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16SrRNA sequencing of dye decolorizing bacteria isolated from soil

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Abstract:
Dye's residues in textile effluents are hazardous for humans and animals health. Such pollutants can be degraded into non-harmful molecules using biological approaches that are considered cheaper and ecologically safer. Isolated 15 bacterial cultures from soil that could be used in biological system were showed decolorization capacity for Acid Green dye (33.9% to 94.0%) using thin layer chromatography and broth culture method. The most promising cultures (AMC3) to decolorize Acid green Dye (94.6%) was re-coded as NSDSUAM for submitting at IMTECH, Chandigarh for sequencing. The 16SrRNA sequencing suggested that it can be a variant of *Pseudomonas geniculata* (99.85% identical similarity) with difference of 2 base pairs to reference strain *Pseudomonas geniculata* ATCC 19374(T). Thus present study proposed dye decolorizing efficiency of the isolated strain of *Pseudomonas geniculata* that was previously unnoticed. The sequence is deposited in NCBI GenBank with the accession number KP238100.

Keywords: Bacteria, pollutants, TLC, biodegradation, 16S rRNA sequencing, Azoreductase.

Background:
Rivers are of special significance in Hinduism, not only for its life-sustaining properties, but also because of its use in rituals and because of the stress given to cleanliness. Throughout India seven principle holy rivers are nourishing flora and fauna of the region. Of the seven, the Ganges (Ganga), Yamuna, and Sarasvati are most important. The river Yamuna in India represents a unique niche for biodiversity. Nowadays, Indian textile Industry is one of the leading textile industries in the world. However, risks of pollution of water and air have been highly increased. Previously, textile effluents have been considered as the most polluting among all in the industrial sectors by volume and composition of effluent **[1-3].** Typically, textile effluents is characterized by high values of BOD, COD, color and pH **[4-5].** In-complete use and the washing operations give the textile effleunts a considerable amount of dyes **[6].** That induces persistent color coupled with organic load leading to disruption of the total ecological/symbiotic balance of the receiving water stream **[7].** Allergic, neurotoxic, carcinogenic effects of these dyes have been reported by several scientists **[8-9].** Dyes may also affect photosynthetic activity in aquatic habitat because of reduced light penetration and induce toxicity due to presence of aromatics compounds, heavy metals, chlorides and other toxic compounds. Humans' non-cancer health hazards are affecting the kidneys, liver, male reproductive organs and developing foetus. Recent approaches are much focused on the use of microorganisms for the elimination of dyes as it is comparatively low cost and easier than other conventional processes of decontamination. The present study was carried out to characterize textile dye degrading bacteria isolated from dye contaminated soil.

Methodology:
Isolation and purification of bacteria from Soil samples
The soil samples (50gm) taken from University campus was seeded with Acid Green dye (0.5%, 1% and 2%) in nutrient broth (10ml) and incubated at 37^{o}C for 14 days in a soil- pot. The soil samples were also seeded with 10ml Acid Green Dye and 10 ml distilled water on alternate days till 14th

day. Serial dilution of this processed soil was poured on nutrient agar to isolate bacterial cultures. The bacterial colonies were characterized on the basis of colony morphology (Color, shape, elevation and optical characteristics) and Gram staining reaction then purified by repeated streaking. Each of the pure bacterial culture was maintained in the slants as a pure or stock culture.

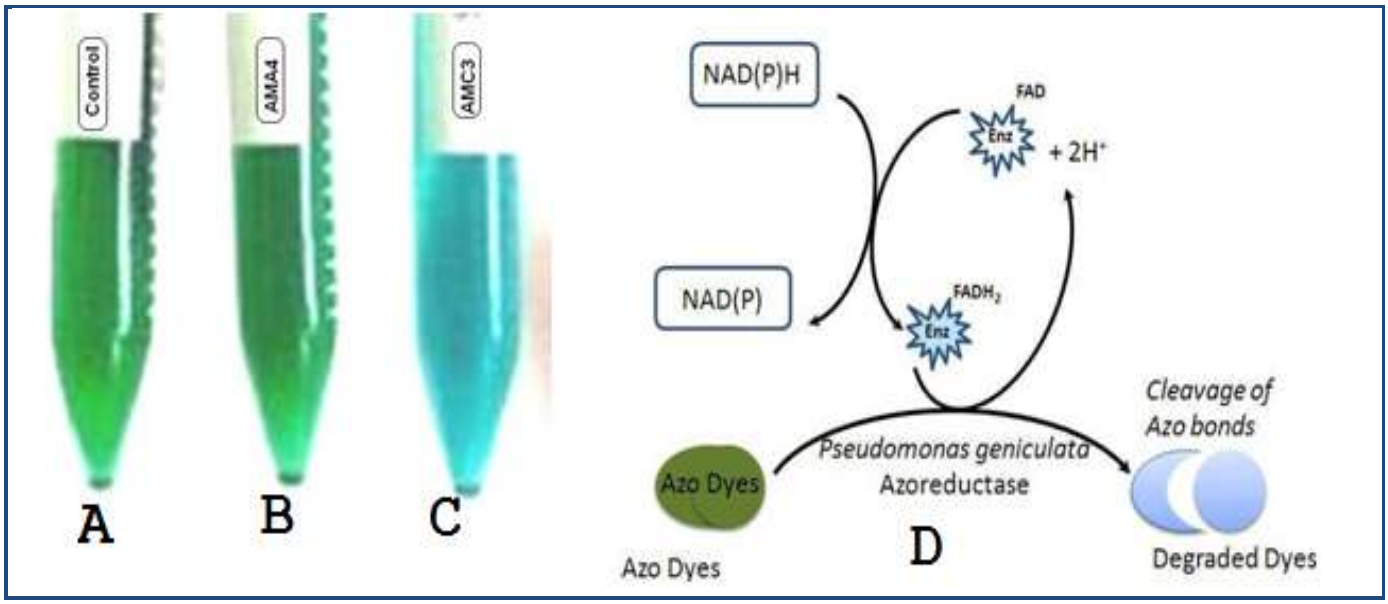

Figure 1: **a)** Control(Green); **b)**Culture tube showing no color change (Green); **c)** Culture tube showing color change from green to blue and **d)** proposed mechanism of color change, the cleavage of Azo bonds of Acid Green Dye by *Pseudomonas geniculata* azoreductase (PgAzoR) enzymes results in smaller molecule with blue color.

Screening of dye decolorizing bacterial cultures

Decolorization in solid culture media

Each cultures (24 hr. old) was streaked on nutrient agar supplemented with Acid Green dye at the concentration 100 mg/l and incubated at 37°C for 14 days. Uninoculated petriplates were incubated as control. On 14th day plates were observed for disappearance/change of color.

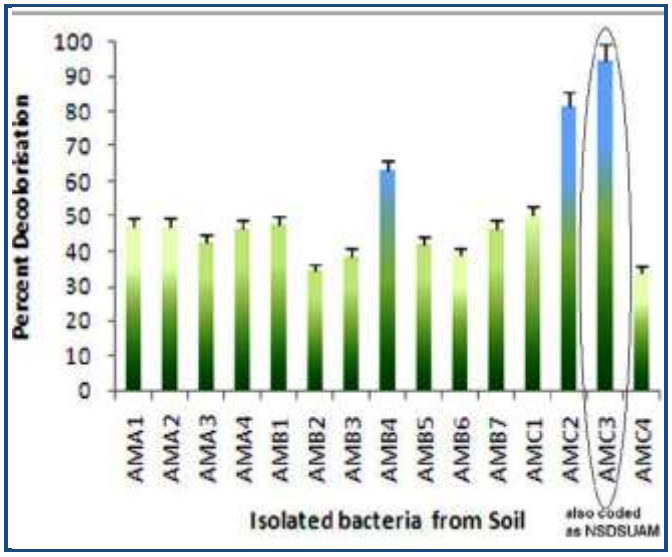

Figure 2: Absorbance values of control and culture supernatant at dye specific wavelengths.

Decolorization in liquid culture media

Nutrient broth (20ml) supplemented with Acid Green dye (100 mg/l) was poured inoculated with 24 hr old bacterial culture (5% inoculum) in culture bottles. Uninoculated culture bottles were taken as control. The inoculated and control bottles were incubated at 37°C for 14 days to observe disappearance/change of color. After 14days 10ml sample was withdrawn from each of the culture as well as control tube and centrifuged at 10,000 rpm for 10 min. The supernatant were analyzed by spectrophotometer and thin layer chromatography (TLC).

Spectrophotometric analysis

Absorbance of the supernatant was recorded at dye specific wavelength: 602nm (Visible range). Sterilized nutrient broth was used as blank. Experiments in triplicate were recorded for mean values along with standard deviation values. Absorbance values of samples and control at 602nm were utilized to calculate Percent decolorization (a measure of decolorization efficiency) for the bacterial cultures according to the following formula:

Percent decolorization = (Ao – A) ×100/Ao

Here: Ao = Absorbance of control at the λmax (nm) of the dye (602nm); A= Absorbance of culture supernatant after incubation period (14 days) at the λmax (nm) of the dye (602nm).

TLC analysis

To perform TLC analysis, stationary phase of silica gel (50gm; Merck India) with gypsum (7.5gm; Qualigens) and mobile phase solvent system ammonium hydroxide and 1-propanol (1:6) was used to separate degraded dye from cultures with highest degrading potential in experiment. The separated spots were visualized after spraying with conc. HCl and incubated at 110^{o}C for 8 min.

Bacterial species identification using 16SrRNA sequence analysis

The sequencing of 16S rRNA gene culture was done from Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh, India (http:// www.mtcc .imtech. res. in). Pairwise sequence alignment of 16S rRNA gene sequence was performed to identify closely related homologs with the help of search tool available at EZTaxon webserver (http://www. eztaxon.org). Bacteria was grown on Nutrient agar medium for 24 hr at 37^{O}C and genomic DNA was isolated from the cell mass according to the method described by ZR Fungal/ Bacterial DNA Miniperp kit manufacturer. The 16S rRNA gene was selectively amplified by PCR using universal primers. Amplification was carried out with an initial incubation (5 min, 94^{O}C) followed by denaturation (35 cycles of 1 min at 94^{O}C), annealing (1 min, 58^{O}C) and elongation (1min, 72^{O}C). The reaction was terminated with a final extension at 72^{O}C for 10 minutes. The 16S rRNA gene sequencing of PCR product was achieved using three different internal primers with three different sequencing PCRs.

Figure 3: TLC plate did not show any spots for supernatant of dye sample treated with **B)** AMC3 and **C)** AMC2 while **A)** Acid Green Dye Sample showed 3 spots.

Result & Discussion:

Isolation and purification of bacteria from soil sample

The bacterial cultures obtained from soil samples pretreated with 0.5%, 1% and 2% Acid Green Dye, were characterized on the basis of colony characteristics and Gram's reaction. The isolates were quite dissimilar on comparison of colony characteristics. There were 15 bacterial cultures were isolated from soil sample **Table 1 (see supplementary material)** and classified as per appearance of colony morphology and Gram staining. On the basis of colony morphology it was observed that the cultures were either white or pale yellow in color, whereas on the basis of the remaining parameters (shape, elevation & optical characteristics) the cultures were showing variable morphology. These isolates were further screened for their dye decolorization capabilities.

Dye decolorizing bacterial cultures

Decolorization of dye solution may take place in two ways, either adsorption on the microbial biomass or biodegradation of the dye molecules by the bacterial cells. Dye adsorption may be evident from the inspection of the bacterial growth, those adsorbing the dye will be deeply colored (similar in color to the adsorbed dye), while those degrading the dye will remain colorless. In this experiment all of the isolate were found to be colored with dye Acid green after completion of decolorization experiment, indicating that these isolates were decolorizing the dye mainly by adsorption but the biodegradation also cannot be ruled out. The dye supplemented nutrient broth supported growth of bacteria that able to use dye as energy source using Azoreductase enzymes. On the visual analysis of the culture tubes, all culture tubes were showing slight to almost complete change in color from green to blue **(Figure 1)**. The bacteria adsorbed dyes to reduce using Azoreductase that relies upon tautomerisation of the substrate to the hydrazone form **[10].** Azo-reductase activity is associated with various Gram positive and Gram negative bacteria. The bacterium *Pseudomonas aeruginosa* has three azoreductase genes, paAzoR1, paAzoR2 and paAzoR3, which as recombinant enzymes have been shown to have different substrate specificities **[11].**

The efficacy of microorganisms in degrading certain dye components of the effluent is somewhat compromised by the complexity of the effluent composition itself, in addition to other conditions, such as extreme alkaline pHs, high salt concentrations, and high temperatures **[12-16].** Bacteria from the *Pseudomonas* genus have shown noteworthy metabolic robustness and versatility in soil and wastewater bioremediation.

Azo dye are not carbon or energy source for bacterial metabolism, but required some other organic compounds (co-substrates), to act as acceptors of electrons that are supplied through the reducing equivalents that are generated by the electron transport chain. Brige´*et al.* **[17]** demonstrated that dye decolorization is an extracellular reduction process requiring a multicomponent electron transfer pathway that consists of cytoplasmic membrane, periplasmic, and outer membrane components. Similarly, we have obtained the ability of bacteria to decolorize Acid Green dye from Nutrient Agar, which suggested the accumulation of redox active enzymes or biochemical substances that were released into the medium during growth of the bacterial cells **[18].** These studies imply that reducing equivalents are transferred from an intracellular electron transport chain to the mediators **(Figure 1d),** which consequently reduces the extracellular dye non-enzymatically. Another possibility is that the bacteria establish a link between their intracellular electron transport systems and the extracellular dye via electron transferring proteins in the outer

membrane **[19].** Spectrophotometric analysis indicated the most promising cultures were AMC2 and AMC3 showing 81.3% and 94.6%, respectively. Dye Decolorizing efficiency: Spectrophotometric analysis showed that all the cultures were showing absorbance values lower than the control, at both the dye specific wavelengths i.e.; 602nm **(Figure 2).**

All the 15 cultures were showing decolorization capacity 33.9% to 94.6% **(Figure 2).** The culture no. AMC4, AMB2, AMB3, AMB6, AMB5, AMA3, AMA4, AMB7, AMA2, AMA1, AMB1 were not showing any color change in comparison to control **(Figure 2)** but on the basis of spectrophotometric analysis they were showing slight decolorization (33.9% to 47.8%), respectively. The cultures showing color change from green to blue in comparison to control included culture AMC1, AMB4, AMC2, and AMC3. Only culture AMC3 was able to decolorize up to 94.6% at 602nm and considered as the most promising cultures. Cultures AMC2 could be also considered as good cultures since it was showing more than 80% decolorization. After this AMC3 was coded as NSDSUAM and selected for 16SrRNA sequencing analysis. The thin layer chromatographic analysis of supernatant from dye sample degraded by culture and Acid Green dye sample showed 3 spots having Rm value 0.258, 0.516 and 0.761 respectively. In case of samples AMC3 and AMC2 no spot was obtained after development **(Figure 3A, 3B & 3C).** Observations suggest that the Acid Green dye might have been degraded by the respective bacterial cultures during the incubation period.

TLC analysis of AMC2 and AMC3 was confirmed the decolorization of dye by adsorption as well as metabolic enzymes like azoreductase since no dye specific spot could be obtained after incubation in comparison to dye sample. 16SrRNA sequencing: A total of 1383 bp of the 16S rRNA gene was sequenced and used for the identification of isolated bacterial strain. The 16S rRNA sequencing suggested that it belongs to *Pseudomonas geniculata* (99.85% pairwise similarity) with difference of 2 base pairs to reference strain *Pseudomonas geniculata* ATCC 19374(T) (bp1358). The identified strain could be a variant as having property of degrading Acid Green Dyes. The 16SrRNA gene sequence was also submitted to NCBI genebank with accession number KP238100.

Conclusion:

Pseudomonas genus having noteworthy metabolic robustness and versatility in soil and wastewater bioremediation. This has earned *Pseudomonas* species preference as a laboratory model for pure culture systems, and with azoreductase genes they can be a component of mixed bacterial consortia for azo dye treatment under both aerobic and anaerobic conditions. Therefore, such bacteria can be used to develop an effective biological treatment system for the wastewaters contaminated with dyes.

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Supplementary material:

Table 1: No. of bacterial cultures isolated from soil samples

Sample no	Type of cultures isolated	Name of isolated cultures
A (0.5% dye)	4	AMA1, AMA2, AMA3, AMA4
B (1% dye)	7	AMB1, AMB2, AMB3, AMB4, AMB5, AMB6, AMB7
C (2% dye)	4	AMC1, AMC2, AMC3, AMC4