BIOREMEDIATION OF TEXTILE DYE WASTEWATER BY *KLEBSIELLA PNEUMONIAE* STRAIN P FROM DYE EFFLUENT

Berde C.V.[1] and Salvi S.P.[2]
Assistant Professor and Head[1], Assistant Professor[2]
Department of Microbiology
Gogate Jogalekar College, Ratnagiri. 415612

Abstract: Textile industries are the major users of dyes in the world. A huge fraction of dyes are discharged out from the textile industries, causing serious damage to the environment such as water resources, soil fertility, aquatic organisms and ecosystem integrity. Bioremediation based technologies has been proved to be the most desirable and cost- effective method to counter textile dye pollution. The ability of the microorganisms to decolorize and metabolize dyes can be employed to treat the environment polluted by textile dyes. In this work, a total of 21 bacterial strains were isolated from textile dye effluent sites Pawne region, Mumbai, Maharashtra and screened for their ability to decolorize three widely used textile dyes- Blue , Reactive Red and Reactive Yellow. The 16S rRNA sequencing of isolate B11 showed high similarity (97%) with *Klebsiella pneumoniae* and designated as strain P. Use of aerobic treatment by consortium was successful decolorization and degradation of dye house effluent. Bioremediation has proved to be very effective method in countering the textile dye pollution in an eco-friendly way.

Index terms - **Bioremediation, Dyes, Textile effluent, Wastewater treatment.**

I. INTRODUCTION

Environmental pollution is one of the major problems of the modern world. Industrialization is necessary to satisfy the needs of the world's growing population but it threatens life on earth by polluting the environment. The problem of environmental pollution is increasing day by day due to the release of recalcitrant substances like pesticides, dyes, polymers and heavy metals. The continuous dumping of such hazardous compound in the form of waste has lead to contaminate soil, ground water, sediments, surface water etc. at alarming level. The roots of such waste products found to be an industrial, agriculture, energy production industries, textile industries, and printing houses that produce huge amount of wastes, which ultimately find its way in the environment. A dye may be defined as an organic compound containing both chromophore and auxochrome groups linked to benzene ring. Chromophore is responsible for imparting color to the compound and auxochrome imparts the property of electrolytic dissociation. The chromogen-chromophore structure is often not sufficient to impart solubility and cause adherence of dye to fiber. The auxochrome or bonding affinity groups are amine, hydroxyl, carboxyl, and sulfonic radicals, or their derivatives (Bhimani, 2011).

Dyes are colored substance that are used in several substrates such a paper, fabrics, cosmetics etc. They are potentially capable of retaining in the substrates by means of physical absorption and also by making covalent bonding with the metals and salts. Dyes are majorly used in textile and in the printing industries. The paper and textiles are washed for removing the excess of dye present in the material and the water is ultimately released into the water bodies which turns out to be hazardous to the water–thriving creatures thus leading to their to extinction. This has become a major environmental pollution. It has been estimated that about 25% dyes from these industries are being released and this affects the processing of water for drinking purposes (Hemalatha et al. 2016). In India, for the annual production of approximately 30 million tonnes of textiles, 70000 tonnes of dyes are required. These dyes were discharged into the environment after processing causes serious environmental problems. The strong color of discharged dyes even at very small concentrations has a huge impact on the aquatic environment caused by its turbidity and high pollution strength. Removal of dyes from textile effluents has been carried out by physical and chemical methods, such as flocculation, membrane filtration, electrochemical techniques, ozonation, coagulation and adsorption. These methods are effective but they are expensive and involve the formation of a concentrated sludge that creates a secondary disposal problem. Considering drawbacks in above mentioned conventional treatment methods, microbial remediation techniques have gained much attention in the last few decades. A wide variety of microorganisms capable of decolorizing various dyes including bacteria, fungi were screened.

Bioremediation is the use of biological systems (mainly microorganisms and plants) for the treatment of polluted air, aquatic or terrestrial component of environment. The textile industry is major user of water. Reduced water resources due to rapid population growth and industrial development has triggered need to reuse of municipal and industrial waste water after proper treatment and elimination of potential pollutants (Keharia and Madamwar, 2003). Microorganisms can breakdown most compounds for their growth and/or energy need. In some cases, metabolic pathways which organisms follow for its own normal growth and development may also be used to break down pollutant molecules. In this process microorganisms do not benefit directly, but researchers have taken advantages of this phenomenon and use it for the process of bioremediation (Alleman and Lesson, 1999).

II. MATERIALS AND METHODS

2.1Collection of Sample

Textile wastewater samples was collected from industrial outfalls of textile industries in Pawne region, Mumbai, Maharashtra. Wastewater samples were collected in sterile container from different locations and sampling sites treated and untreated sample were collected from outlets of textile units. Collected water samples was brought to the laboratory in sterile condition and was stored at 4^0C in refrigerator till further use.

2.2 Physico-Chemical Characterization of Samples

The effluent samples, mainly before treatment and after treatment were tested for its physico-chemical characteristics like, color, pH, etc. Color was noted visually and pH was measured using pH meter.

2.3Dyes Used In The Study

All dyes including Blue, Orange, Yellow and Basic fuchsin were procured from local dye manufacturing unit Mumbai, Maharashtra. The main dye in this study Blue also known as Navy blue.

2.4Enrichment and Isolation of Dye Degrading Bacteria

Textile effluent samples were used for isolation of dye decolorizing bacterial cultures by enrichment culture techniques using NB medium containing dye (Basic fuchsin) with the final dye concentration of 100 mg l^{-1}. The enrichment was carried out in 250 ml Erlenmeyer flasks containing 100 ml of dye containing medium was inoculated with 10 ml of effluent sample and incubated at R.T. for 48 hrs under static condition. The acclimatised culture so obtained was serially diluted with autoclaved saline (0.85% NaCl) from 10^{-1} to 10^{-10}. From 10-^{8}, 10^{-9}, 10^{-10} dilution, 100 µl of culture was spread on nutrient agar plates and incubated at R.T. for 24 hrs. After 24 hrs of incubation, morphologically distinct colonies were selected and screened for textile effluent decolorization.

2.5Screening of Dye Decolorizing Bacteria

The 21 morphologically distinct bacterial isolates was tested for their ability to decolorize textile azo dyes. Overnight grown culture broth of these 21 isolates was inoculated 1ml in tubes containing 10 ml NB supplemented with blue, orange, yellow and Basic fuchsin (0.005%) dye. The inoculated tubes were incubated under static conditions. 2 ml sample was taken out aseptically and centrifuged at 5000 rpm for 15 min. The cell free supernatant was used to determine the percentage decolorization of the added dye. Bacterial isolates showing rapid more decolorization of the added dye were selected for further studies.

2.6 Assay of Decolorization

Decolorization activity was expressed in terms of percentage decolorization and was determined by monitoring the decrease in absorbance at absorption maxima (λmax) of respective dyes (i.e. 420 nm for Blue, 530 nm for Yellow, 620 nm for Orange and Basic fuchsin). The uninoculated NB supplemented with respective dye was used as reference. The culture suspension was centrifuged at 5,000 rpm for 20 min for removal of the biomass. The degree of decolorization of the tested dye was measured at its respective maximum absorbance wavelength using supernatant by colorimeter. The biomass was determined by resuspending the biomass pellet in 3 ml sterile distilled water and at λ 620nm. The decolorization assay was calculated according to the following formula.
Decolorization activity (%) = (A-B)/A x 100
Where, A = Initial absorbance
B = Observed absorbance

2.7 Decolorization and Growth Measurement

Five selected cultures were inoculate in 250ml Erlenmeyer flask containing 100 ml NB, and 0.5% blue dye. At 0 hr and at the intervals of 2 hr, 5 ml sample was harvested from each flask. These samples were collected in centrifuge tube of 12 ml volume capacity. These tubes was centrifuged in laboratory centrifuge at 5000 rpm for 15 minutes and cell free supernatants was collected and analyzed for residual dye by determining absorbance at 530 nm. The pellets of bacterial cells were resuspended in 5 ml sterile saline and its absorbance were measured at 620nm to assess the biomass of cells. Difference between absorbance of final and initial growth of bacteria was represented as net increase in biomass in terms of Absorbance Unit (A.U.).

2.8 Utilization of Dye as Sole Carbon Source

Mineral Salt Medium (MSM) supplemented with 0.01% concentration of Blue dye was prepared and inoculated with loopful potential bacterial strains such as B1, B4, B5, B10, and B11 in test tubes and incubated at R.T. for 72 hrs under static condition. At the end of incubation period, samples were withdrawn and centrifuged at 5000 rpm for 15 minutes. Decolorization was determined by measuring absorbance of culture supernatants at 620 nm. The Percentage of decolorization was calculated by using the above mentioned formula.

2.9 Bacterial Identification By 16S rRNA Sequencing

PCR Amplification of 16S rRNA Gene

The PCR primers used to amplify 16S rRNA fragments were the bacteria-specific primers (Lane, 1991) a forward primer F27 (5'-AGAGTTTGATCMTGGCTCAG-3'); and a reverse primer R1492 (5'-TACGGYTACCTTGTTACGACTT-3'). A total of 25 µl of reaction mixture consisted of 10 pmol of each primer, 5 ul from colony suspension as template DNA, 12.5 µl of Master mix. The PCR amplification was performed by Thermal Cycler using the following program: Denaturing at 95°C for 5 minutes, followed by 30 cycles of 30 seconds of denaturing at 95°C, 30 seconds of annealing at 50°C and 2 minutes of elongation at 72°C with a final extension at 72°C for 10 minutes for first set. The PCR product (bp) was cleaned by using a DNA Gel Extraction Kits in accordance with the directions of the manufacturer.

Sequencing

Sequencing was performed (purified PCR product) by using above primer (above PCR conditions) with an 3130 XL DNA analyzer (Thermo chemistry) using BigDye Terminator (version 3.1) at Chromgene Biotech. Private Limited, Bengaluru, India.

Phylogenetic Analysis and Sequence Homology

The 16S rRNA sequences were initially analyzed at NCBI server (http://www.ncbi.nlm.nih.org) using BLAST tool and corresponding sequences were downloaded. Evolutionary history was inferred using the Neighbor-joining method. The tree was drawn to the scale, with branch lengths in the same units as those of the evolutionary distance used to infer the phylogenetic tree.

III. RESULTS

3.1 Collection of Sample

The effluent samples was collected in sterilized container from textile dye effluent sites (Pawne region, Mumbai, Maharashtra) (19.0829213 ºN, 73.0063196 ºE). The color of the samples was recorded on the site and samples was transported to the laboratory by storage at 4^0 C.

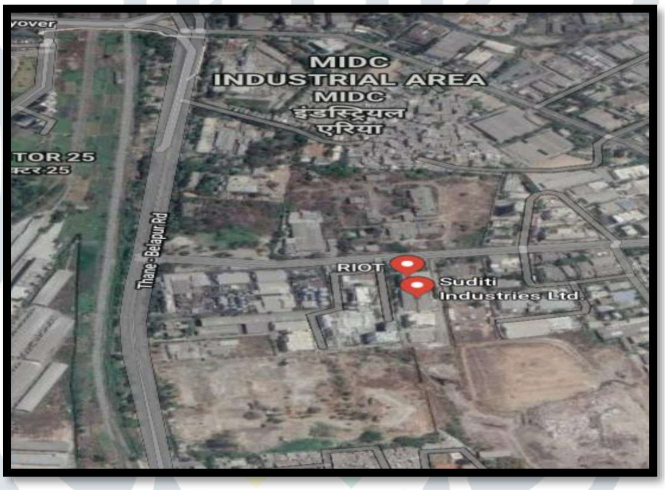

Figure 1: Sample collection from Textile industry effluents (Pawne, Mumbai, Maharashtra, Google image).

3.2 Physico-Chemical Characterization of Collected Samples

The samples were collected in sterilized container from respective sites (Fig. 1). The color and pH of the sample were recorded on the site and samples were transported to the laboratory by storage at 4ºC. The raw sewage was dark blue in color while treated effluent was light brown. The pH of the untreated effluent was 9.2, which reduced during treatment to neutral 7.6 (Table 1).

Table 1: Characteristics of samples collected from different stages of Pawne, Mumbai.

Sr. No.	Samples	Nature of Sample	Color	pH
1	Inffluent drainage line	Liquid	Dark Blue	9.2
2	After aeration (Effluent)	Liquid	Light Brown	7.6

3.3 Isolation and Screening of Bacterial Strains

The selective enrichment of liquid effluent sample collected from the waste disposal sites, led to isolation bacterial isolates on the media plates. Morphologically distinct colonies were picked from these plates for further analysis. Thus, 21 bacterial colonies were selected. Cultures were purified and maintained at 4^0 C.

3.4 Colony Characteristics of Isolated Colonies

Organisms was isolated from dye effluent and spread on nutrient agar plate. Colony characteristics were studied for selected 21 isolates from effluent sample after 24 hrs of incubated at R.T.

3.5 Morphological Characteristics

The Gram's staining indicated that out of 21 bacterial isolates. Among these most of the isolates are Gram negative in nature and some of these are Gram positive, out of these 11 isolates are Gram negative and 10 isolates are Gram positive. The percentage of Gram negative isolates was 52.38 % and Gram positive isolates was 47.62%. Gram positive rods- 5, Gram positive cocci- 5, Gram negative cocci- 5, and Gram negative rod- 6. The additional information from Gram staining and monochrome staining was in the form of cell morphology and arrangement. The organisms grown on nutrient agar plate, their characteristic pigmentation of colonies like white, off white, yellowish, yellow. Two isolates B13 and B1D was found to produce dark pigmentation of yellow. Size of colonies varied from small to moderate to large having smooth or rough texture with entire, irregular margins and circular, filamentous forms.

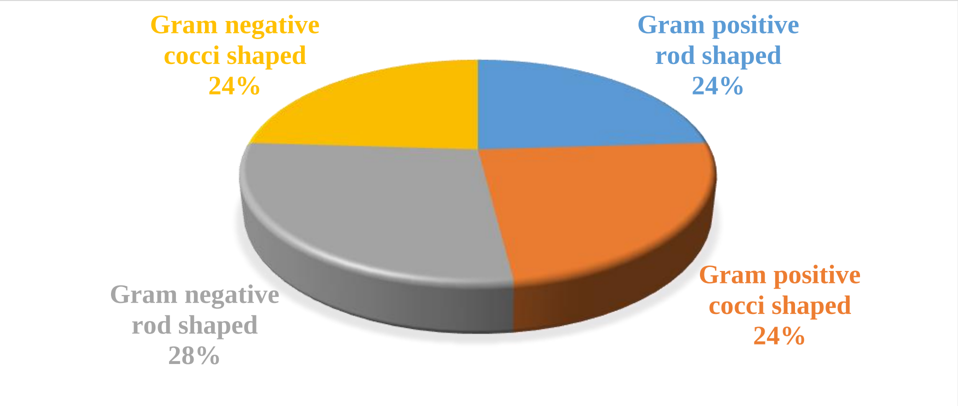

Figure 2: Diversity of bacterial isolates at Pawne, Maharashtra.

3.6 Decolorization Assay

All 21 bacterial isolates was tested individually for their ability to decolorize basic fuchsin, blue, orange, and yellow dye separately at the concentration of 0.005%, 0.01%, 0.1%, 0.25% and 0.5%. All isolates decolorize all dyes with different capacity ranging from many of lowest concentration and others are higher concentration of dyes in vary incubation time (After inoculation of 2 days to 10 days). Decolorization of Blue dye was around 98% by B1 and B11. B4, B5, and B10 decolorized this dye at, 96%, 93% and 90%, respectively. The dye that has been mainly studied, Blue was decolorize more than 90% by selected the isolates whereas this dye was decolorize up to 98% by B1 and B11, the most studied organism in this study. The lowest and highest decolorization of different dyes by selected organisms were in the range of 42% to 78% for Orange, 35% to 92% for Yellow, and 14% to 65% for Basic fuchsin. The isolation of different microorganisms from the sample indicates decolorization of yellow dye within 5 days, blue and orange in one week and basic fuchsin in 10 days (Fig. 3).

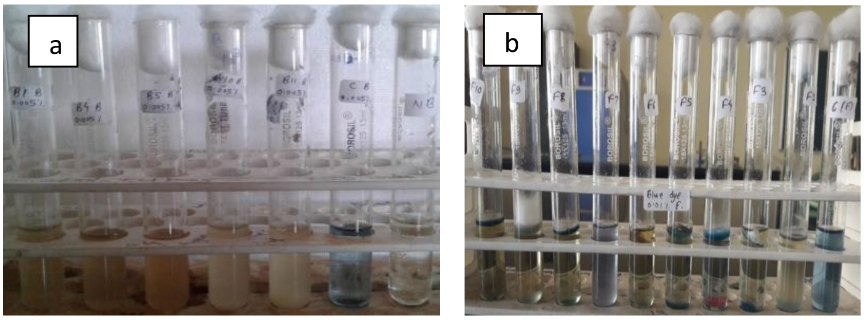

Figure 3: Decolorization of 0.005% (a) and 0.01% (b) Blue dye by bacterial isolates respectively.

3.7 Growth and Decolorization

Decolorizations of Blue dye by shake culture of selected five bacterial isolates in medium NB were monitored for concomitant increase in growth. Under shaking conditions, aerobically growing biomass of bacteria decolorize blue dye within 24 hrs. In shaking incubations there was lag period observed in 2 hrs during Bacterial growth. However stationary phase was started after 10 hrs of growth in shaking incubation. These results indicate the necessity of aerobic conditions for decolorization of azo dyes by bacteria.

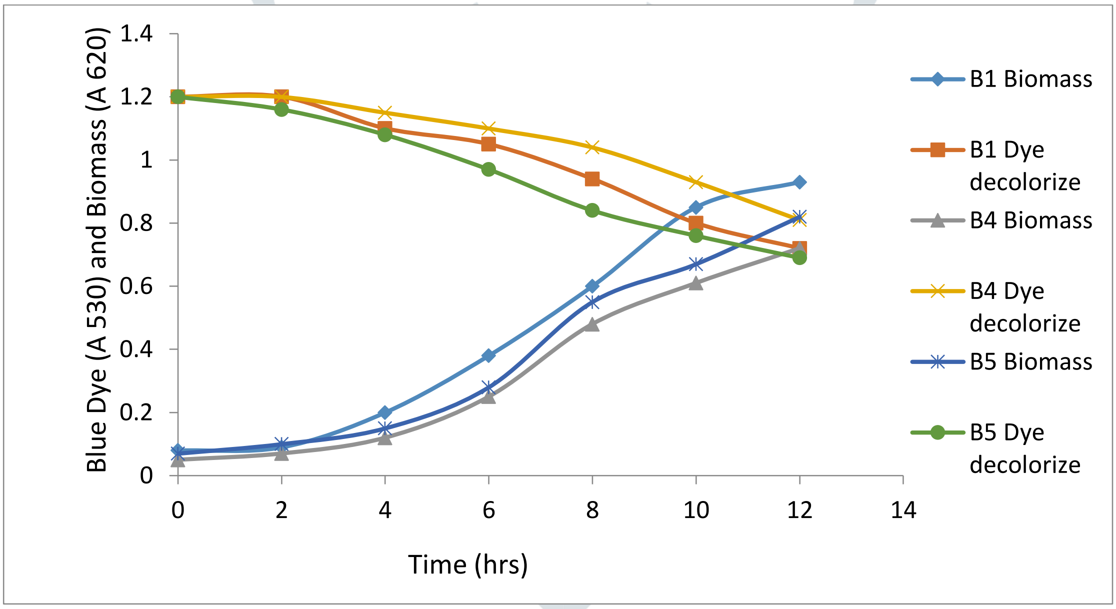

Figure 4: Decolorization of Blue dye (0.5%) and growth of isolated bacteria B1, B4, and B5 with Shaking conditions of incubation in NB.

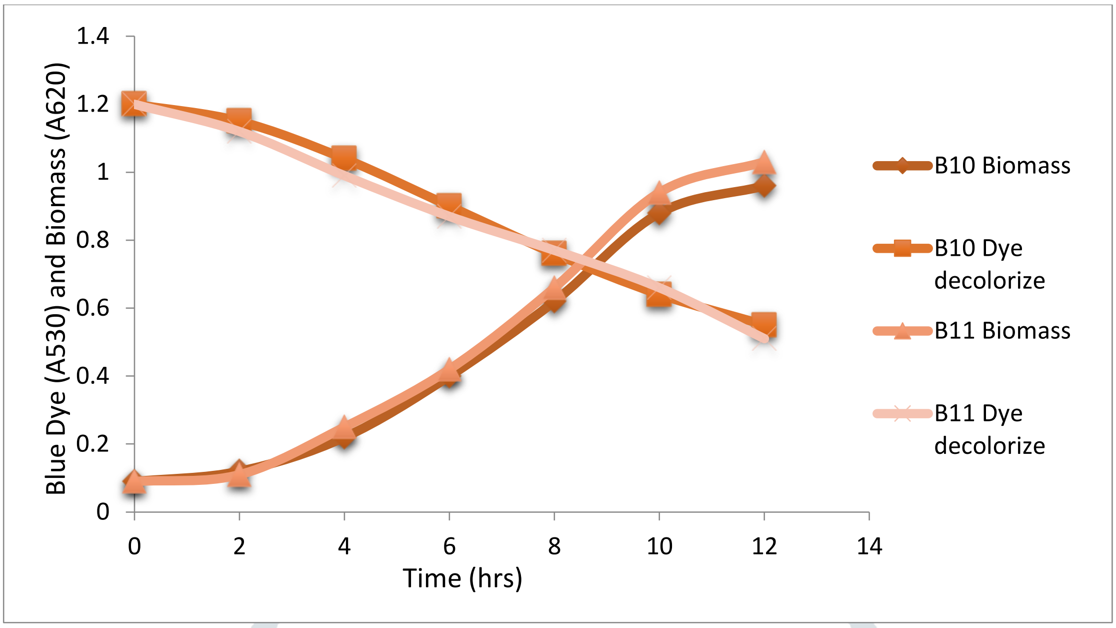

Figure 5: Decolorization of Blue dye (0.5%) and growth of isolated bacteria B10 and B11with Shaking conditions of incubation in NB.

3.8 **Utilization of Dye as Sole Carbon Source**

Decolorization experiment was carried out in test tubes containing 5ml of MSM with Blue dye concentration (0.01%) for the five strains (B1, B4, B5, B10, and B11) were represented. The results were showing that B4 have the ability to decolorize Blue dye about 67% decolorization is observed by B4 followed by B5 (62%), B11 (54%), B1 (21%) and B10 (13%) (Fig. 6).

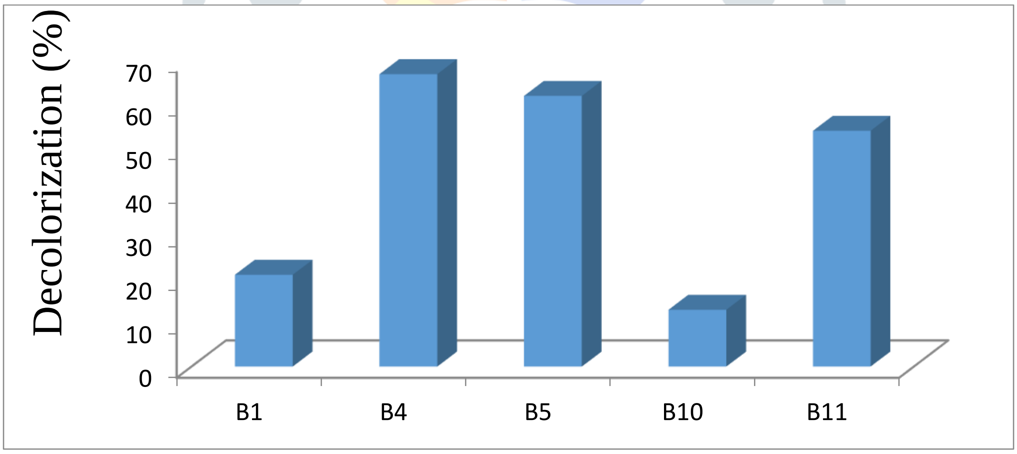

Figure 6: Utilization of sole carbon source from Blue dye and decolorization.

3.9 Bacterial Identification by 16S rRNA sequencing

To identify the experimental strain according to 16S rRNA sequence analysis as well as taxonomical studies, 16S rRNA was used as template to amplify partial genomic DNA of the organism. The chosen positive clones were sequenced. Finally, the obtained partial 16S rRNA sequence of this strain was analysed with BLAST. It was found to have 90-96% identity with different strains of *Klebsiella*. Among them, it showed high similarity (97%) with *Klebsiella pneumoniae*.

The construction of phylogenetic tree based on 16S rRNA analysis

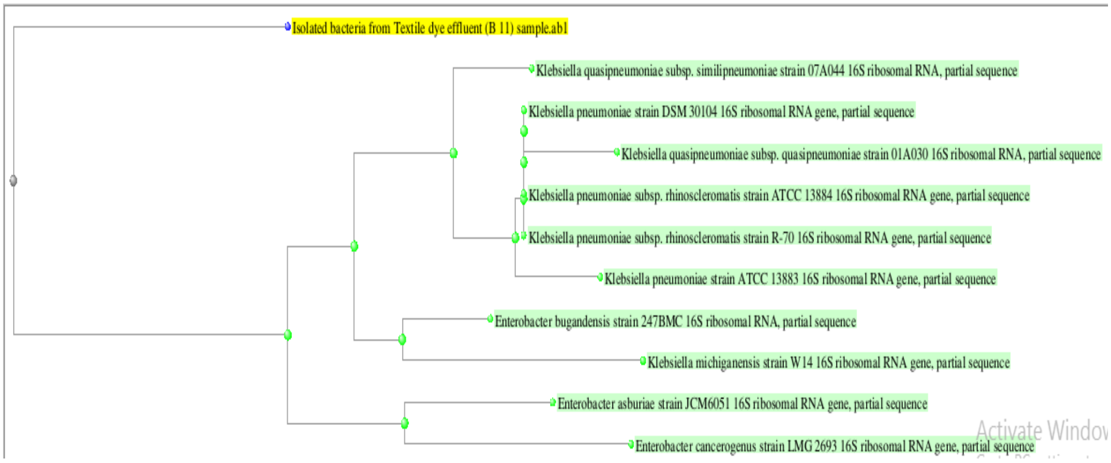

Figure 7: Phylogenetic tree for isolate B11.

IV. DISCUSSION

The present work focuses on the use of microorganisms to remediate the environment contaminated by azo dyes, which are extensively used in the textile industries. The bluish color of the incoming effluent is due to wide use of blue color dye in dyeing and printing industries, thus, it contributes more to the effluent's color compared to other dyes. The light brown color of the finally released effluent after treatment may be due to the dirty water condition. The presence of high salt content in the textile dye effluents hinders the development of efficient bio-treatment system to bioremediate the textile azo dyes. Prabhakar (2013) reported collection of soil samples from the near by Kelambakkam solar salt crystallizer ponds, Tamil nadu, India. Bhimani (2011) collected samples from in and around Jetpur common effluent treatment plant. They also recorded the color, temperature and pH of the samples.

In the present work, 21 bacterial isolates were picked based on colony morphology out of which 11 were Gram negative and 10, Gram positive. Bhimani (2011) reported the isolation of 37 morphologically different bacterial isolates using the enrichment culture method. Gram staining of all isolates indicated the presence of 20 Gram positive and 17 Gram negative organisms including Gram positive rods – 16, gram positive short rods- 3, Gram positive cocci- 1, Gram negative rod- 11 and gram negative short rod- 6. Prabhakar (2013) isolated total of 84 bacterial strains among which 32 strains belong to rhizosphere soil and 52 strains belong to non- rhizosphere soil. Rani *et al*, (2014) has reported isolation of 61 fungal strains and two fungal isolates were selected after comprehensive screening of the textile dyes biodegradation. Rajeswari *et al*, (2014) also isolated 112 bacterial strains from dye waste effluent and of these two most competent strains were selected. The strains were identified as *Lysinibacillus sphaericus* RSV-1 and *Stenotrophomonas maltophilia* RSV-2 based on morphology, physiochemical properties and the results of 16 S ribosomal RNA (rRNA) gene sequence analysis. Out of the 21 dye tolerant isolates in the present study, five strains showed good decolorisation activity and the best among these was identified as *Klebsiella pneumoniae* designated as Strain P.

Previously Tony *et al*, (2009) reported that decolorization of Remazol golden yellow dye by different *Bacillus* sp., Interestingly least decolorization was observed with Remazol golden yellow concentration 10 mg/l upto 9.8% decolorization at the end of 96 hours showed moderate growth in the presence of Remazol golden yellow dye in basal medium indicating that they were not toxic to the culture compared to the above reports. *Pseudomonas* sp., Produces good decolorization upto 92% of dye concentration of 100 mg/ l at 48 hours. Similarly Padamavathy *et al*, (2003) reported that the Remazol golden yellow decolorization by *Pseudomonas* sp., upto 77% with dye concentration of 250 mg/l. This was the only report supporting the current study.

Khehra *et al*, (2005) suggested that the decrease in decolorization efficiency might be due to the toxic effect of dyes. Initial concentration provides an important driving force to overcome all mass transfer resistance of the dye between the aqueous and solid phases (Parshetti *et al*, 2006). The dye concentration in effluent from textile printing house is approximately in the range of 50 to 200 mg L^{-1}. This value is typical of those used in studies on treatment for azo dyes containing effluent. However, change in operating processes may lead to still high concentration of dye in effluent. The 0.005% to 0.5% concentration of dye was used to check their ability to decolorize different dyes.

The difference in decolorization pattern is due to the dissimilarity in specificities, structure and complexity, particularly on the nature and position of substituent in the aromatic rings and the interaction with azo bond with different dyes as reported by many authors. The isolation of different microorganisms from the sample indicates the natural adaptation of microorganisms to survive in the presence of toxic dyes. The difference in their rate of decolorizaton may be due to the loss of ecological interaction, which they might be sharing with each other under natural conditions. The higher concentration of azo dye inhibits nucleic acid biosynthesis and cell growth (Chen et al. 2003), so the effect of dye concentration on growth of organisms is an important consideration for its field application. Halotolerant bacteria has been reported to decolorize textile azo dyes (Khalid et al. 2008).

Khaled et al. (2010) determined the plasmid harboring the catabolic genes involved in azo dye degradation. Isolates Lab 2 and Lab 11 were similar in their plasmid profiles containing four different plasmids with molecular weights of 16, 8, 3 and 2.5 Kb. *S. cerevisiae* SY proved to be able to decolorize various azo dyes in the complex and chemically defined growth medium. They assessed decolorization of these samples by *S. cerevisiae* SY by respective maximum absorption wavelength and percentage wavelength (Ambasana, 2006).

Five potential bacterial isolates namely; B1, B4, B5, B10 and B11 showed good decolorization efficiency in all four dyes within less time. Bhimani (2011) has examined the ability of *Lysinibacillus fusiformis* JTP-23 to decolorize industrial effluent. Rajeswari et al. (2014) determined decolorization and degradation ability of RSV-1, RSV-2 and consortium on real textile effluent. Microorganisms capable of utilizing a variety of complex chemicals including dye as their sole carbon source or either nitrogen source. Only few researches were successful in isolating culture capable of utilizing dyes as sole source as carbon (Sarnaik and Kanekar, 1999). Microorganism require organic carbon sources without any extra carbon source the biodegradation of dye was very difficult because as they cannot utilize dye as the growth substrate. However, the aerobic decolorization of azo dyes can also be carried out in the presence of external carbon source and presumably does not use azo dyes as the sole carbon source or energy source (Padmavathy et al. 2003).

In the present study, six strains *Bacillus* sp., *Micrococcus* sp., *Staphylococcus* sp., *Pseudomonas* sp., and *Lactobacillus* sp., have capability to utilize the dye as a sole carbon source without any external carbon in the presence of nitrogen source under anoxic condition was found. This reports was contrast with the previous reports that under aerobic condition, the azo dyes were non degradable by most of the bacteria which use dye as sole source of carbon was proved to be difficult (Zimmermann et al. 1982).

The 16S rRNA analysis revealed that the isolated strain B11 is *Klebsiella pneumoniae* designated as strain P. It is a Gram- negative bacterium isolated from the textile dye effluent, Pawne, Mumbai. The *Klebsiella* decolorized and degraded all the 4 dyes (Blue, Orange, Yellow, and Basic fuchsin) efficiently. However, there is no available literature on azo dye degradation by *Klebsiella*. Previous studies have shown that the strains of *Halomonas* sp. isolated from textile industries effluents were able to decolorize seven azo dyes- Remazol Black B, Remazol Black N, Sulfonyl Scarlet BNLE, Sulfonyl Blue TLE, Maxilon Blue and Entrazol Blue IBC. The strains were identified as *Halomonas aquamarina, Halomonas meridian, Halomonas salina* (Asad et al. 2007). Guo et al. (2008) isolated *Halomonas* strains from coastal sediments contaminated by chemical wastewater. They showed that under high salt concentration the isolated *Halomonas* sp. decolorized five azo dyes in 24 hours with a decolorization above 90%. Moreover, lot of future work is needed to isolate new microorganisms capable of effectively degrading wide range of textile dyes and to create an environment free from textile dye pollution.

V. CONCLUSION

Continuous dumping of dye stuffs and dye waste water has created environmental pollution as well as medical and aesthetic problems associated with human health and agriculture, thus bioremediation of contaminated site is of prime importance. Due to adaptation of various bacterial species, the effluent had high count of Gram positive and Gram negative dye decolorizing bacteria. Use of aerobic treatment by consortium was successful in decolorization and degradation of dye house effluent. The difference in decolorization capacity of different azo dyes by individual bacteria was due to dissimilarity in specificities, structure and complexity, and the interaction with azo bond with different dyes. Gram negative bacteria dominated in sample collected from textile dye effluent. Best growth and decolorization was achieved in mesophilic range of temperature, while alkaline pH (8 to 10) favored decolorization. The 16S rRNA sequencing of the most efficient bacterial strain B11 showed 97% similarity to *Klebsiella*. The isolated bacteria, *Klebsiella pneumoniae* strain P, could effectively be used as an alternative to physical and chemical process used for textile effluent treatment. This approach creates a promising hope to remediate the environments polluted by textile azo dyes.

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