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Decolourization of textile dye effluent by Efficient Microbe (EM) Technology

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Abstract

With rapid industrialization all over the world, pollution is on the increase and India is no exception. One of the modes through which pollutants enter the biosphere is that of industrial effluents. A variety of synthetic dyestuffs released by the textile industry pose a threat to environmental safety. Novel dye-degrading bacterial strains were isolated from textile effluent and soil samples collected from Tiruppur, Tamil Nadu. Taxonomic identification including morphological and cultural characterization indicated that isolates was capable of decolorizing direct dyes efficiently. Four bacterial strains screened out of total 11 isolates and they were able to decolorize direct dye Direct Red 23 cultured in nutrient broth containing 100ppm direct dyes. The decolourization percentage was found to be ranging from 67% - 85% among the four isolates . The isolates were used to prepare a consortium and develop an efficient microorganism (EM) solution. Then this EM solution is used for the decolourization of textile effluents and the percentage color removal was 70%. Hence EM methodology can be effectively used for decolourising textile effluents.

Key words: Synthetic dyes; Direct Red 23; Dye decolourising bacterial strains; Decolourisation; EM technology

1. Introduction

Approximately 70% of all dyestuffs used worldwide are azo dyes by weight *(Zollinger, 1987),* making them the largest group of synthetic colorants and the most common synthetic dyes released into the environment (*Chang et al., 2001b; Saratale et al., 2009a; Zhao and Hardin, 2007).* Synthetic dyes used in many industries which include tanning industries, textile industries, leather industries, etc. dispose their effluent without any treatment or by partial physical or chemical treatments. Due to this the water pollution is a serious problem nowadays. Improper discharge of textile dye effluent containing azo dyes and their metabolites in aqueous ecosystems is aesthetically unpleasant and leads to a reduction in sunlight penetration, which in turn decreases photosynthetic activity, dissolved oxygen concentration, and water quality, and had acute toxic effects on aquatic flora and fauna, causing severe environmental problems worldwide *(Vandevivere et al., 1998).* In addition, azo dyes also have an adverse impact in terms of total organic carbon (TOC), biological oxygen demand (BOD) and chemical oxygen demand (COD) *(Saratale et al., 2009b).* Many synthetic azo dyes and their metabolites are toxic, carcinogenic, and mutagenic *(Myslak and Bolt, 1998).* Moreover, numerous reports indicate that textile dyes and effluents have toxic effects on the germination rates and biomass of several plant species which have important ecological functions, such as providing a habitat for wildlife, protecting soil from erosion and providing the organic matter that is so significant to soil fertility *(Ghodake et al., 2009a; Kapustka and Reporter, 1993).* Therefore, treatment of industrial effluents containing azo dyes and their metabolites is necessary prior to their final discharge to the environment.

The samples are collected from Tiruppur city, Tamilnadu. Tiruppur is a textile city located on the banks of Noyyal River. Tirupur has emerged as the knitwear capital of the country in three decades Exports from Tirupur, which provides employment to over five lakh people has crossed the Rs.12,000 crore mark last year. There are nearly about 3000 sewing units, 450 knitting units, hundreds of dyeing units and other ancillary units which are un-countable. The annual for-ex business for the past year 2008 stands at Rs. 8,000 crore. Even though the city is developing at a faster rate, the mechanism of dye industry discharge disposal is not properly constituted and the waste water is literally let out into nearby lakes which has heavily polluted water resources around the city. Recently in April 2011 all manufacturing works has been stopped due to major pollution problems and it's yet to be resolved. Hence this work involves a step to identify dye degrading bacteria from contaminated soil and to find a effective way to treat waste water coming out of the dyeing industry.

EM is a natural, probiotic technology developed for over 25 years around the world. It is based on beneficial and effective microorganisms ("EM"). The microbes in EM are non-harmful, non-pathogenic, not-genetically-engineered or modified (non-GMO), and not-chemically-synthesized. The basic groups of microorganisms in EM are lactic acid bacteria (commonly found in yogurt, cheeses), yeast (bread, beer), and phototrophic bacteria ("cousins" of blue-green algae).One of the most important concepts of EM is not the overall number of microorganisms present but the interactive relationship between the different species. Based on research and development activities in many countries, EM is increasingly viewed as a means of providing solutions to many problems of food production,

depletion of natural resources, environmental pollution, food safety and nutrition, and human and animal health. Originally, EM was developed for use in agriculture (crop farming) as an alternative to agricultural chemicals such as pesticides and fertilizers. From crop farming, its application flowed naturally into livestock. EM is actively used in livestock operations, including hog, cattle/dairy, and poultry. From livestock, the positive effects on the livestock waste and effluent into lagoons and rivers led to the use of EM for environmental purposes: from land/soil remediation to water purification. EM environmental applications throughout the world have included cleaning polluted waterways, lakes and lagoons, in septic systems, municipal wastewater treatment plants, and landfills/dump sites.

2. Materials and Methods

2.1 Dye stuffs and chemicals

Direct Red 23 was procured from Vijayalakshmi Color Company, Tiruppur. All the other chemicals were procured from Hi-media (India) and Sigma Aldrich, Bangalore.

2.2 Sampling

Soil samples and textile effluent were collected from a contaminated of a textile industry from Tiruppur in a sterile container. The samples were brought to the laboratory processed for the isolation of bacterial populations as per APHA (2005).

2.3 Enrichment and Isolation of Dye degrading bacteria

Collected samples were serially diluted and inoculated onto Nutrient broth medium supplemented with 100 mg/L of Direct Red 23 dye and incubated at 30°C for 4 to 6 days under shaking (150 rpm) to enrich the dye tolerant bacterial populations.

Further isolation was done from the enriched culture on Nutrient agar medium supplemented with corresponding dye. The enriched culture broth was diluted into a series of dilution sequences and used for isolation of dye tolerant bacteria in Nutrient agar plates supplemented with 100 mg/L of Direct Red 23 dye. The plates were incubated at 30° C for 2-4 days.

Enumerations of total bacterial populations were done on Nutrient agar medium. A control without dyes has been done before enrichment to compare the normal bacterial populations and the dye resistant bacteria in the collected samples.

Discrete bacterial colonies that grew on agar plates were initially grouped on the basis of gram staining and different morphological characteristics, such as pigmentation, motility and colony forms. From the agar plate pure culture of the isolates were obtained using streak plate technique and used for further screening.

2.4 Selection and Characterization of Effective Isolates

Based on gram staining and different morphological characteristics, such as pigmentation, motility and colony forms, five morphologically distinct bacterial isolates were screened for the adaptation and effective dye decolorizing ability. The bacterial strains with the ability to decolorize the dye were selected for identification. The selected isolates were characterized morphologically, biochemically.

2.5 16S rDNA Amplification and Sequencing

2.5.1 PCR Conditions

Initial denaturation, 95°C for 7min.; 4 cycles of denaturation at 95°C for 1 min, annealing at 35°C for 3 min, extension at 72°C for 5 min; 40 cycles of denaturation at 95°C for 1 min, annealing at 36°C for 2 min, extension at 72°C for 3 min and final extension at 72°C for 10 min.

2.5.2 Primers

The sequencing templates of 16S ribosomal DNA (rDNA) was amplified from genomic DNA by PCR as described previously (Stach *et al*, 2003). Bacterial 16S rRNA gene primers forward primer 27 F - 5'CGGCTACCTTGTTACGACT3' and reverse primer 1492 R- 5'GAGTTTGATCCTGGCTCAG3'.

2.5.3 Amplification of 16S rDNA and Sequencing

Reaction mixture (50µL) contained genomic DNA extract (1µL), Thermopol buffer, DMSO (2µL), bovine serum albumin (2µL), deoxynucleoside triphosphate mixture (2.5 pmol), each primer (20 pmol), and Taq DNA polymerase (2.5 U). All sequencing reactions were carried out with an ABI PRISM 377TM DNA sequencer at the Invitrogen lab, Bangalore, India. The 16S rDNA sequences obtained were used to search the GenBank database by using BlastN algorithm to identify the closest matches among the known species. Sequences were aligned with representative actinomycete 16S rDNA sequences and a phylogenetic tree was constructed using the Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (Kumar *et al.,* 2001).

2.6 Growth Kinetics

The growth kinetics of the isolates was studied by culturing it in 250 ml Erlenmeyer flask containing 100 ml nutrient broth and incubated at 30°C for 24 h under shaking conditions (150rpm).The growth was determined by noting down the optical density values for every two hours from a spectrophotometer by setting its wavelength at 510nm and the growth curve was plotted. Each of the five isolate's growth was measured along with a consortium containing all the five isolates.

2.7 Decolorization Experiment

A loopful of microbial culture was inoculated in 250 ml Erlenmeyer flask containing 100 ml nutrient broth and incubated at 30°C for 24 h under shaking conditions (150rpm). After 24 h of incubation, Direct Red 23 dye was added in flasks at a concentration of 100mgl/1. Five ml sample was withdrawn at different time intervals, centrifuged at 5000×g for 15 min. The clear supernatant was used to measure the decolorization at the absorbance maxima of the dye, i.e. 510 nm and then percentage decolorization was calculated. Un-inoculated control was used to compare colour loss during the experiment.

(%) Decolorization = Initial absorbance - Observed absorbance / Initial absorbance ×100

2.8 EM Solution Formulations

EM solution was formulated by culturing all the four isolates in nutrient broth 250 ml Erlenmeyer flask containing 100 ml nutrient broth and incubated at 30°C for 24 h under shaking conditions (150rpm). Compatibility testing was done to the cultures to check if they can be cultured together and then the consortium culture is used as EM solution to treat the textile effluents. After 24 h of incubation, Direct Red 23 dye was added in flasks at a concentration of 100mgl/1. Five ml sample was withdrawn at different time intervals, centrifuged at 5000×g for 15 min. The clear supernatant was used to measure the percentage decolorization.

3. Results & Discussion

ISOLATION AND CHARACTERIZATION OF BACTERIAL STRAINS

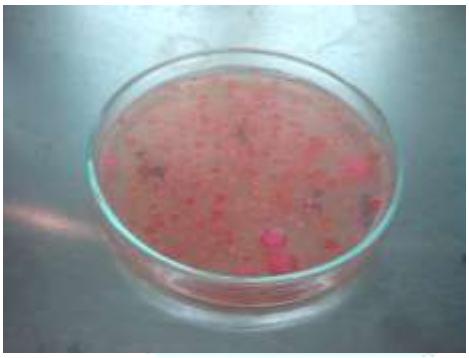
Isolation from soil sample was carried out by the enrichment technique using nutrient broth and synthetic textile dyes as source of carbon and nitrogen that has decolorization capacity. Four bacterial isolates were screened from 11 isolates which has effective decolorizing ability from plates containing four synthetic dyes namely Direct Green 2 , Direct Red 23 , Direct Yellow 12 , Direct Blue 80. An identification of two isolates indicated the cultures were of Bacillus sp., was based on biochemical tests and 16S rDNA analysis done. The nucleotide alignment of this strain showed it was most phylogenetically similar to the genus Bacillus. It has a significant potential for decolorization of direct dyes. When decolorization pattern for Direct Red 23 was studied by using four bacterial isolates and its consortium culture in the nutrient broth, it showed ability to decolorize reactive Direct Red 23 (%) at the dye concentration 100 mg /l. Visible spectrum of Direct Red 23 showed decrease in optical density at 510 nm indicating the complete decolorization of the dye. In further studies, the effect of various temperature and pH conditions on the decolorization of Direct Red 23 isolates showed optimal growth and efficient decolorization at 30°C and pH of 7.0 under shaking conditions.

PHYSICOCHEMICAL ANALYSIS OF THE SOIL SAMPLE

Table.1. Analysis of physicochemical parameters of the soil sample

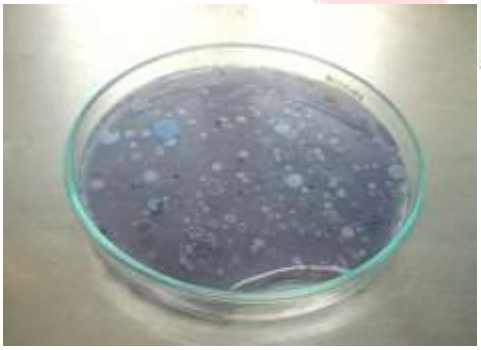
Results of Soil Analysis

S.No.	Parameter	Unit	Results
1	**pH**	-	9.14
2	**Electrical Conductivity**	mS/cm	32.6
3	**Organic Matter**	%	5.18
4	**Nitrate** Nitrogen	ppm	31.4
5	Available **Phosphorous**	ppm	70.8
6	**Potassium** Exchangeable K	ppm	1392



(a)

(b)



(c)

(d)

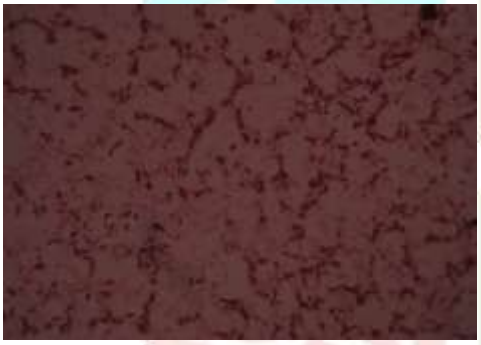
Fig.3. Plates showing growth of various bacterial colonies on nutrient agar medium, containing various direct dyes.

a. **Plate containing Red dye plated from enriched culture.**
b. **Plate containing Green dye plated from enriched culture.**
c. **Plate containing Blue dye plated from enriched culture.**
d. **Plate containing Yellow dye plated using enriched culture.**

GRAM STAINING

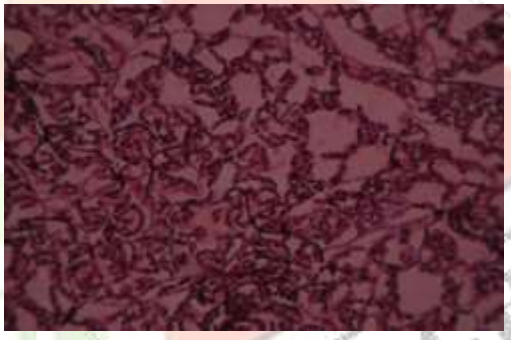
Gram staining results showed out of the four isolates only one was gram positive rod (TSB 7) and the others were found to be gram negative bacilli**.**

TSY 1



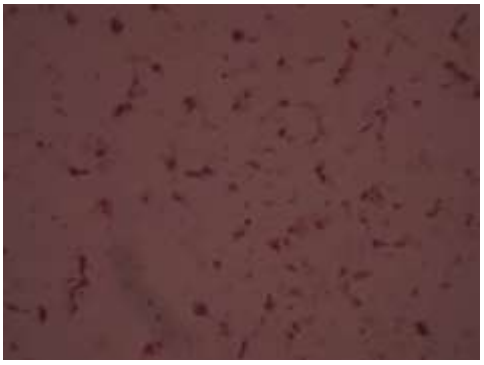
(a)

TSR 4



(b)

TSB 6



(c)

TSB 7

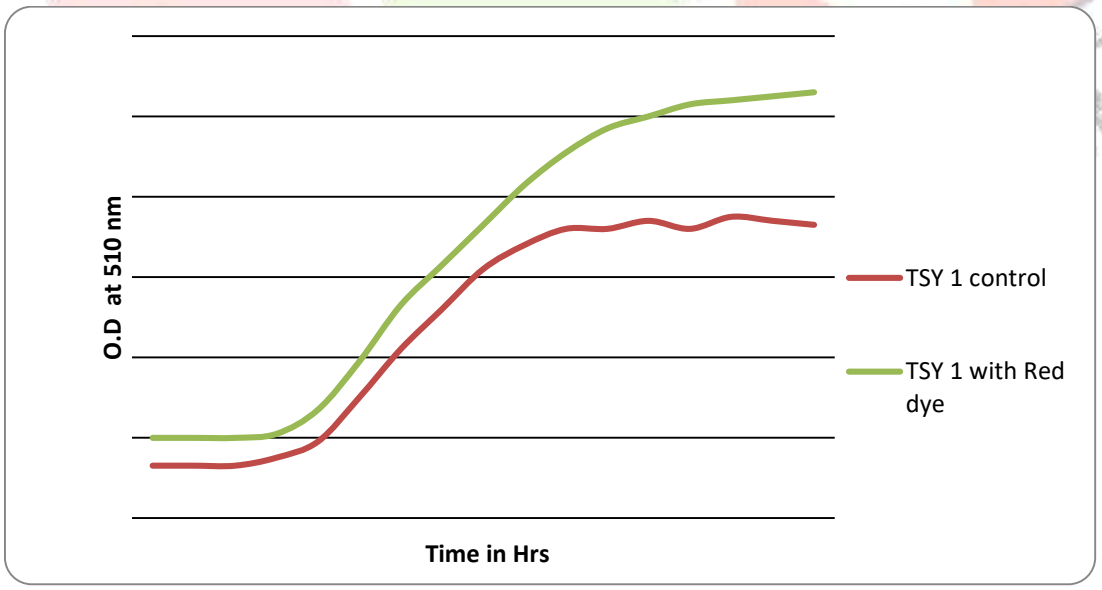
(d)

Fig.4. Gram staining results

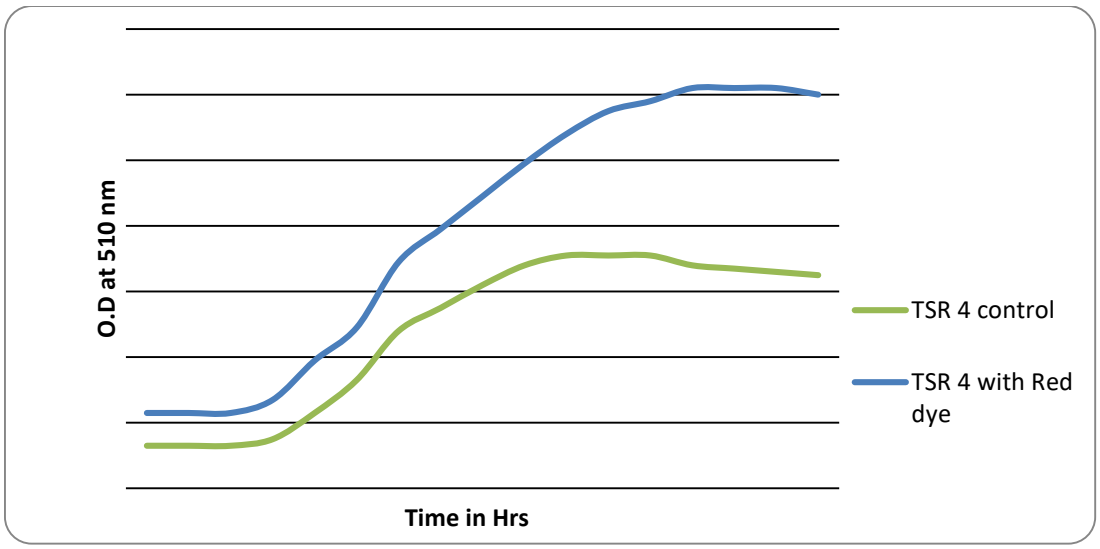
a. **TSY 1 Gram negative bacilli**
b. **TSR 4 Gram negative bacilli**
c. **TSB 6 Gram negative bacilli**
d. **TSB 7 Gram positive rods**

GROWTH PROFILE OF THE SELECTED ISOLATES

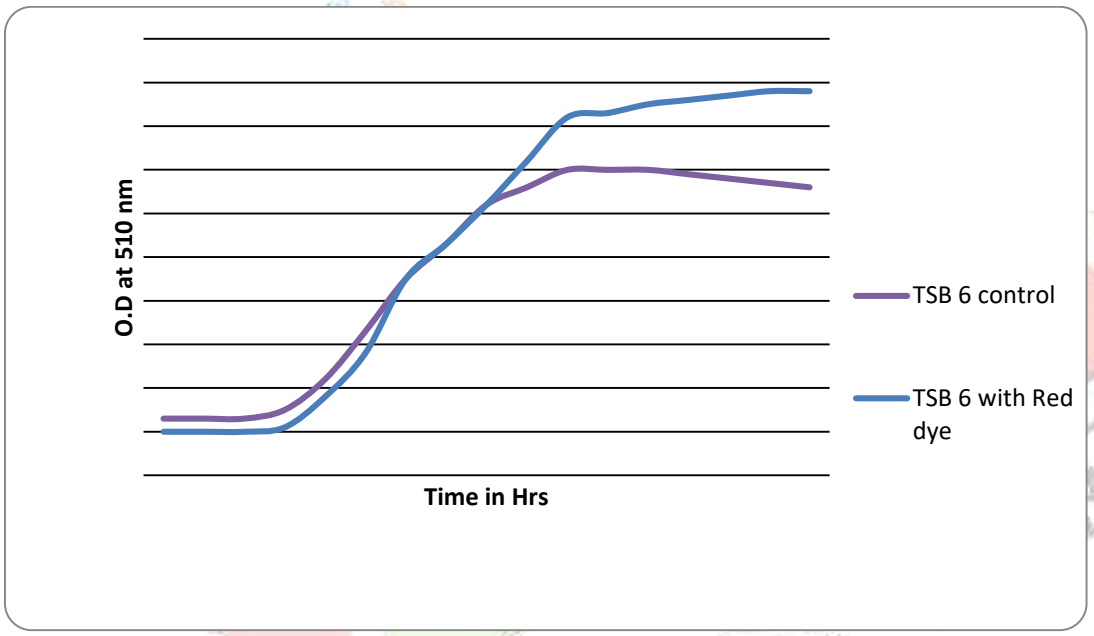
The growth profile was determined for the four isolates in nutrient broth (control) and another set containing nutrient broth and Direct Red 23(100mg/l) dye to compare their growth. It is determined that the bacteria grow efficiently on dye containing medium. The growth profile of the bacteria is directly related to cell density. The reduction in the growth profile indicates reduction in cell density. The exponential phase started at 8 h for four isolates in nutrient broth alone and the exponential phase was at 6 h for the four isolates. And the stationary phase was attained after 24 hrs. The absorbance value was very more in the presence of Direct Red 23 dye compared with nutrient broth alone. This indicated that the isolates grow efficiently in the presence of the dye by decolorizing it. The growth profile was also recorded for the consortium containing all the four isolates.



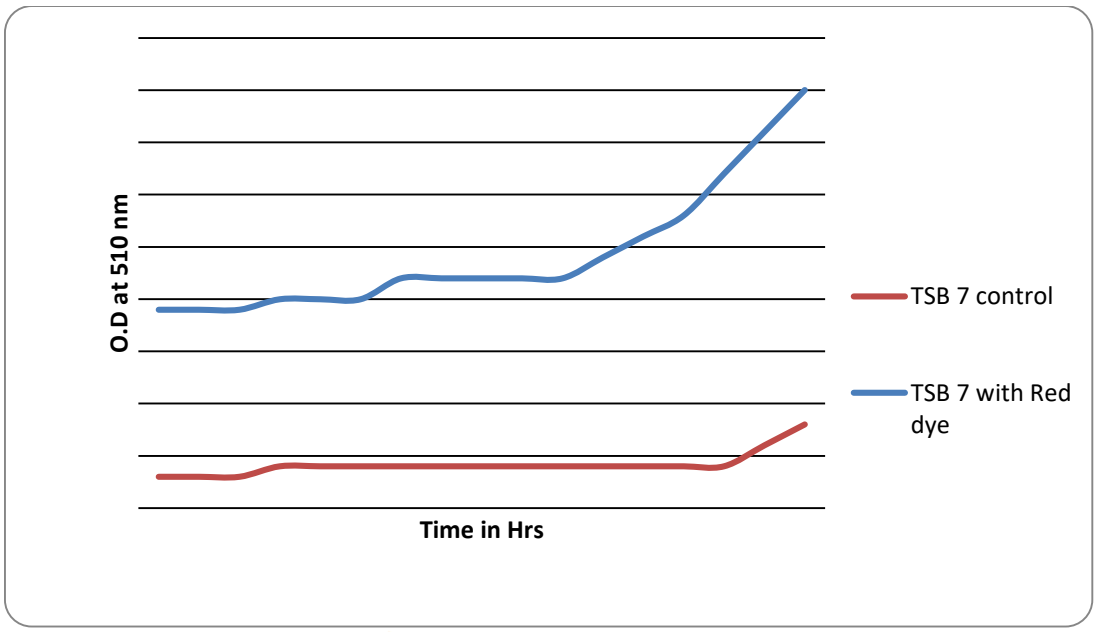
(a)


O.D at 510 nm
Time in Hrs
TSR 4 control
TSR 4 with Red dye

(b)


O.D at 510 nm
Time in Hrs
TSB 6 control
TSB 6 with Red dye

(c)



(d)

Fig.5. Growth profile of all the four isolates with and without dye (a) TSY 1 (b) TSR 4 (c) TSB 6 and (d) TSB 7.

MOLECULAR CHARACTERIZATION

The cultural and morphological characteristics of TSR 4 and TSB 7 indicate that both the strains belong to the genus *Bacillus*. The molecular characterization by 16S rRNA sequencing was performed using the forward and reverse primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'- GGTTACCTTGTTACGACTT-3'). The partial sequencing of 16S rRNA gene of the strains TSR 4 and TSB 7 on both directions yielded 16S rDNA nucleotide sequence with 1404 and 1419 base pairs respectively. The 16S rDNA sequences of the strains were deposited in the GenBank (NCBI, USA) and waiting to get our accession numbers.

The BLAST search of 16S rDNA sequence of the strain TSR 4 showed highest similarity (99%) with *Bacillus pumilus* and phylogenetic tree was constructed with bootstrap values. A neighbor-joining tree based on 16S rDNA sequences showed that the isolate occupies a distinct phylogenetic position within the radiation including representatives of the *Bacillus* family. Based on the molecular taxonomy and phylogeny the strain was identified as novel *Bacillus pumilus* stain.

The BLAST search of 16S rDNA sequence of the strain TSB 7 showed highest similarity (98%) with *Bacillus foraminis* and phylogenetic tree was constructed with bootstrap values. A neighbor-joining tree based on 16S rDNA sequences showed that the isolate occupies a distinct phylogenetic position within the radiation including representatives of the *Bacillus* family. Based on the molecular taxonomy and phylogeny the strain was identified as novel *Bacillus foraminis* stain.

DECOLORISATION EXPERIMENT

The decolorization experiment was carried out for all the four isolates individually in 250 ml Erlenmeyer flask containing 100ml nutrient broth supplemented with 100mg/l of Direct Red 23 dye. The percentage removal was calculated using the above mentioned formula and it was found that TSR 4 and TSB 6 has the highest value of 85% after 96 hrs.

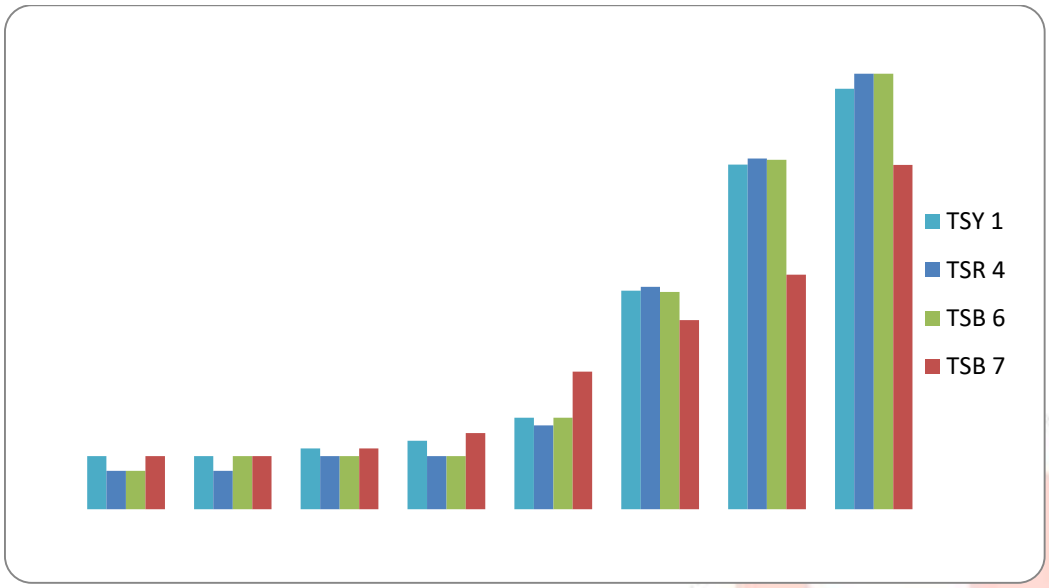


Fig.6. Percentage decolourisation of Direct Red 23 dye for all the four isolates

APPLICATION OF EM SOLUTION TO TEXTILE EFFLUENTS

EM FORMULATION

The nutrient broth media was slightly altered to get an optimized media so that the EM solution can be prepared. The optimized media contains Peptone- 5g/l, NaCl- 7g/l, Beef extract- 4g/l, Yeast extract- 5g/l. The consortium is cultured in above mentioned optimized media and incubated for 24 H. after 24 H, it is used as the EM solution to decolorize the textile effluents.

DECOLORISATION EXPERIMENT IN TEXTILE EFFLUENT

In a 250ml Erlenmeyer flask, 50 ml of the textile effluent and 50ml of EM solution was added and kept in a orbital shaker (150rpm) at 30°C and at a pH of 7.0. Five ml sample was withdrawn at different time intervals, centrifuged at 5000×g for 15 min. The clear supernatant was used to measure the decolorization was checked at different time intervals by measuring their absorbance at 510nm using a spectrophotometer. The decolorization experiment was carried out at various temperature (30°C, 40°C, 50°C and 60°C) and pH(6.0, 7.0, 8.0 and 9.0) conditions and the maximum decolorization was found to occur at 30°C and at a pH of 7.0.

The EM solution was also used to decolorize synthetic dye Direct Red 23 by adding 90ml of EM solution to 10ml of 100mg/l Direct Red 23 dye in an 250ml Erlenmeyer flask. and kept in a orbital shaker (150rpm) at 30°C and at a pH of 7.0. Five ml sample was withdrawn at different time intervals, centrifuged at 5000×g for 15 min. The clear supernatant was used to measure the decolorization was checked at different time intervals by measuring their absorbance at 510nm using a spectrophotometer.

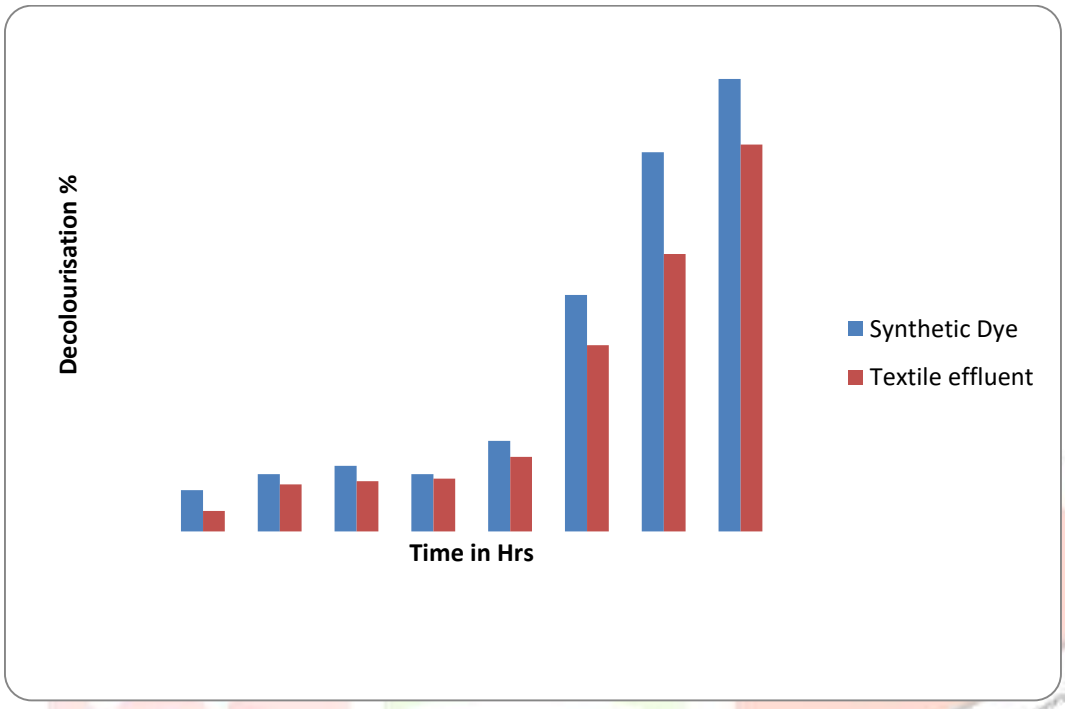


Fig.7. Percentage decolourisation in synthetic Red dye and textile effluent using EM solution at various time intervals

4. Conclusion

Accumulation of dyestuff and dye wastewater creates not only environmental pollution, but also medical and aesthetic problems. As regulations are becoming even more stringent, there is an urgent need for technically feasible and cost-effective treatment methods. Microbial and enzymatic decolorization and degradation of azo dyes have significant potential to address this problem due to their environmentally-friendly, inexpensive nature, also because they do not produce large quantities of sludge and they can grow faster too.

From the present study the following conclusions were drawn:

- Physiochemical characteristics of the soil were altered by the effluent. The soil sample had dark colouration, alkaline pH and high nitrogen content.
- From the soil sample, four isolates were screened and they were able to decolorize Direct Red 23 dye effectively up to 85%.
- Molecular characterization of two of the isolates, showed that they are of Bacillus sp., and was found to be *Bacillus pumilus* and *Bacillus foraminis* by constructing a Phylogenetic tree.
- The optimal pH and temperature for decolourisation of Direct Red 23 were found to be 7.0 and 30°C, respectively.
- Textile effluent was treated with formulated EM solution and 70% decolourisation was observed.
- Efficient Microorganism technology has proved to be a useful technology for dye decolourisation studies.
- Further studies have to be done on the enzymatic part to clearly understand the nature of the intermediates formed during decolorisation. And if the isolated bacteria can be genetically engineered, they can be effectively used for bioremediation studies.

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