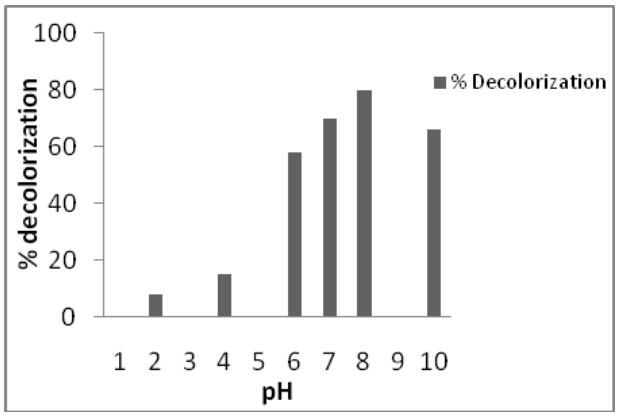

Figure 3. Effect of pH on decolorization by *Pseudomonas spp.* ETL-M.

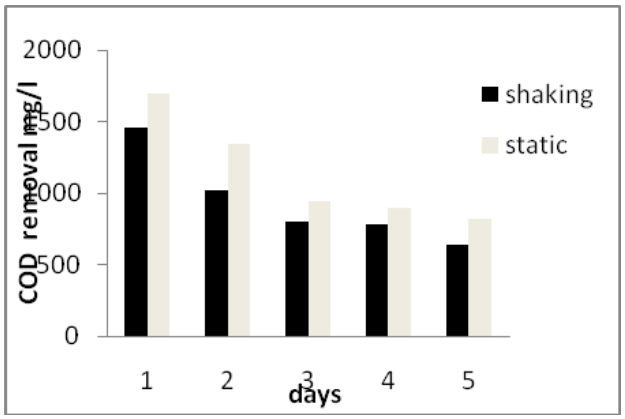

Figure 4. Effect of Static & Shaking condition on decolorization by *Pseudomonas spp.* ETL-M.

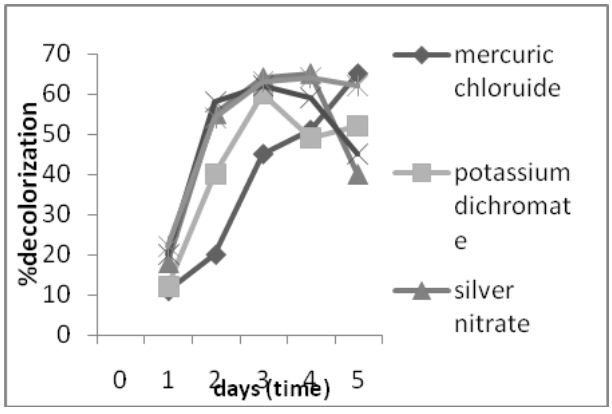

Figure 5. Effect of heavy metals on rate of uptake of dye during decolorization by *Pseudomonas spp.* ETL-M.

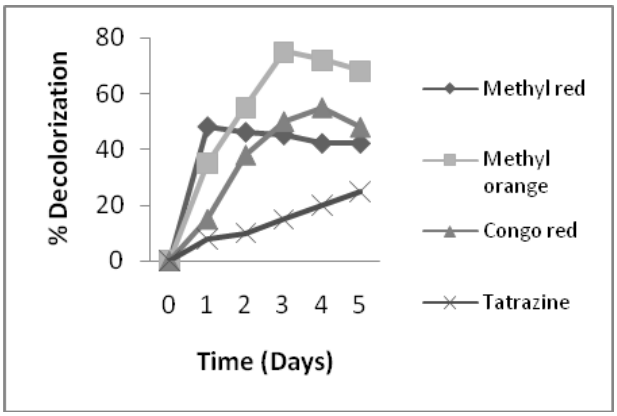

Figure 6. Decolorization of different azo dyes by *Pseudomonas spp.* ETL-M.

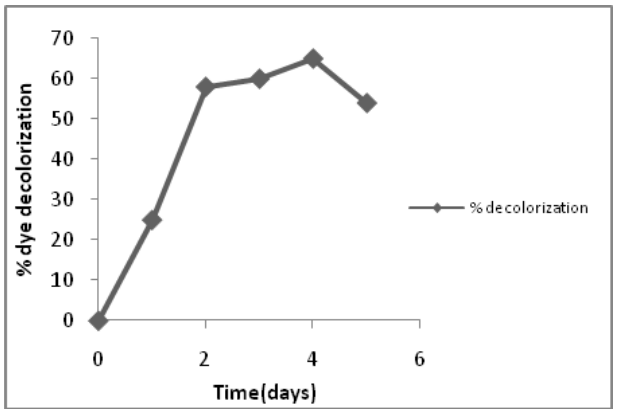

Figure 7. Decolorization of azo dyes in consortium by *Pseudomonas spp.* ETL-M.

4. Conclusion

In this study, a decolorizing bacterial strain, *Pseudomonas spp.*, ETL-M was isolated from dye contaminated soil. *Pseudomonas spp.* ETL-M showed decolorizing activity through a degradation mechanism rather than adsorption, and it could tolerate high concentrations (up to 500 mg^{-1}) of Methyl Orange. With high degradative and decolorizing activity against various reactive dyes commonly used in the textile industries, it is proposed that *Pseudomonas spp.* ETL-M has a practical application potential in the biotransformation of various dye effluents. The effects of oxygen, pH, Temperatures, and dye concentration on the decolorization of methyl orange were investigated. Examination of the mechanism of the decolorization process indicated that it proceeded primarily by biological degradation associated with a minor portion of the dye adsorbing onto the cell surface. Identification and toxicity study of the products from the degradation of Methyl orange dye by *Pseudomonas spp.* ETL-M is now in progress. This observation has established that the bacteria are adaptive in nature and can degrade contaminants. The ability of the strain to tolerate, decolorize azo dyes at high concentration gives it an advantage for treatment of textile industry waste waters. However, potential of the strain needs to be demonstrated for its application in treatment of real dye bearing waste waters using appropriate bioreactors.

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