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Microbial Decolourization of Crystal Violet by *Bacillus subtilis*

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ABSTRACT : The physicochemical characterization of the textile industry effluent collected from Oswal Textile Industries, Ludhiana (Punjab.) India has been carried out and the results showed that the temperature (40°C), pH (8.00), Biological Oxygen Demand (260 mgl^{-1}), Chemical Oxygen Demand (790 mgl^{-1}), Total Suspended Solids (2000 mgl^{-1}), Total Dissolved Solids (7000 mgl^{-1} and colour over the prescribed fresh water limits. A potential bacterial strain was isolated and selected from the textile effluent on the basis of rapid azo dye Crystal violet (100mgl^{-1}) decolorization and later identified as belonging to genus *Bacillus* based on Phenotypic characterization and phylogenetic analysis of the 16s rRNA gene sequence. Effects of physicochemical parameters (pH, Temperature, etc.) on the Crystal violet decolorization by the *Bacillus* were studied. Decolorization was effective at pH 8, 35°C with starch and peptone as carbon and nitrogen sources and in static conditions. This decolorization potential increased the applicability of this microorganism for the dye removal.

Keywords : Textile Industry effluent: *Bacillus subtilis*; Crystal Violet; decolorization.

INTRODUCTION

The synthetic dyes are used extensively in textile dyeing and paper printing. First synthetic dye was reported in 1856. There are more than 40,000 dyes and pigments with some 7000 different chemical structures, out of which more than 3500 dyes are of practical use. Based on the chemical structure of the chromophoric group, the synthetic dyes are classified as azo dyes, nitroso dyes, triphenylmethane dyes, xanthane dyes and anthraquinone dyes (Shenai, 1994). India is now the largest producer of dyes and intermediaries in World. Because these dyes are mutagenic and carcinogenic and also cannot be completely removed by conventional wastewater treatment systems, before disposal and discharge of dye-containing effluents, they are to be treated to reduce their levels of toxicity and thus, to minimize their pollution impact. Some of the triphenylmethane dyes are used as dermatological agents, the best among them being gentian violet, which is a mutagen, a mitotic poison and clastogen (Kapdan *et. al.*, 2000). Bioremediation is an expensive mean to remove hazardous metal ions from the contaminated effluent (Faryal and Hammed, 2005). Presently, it was estimated about 10,000 of different commercial dyes and pigments exists and over 7 × 10 tones are produced annually worldwide (Guendy, 2007). Biotechnological tools also have been applied for the degradation of various textile dye and it was found that upto 70% color removal was noticed with different microflora (Khadijah *et al.*, 2009). The biological methods being simple to use and low in cost have become main focus in recent studies on dye biodegradation. Keeping the above facts in mind, the present study was envisaged.

Indiscriminate disposal of sewage and industrial effluents is a major cause of pollution of water bodies and rivers into which they are discharged. The use of industrial effluents carrying a heavy load of heavy metals such as Pb, Zn, Cr, Ni and Hg for irrigation of crops also produces adverse effects on plant growth. Waste water generated by different production steps of a textile industry have high pH, temperature, detergents, oil, suspended and dissolved solids, dispersants, leveling agents, toxic and non-biodegradable matter, color and alkalinity. The effluents also consist of high concentrations of dye stuff, biochemical oxygen demand, total dissolved solids, sodium, chloride, sulphate, hardness, heavy metals and carcinogenic dye ingredients which pose serious environmental problems.

The mechanism of biodegradation depends in part, on the compound being degraded, but there are some consistent steps in the process regardless of the substrate. When an electron is added or removed from the ground state of a chemical it becomes highly reactive, allowing it to give or take electrons from other chemicals. This provides the basis for the non specificity of the enzymes and the ability of the enzymes to degrade xenobiotics, chemicals that have never been encountered in nature. The main reactions that are catalyzed by the lignolytic enzymes include depolymerization, demethoxylation, decarboxylation, hydroxylation and aromatic ring opening. Many of these reactions result in oxygen activation, creating radicals that perpetuate oxidation of the organopollutants. Once the peroxidases have opened the aromatic ring structures by way of introducing oxygen, other more common species of fungi and bacteria can mineralize the products intracellularly into products such as CO_2 and other benign compounds. (Weigel, 1999) further reported that anaerobic dehalogenation of polychlorinated biphenyls in sediment slumes dehalogenales all flanking chlorines. Faryal and Hameed (2005) carried out the textile effluent analysis for presence of Mn, Zn, Mg etc. and reported subsequent decolourising bacteria.

A very small amount of dye in water (10-50 mg L^{-1}) affects the aesthetic value, transparency of water and gas solubility of water bodies. The presence of even very low concentrations of dyes in effluent is highly visible and degradation products of these textile dyes are often

carcinogenic (Kim *et al.*, 2003). Further, the adsorption of light by these textile dyes creates problems for photosynthetic aquatic plants and algae (Singh and Singh, 2006). Guendy (2007) discovered a method for treatment of wide conc. range of dye waste water through ozonization. Khadijah (2009) repoted 1540 bacterial isolates and screened for their ability to degrade selected azo dyes.

MATERIALS AND METHODS

(*a*) Sampling and analysis of effluent : Oswal Textile Industries, Ludhiana is one of the most industrialized cities in India. It is known as the textile capital of North India and was chosen for effluent sample collection. The Effluent sample was collected from the middle point of the area. Standard procedures (Spot and Grab) were followed during sampling. The Temperature and pH were determined at the sampling site. The pH was determined by using pH meter (Hanna digital pH meter, model-671-p) and temperature with laboratory thermometer. The sample was transported to laboratory at 4°C as in accordance with the standard methods (Yatome *et al.*, 1981). The physicochemical parameters such as (Colour, Biological Oxidation Demand (BOD) Chemical Oxygen Demand (COD), Total Suspended Solids (TSS), and Total Dissolved Solids (TDS) were determined as soon as the sample was brought to the laboratory. Sample colour was analysed by spectrophotometer. BOD was determined by employing evaporation method by DO meter while COD was measured by COD instrument directly.

(*b*) Chemicals: The textile dye, Crystal violet (λ_{max} 523 nm) was obtained from Oswal Textile Industries, Ludhiana. Nutrient broth (gL^{-1}Peptone-5, Meat extract-1, Yeast extract-2, NaCl-5, pH-7) A stock solution of the dye (1000mg L^{-1}) was prepared in de-ionized water and used for all studies.

(*c*) Isolation, screening and identification of dye decolorizing bacteria from effluent: The Textile Effluent was collected in sterile collection tubes from the sludge and wastewater of the ditches at industrial site located in Oswal Textile Industries, Ludhiana. The sample collected from the textile mill was screened for azo dye (Crystal violet) decolorizing bacterial strains by inoculating 10 ml. of sludge solution into 250ml. Erlenmeyer flask containing 100ml. nutrient broth (gL^{-1} Peptone-5, Meat extract-1, Yeast extract-2, NaCl-5, pH-7). The flasks were incubated at 35°C under shaking conditions (130rpm). After 48h of incubation, 1.0ml. of the culture broth was appropriately diluted and plated on Nutrient Agar (gL^{-1} Peptone-5, Meat extract-1, Yeast extract-2, NaCl-5, Agar-15, pH-7.0) containing 20 mg L^{-1} Crystal violet.The Morphologically distinct bacterial isolates showing clear zones around their colonies due to decolorization of dye were selected for further studies. The pure culture stocks of these isolates were stored at 4°C on Nutrient Agar slopes containing 1000 mg L^{-1} of Crystal violet. These isolates were screened for their ability to decolorize Crystal violet in liquid culture.

The Screening process in liquid media was carried out by inoculating a loop full of cultures exhibiting clear zones into Nutrient broth containing Crystal violet under static conditions. After 24h of incubation, 1ml. of cell suspension was transferred to fresh nutrient broth containing Crystal violet to screen the strains with color removing ability. The Screening procedure in liquid medium was continued until complete decolorization of broth. A small amount of decolorized broth was transferred to nutrient agar plates containing Crystal violet (50 mg L^{-1}). The bacterial isolate which tolerated higher concentration of the Azo dye was isolated by streak plate method. The Azo dye decolorizing bacteria was identified from several aspects including morphology characters, biochemical tests as described in Bergey's manual of determinative bacteriology (Indole, Methyl Red, Voges-Proskauer test, Citrate, Catalase, Oxidase, Nitrate Reduction test, Hydrolysis of Casein, Starch, Urea and Gelatin). Assimilation of various sugars such as D-glucose, D-fructose, galactose, mannitol and D-maltose as sole carbon source was determined by inoculating the isolate into carbohydrate broth supplemented with respective carbon source. After inoculation the tubes were incubated at 37°C for 24-48h.

(*d*) Decolorization assay: The decolorizing activity was expressed in terms of the percentage decolorization by the modified method described previously (Deepak *et al.*, 2004). The Decolorization process was carried out using shaking culture and static culture by inoculating 1ml. of precultured (O.D 0.8-1) *Bacillus subtilis* into 100ml. of sterilized Nutrient broth in 250 ml. Erlenmeyer flask and incubated on rotary shaker (130 rpm) at 35°C for 24h (Kalyani *et al.*, 2009). Filter sterilized (0.22 λm) Crystal violet (100 mgL^{-1}) was added to the culture and incubated in shaking conditions at 130rpm and in static conditions at room temperature for decolorization to occur. At regular intervals, 4 ml.sample was withdrawn aseptically and centrifuged at 10,000 rpm for 15min. The cell free supernatant was used to determine the percentage decolorization of Crystal violet. Decolorization of dye was determined by monitoring the decrease in absorbance at the maximum wavelength of Crystal violet (λ_{max} 523 nm) by using a UV-Visible spectrophotometer (UV-1700 pharmaspec, shimadzu). The uninoculated dye Medium supplemented with respective dye was used as blank (Jacob Thomson, 1998). Decolorization activity (%) was calculated by the following formula and all assays were done in triplicate :

$$\% \text{ decolorization} = \frac{\text{Initial absorbance} - \text{Final Absorbance}}{\text{Initial Absorbance}} \times 100$$

(*e*) Decolorization of crystal violet under different culture conditions: The decolorization efficiency of *Bacillus subtilis* strain was compared over a wide range of pH (5-9) by adjusting the pH with hydrochloric acid or sodium hydroxide. Decolorization at different Temperatures (RT, 35°C, 37°C, 40°C, 45°C, 50°C) was carried out by adjusting the pH to 8. Varying Carbon sources 1% each (dulcitol,

starch, maltose, sucrose, dextrose, mannitol, d-xylose, lactose, mannose)and Nitrogen sources 1% each (urea, potassium nitrate, sodium nitrate, malt extract, ammonium sulphate, ammonium nitrate, ammonium chloride, peptone) were used to check the decolorizing potential of the strain. All the flasks were incubated in static conditions at pH 8 and at 35°C.

(f) **Statistical analysis:** Data was statistically defined by one-way ANOVA using Microsoft excel. Results in each experiment were interpreted depending upon probabilities. Probability (p-value) was less than 0.05 which was found to be significant.

RESULTS

Physico-chemical characterization of textile effluent: The effluent sample collected from a small scale Oswal Textile Industries, Ludhiana, India, was black in colour, with pungent smell and pH of slightly above neutral level and was within the permissible limits. The temperature of the effluent was high. Total Suspended Solids (TSS) and Total Dissolved Solids (TDS) in the textile effluent were very high. The solids present in ground water, besides effecting the growth of the plants directly, also affect the soil structure, permeability and aeration, indirectly effecting the plant growth. The Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD) values were within the permissible limits in the effluent sample. Different bacterial strains isolated from the textile effluent were screened for their ability to decolorize the textile Azo dye (Crystal violet) and the potential strains were characterized morphologically, biochemically and at molecular level for identification. The bacterial count (CFU/ml) was significantly high.

Table 1: Physico-chemical characterization of the textile effluent collected from Oswal Textile Industries, Ludhiana.

S. No	*Parameter*	*Units*	*Effluent*
1.	Colour	-	Black
2.	Smell	-	Pungent
3.	Temperature	° C	40
4.	pH	-	8.00
5.	TSS	mg l^{-1}	2000
6.	TDS	mg l^{-1}	7000
7.	COD	mg l^{-1}	790
8.	BOD	mg l^{-1}	260

Isolation and identification: The study was started by screening for potential textile Azo dye decolorizing bacteria isolated from the textile industry effluent. Colonies surrounded by a nearly decolorized zone were isolated and then tested for dye removal capability using submerged culture. Strains isolated from the white colonies were inoculated in 100ml. of Nutrient broth in a 250ml. conical flask and incubated at 35°C under static conditions. One strain exhibiting highest decolorizing activity was chosen for further studies. The gram staining test showed the isolate to be non-motile, gram positive, spore forming, and rod-shaped bacteria. The spore was terminally located and ellipsoidal in shape. Biochemical characterization of the isolate revealed it to be negative for Indole, Methyl Red test, Voges-Proskauer, Citrate, Catalase, oxidase test and Nitrate Reduction test. The isolate showed negative result for the hydrolysis of casein, gelatin, starch and urea. The strain utilized various sugars, D-Maltose, D-Glucose, D-Fructose, Mannitol and Galactose as sole carbon sources and was found to be positive.

Effect of pH and temperature on decolorization: The decolorization efficiency of *Bacillus subtilis* was compared across a range of pH (5-9). The maximum decolorization (90%) was recorded at pH 8. At neutral pH the strain exhibited percentage decolorization value of 77%.Where as it was 47% and 44% at pH 6 and 9.The percentage decolorization decreased markedly at pH 5 (8%) due to acidic conditions (Fig. 1). The optimum pH for growth and decolorization was found to be 8. The dye decolorization activity of the strain was found to decrease with increasing incubation temperature. Highest decolorization was achieved at 35°C (90%) and least percentage decolorization was at Room Temperature (RT) (27%). At 37°C there was 82% decolorization noted followed by 67%, 50% and 29% at 40°C, 45°C and 50°C respectively at the end of 24h incubation (Fig. 2). No specific decolorization was observed in shaking conditions (130 rpm).

Effect of different carbon and nitrogen sources on crystal violet decolorization: Results of Crystal violet decolorization by with different Carbon (Fig. 3) and Nitrogen sources (Fig. 4) are depicted. Dextrose resulted in better decolorization efficiency with 91% followed by starch (78%) and mannose (62%) at the end of 24h incubation period. The decolorization efficiency decreased with dulcitol (56%), mannitol (42%), lactose (37%), d-xylose (34%) and sucrose (28%). Least decolorization was observed with maltose (11%). Maximum decolorization with nitrogen sources was achieved with Peptone (87%) and least was with Malt extract (16%). Urea and Ammonium sulphate exhibited good decolorization with 77% and 61%. The decolorization efficiency decreased markedly with Ammonium nitrate (57%), Sodium nitrate (26%), Potassium nitrate (22%) and Ammonium chloride (21%).

DISCUSSION

Industrial effluent is not stable and it varies often in a wide range depending upon the process practiced. South Asian countries are experiencing severe environmental problems due to rapid industrialization. This phenomenon is very common where the polluting industries like textile dyeing, leather tanning, paper and pulp processing, sugar manufacturing, etc.thrive as clusters. Among these the Textile industries are large industrial consumers of waters as well as producers of wastewater. The effluent discharged by this industry leads to serious pollution of groundwater and soils and ultimately affects the livelihood of the poor (Jiunkins *et al.*, 1982). The physico-chemical characterization of the collected textile effluent sample from Oswal Textile Industries, Ludhiana showed a high load of pollution indicators. Colour is contributed to a water body by the dissolved compounds (dyes and pigments). The effluent color was black due to mixture of various dyes and chemicals used in the dyeing process. The pH of the study

sample was slightly alkaline when compared to the acidic pH of the dyeing effluent in a previous study (Tyagi *et al.*, 1990). The pH of the effluent alters the physico-chemical properties of water which in turn adversely affects aquatic life, plant and humans. The soil permeability gets affected resulting in polluting underground resources of water (Vandevivre *et al.*, 1998). The temperature of the effluent was high in comparison with the temperature of another effluent in one study (Kumar *et al.*, 1989). High temperature decreases the solubility of gases in water which is ultimately expressed as high BOD/COD. The values of BOD and COD were within the permissible limits in the present sample in comparison to the very high values of BOD and COD in one effluent study.

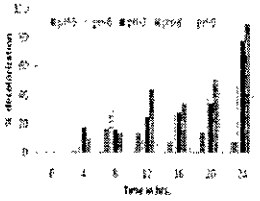

Fig. 1. Crystal violet decolorization at different pH.

TDS and TSS values of effluent sample was high than the permissible limits but when compared to a textile effluent collected from a mill near Jalandhar, Punjab was found to be low (Senthilnathan *et al.*, 1999). Sediments rate is drastically increased because of High value of Total Dissolved Solids which reduces the light penetration into water and ultimately decrease the photosynthesis. The decrease in photosynthetic rate reduces the DO level of wastewater which results in decreased purification of wastewater by microorganisms (Delee *et al.*, 1998). The current sample exhibited high values of heavy metals which was of the same order of magnitude reported in another effluent sample (Kim *et al.*, 1994). The nutrients of the surrounding soils are depleted as a result of high value of heavy metals thereby affecting soil fertility. High chloride contents are harmful for agricultural crops if such wastes containing high chlorides are used for irrigation purposes. (Agarwal *et al.*, 1996). Majority of the textile effluent samples have permissible limits of sulphate ions. The effluent showed phenolic contents greater than 0.1 ppm which is though permissible limit of the phenolic compounds still these compounds are very toxic to fish even at very low concentrations (Coughlin *et al.*, 1997). The bleaching and dyeing process are the main causes of pollutants which include caustic soda, hypochlorite and peroxides.

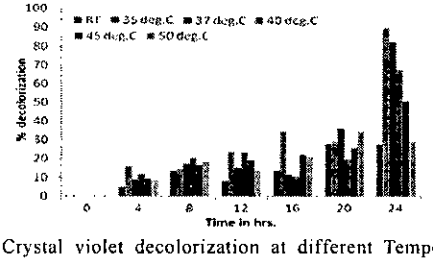

Fig. 2. Crystal violet decolorization at different Temperatures.

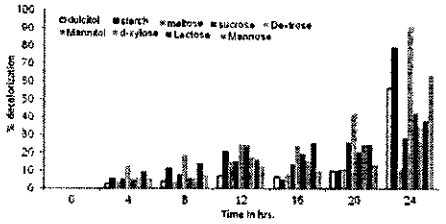

Fig. 3. Crystal violet decolorization in different C-sources.

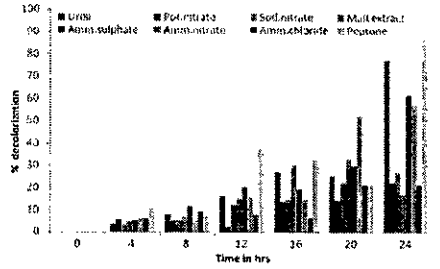

Fig. 4. Crystal violet decolorization in different N-sources.

The isolation of different microorganisms from the effluent sample collected from the Oswal Textile Industries, Ludhiana indicates to natural adaptation of microorganisms to survive in the presence of toxic chemicals and dyes. Interest in the bioremediation of pollutants using bacteria has intensified in recent years, as many researches demonstrated the efficacy of bacterial bioremediation over fungal and Actinomycetes. Many bacteria capable of reducing Azo dyes reported were isolated from Textile effluent contaminated sites (Dawkar *et al.*, 2008). A strain of bacterium *Bacillus subtilis* with strong decolorizing ability was isolated from Textile effluent to decolorize the textile Azo dye Crystal violet (100 mgL^{-1}) within 24h in aerobic and static conditions. The reason for the decreased decolorization under shaking conditions could be competition of oxygen and dye compounds for the reduced electron carriers under aerobic conditions (Junnarkar *et al.*, 2006). The percentage decolorization of Crystal violet by *Bacillus subtilis* strain under static conditions was 90% within 24h of incubation which was equal to a similar study but with 35h of incubation period (Khehra *et al.*, 2005). In another study conducted with *Pseudomonas putida, P. fluorescence, Bacillus cereus and Stentrophomonas acidaminiphila* to decolorize Acid Red 88 showed their efficiencies at 35%, 31%, 40% and 50% respectively (Hu *et al.*, 1998). Under aerobic conditions azo dyes are generally resistant to attack by bacteria (Daneshvar *et al.*, 2007). The optimal pH for complete decolorization of Crystal violet was at 8 which is slightly in accordance with Cosmarium sp. Decolorizing malachite green at pH 9 (Wong and Yuen, 1998) and *Klebsiella pneumonia* RS-13 which completely degraded Methyl Red in pH range of 6 to 8 (Mali *et al.*, 2000). Optimal growth temperature of was found to be 35°C which is consistent with the highest decolorization temperature in our study. Maximum potential of Pseudomonas sp. to decolorize Malachite green, Fast green was noticed at 37°C (Adedayo *et al.*, 2004). *Vibrio logei* and *Pseudomonas nitroreducens* showed the highest Methyl Red degradation activity at 30-35°C (Kapdan *et al.*,

2000). Starch and Peptone were found to be most effective carbon and nitrogen sources for decolorization of Crystal violet by in the present study compared to Lactose and Yeast extract in another similar study for decolorization of Everzol Red RBN (Panswed and Wongehaisuwan, 1986).

CONCLUSION

Although decolorization is a challenging process to both the textile industry and the waste water treatment, the result of this findings and literature suggest a great potential for bacteria to be used to remove color from dye wastewaters. Interestingly, the bacterial species used in carrying out the decolorization of Azo dye Crystal violet in this study was isolated from the textile dye industry waste effluent. The bacterial strain *Bacillus subtilis* showed decolorizing activity through a degradation mechanism rather than adsorption. This observation has established that the bacteria are adaptive in nature and can degrade contaminants. The ability of the strain to tolerate, decolorize azo dyes at high concentration gives it an advantage for treatment of textile industry waste waters. However, potential of the strain needs to be demonstrated for its application in treatment of real dye bearing waste waters using appropriate bioreactors.

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Microbial Decolourization of Crystal Violet by *Bacillus subtilis*

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ABSTRACT : The physicochemical characterization of the textile industry effluent collected from Oswal Textile Industries, Ludhiana (Punjab.) India has been carried out and the results showed that the temperature (40°C), pH (8.00), Biological Oxygen Demand (260 mgl^{-1}), Chemical Oxygen Demand (790 mgl^{-1}), Total Suspended Solids (2000 mgl^{-1}), Total Dissolved Solids (7000 mgl^{-1} and colour over the prescribed fresh water limits. A potential bacterial strain was isolated and selected from the textile effluent on the basis of rapid azo dye Crystal violet (100mgl^{-1}) decolorization and later identified as belonging to genus *Bacillus* based on Phenotypic characterization and phylogenetic analysis of the 16s rRNA gene sequence. Effects of physicochemical parameters (pH, Temperature, etc.) on the Crystal violet decolorization by the *Bacillus* were studied. Decolorization was effective at pH 8, 35°C with starch and peptone as carbon and nitrogen sources and in static conditions. This decolorization potential increased the applicability of this microorganism for the dye removal.

Keywords : Textile Industry effluent; *Bacillus subtilis*; Crystal Violet; decolorization.

INTRODUCTION

The synthetic dyes are used extensively in textile dyeing and paper printing. First synthetic dye was reported in 1856. There are more than 40,000 dyes and pigments with some 7000 different chemical structures, out of which more than 3500 dyes are of practical use. Based on the chemical structure of the chromophoric group, the synthetic dyes are classified as azo dyes, nitroso dyes, triphenylmethane dyes, xanthane dyes and anthraquinone dyes (Shenai, 1994). India is now the largest producer of dyes and intermediaries in World. Because these dyes are mutagenic and carcinogenic and also cannot be completely removed by conventional wastewater treatment systems, before disposal and discharge of dye-containing effluents, they are to be treated to reduce their levels of toxicity and thus, to minimize their pollution impact. Some of the triphenylmethane dyes are used as dermatological agents, the best among them being gentian violet, which is a mutagen, a mitotic poison and clastogen (Kapdan *et. al.*, 2000). Bioremediation is an expensive mean to remove hazardous metal ions from the contaminated effluent (Faryal and Hammed, 2005). Presently, it was estimated about 10,000 of different commercial dyes and pigments exists and over 7 × 10 tones are produced annually worldwide (Guendy, 2007). Biotechnological tools also have been applied for the degradation of various textile dye and it was found that upto 70% color removal was noticed with different microflora (Khadijah *et al.*, 2009). The biological methods being simple to use and low in cost have become main focus in recent studies on dye biodegradation. Keeping the above facts in mind, the present study was envisaged.

Indiscriminate disposal of sewage and industrial effluents is a major cause of pollution of water bodies and rivers into which they are discharged. The use of industrial effluents carrying a heavy load of heavy metals such as Pb, Zn, Cr, Ni and Hg for irrigation of crops also produces adverse effects on plant growth. Waste water generated by different production steps of a textile industry have high pH, temperature, detergents, oil, suspended and dissolved solids, dispersants, leveling agents, toxic and non-biodegradable matter, color and alkalinity. The effluents also consist of high concentrations of dye stuff, biochemical oxygen demand, total dissolved solids, sodium, chloride, sulphate, hardness, heavy metals and carcinogenic dye ingredients which pose serious environmental problems.

The mechanism of biodegradation depends in part, on the compound being degraded, but there are some consistent steps in the process regardless of the substrate. When an electron is added or removed from the ground state of a chemical it becomes highly reactive, allowing it to give or take electrons from other chemicals. This provides the basis for the non specificity of the enzymes and the ability of the enzymes to degrade xenobiotics, chemicals that have never been encountered in nature. The main reactions that are catalyzed by the lignolytic enzymes include depolymerization, demethoxylation, decarboxylation, hydroxylation and aromatic ring opening. Many of these reactions result in oxygen activation, creating radicals that perpetuate oxidation of the organopollutants. Once the peroxidases have opened the aromatic ring structures by way of introducing oxygen, other more common species of fungi and bacteria can mineralize the products intracellularly into products such as CO_2 and other benign compounds. (Weigel, 1999) further reported that anaerobic dehalogenation of polychlorinated biphenyls in sediment slumes dehalogenales all flanking chlorines. Faryal and Hameed (2005) carried out the textile effluent analysis for presence of Mn, Zn, Mg etc. and reported subsequent decolourising bacteria.

A very small amount of dye in water (10-50 mg L^{-1}) affects the aesthetic value, transparency of water and gas solubility of water bodies. The presence of even very low concentrations of dyes in effluent is highly visible and degradation products of these textile dyes are often

carcinogenic (Kim *et al.*, 2003). Further, the adsorption of light by these textile dyes creates problems for photosynthetic aquatic plants and algae (Singh and Singh, 2006). Guendy (2007) discovered a method for treatment of wide conc. range of dye waste water through ozonization. Khadijah (2009) repoted 1540 bacterial isolates and screened for their ability to degrade selected azo dyes.

MATERIALS AND METHODS

(*a*) Sampling and analysis of effluent : Oswal Textile Industries, Ludhiana is one of the most industrialized cities in India. It is known as the textile capital of North India and was chosen for effluent sample collection. The Effluent sample was collected from the middle point of the area. Standard procedures (Spot and Grab) were followed during sampling. The Temperature and pH were determined at the sampling site. The pH was determined by using pH meter (Hanna digital pH meter, model-671-p) and temperature with laboratory thermometer. The sample was transported to laboratory at 4°C as in accordance with the standard methods (Yatome *et al.*, 1981). The physicochemical parameters such as (Colour, Biological Oxidation Demand (BOD) Chemical Oxygen Demand (COD), Total Suspended Solids (TSS), and Total Dissolved Solids (TDS) were determined as soon as the sample was brought to the laboratory. Sample colour was analysed by spectrophotometer. BOD was determined by employing evaporation method by DO meter while COD was measured by COD instrument directly.

(*b*) Chemicals: The textile dye, Crystal violet (λ_{max} 523 nm) was obtained from Oswal Textile Industries, Ludhiana. Nutrient broth (gL^{-1}Peptone-5, Meat extract-1, Yeast extract-2, NaCl-5, pH-7) A stock solution of the dye (1000mg L^{-1}) was prepared in de-ionized water and used for all studies.

(*c*) Isolation, screening and identification of dye decolorizing bacteria from effluent: The Textile Effluent was collected in sterile collection tubes from the sludge and wastewater of the ditches at industrial site located in Oswal Textile Industries, Ludhiana. The sample collected from the textile mill was screened for azo dye (Crystal violet) decolorizing bacterial strains by inoculating 10 ml. of sludge solution into 250ml. Erlenmeyer flask containing 100ml. nutrient broth (gL^{-1} Peptone-5, Meat extract-1, Yeast extract-2, NaCl-5, pH-7). The flasks were incubated at 35°C under shaking conditions (130rpm). After 48h of incubation, 1.0ml. of the culture broth was appropriately diluted and plated on Nutrient Agar (gL^{-1} Peptone-5, Meat extract-1, Yeast extract-2, NaCl-5, Agar-15, pH-7.0) containing 20 mg L^{-1} Crystal violet.The Morphologically distinct bacterial isolates showing clear zones around their colonies due to decolorization of dye were selected for further studies. The pure culture stocks of these isolates were stored at 4°C on Nutrient Agar slopes containing 1000 mg L^{-1} of Crystal violet. These isolates were screened for their ability to decolorize Crystal violet in liquid culture.

The Screening process in liquid media was carried out by inoculating a loop full of cultures exhibiting clear zones into Nutrient broth containing Crystal violet under static conditions. After 24h of incubation, 1ml. of cell suspension was transferred to fresh nutrient broth containing Crystal violet to screen the strains with color removing ability. The Screening procedure in liquid medium was continued until complete decolorization of broth. A small amount of decolorized broth was transferred to nutrient agar plates containing Crystal violet (50 mg L^{-1}). The bacterial isolate which tolerated higher concentration of the Azo dye was isolated by streak plate method. The Azo dye decolorizing bacteria was identified from several aspects including morphology characters, biochemical tests as described in Bergey's manual of determinative bacteriology (Indole, Methyl Red, Voges-Proskauer test, Citrate, Catalase, Oxidase, Nitrate Reduction test, Hydrolysis of Casein, Starch, Urea and Gelatin). Assimilation of various sugars such as D-glucose, D-fructose, galactose, mannitol and D-maltose as sole carbon source was determined by inoculating the isolate into carbohydrate broth supplemented with respective carbon source. After inoculation the tubes were incubated at 37°C for 24-48h.

(*d*) Decolorization assay: The decolorizing activity was expressed in terms of the percentage decolorization by the modified method described previously (Deepak *et al.*, 2004). The Decolorization process was carried out using shaking culture and static culture by inoculating 1ml. of precultured (O.D 0.8-1) *Bacillus subtilis* into 100ml. of sterilized Nutrient broth in 250 ml. Erlenmeyer flask and incubated on rotary shaker (130 rpm) at 35°C for 24h (Kalyani *et al.*, 2009). Filter sterilized (0.22 λm) Crystal violet (100 mgL^{-1}) was added to the culture and incubated in shaking conditions at 130rpm and in static conditions at room temperature for decolorization to occur. At regular intervals, 4 ml.sample was withdrawn aseptically and centrifuged at 10,000 rpm for 15min. The cell free supernatant was used to determine the percentage decolorization of Crystal violet. Decolorization of dye was determined by monitoring the decrease in absorbance at the maximum wavelength of Crystal violet (λ_{max} 523 nm) by using a UV-Visible spectrophotometer (UV-1700 pharmaspec, shimadzu). The uninoculated dye Medium supplemented with respective dye was used as blank (Jacob Thomson, 1998). Decolorization activity (%) was calculated by the following formula and all assays were done in triplicate :

$$\% \text{ decolorization} \quad \frac{\text{Initial absorbance} \quad \text{Final Absorbance}}{\text{Initial Absorbance}} \quad 100$$

(*e*) Decolorization of crystal violet under different culture conditions: The decolorization efficiency of *Bacillus subtilis* strain was compared over a wide range of pH (5-9) by adjusting the pH with hydrochloric acid or sodium hydroxide. Decolorization at different Temperatures (RT, 35°C, 37°C, 40°C, 45°C, 50°C) was carried out by adjusting the pH to 8. Varying Carbon sources 1% each (dulcitol,

starch, maltose, sucrose, dextrose, mannitol, d-xylose, lactose, mannose)and Nitrogen sources 1% each (urea, potassium nitrate, sodium nitrate, malt extract, ammonium sulphate, ammonium nitrate, ammonium chloride, peptone) were used to check the decolorizing potential of the strain. All the flasks were incubated in static conditions at pH 8 and at 35°C.

(f) Statistical analysis: Data was statistically defined by one-way ANOVA using Microsoft excel. Results in each experiment were interpreted depending upon probabilities. Probability (p-value) was less than 0.05 which was found to be significant.

RESULTS

Physico-chemical characterization of textile effluent: The effluent sample collected from a small scale Oswal Textile Industries, Ludhiana, India, was black in colour, with pungent smell and pH of slightly above neutral level and was within the permissible limits. The temperature of the effluent was high. Total Suspended Solids (TSS) and Total Dissolved Solids (TDS) in the textile effluent were very high. The solids present in ground water, besides effecting the growth of the plants directly, also affect the soil structure, permeability and aeration, indirectly effecting the plant growth. The Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD) values were within the permissible limits in the effluent sample. Different bacterial strains isolated from the textile effluent were screened for their ability to decolorize the textile Azo dye (Crystal violet) and the potential strains were characterized morphologically, biochemically and at molecular level for identification. The bacterial count (CFU/ml) was significantly high.

Table 1: Physico-chemical characterization of the textile effluent collected from Oswal Textile Industries, Ludhiana.

S. No	*Parameter*	*Units*	*Effluent*
1.	Colour	-	Black
2.	Smell	-	Pungent
3.	Temperature	° C	40
4.	pH	-	8.00
5.	TSS	mg l^{-1}	2000
6.	TDS	mg l^{-1}	7000
7.	COD	mg l^{-1}	790
8.	BOD	mg l^{-1}	260

Isolation and identification: The study was started by screening for potential textile Azo dye decolorizing bacteria isolated from the textile industry effluent. Colonies surrounded by a nearly decolorized zone were isolated and then tested for dye removal capability using submerged culture. Strains isolated from the white colonies were inoculated in 100ml. of Nutrient broth in a 250ml. conical flask and incubated at 35°C under static conditions. One strain exhibiting highest decolorizing activity was chosen for further studies. The gram staining test showed the isolate to be non-motile, gram positive, spore forming, and rod-shaped bacteria. The spore was terminally located and ellipsoidal in shape. Biochemical characterization of the isolate revealed it to be negative for Indole, Methyl Red test, Voges-Proskauer, Citrate, Catalase, oxidase test and Nitrate Reduction test. The isolate showed negative result for the hydrolysis of casein, gelatin, starch and urea. The strain utilized various sugars, D-Maltose, D-Glucose, D-Fructose, Mannitol and Galactose as sole carbon sources and was found to be positive.

Effect of pH and temperature on decolorization: The decolorization efficiency of *Bacillus subtilis* was compared across a range of pH (5-9). The maximum decolorization (90%) was recorded at pH 8. At neutral pH the strain exhibited percentage decolorization value of 77%.Where as it was 47% and 44% at pH 6 and 9.The percentage decolorization decreased markedly at pH 5 (8%) due to acidic conditions (Fig. 1). The optimum pH for growth and decolorization was found to be 8. The dye decolorization activity of the strain was found to decrease with increasing incubation temperature. Highest decolorization was achieved at 35°C (90%) and least percentage decolorization was at Room Temperature (RT) (27%). At 37°C there was 82% decolorization noted followed by 67%, 50% and 29% at 40°C, 45°C and 50°C respectively at the end of 24h incubation (Fig. 2). No specific decolorization was observed in shaking conditions (130 rpm).

Effect of different carbon and nitrogen sources on crystal violet decolorization: Results of Crystal violet decolorization by with different Carbon (Fig. 3) and Nitrogen sources (Fig. 4) are depicted. Dextrose resulted in better decolorization efficiency with 91% followed by starch (78%) and mannose (62%) at the end of 24h incubation period. The decolorization efficiency decreased with dulcitol (56%), mannitol (42%), lactose (37%), d-xylose (34%) and sucrose (28%). Least decolorization was observed with maltose (11%). Maximum decolorization with nitrogen sources was achieved with Peptone (87%) and least was with Malt extract (16%). Urea and Ammonium sulphate exhibited good decolorization with 77% and 61%. The decolorization efficiency decreased markedly with Ammonium nitrate (57%), Sodium nitrate (26%), Potassium nitrate (22%) and Ammonium chloride (21%).

DISCUSSION

Industrial effluent is not stable and it varies often in a wide range depending upon the process practiced. South Asian countries are experiencing severe environmental problems due to rapid industrialization. This phenomenon is very common where the polluting industries like textile dyeing, leather tanning, paper and pulp processing, sugar manufacturing, etc.thrive as clusters. Among these the Textile industries are large industrial consumers of waters as well as producers of wastewater. The effluent discharged by this industry leads to serious pollution of groundwater and soils and ultimately affects the livelihood of the poor (Jiunkins *et al.*, 1982). The physico-chemical characterization of the collected textile effluent sample from Oswal Textile Industries, Ludhiana showed a high load of pollution indicators. Colour is contributed to a water body by the dissolved compounds (dyes and pigments). The effluent color was black due to mixture of various dyes and chemicals used in the dyeing process. The pH of the study

sample was slightly alkaline when compared to the acidic pH of the dyeing effluent in a previous study (Tyagi *et al.*, 1990). The pH of the effluent alters the physico-chemical properties of water which in turn adversely affects aquatic life, plant and humans. The soil permeability gets affected resulting in polluting underground resources of water (Vandevivre *et al.*, 1998). The temperature of the effluent was high in comparison with the temperature of another effluent in one study (Kumar *et al.*, 1989). High temperature decreases the solubility of gases in water which is ultimately expressed as high BOD/COD. The values of BOD and COD were within the permissible limits in the present sample in comparison to the very high values of BOD and COD in one effluent study.

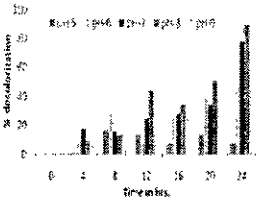

Fig. 1. Crystal violet decolorization at different pH.

TDS and TSS values of effluent sample was high than the permissible limits but when compared to a textile effluent collected from a mill near Jalandhar, Punjab was found to be low (Senthilnathan *et al.*, 1999). Sediments rate is drastically increased because of High value of Total Dissolved Solids which reduces the light penetration into water and ultimately decrease the photosynthesis. The decrease in photosynthetic rate reduces the DO level of wastewater which results in decreased purification of wastewater by microorganisms (Delee *et al.*, 1998). The current sample exhibited high values of heavy metals which was of the same order of magnitude reported in another effluent sample (Kim *et al.*, 1994). The nutrients of the surrounding soils are depleted as a result of high value of heavy metals thereby affecting soil fertility. High chloride contents are harmful for agricultural crops if such wastes containing high chlorides are used for irrigation purposes. (Agarwal *et al.*, 1996). Majority of the textile effluent samples have permissible limits of sulphate ions. The effluent showed phenolic contents greater than 0.1 ppm which is though permissible limit of the phenolic compounds still these compounds are very toxic to fish even at very low concentrations (Coughlin *et al.*, 1997). The bleaching and dyeing process are the main causes of pollutants which include caustic soda, hypochlorite and peroxides.

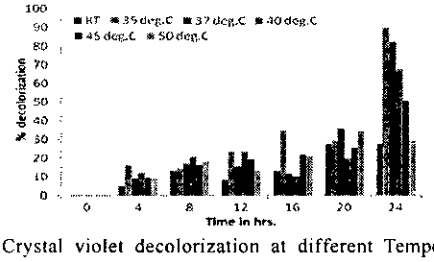

Fig. 2. Crystal violet decolorization at different Temperatures.

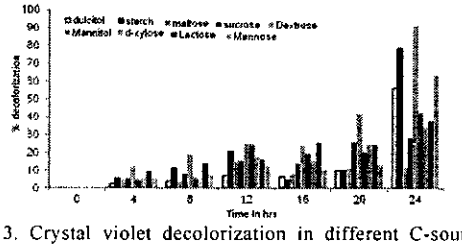

Fig. 3. Crystal violet decolorization in different C-sources.

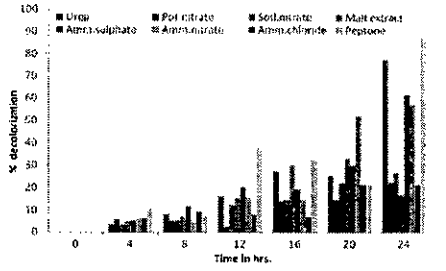

Fig. 4. Crystal violet decolorization in different N-sources.

The isolation of different microorganisms from the effluent sample collected from the Oswal Textile Industries, Ludhiana indicates to natural adaptation of microorganisms to survive in the presence of toxic chemicals and dyes. Interest in the bioremediation of pollutants using bacteria has intensified in recent years, as many researches demonstrated the efficacy of bacterial bioremediation over fungal and Actinomycetes. Many bacteria capable of reducing Azo dyes reported were isolated from Textile effluent contaminated sites (Dawkar *et al.*, 2008). A strain of bacterium *Bacillus subtilis* with strong decolorizing ability was isolated from Textile effluent to decolorize the textile Azo dye Crystal violet (100 mgL^{-1}) within 24h in aerobic and static conditions. The reason for the decreased decolorization under shaking conditions could be competition of oxygen and dye compounds for the reduced electron carriers under aerobic conditions (Junnarkar *et al.*, 2006). The percentage decolorization of Crystal violet by *Bacillus subtilis* strain under static conditions was 90% within 24h of incubation which was equal to a similar study but with 35h of incubation period (Khehra *et al.*, 2005). In another study conducted with *Pseudomonas putida, P. fluorescence, Bacillus cereus and Stentrophomonas acidaminiphila* to decolorize Acid Red 88 showed their efficiencies at 35%, 31%, 40% and 50% respectively (Hu *et al.*, 1998). Under aerobic conditions azo dyes are generally resistant to attack by bacteria (Daneshvar *et al.*, 2007). The optimal pH for complete decolorization of Crystal violet was at 8 which is slightly in accordance with Cosmarium sp. Decolorizing malachite green at pH 9 (Wong and Yuen, 1998) and *Klebsiella pneumonia* RS-13 which completely degraded Methyl Red in pH range of 6 to 8 (Mali *et al.*, 2000). Optimal growth temperature of was found to be 35°C which is consistent with the highest decolorization temperature in our study. Maximum potential of Pseudomonas sp. to decolorize Malachite green, Fast green was noticed at 37°C (Adedayo *et al.*, 2004). *Vibrio logei* and *Pseudomonas nitroreducens* showed the highest Methyl Red degradation activity at 30-35°C (Kapdan *et al.*,

2000). Starch and Peptone were found to be most effective carbon and nitrogen sources for decolorization of Crystal violet by in the present study compared to Lactose and Yeast extract in another similar study for decolorization of Everzol Red RBN (Panswed and Wongehaisuwan, 1986).

CONCLUSION

Although decolorization is a challenging process to both the textile industry and the waste water treatment, the result of this findings and literature suggest a great potential for bacteria to be used to remove color from dye wastewaters. Interestingly, the bacterial species used in carrying out the decolorization of Azo dye Crystal violet in this study was isolated from the textile dye industry waste effluent. The bacterial strain *Bacillus subtilis* showed decolorizing activity through a degradation mechanism rather than adsorption. This observation has established that the bacteria are adaptive in nature and can degrade contaminants. The ability of the strain to tolerate, decolorize azo dyes at high concentration gives it an advantage for treatment of textile industry waste waters. However, potential of the strain needs to be demonstrated for its application in treatment of real dye bearing waste waters using appropriate bioreactors.

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