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Research Paper

Microbial decolorization of Methyl Orange by *Klebsiella* spp. DA26

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Abstract

Synthetic dyes have increasingly been used in the textile and dyeing industries because of their ease and cost-effectiveness in synthesis, firmness, high stability to light, temperature, detergent and microbial attack and variety in color compared with natural dyes. This has resulted in the discharge of highly polluted effluents. physicochemical treatment methods are not economically feasible as they produce large volume of sludge. Besides the conventional physico-chemical methods, microbial degradation of azo dyes has been attracted significant attention. In present study methyl Orange as an azo dye was used for biodegradation study by using indigenous bacterial isolates from contaminated soil. The potent decolorizing bacterial strain, was isolated and screened for dye decolorization and identified as Klebsiella spp. DA26 on the basis of morphology, cultural and biochemicals and further 16s rRNA sequencing was carried out. In Phlogenetic tree, isolate had shown 95% similarity with *Klebsiella oxytoca*. The isolate had showed 86.9% decolorizing activity under optimized condition through a degradation mechanism rather than adsorption as seen by decrease in peak at 530nm within 48 hrs. In presence of penicillin and Chloramphenicol- 61.68% and 14.83% dye decolorization was seen. The Rf values 0.79 and 0.85 for the dye degraded products and 0.93 for parental dye compound was observed during TLC of biodecolorized broth. In FTIR spectra of biodecolorized broth, absence of peaks at 1600.92/cm and 1517.98/cm indicate stretching of –C=C- aromatic skeletal bond and –N=N– bond. On ANOVA test, all the data obtained was significant at 0.5% level. The toxicity of dye and degraded products is under study. These observations has established that the bacteria are adaptive in nature and can degrade dye contaminants. The novel dye degrading bacterial isolate has been isolated. However, potential of the strain needs to be demonstrated for its application in treatment of real dye bearing waste waters using appropriate bioreactors.

Keywords: Azo dyes, Bioremediation, Decolorization, Dye degradation, Methyl Orange.

Introduction

Rapid industrialization has necessitated the manufacture and use of different chemicals in day to day life**.** The textile industry is one of them which extensively use synthetic chemicals as dyes. Synthetic dyes have increasingly been used in the textile and dyeing industries because of their ease and cost-effectiveness in synthesis, firmness, high stability to light, temperature, detergent and microbial attack and variety in color compared with natural dyes. This has resulted in the discharge of highly polluted effluents. Normally colour is noticeable at a dye concentration higher than 1 mg/L and an average concentration of 300 mg/L has been reported in effluents from textile manufacturing processes. 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.

Biological treatment is a more natural wastewater treatment process than other treatment methods. Microorganisms feed on the complex materials present in the wastewater and turn them into simpler substances, preparing the water for further treatment. Biodegradation is the chemical dissolution of materials by bacteria or other biological means. Azo dyes, characterized by nitrogen to nitrogen double bonds (–N=N–), account for up to 60-70% of all textile dyestuffs produced[1], and are the most common chromophore in reactive dyes. Reactive azo dyes are very soluble by design and, as a result, not all are exhausted by textile fibers during the dyeing process and therefore end up in the discharge from dye houses. The reactive azo dyes-containing effluents from these industries have caused serious environment pollution[1-4]. There are several ways in which colorants cause problems in waters. (1) Depending on the exposure time and dye concentration, dyes can have acute and /or chronic effects on exposed organisms. (2) Even minor releases of effluents may cause abnormal coloration of surface water, which captures the attention of both public and the authorities. (3) Dyes can remain in the environment for an extended period of time, because of high thermal and photo stability. For instance, the half life of hydrolyzed Reactive Blue 19 is about 46 years at pH 7 and 25°C. (4) Many dyes and their breakdown products are carcinogenic, mutagenic and / or toxic to life. Dyes are mostly introduced into the environment through industrial effluents. (5) Textile dyes can cause allergies such as contact dermatitis and respiratory diseases, allergic reactions in eyes, skin irritation to mucous membrane and the upper respiratory tract. (6) The greatest environmental concern with dyes is their absorption and reflection of sunlight entering the water. This interferes with the growth of bacteria to levels sufficient to biologically degrade impurities in the water and start the food chain.

Physical and chemical methods[1,2] for e.g. Ozonation, Photochemical, Activated carbon, Peat , Membrane filtration, Electro kinetic coagulation etc[5], But due to demerits of physicochemical methods viz. cost, time consuming, methodological demanding, sludge disposal and coagulation, they are not in use. Bioremediation is an only option that offers the possibility to destroy or render harmless various contaminants using natural biological activity. As such, it uses relatively low- cost, low technology techniques, which generally have a high public acceptance and can often of carried out on site.

Table 1: Advantages and Disadvantages of Physicochemical methods for dye removal [5]

Physical / Chemical methods	Advantages	Disadvantages
Fentons reagent	Effective decolorization of both Soluble and insoluble dyes	Sludge generation
Ozonation	Applied in Gaseous state: No alteration of volume	Short haif life (for 20 Min.)
Photochemical	No sludge production	Formation of by- products
NaOCl	Initiates and accelerates azo bond cleavage	Release of aromatic amines
Cucurbutir	Good sorption capacity for various dyes	High cost
Electrochemical destruction	Breakdown compounds are not hazardous	High cost of electricity
Activated carbon	Good removal of wide variety of dyes	Very expensive
Peat	Good adsorbant due to cellular structure	Specific surface area for adsorption are lower than activated carbon
Wood Chips	Good sorption capacity for acid dyes	Requires long retention time

Bioremediation is defined as the process whereby organic and /or inorganic wastes are biologically degraded under controlled conditions to an innocuous state, or to levels below concentration limits established by regulatory authorities. It is a non- destructive, cost and treatment effective and sometimes logistically favorable clean up technology, which attempts to accelerate the naturally occurring biodegradation of contaminants through the optimization of limiting conditions. It uses naturally occurring bacteria and fungi or plants to degrade or detoxify substances hazardous to human health and / or the environment[4]. The microorganisms may be indigenous to a contaminated area or they may be isolated from elsewhere and brought to the contaminated site. It also involves use of enzymes extracted from microorganisms used in an free / immobilized form. Contaminant

compounds are transformed by the living organisms through reactions that take place as a part of their metabolic processes. They generally use the contaminant compound as a source of carbon and energy for growth and multiplication. However some compounds may be used only as a source of energy (electron donor) or may be degraded by co-metabolism. The bacteria synthesize an azo reductase which under controlled reductively cleave the azo group in presence of oxygen. The reduction of azo dyes result in the formation of aromatic amines which accumulate after azo cleavage are not mineralized anaerobically with the exception of a few aromatic amines substituted with hydroxyl and carboxyl groups which were fully degraded under anaerobic conditions. Generally bacterial azo dye biodegradation proceeds in 2 stages[4,6,8] (Figure 2). The 1st stage involves reductive cleavage of the dyes. Azo linkages resulting in the formation of generally colorless but potentially hazardous aromatic amines. The second stage involves degradation of the aromatic amines. azo dye reduction usually requires anaerobic conditions, where as bacterial biodegradation of aromatic amines is an almost exclusively aerobic process.

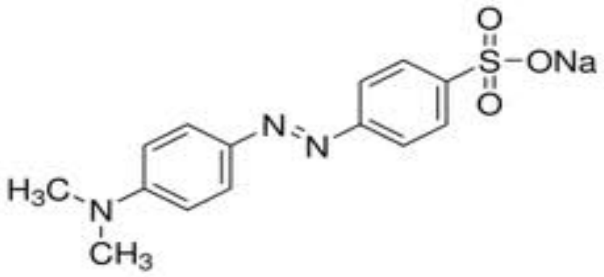

Figure 1: Structure of Methyl Orange dye

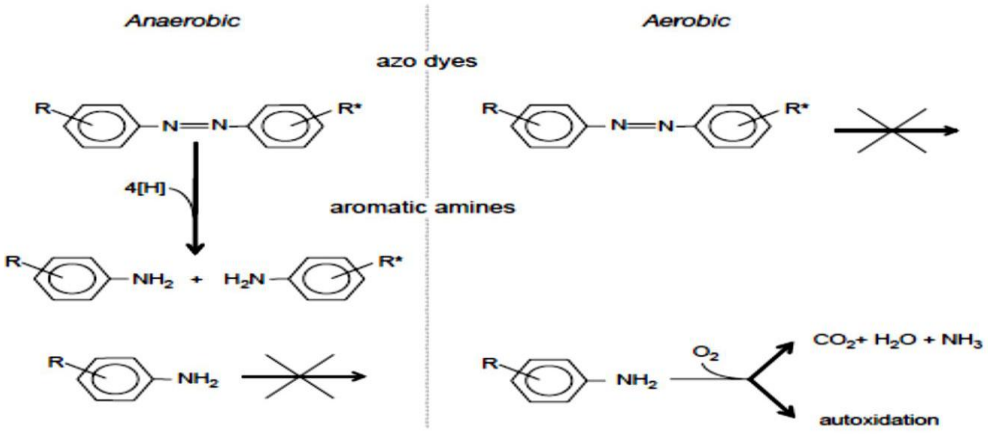

Figure 2: Mechanism of azo dye degradation[3]

In the present study, a bacterium was isolated from dye contaminated environment capable of decolorizing and degrading a textile azo dye Methyl Orange. The decolorization of the dye was monitored spectrophotometrically (Systronics) at its specific absorbance maxima (λmax) 465nm. Methyl Orange is an azo dye with chemical structure formula Sodium 4-(4- dimethyl aminophenylazo) benzene sulphonate (Figure 1) $(CH_3)_2NC_6H_4N{=}N\ C_6H_4\ SO_3$ Na and Mol. wt. 327.33 g/mol with CAS No. 502-02-3 and C.I. No. 13025.

Materials and Methods

Dye and chemicals were purchased from Loba Pvt. Ltd., Mumbai. and Hi media pvt. Ltd., Mumbai.

Screening for dye decolorizing bacteria

Soil near to textile industrial outlet was used as source for enrichment and isolation of decolorizers. The mineral salt medium (MSM) of following composition (g/L) Na_2HPO_4 (3.6), $(NH_4)SO_4$ (1.0),

KH_2PO_4 (1.6), $MgSO_4$ (1.0),Fe (NH_4) Citrate (0.01), $CaCl_2$ $_2H_2O$ (0.1) and 10.0ml of trace elements soln per liter was used for all studies. The trace element solution used was of following composition (mg/l) $ZnSO_4$ $7H_2O$ (10.0), $MnCl_2$ 4 H_2O (3.0), $CoCl_2$ $6H_2O$ (1.0), $NiCl_2$ $6H_2O$ (2.0), No_2MoO_4 $2H_2O$(2.0), H_3BO_3 (3.0) and $Cucl_2$ $2H_2O$ (1.0). the final PH of the medium was adjusted to 7.0± 0.2. The MSM was supplemented with 0.1% (w/v)Yeast extract, 0.2% Glucose and 100 mcg/ml of dye . 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. Five grams of soil was then added to a 500-ml Erlenmeyer flask containing 100 ml of medium. The cultures were incubated at room temperature. For Every experiment, Mineral Salt medium supplemented with Yeast Extract, Glucose and dye was used as decolorization medium otherwise in case of any change it is mentioned. The broth of the decolorized flask was transferred to fresh medium and incubate for 3 days to screen the strain having color removing ability The enrichment process was repeated for five times.

In between every enrichment, enriched broth was streaked on the solidified media to get dye decolorizing isolates. The dye decolorizing bacterial isolates were streaked on Nutrient agar plate (Composition (g/l , Peptone 10.0, Yeast extract 3.0 , NaCl 5.0 and Agar 15.0 pH 7.2±0.2) . The screening procedure was carried out using Mineral salt medium supplemented with dye as a carbon and /or Nitrogen source and able to decolorize high concentration of dye[7]. The potent decolorizing isolate was, identified on the basis of Morphology, Cultural and Biochemicals as per Bergey's manual of Determinative Bacteriology (9^{th} Ed.2000) and the final identification of isolate was based on 16S rRNA sequencing and BLAST analysis.

Bacterial identification by 16S rRNA gene amplification and sequencing with BLAST analysis

DNA Extraction was carried out using HiPurA Bacterial Genomic DNA Purification Kit (Himedia, MB505). Concentration of DNA was determined using UV-1800 spectrophotometer (Schimadzu Corporation A11454806498). The DNA was stored at -20^0C for further use. The DNA isolated from bacteria was subjected to polymerase chain reaction (PCR) amplification using Biometra thermal cycler (T-Personal 48) The reagents used are procured from GeNei. Gel electrophoresis was performed using 1.0% agarose (Seakem, 50004L) to analyze the size of amplified PCR product. The PCR product was purified using AxyPrep PCR Clean up kit (Axygen, AP-PCR-50). The sample was sequenced using Applied Biosystems 3730xl DNA Analyzer USA and chromatogram was obtained. The DNA sequences were analyzed using online BLASTn (nucleotide Basic Local Alignment Search Tool) facility of National Center for Biotechnology Information (NCBI). The tree was constructed by using MEGA 5 software [10-12]

Effect of Penicillin and Chloramphenicol on dye decolorization

To study the effect of antibiotics on dye decolorization, two antibiotics were selected - Penicillin affecting cell wall synthesis and Chloramphenicol affecting protein synthesis. Before adding in media, the Minimum Inhibitory Concentration (MIC) of the antibiotics for the given isolate was checked. According to the MIC values, antibiotic concentration was maintained in the media to study its effect on dye decolorization by isolate. The Control experiment was carried out by keeping all conditions similar except antibiotic additions [12].

Thin Layer chromatography

The bacterial strain was inoculated in minimal medium containing 100 mg/l of Methyl Orange and incubated under optimized conditions. After complete decolorization, 40 ml of dye degraded sample was taken and centrifuged at 10,000 rpm for 10 minutes. Then the supernatant was filtered through whattman No.41 filter paper. The Filtrate was extracted three times with diethyl ether, pooled and evaporated to dryness. The degraded products were analysed by TLC and FTIR. The solvent system Butenol : Water:acetic acid 5:3:2 was poured into the TLC tank. The extracted sample was dissolved in 0.5 ml of methanol and was placed on TLC plate of 3.5x9.0 cm and allowed to run in a TLC tank. After the run was over, the TLC plate was taken out and air dried. The plate was observed under UV light and the Rf values of the bands were recorded.[7]

Change in UV-Visible spectra during dye decolorization

The decolorization experiment was performed in three sets. The isolate with 4% inoculant with OD 0.699 at 540 nm was inoculated in 250 ml Erlenmeyer flask containing 100ml Decolorization medium

and incubated under optimum conditions viz. pH-7.0, Temperature 30ºC, under static conditions with a Incubation period of 120 Hrs, and 86.9% decolorization of methyl orange(Data not shown). At interval of 24h of incubation, 5 ml of the culture media was withdrawn and centrifuged at 6000 rpm for 10 minutes to separate the bacterial cell mass. The clear supernatant was used to measure the decolorization at the absorbance maxima of the dye. Abiotic control (without microorganism) was always included[12].

Percentage decolorization was calculated as follows

$$\text{Decolorization (\%)} = 100 \times \frac{\text{Initial OD Observed} - \text{OD after Decolorization}}{\text{Initial OD}}$$

Biodegradation analysis by FTIR

After complete decolorization, the decolorized medium was centrifuged at 10,000 rpm for 20 min. and supernatant obtained was used to extract metabolites with equal volume of Dichloromethane. The extract was dried over anhydrous Na_2SO_4 and evaporated to dryness in rotary evaporator. The crystals obtained were dissolved in small volume of methanol and used for UV – Visible spectral analysis using Systronic UV- Visible spectrophotometer 117 and changes in absorption spectrum of the decolorized medium was recorded (300-700 nm) and compared with control. The FTIR analysis of extracted metabolites was done on Shimadzu, spectrum and compared with control dye in the mid IR region of 500-4500 cm^{-1} with 16 scan speed. The samples was mixed with spectroscopically pure KBR in the ratio of 5:95[12].

Statistical analysis

Experiments were performed at least 3 times and results were presented as mean ±SD (standard deviation).The ANOVA was used to evaluate significant differences between groups at a 0.05 significance level.

Results and Discussion

The total 72 isolates were obtained on primary isolation using Mineral salt medium supplemented with 0.1%Yeast extract, 0.2%Glucose and 50 mcg/ml methyl Orange dye after enrichment. From 72 isolates, the total 39 isolates were selected based on their luxuriant growth and using dye as Carbon & / or Nitrogen source. On secondary screening, 07 isolates were selected on the basis their ability of dye decolorization. The potent dye decolorizing isolate Number DA26 showed luxuriant growth, No lag period during growth / decolorization and highest dye decolorization and selected for further studies. The potent methyl orange dye decolorizing isolate was morphologically, and biochemically identified as *Klebsiella* spp.with reference to Bergey's manual of Systematic Bacteriology[13](Table 2).

Table 2: Biochemical Identification of Isolate DA26

Test	**Isolate - DA26**
Morphology	Short rods/coccobacilli
Gram nature	Gram negative
Cultural characteristics on Nutrient agar medium	Big, Mucoid, Dirty white, Glistening colony
Mannose	A
Arabinose	A
Trehalose	A
Galactose	A
Lactose	A
Fructose	A
Dulcitol	A
Cellobiose	A
Adonitol	-
Sorbitol	A
Dextrose	A
Rhamnose	A
Raffinose	-

Inulin	-
Inositol	A
Mannitol	A
Sucrose	A
Maltose	A
Melibiose	A
Xylose	-
Salicin	A
Indole production	+ve
Lysine Decarboxylase	+ve
Ornithine Decarboxylase	-ve
Nitratase production	+ve
Urease production	+ve
Oxidase production	+ve
Desulphurase production	-ve
Citrate utilization	+ve
Catalase production	+ve
Identification as per Bergey's manual	*Klebsiella* spp. DA26

Key: A- Acid Production, +ve – Positive results, -ve- Negative results.

The evolutionary history was inferred using the Neighbor-Joining method (Figure 3). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura-Nei method and are in the units of the number of base substitutions per site. The analysis involved 21 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 880 positions in the final dataset. Evolutionary analyses were conducted in MEGA5. The final identification was carried out by using 16s rRNA sequencing and BLAST analysis where isolate *Klebsiella* spp. DA26 had shown 95% similarity in phylogenetic tree ,with *Klebsiella oxytoca* with accession number NR0417449.1 [10-12].

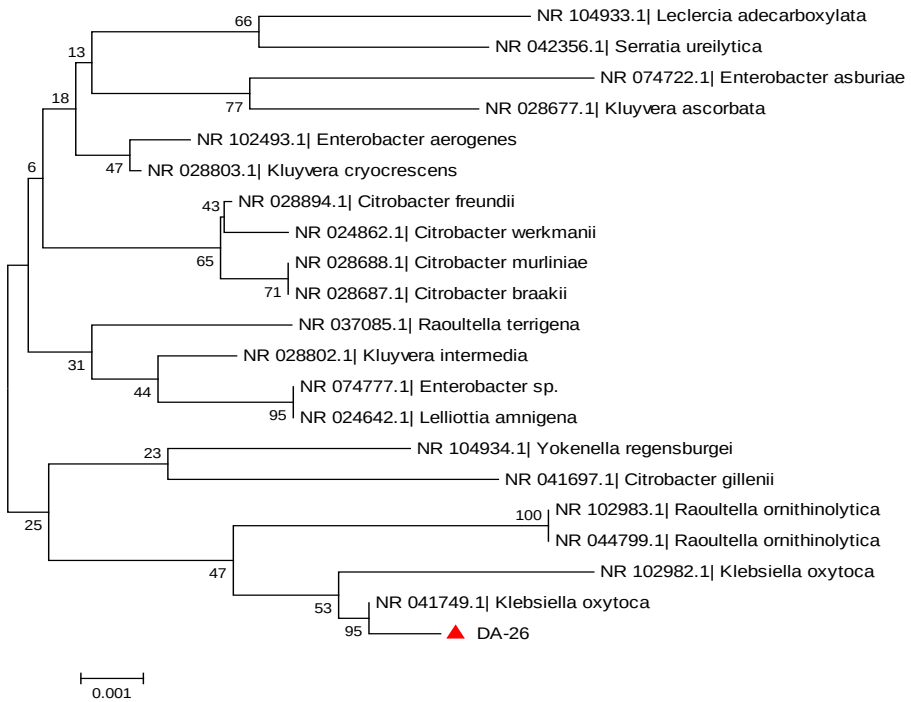

Figure 3: Phylogenetic tree for isolate DA26

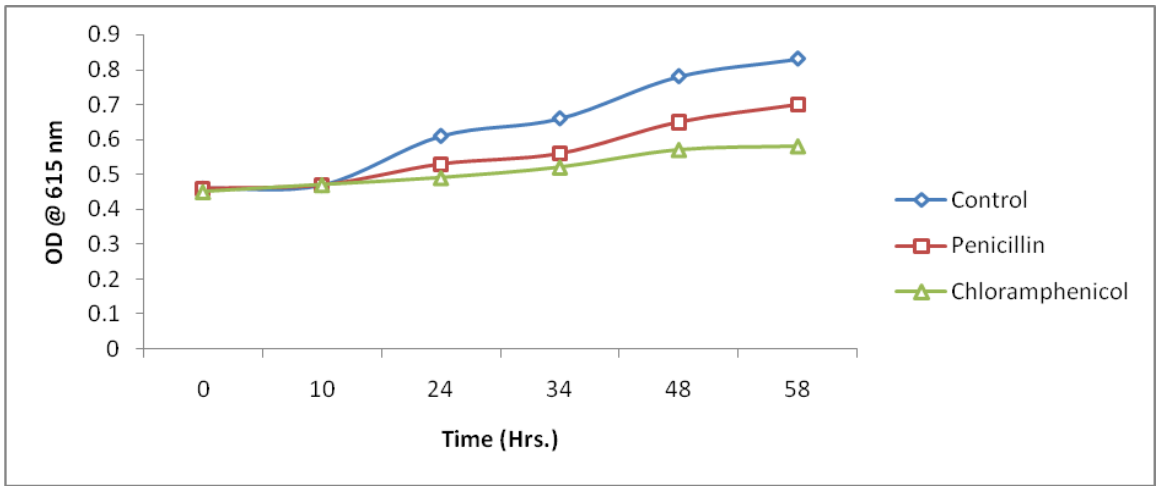

Figure 4: Effect of antibiotics on Biomass production by *Klebsiella* spp. DA26

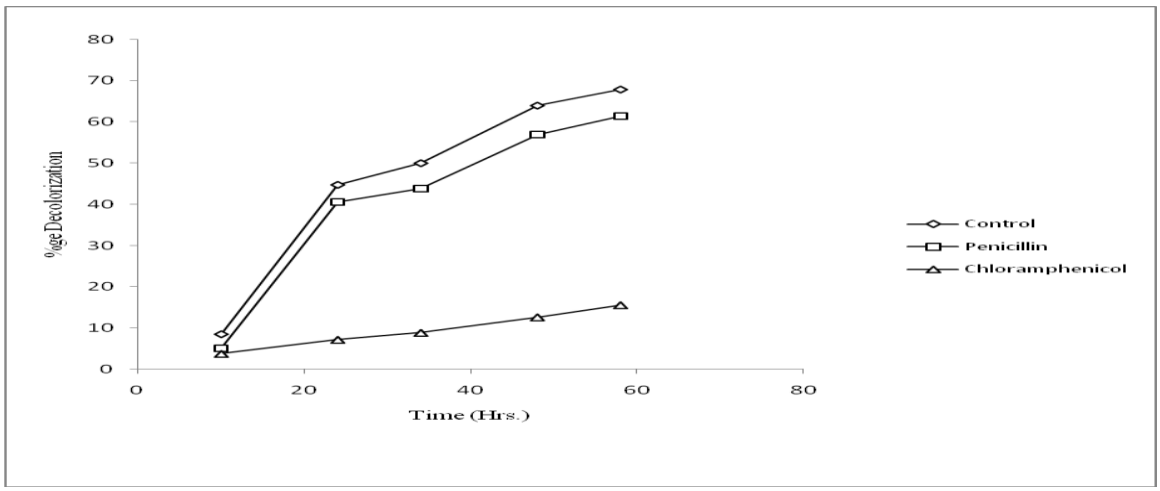

Figure 5: Effect of antbiotics on decolorization by *Klebsiella* spp. DA26

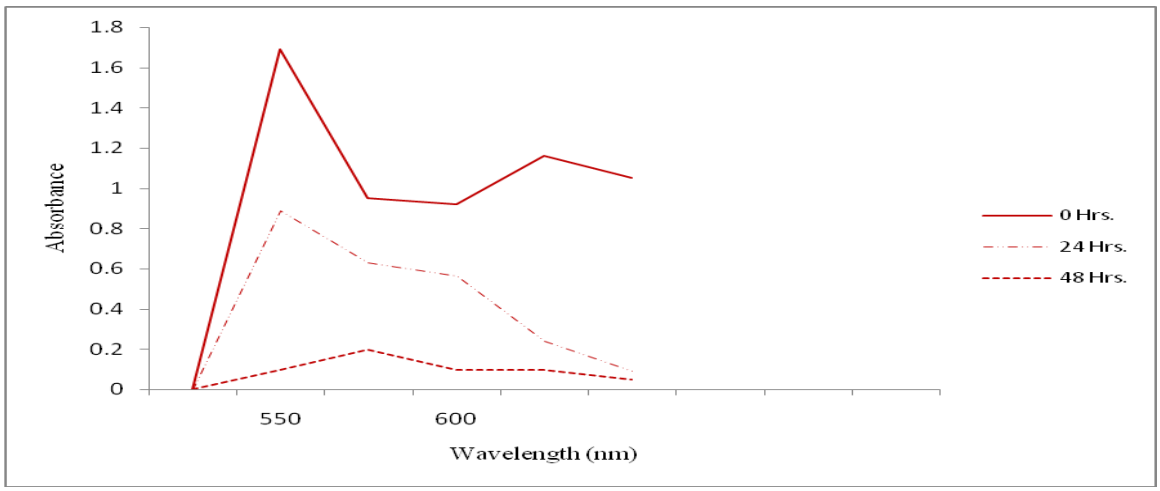

Figure 6: Change in UV – Visible spectra of Methyl Orange during decolorization by *Klebsiella* spp. DA26

Growth and decolorization in presence of antibiotics was studied by using penicillin G and Chloramphenicol in 2.5 u/ml and 15 mcg/ml with respect to their MIC for the isolate. Penicillin G inhibit

cell wall synthesis and Chloramphenicol inhibits protein synthesis. Cell growth impaired greatly in Chloramphenicol and cell grew in presence of penicillin G. Effect of antibiotics on decolorization was observed in Fig.-5, which reveals about 23.84%of dye removal in the presence of Chloramphenicol and76.69 % in presence of Penicillin G. These findings indicate the major role of enzymes in dye decolorization. Our results are in comparison to the findings of Chen et al., (1999) who had reported the drastic effect of Chloramphenicol on Methyl orange decolorization during their study on Microbial decolorization of azo dyes by *Proteus mirabilis*[9].

UV- Visible analysis has been used to confirm the decolorization of dye. In the present study, the decolorization of Methyl Orange was carried out by isolate *Klebsiella* spp. under optimized conditions and due to the biodegradation and is not merely a visible decolorization. Spectrophotometric analysis of Methyl Orange showed a maximum absorbance at 550nm. On incubation for 24 Hrs, decrease in original peak at 550nm and generation of new peaks were seen (Figure 6).The removal of dye is attributed to the process of biodegradation, when, a major visible light absorbance peak would completely disappear or a major peak appear . Our results are matching with the findings of Chen et al., (1999),who had reported the disappearance of original peaks with generation of new peaks during study of microbial decolorization of azo dyes by *Proteus* spp[9].

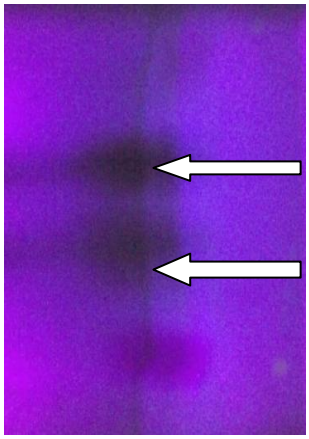

Figure 7: Thin Layer Chromatography analysis of biodegraded Methyl Orange by *Klebsiella* spp. DA26

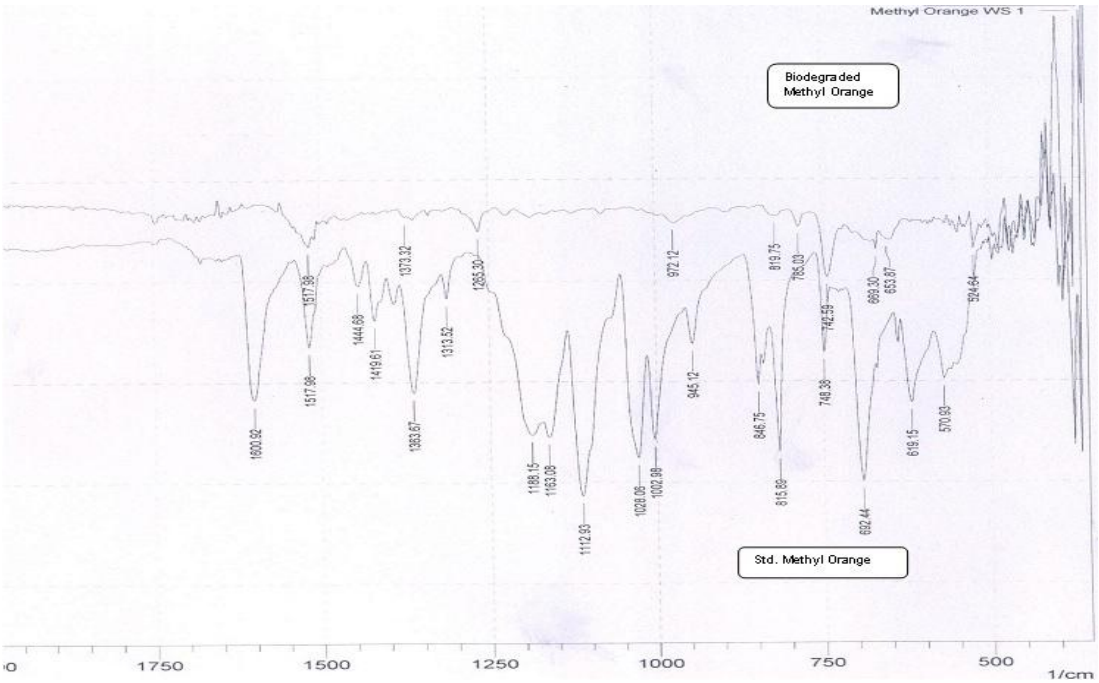

Figure 8: FTIR spectra analysis of Methyl Orange dye and its biodegraded products

The appearance of two different spots in the degraded sample (Fig.7) with R_f values of 0.79 and 0.85 as compared with the R_f value (0.93) of original dye. Our findings are in comparison with findings by other authors Franciscon Elisangela, etal.,(2012), who studied about Decolorization and biodegadation of reactive sulfonated azo dyes by a newly isolated *Brevibacterium* spp. Strain VN-15. [7]. The FTIR spectrum of standard dye compared with degraded product is shown in figure 8. In case of standard dye, peak at 1600.92 /cm (-N=N- vibration), peak at 1517.98/cm and 1444.68/cm may be characteristic of-C=C- aromatic skeletal vibration and azo linkages on aromatic structures, band at region 1363.67/cm and 1419.61 /cm originate from CH_3 symmetric vibration and $–CH_2$ asymmetric stretching vibration. The peaks at 1112.93, 1163, 1165.15, 1002.98 and 1028.06 can be assigned to CO, CN or phenolic C-O vibration, all these mentioned peaks were absent in biodegraded product and show the stretching of attached groups on the benzene ring structure. The FTIR spectrum of degraded product by a *Klebsiella* spp. DA26 display peak at 1112.90 /cm which points towards the formation of C=O stretching. This C=O group may be generated by the oxidizing action of laccase enzyme which may have converted the aromatic structure to quinine like structure. The peak generated at 815.80/cm, 945.12/cm, 845.75/cm represent stretching C-O-C stretching. The FTIR pattern of degraded metabolite does not have a peak at 1600.92/cm, which indicates loss of azo bond [15,16]. Telke A. et al., (2010) had reported the similar results for disappearance of peaks during study of Biochemical characterization and potential for textile dye degradation of Blue Laccase from *Aspergillus ochraceus* NCIM-1146[16]. On ANOVA analysis, all the values were significant at 0.05% level with obtained F- value less then F critical value, and the P-value was less then 0.5.

Conclusion

In this study, a potent Methyl orange decolorizing bacterial strain was isolated, screened for dye decolorization, and identified as *Klebsiella spp.* DA26 on the basis of morphology, cultural, and Biochemical study and on 16s rRNA identification. The *Klebsiella* spp. DA26 had showed 95% similarities with *Klebsiella oxytoca* with accession number NR041749-1.the isolate had showed decolorizing activity through a degradation mechanism rather than adsorption. With high degrading and decolorizing activity against Methyl Orange dye commonly used in the textile industries, It is proposed that *Klebsiella* oxytoca. DA26 has a practical application potential in the biotransformation of various dye effluents. 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. The toxicity study of the Methyl orange dye degraded by isolate and optimization of various environmental and nutritional factors affecting decolorization are in progress. This observation has established that the bacteria are adaptive in nature and can degrade contaminants. However, potential of the strain needs to be demonstrated for its application in treatment of real dye bearing waste waters using appropriate bioreactors. Degraded products can be identified to draw pathway followed by isolate in methyl orange degradation.

Acknowledgement

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