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Bioremedial Application of Bacillus Megaterium PMS82 in Microbial Degradation of Acid Orange Dye

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Abstract A potential bacterial strain PMS82, capable of degrading an azo dye Acid orange as a sole source of carbon was isolated from common effluent treatment plant of Ankleshwar, India. The 16S rDNA sequence and phenotypic characteristics indicated that an isolated organism as Bacillus megaterium PMS82. This strain exhibited complete decolorization of Acid orange (100 mg/L) within 16 h, while maximally it could decolorize 800 mg/L of dye within 38 h with 73% decolorization under static condition. For color removal, the most suitable pH and temperature were pH 6.0-9.0 and 25-40°C respectively. The organism has shown more than 70% decolorization activity against five structurally different azo dyes within 38 h and tolerates high salt concentration up to 2% (w/v) respectively with more than 90% decolorization. UV-Visible absorption spectra before and after decolorization suggested that decolorization was due to biodegradation and was further confirmed by FT-IR spectroscopy. Overall results indicating the effectiveness of the strain PMS82 explored for the treatment of common effluent treatment plants containing various azo dyes. To our knowledge, this could be the first report on biodegradation of Acid oragne by Bacillus megaterium PMS82.

Keywords: *biodegradation, decolorization, bacillus megaterium, acid orange, textile azo dye*

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1. Introduction

Synthetic dyes are extensively used in textile dyeing, paper printing, color photography, pharmaceutical, food, cosmetics and other industries. During textile dyeing, the amount of dye lost in the effluents is dependent upon the class of dye used, varying from only 2% loss when using basic dyes to a 50% loss when reactive dyes are used [1]. Approximately 20% of the losses enter the environment through effluents from wastewater treatment plants [2]. Azo compounds are a broad class of organic compounds with formula R-N=N-R0, in which R and R0 can be either aryl or alkyl groups. Azo compounds are solids of varying color from yellow to red and violet to blue. The N=N group is called an azo group and parent compound, HNNH, is called diimide. Acid orange, an azo reactive dye, is a representative of a dye class to be recalcitrant with a conventional wastewater treatment system. Reactive dyes are easily soluble in water, therefore they have little affinity to be adsorbed on biomass and generally pass through activated sludge systems [3]. It is quite undesirable to discharge azo dyes with different colors into the environment due to their color pollution, biorecalcitrance and toxic intermediates, since the cleavage of azo bonds produces aromatic amines which are considered mutagenic and carcinogenic [4,5]. In addition, some azo dyes or their metabolites because of low biodegradability may be mutagens or carcinogens to humans as well as to other animals [5,6]. Therefore, considerable attention has been given to evaluating the fate of azo dyes during wastewater treatment and in the natural environment. The effluents of these industries are highly colored and disposal of these wastes into natural waters causes damage to the environment [7,8]. Decolorization of these dyes by physical or chemical methods including flotation, hyper filtration, [9], adsorption, coagulation-flocculation, ion-exchange, oxidation, electrochemical methods [10,11] and precipitation methods, chemical degradation or photodegradation is financially and often also methodologically demanding, time-consuming and mostly not very effective [12]. The above mentioned ways for clean-up are expensive, coupled with the formation of large amount of sludge and the emission of toxic substances [13], which limit their application [14]. Compared with chemical and physical methods, a number of studies have focused on biological treatment, in which microorganisms which are able to decolorize and biodegrade these azo dyes are used, thus producing lower costs and fewer toxic resultants [15]. Several combined anaerobic and aerobic microbial treatments have been suggested to enhance the degradation of azo dyes [5]. Alternatively, dye decolorization using microbial enzymes has received great attention in recent years due to its

efficient application [16,17,18]. Color removal processes with active microorganisms have two different simultaneous steps: adsorption of dyes on the surface of the organisms and degradation of dyes by the enzymes produced by these organisms [19,20,21]. Decolorization of textile dye effluent does not occur when treated aerobically by municipal sewage systems [22]. Brightly colored, water-soluble reactive and acid dyes are the most problematic, as they tend to pass through conventional treatment systems unaffected [22]. This study was aimed to isolate and characterize an efficient bacterial strain, which exhibited the remarkable ability to degrade Acid orange as a sole source of carbon. Various physico-chemical parameters have been optimized for efficient dye decolorization. The dye degraded products were characterized by ultraviolet–visible (UV-Vis) and Fourier transformed infrared spectroscopy (FT-IR) techniques. Very few reports are available on Acid orange degradation. After survey of the literature, this could be the first report on biodegradation of Acid orange by Bacillus megaterium PMS82.

2. Materials & Methods

2.1. Dyestuffs, Chemicals & Microbiological Media

Six textile azo dyes Acid Orange, Reactive Red 2, Reactive Orange 16, Reactive Blue 4, Reactive Black 5 and Reactive Green A were generous gifts from local textile industry of Ankleshwar, India. All these dyes were of industrial grade and are widely used in textile industries. In order to obtain a high-performance bacterial decolorization, Acid orange dye was chosen for the screening of dye degrading bacteria. All the chemicals used were of highest purity and of analytical grade. Reagents and other fine chemicals were obtained from Sigma – Aldrich & Himedia Laboratory, India.

2.2. Culture Medium

The Mineral Salts Medium (MSM) was prepared as per Brilon et al. [23] with some 117 modifications. The MSM contained of following constituents (g/L): $Na_2HPO_4.2H_2O$ (12.0), KH_2PO4 (2.0), NH_4NO_3 (0.50), $MgCl_2.6H_2O$ (0.10), Ca $(NO3)_2.4H_20$ (0.050), $FeCl_2.4H_20$ (0.0075) with 10 mL of trace element solution per liter. The trace element solution was prepared as follows (mg/L): $ZnSO_4.7H_2O$ (10.0), $MnCl_2.4H_2O$ (3.0), $CoCl_2.6H_2O$ (1.0), $NiCl_2.6H_2O$ (2.0), $Na_2MoO_4.2H_2O$ (3.0), H_3BO_3 (30.0), $CuCl_2.2H_2O$ (1.0). Further, MSM was blended with different concentrations of Acid orange and used throughout the study as a test medium and uninoculated flasks were also incubated as control. The final pH of the medium was adjusted to 7.0 ± 0.2. The MSM with agar (1.9% w/v) was used for isolation and maintenance of pure culture. The media were sterilized at 121°C for 20 min before use.

2.3. Isolation and Identification

Activated sludge (10 mL) was added to Tanner mineral medium [24], containing 100 mg/L of Acid orange. This was then added as an enrichment substrate.The suspension was then incubated under continual shaking at 150 rpm at 30°C in the dark. After 6 days, a 10 mL aliquot was transferred to a 200 mL fresh MM containing 100 mg/L Acid orange and incubated. This process was repeated five times. Pure cultures were obtained by diluting 1 mL of culture by 10 thousand and spreading a 100 µL aliquot on solid MM plates. Colonies were harvested from dilution plates based on dis-tinct colony morphology, and transferred to fresh LB agar plates several times in order to ensure culture purity. The isolated strain was identified by color and morphology. Gram staining, flagellum staining, capsule staining, spore stain, catalase, gelatin liquefaction, methyl red (M.R), nitrate reduc-tion, acetyl methyl carbinol (V.P), tetracycline resistance (TetR) were tested as described previously [25,26]. All tests were per-formed twice and incubated at 37°C [27]. Further identification was done using the 16S rDNA gene sequencing. The fragment of rDNA was amplified using a Gene Amp PCR System (PE, USA) under the following conditions [28]: 1 µL template DNA, 2.5 µL 10× PCR buffer, 1.5 mM $MgCl_2$, 500 mM KCl, and 0.1% (w/v) glutin, 0.25 U of rTaq polymerase, included 1’ PCR buffer containing 1.5 mM $MgCl_2$, 200 mM of each deoxynucleoside triphosphate, 10 pmol of each primer, 0.25 U of rTaq polymerase, 1 µL bacteria liquid, 0.5 mM upstream primers 27F (5’-AGAGTTTGATCCTGGCTCAG-3’), 0.5 mM downstream primer 1492R (5’-TACCTTGTTACGACTT-3’), 2.5 mM dNTPs and double distilled water contained in a total volume of 50 mL. The tubes were incubated at 95°C for 5 min and then subjected to the following thermal cycling program: denaturation at 94°C for 1 min, primer anneal at 55°C for 1 min, chain extension at 72°C for 1 min 30 s and a final extension at 72°C for 30 min, for a total of 30 cycles. Sequencing on both strands of PCR-amplified fragments was carried out using dideoxy chain termination method in automated sequencer. Using the Basic Local Alignment Search Tool (BLAST) program [29], 16S rDNA homology searches against the NCBI database were carried out in order to find the 16S rDNA database for similar sequences.

2.4. Decolorization Experiment

The dye decolorization experiments were performed in 250 mL Erlenmeyer flasks containing 100 mL of sterilized MSM broth supplemented with yeast extract (0.1% w/v) and Acid orange (100 mg/L). We observed that complete decolorization of Acid orange in MSM medium within 16 h as compared to 56 h without yeast extract under the static condition (data not shown). Reports suggests the inclusion of yeast extract was found to be most effective supplement for growth of azo dye degrading bacteria as well as increases the dye decolorization efficiency [30]. Therefore all further decolorization experiments were performed using MSM broth supplemented with 0.1% (w/v) yeast extract as a cosubstrate. The flasks were inoculated with 5 mL of cultures broth in test and uninoculated controls were used to compare abiotic color loss during the decolorization studies. The flasks were incubated at 30°C under static as well as shaking (120 rpm) conditions till the decolorization was completed. The 5 mL of cultures were withdrawn at different intervals for color measurement. The supernatant was collected by

centrifuging at 10,000 rpm for 15 min. Decolorization was monitored spectrophotometrically by measuring absorbance of culture supernatant at 558 nm. Growth of bacteria in dye containing medium was determined spectrophotometrically. The cell pellet obtained upon centrifugation of 5 mL culture was resuspended in 5 mL distilled water and its absorbance was studied at 660 nm. The percentage of decolorization was calculated as mentioned by Dave and Dave [31].

$$Decolorization\ (\%) = \frac{I - F}{I} \times 100$$

Where I = Initial absorbance and F = Absorbance of decolorized sample.

2.5. Optimization of Physico-chemical Parameters

The decolorization efficiency of *Bacillus megaterium* PMS82 on Acid orange was studied at different pH (4-10) and temperatures values (20-50°C). The obtained optimum pH 7.0 and temperature at 30°C were selected to study the decolorization activity under various physico-chemical factors such as initial dye concentration (100-800 mg/L), salt concentration (1-6%) and yeast extract concentration (0.1-2.0 g/L). Further, the decolorization of various azo dyes was studied by incubating MSM containing respective dye with bacterial strain PMS82 under static condition.

2.6. Decolorization and Biodegradation Studies

The Acid orange degraded products formed during biodegradation after 16 h of incubation under static condition was studied by following the change in the UV-Vis spectra (200 to 800 nm) using a UV-Vis spectrophotometer (Shimadzu, UV-1800, Japan). To know the decolorization was due to biodegradation of Acid orange was confirmed by FT-IR by analyzing dye degraded products in the decolorized medium. After complete decolorization, culture medium was centrifuged at 10,000 rpm for 15 min to remove the suspended particles. The supernatant was once again centrifuged to ensure the supernatant was free off bacterial cells and was used for extraction of metabolites using an equal volume of ethyl acetate. The extracts were dried over anhydrous Na_2SO_4 and concentrated in a rotary evaporator. The crystals obtained were dissolved in a small volume of high performance liquid chromatography (HPLC) grade methanol and the same sample was used for FT-IR analysis. The FT-IR analysis of extracted metabolites was done using Fischer Scientific (Nicolet, iH5), Spectrophotometer and compared with control dye in the IR region of 550–4000 cm−1 with 32 scan speed.

3. Results & Discussion

3.1. Isolation and Identification of Acid Orange Degrading Strain

The strain PMS82 was rod-shaped and formed entire, smooth, convex, opaque and wet colonies, that became slightly yellow in color and circular with a diameter of 0.5–1 mm within 2–3 days. Within 5–6 days they were dark yellow and circular with a diameter of 1–2 mm. As shown in Table 1, this strain was gram positive, had spore and flagellum, but no capsule. Both catalase and gelatin liquefaction tests were positive. The strain PMS82 was negative for M.R and V.P tests and was unable to reduce/restore nitrate. The strain was sensitive to tetracycline and could not grow at tetracycline concentrations of 1µg/mL, similar to that reported by Fergus et al. [26]. By comparing the 16S rDNA gene sequence, this strain was identified as Bacillus megaterium (Figure 1) with 99% identity compared to the NCBI database.

Table 1. The biochemical characters of the strain PMS82

Test	Result
Gram stain	Positive
Flagellum stain	Positive
Capsul stain	Negative
Spore stain	Positive
Catalase stain	Positive
Gelatin liquification	Positive
M.R	Negative
Nitrate reduction	Negative
V.P	Negative

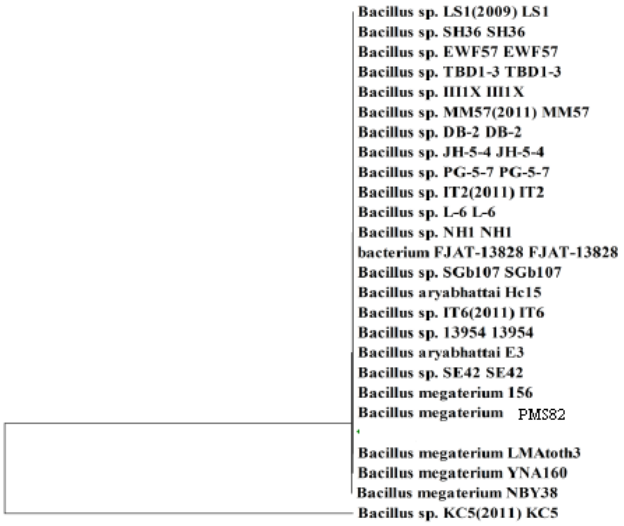

Figure 1. Phylogenetic tree of isolate PMS82 in relation to genus *Bacillus*

3.2. Effect of Static and Shaking Conditions

Bacillus megaterium PMS82 showed 100% decolorization of added Acid orange (100 mg/L) within 16 h under static condition when compared to only 16% decolorization was observed under shaking condition, while the growth of bacterium was greater under shaking condition as compared to static condition (Figure 2). To confirm whether this decolorization was due to microbial action or due to change in pH, the change in pH was recorded, which was in the range of 6.0-7.5 at static condition. Thus, confirming that the biodegradation of dye was due to microbial action. Under aerobic conditions azo dyes are generally resistant to attack by bacteria [32]. Similar findings were reported by other researchers [33]. During dye decolorization in shaking environment electrons released by oxidation of electron donors are preferentially utilized to reduce free oxygen rather than

azo dyes [34]. Hence, in this study static conditions were maintained in the following experiments.

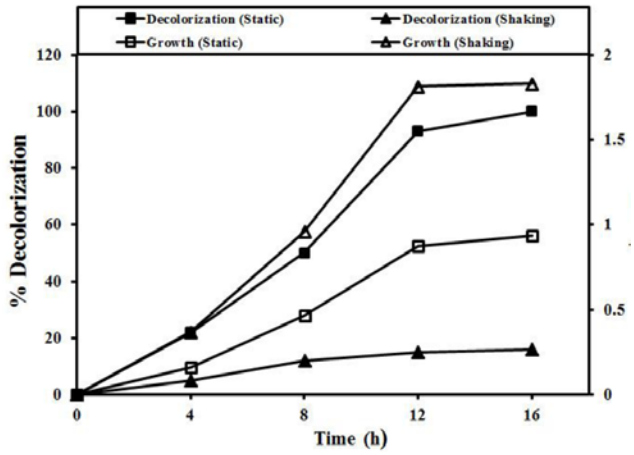

Figure 2. Decolorization of Acid orange by *Bacillus megaterium* PMS82 in MSM under static and shaking condition (120 rpm) at 30°C.

3.3. Effect of pH

The effect of pH on decolorization of Acid orange by *Bacillus megaterium* PMS82 was determined over a wide range of pH 4.0 to 10.0 with an interval of pH 1. The isolate showed the maximum of 100% decolorization at pH 7.0 at 30°C. Following increases from either side of neutral pH, the percentage of decolorization decreased steadily from 97% to 40% on the alkaline side while steep decline in percent decolorization from 89% to less than 15% on acidic side was found. More than 88% of decolorization was observed in a wide range pH 6.0 to 8.0 (Figure 3). Chan and Kuo [35] reported that the neutral pH would be more favorable for decolorization of the azo dyes and is suitable for industrial applications.

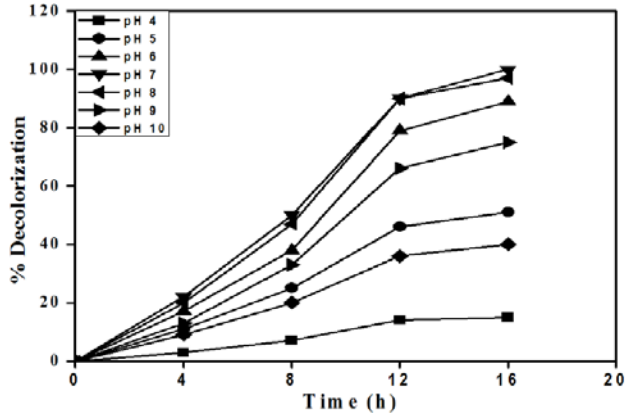

Figure 3. Effect of pH on decolorization of Acid orange

3.4. Effect of Temperature

Similarly in the temperature optimization study, the dye decolorization activity of *Bacillus megaterium* PMS82 was found to increase with increase in incubation temperature from 20 to 30°C. Further increase in temperature, decolorization was decreased the decolorization by 23% and 44% at 40°C and 45°C respectively and almost no activity was found at 50°C (Figure 4). This might be attributed to the adverse effect of high temperature on enzyme activities [36]. Tamboli et al. [37] also found that the decrease in dye decolorization efficiency of *Sphingobacterium* sp. ATM for color removal beyond 30°C, which may be due to the thermal inactivation of the decolorizing enzymes.

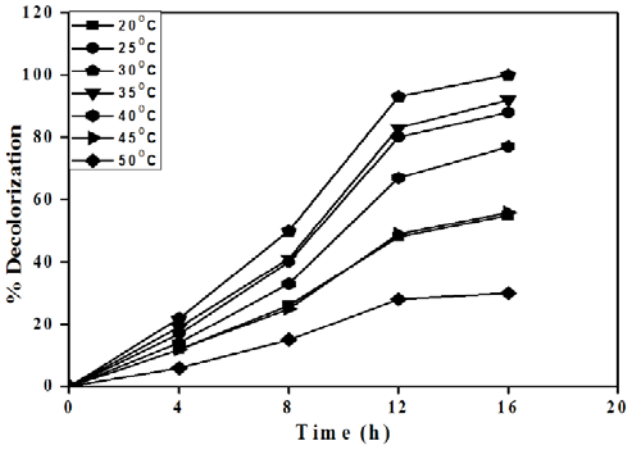

Figure 4. Effect of temperature on decolorization of Acid orange

3.5. Effect of Initial Dye Concentration

The decolorization performance of Acid orange by the strain PMS82 was studied by increasing initial dye concentration (100-800 mg/L). We observed that the percentage of decolorization was decreased slowly with increasing dye concentration (Figure 5). It could effectively decolorize up to 100 mg/L of Acid orange (100%) within 16 h and is decreased to 63%, when dye concentration increased to 800 mg/L and decolorization time increases from 16 h to 38 h respectively. Lower percentage of decolorization and enhanced time period at high dye concentration may be attributed to the presence of four sulfonic acid groups on Acid orange which acts as detergent exerting inhibitory effect on growth of *Bacillus megaterium* PMS82.

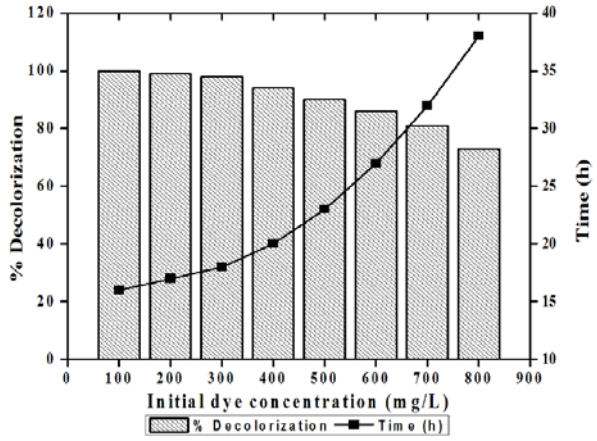

Figure 5. Effect of initial dye concentration on decolorization of Acid orange

3.6. Effect of Salt Concentration

Textile industry effluents generally contain chloride salts of sodium and potassium which are most widely used for salting out of dyes and discharged along with unused dyes. Hence, the present investigation was undertaken to study the effect of salt concentration (1-6% w/v) on decolorization of Acid orange by the strain PMS82. The

organism showed satisfactory decolorization up to maximum of 2% salt concentration in MSM under optimum conditions after 16 h of incubation (Figure 6). Previously De Baere et al. [38] has stated that sodium concentration higher than 3 g/L can cause inhibition of most the bacterial metabolism. But, contrary to the above statement we could notice 74% and 45% of decolorization at 3% and 4% of salt concentration respectively. Negligible activity was observed when 6% of salt concentration was employed in to the medium. This may be attributed to the inhibition of bacteria at high salt concentration due to plasmolysis or loss of activity of cells [39].

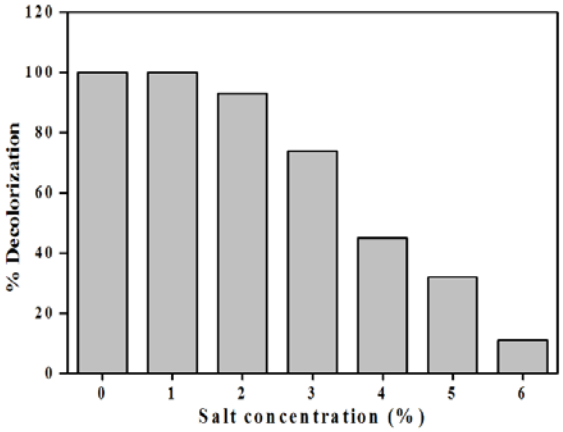

Figure 6. Effect of salt concentration on decolorization of Acid orange

3.7. Effect of Different Concentrations of Yeast Extract

Bacillus megaterium PMS82 was able to degrade Acid orange (100 mg/L) efficiently in the presence of yeast extract as a co substrate. Among all other nitrogen sources, only yeast extract served as better nitrogen source for decolorization of Acid orange within less time was selected for further experiments (data not shown). Effect of different concentrations of yeast extract (0.1-2.0 g/L) in MSM on the decolorization efficacy of PMS82 was evaluated (Figure 8). On addition of 1 g/L of yeast extract in MSM exhibited complete decolorization of Acid orange within 16 h. Further increase in yeast extract concentration has no effect on decolorization activity. Thus, to make the process economical 1 g/L of yeast extract concentration was found to be optimum.

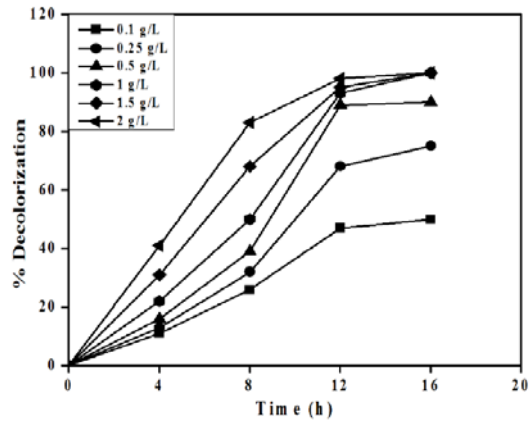

Figure 7. Effect of yeast extract concentration on decolorization of Acid orange

3.8. Decolorization of Structurally Different Azo Dyes

Structurally different azo dyes were most widely used in textile processing industries, and therefore, the effluents from the industry are markedly diverse in composition [40]. Thus, *Bacillus megaterium* PMS82 was tested for its ability to decolorize five other structurally different azo dyes such as Acid orange, Reactive Orange 16, Reactive Black 5, Reactive Blue 4 and Reactive Green 19A. The organism effectively decolorized all structurally different azo dyes within 38 h (data not shown). The efficiency was 100% for Acid orange, followed by 99% for Reactive Orange 16, 92% for Reactive Blue 4, 82% for Reactive Black 5 and 73% for Reactive Green 19A. We presume that decolorization of structurally different azo dyes by *Bacillus megaterium* PMS82 within 38 h might be the first. This variation in the decolorization of different dyes might be attributed to the structural differences, high molecular weight and presence of inhibitory groups like -NO_2 and -SO_3Na in the dyes [40]. The present study confirms the ability of strain PMS82 to decolorize different azo dyes with decolorization efficiency of more than 70%. Thus, the strain PMS82 could be successfully employed for the treatment of textile industry effluents containing various azo dyes.

3.9. Decolorization and Biodegradation Studies

To disclose the possible mechanism of dye decolorization, we also analyzed the degraded products of Acid orange dye by UV-Vis and FT-IR techniques. UV-Vis absorbance of (200-800 nm) of Acid orange in MSM showed single peak in visible region at 480 nm (λmax). In present study we investigated the decolorization potential of bacterium Bacillus megaterium PMS82 towards textile azo dye Acid orange. Bacterium was highly efficient in decolorizing the dye in absence of organic or inorganic nutrients. To confirm the decolorization of dye, UV–vis spectroscopic analysis was carried out. Figure 8 denotes the absorption spectra of Acid orange before and after its bacterial treatment in visible range. Peak responsible for absorption maxima of parent dye (480 nm) was found to be almost completely disappeared in the sample obtained after dye decolorization. Only yeast species S. cerevisiae MTCC 463 has been shown to decolorize the azo dye methyl red without adding any organic or inorganic nutrients [33]. In the present study, Acid orange was found to be adsorbed only initially and no adsorption was observed visually after dye decolorization. Enzymatic analysis in the extracellular sample as well as intracellular filtrate, before and after dye decolorization, exposed the induction in extracellular activity of tyrosinase and NADHDCIP reductase. Intracellular activities of laccase, veratryl alcohol oxidase, NADHDCIP reductase and tyrosinase were also induced (data not shown). These results clearly propose that dye was decolorized both by extracellular enzymatic degradation plus simultaneous adsorption to cell and decolorization by intracellular enzymes. The mechanism of simultaneous adsorption and then decolorization can be seen reported for various microbial species [41,42,43]. Figure 2 represents the optimum pH and temperature for the Acid orange

decolorization which came out to be 8 and 30°C, respectively. When dye decolorization was observed at shaking condition (120 rpm), no dye decolorization occurred. This designates that static condition was necessary as is the case for most of the microbial species for azo reduction. The general mechanism for the azo dye degradation is through reduction of azo bond by azoreductase under anaerobic conditions [44]. However there are certain oxygeninsensitive or aerobic azoreductases that have been reported from aerobic microorganisms [30,31]. But notably in our study, no activity of azoreductase was observed, suggesting different mechanism of this azo dye reduction other than azoreductase. It decolorized reactive, azo, diazo and triphenylmethane dyes. Viability of bacterial strain was the main question in this study, because it was the decolorizing variety of dyes in absence of nutrients. We checked the viability of the strain qualitatively by streaking the loop full of suspension from the dye decolorized flask. We observed normal growth pattern with colonies observed on all the four quadrants of agar plate, indicating that strain remains viable. These studies along with decolorization studies carried out with heat-killed cell suspension signify that dye decolorization was mediated by live culture

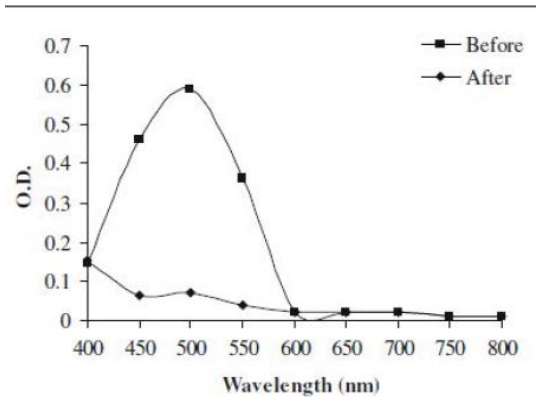

Figure 8. UV-vis spectra of orange 16 before and after decolorization

According to Asad et al. [45] decolorization of dyes by bacteria could be due to adsorption by microbial cells or to biodegradation. In the case of adsorption, the UV-Vis absorption peaks decrease approximately in proportion to each other, whereas in biodegradation, either the major visible light absorbance peak disappears completely, or a new peak appears. The observation of cell mass showed that *Bacillus megaterium* PMS82 retained their natural color after decolorization of Acid orange. The results of FT-IR analysis of Acid orange parent dye and sample obtained after decolorization showed various peaks. The FT-IR spectra of Acid orange parent dye (Figure 9) displays peaks at 3483, 2929, 1660 and 1440 cm^{-1} for –OH stretching vibration, aromatic -CH stretching vibration, -C=C- stretching and -N=N- stretching vibration, respectively. While peak near 1065 cm-1 is for -S=O, indicates sulfoxide nature of the dye. The IR spectra of degradation product displays peak at 3263 cm-1 for –OH stretching. During the degradation of aromatic amines of Acid orange there is formation of aromatic aldehyde as an intermediate which was confirmed by the spot test using 2,4-dinitrophenyl hydrazine reagent which indicated color test due to presence of aldehyde. Besides the signal in IR at 1660 and 2929 cm-1, which corresponds to aldehyde and a signal at 2869 cm-1 for –CH stretching is similar to that of vanillin. Thus aldehyde, one of the intermediate, formed during degradation of Acid orange is confirmed. During the degradation there is asymmetric cleavage of azo bond in Acid orange resulting in formation of phenyl hydrazine, which was confirmed by the standard GC-MS library data, this is further converted to aniline and later on aromatic ring cleavage leading to complete mineralization. While the naphthalene part of the dye was further biodegraded with opening of one ring, the formation of aldehyde as one of the intermediate is confirmed from the IR data. On the basis of above results, it can be concluded that *Bacillus megaterium* PMS82 has ability to mineralize Acid orange completely.

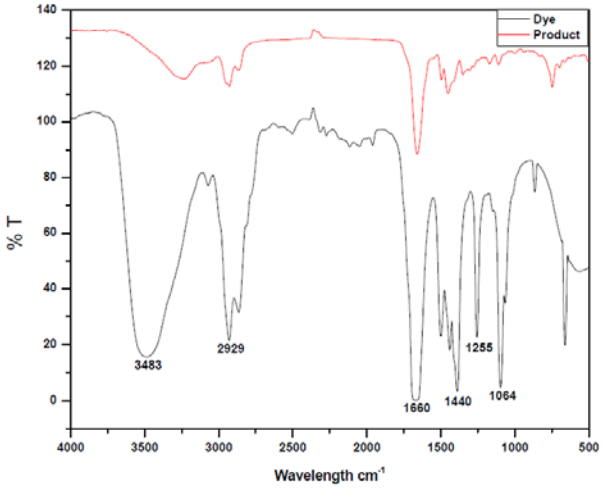

Figure 9. FTIR Spectrum of Acid orange and its degradation product

4. Conclusion

The present study showed that a bacterial strain *Bacillus megaterium*. PMS82 capable of degrading Acid orange as a sole source of carbon with minimal nutritional requirements under static condition. The potential of this strain has ability to decolorize Acid orange in a wide range of pH, temperature, salt and initial dye concentrations, which is significant for its commercial application. The FT-IR results showed that complete loss of the aromatic nature of the dye Acid orange by *Bacillus megaterium*. PMS82. Furthermore, strain PMS82 had the ability to decolorize five other structurally different azo dyes indicate its field applicability in the treatment of textile effluents. Therefore, *Bacillus megaterium*. PMS82 is the highly promising bacterium can be used for the treatment of textile industry effluents containing various azo dyes. Work is in progress to elucidate the complete degradation pathway of Acid orange by *Bacillus megaterium*. PMS82.

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