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An Innovative Approach to Biodegradation of Textile Dye (Remazol Black B) by *Bacillus Spp.*

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Abstract In the present study, an attempt was made to examine the potential of isolated bacterium for decolorization of Remazol Black B dye in batch reactors. A potential bacterial strain was isolated and selected from the textile effluent on the basis of rapid azo dye Remazol Black B ($250mgl^{-1}$) decolorization and later identified as belonging to genus *Bacillus* based on phenotypic characterization. The effect of pH, temperature, carbon & nitrogen source was studied with an aim to determine the optimal conditions required for maximum decolorization and degradation. 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. The results show that the isolated bacterium has a dynamic potential in removal of Remazol Black B dye from wastewater under aerobic conditions.

Keywords: *Remazol Black B, Bacillus, pH, temperature, static condition*

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1. Introduction

Rapid industrialization has necessitated the manufacture and use of different chemicals in day to day life (Moorthi et al., 2007, Baljeet et al., 2001). The textile industry is one of them which extensively use synthetic chemicals as dyes. Wastewaters from textile industries pose a threat to the environment, as large amount of chemically different dyes are used. A significant proportion of these dyes enter the environment via wastewater. Approximately 10,000 different dyes and pigments are used industrially and over 0.7 million tons of synthetic dyes are produced annually, worldwide (Rafi et al., 1990). Pollution due to textile industry effluent has increased during recent years. Moreover, it is very difficult to treat textile industry effluents because of their high BOD, COD, heat, color, pH and the presence of metal ions (Anjali P et al., 2007). The textile finishing generates a large amount of waste water containing dyes and represents one of the largest causes of water pollution (Bhatti HN et al., 2008), as 10-15% of dyes are lost in the effluent during the dyeing process (Zollinger H, 1991). The traditional textile finishing industry consumes about 100 liters of water to process about 1 Kg of textile material. The new closed-loop technologies such as the reuse of microbial or enzymatical treatment of dyeing effluents could help reducing this enormous water pollution (Abadull E et al., 2000). Azo dyes have been used increasingly in industries because of their ease and cost effectiveness in synthesis compared to natural dyes. However, most azo dyes are toxic, carcinogenic and mutagenic (Pinherio HM et al., 2004). Azo bonds present in these compounds are resistant to breakdown, with the potential for the persistence and accumulation in the environment (Talarposhti et al., 2001). However, they can be degraded by bacteria under aerobic and anaerobic conditions (Woong & Yueen., 1996). Several physico-chemical techniques have been proposed for treatment of colored textile effluents. These include adsorption on different materials, oxidation and precipitation by Fenton's reagent, bleaching with chloride or ozone photo degradation or membrane filtration (Robinson et al., 2001). All these physical or chemical methods are very expensive and result in the production of large amounts of sludge, which creates the secondary level of land pollution. Therefore, economic and safe removal of the polluting dyes is still an important issue. Bioremediation through microorganisms has been identified as a cost effective and environment friendly alternative for disposal of textile effluent (Chen KC et al., 2003 , Ponraj et al., 2011). In recent years a number of studies have focused on some microorganisms capable of degrading and absorbing dyes from wastewater. A wide variety of microorganisms are reported to be capable of decolonization of dyes (Chen KC et al., 2003, Chang et al., 2000, Chang et al., 2001, Fu Y, 2002, Saikia N., 2004, Fournier et al., 2004, Aksu, 2003, Gupta et al, 2006, Acuner E, 2004, De-Bashan et al., 2002, Valderama et al., 2002, Yan H, 2004, Gupta et al., 2004, Kumar et al., 2005, Sneath et al, 1984, Arun Prasad et al., 2010, Tripathi A et al., 2012, Modi HA et al., 2010,

Ola et al., 2010, Khan JA et al., 2011, Namdhari BS et al., 2012, Kumar Praveen et al., 2012, Raj Kumar S et al., 2012). The current study has evaluated the potential of isolated bacterial strain from textile effluent for their decolorization efficiency of the textile dye, Remazol Black B under in vitro conditions and optimization of the factors influencing the process.

2. Materials & Methods

2.1. Sampling and Analysis of Effluent

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 and temperature with laboratory thermometer. The sample was transported to laboratory at 4ºC 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.

2.2. Chemicals

The textile dye, Remazol Black B (λmax 595 nm) was obtained from Ankleshwar Textile Industries, Ankleshwar, Gujarat, India. Nutrient broth (gL^{-1}Peptone-5, Meat extract-1, Yeast extract- 2, NaCl-5, pH-7) A stock solution of the dye (1000mgL^{-1}) was prepared in de-ionized water and used for all studies.

2.3. 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 Ankleshwar Textile Industries, Gujarat, India. The sample collected from the textile mill was screened for azo dye (Remazol Black B) 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 (120rpm). After 48h of incubation, 1.0 ml. 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 25 mg L^{-1} Remazol Black B. 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 Remazol Black B. These isolates were screened for their ability to decolorize Remazol Black B 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 Remazol Black B under static conditions. After 24h of incubation, 1ml. of cell suspension was transferred to fresh nutrient broth containing Remazol Black B 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 Remazol Black B (250 mgL^{-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 37ºC for 24-48h.

2.4. 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 pre cultured(O.D 0.8-1) *Bacillus spp.* into 100ml. of sterilized Nutrient broth in 250 ml. Erlenmeyer flask and incubated on rotary shaker (120 rpm) at 35ºC for 24h (Kalyani *et al.*, 2009). Filter sterilize Remazol Black B (250 mgL^{-1}) was added to the culture and incubated in shaking conditions at 120rpm and in static conditions at room temperature for decolorization to occur. At regular intervals, 4 ml.sample was withdrawn aseptically and centrifuged at 8,000 rpm for 10min. The cell free supernatant was used to determine the percentage decolorization of Remazol Black B. Decolorization of dye was determined by monitoring the decrease in absorbance at the maximum wavelength of Remazol Black B (λmax 595 nm) by using a UV-Visible spectrophotometer (UV-1800 pharmaspec, shimadzu, Japan). 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:

$$\%Decolorization = \frac{Initial\ OD\ Observed\ OD \times 100}{Initial\ OD}$$

2.5 Decolorization of Crystal Violet under Different Culture Conditions

The decolorization efficiency of *Bacillus spp.* 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.

3. Results

3.1. Physico-chemical Characterization of Textile Effluent

The effluent sample collected from Ankleshwar Textile Industries, Ankleshwar, Gujarat, India, was black in color, 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 (Remazol Black B) and the potential strains were characterized morphologically, & biochemically for identification.

3.2. 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, DFructose, Mannitol and Galactose as sole carbon sources and was found to be positive.

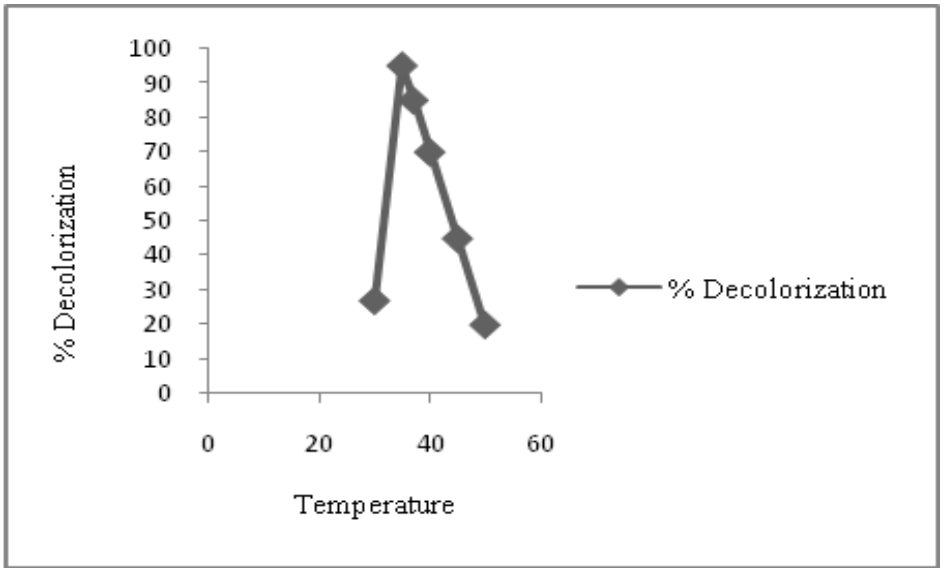

Figure 1. Effect of pH on degradation of Remazol Black B

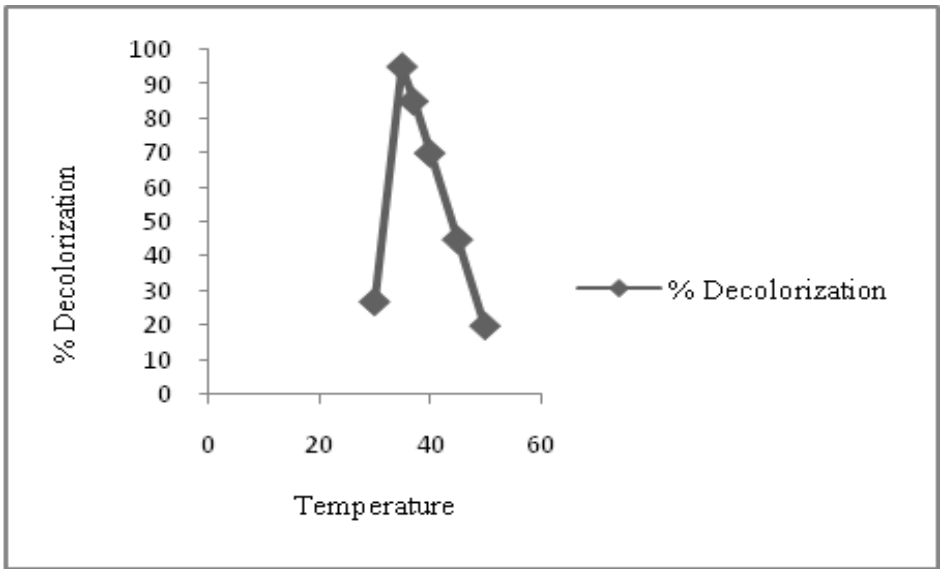

Figure 2. Effect of temperature on degradation of Remazol Black B

3.3. Effect of pH and Temperature on Decolorization

The decolorization efficiency of *Bacillus spp.* was compared across a range of pH (5-9). The maximum decolorization (95%) was recorded at pH 8. At neutral pH the strain exhibited a percentage decolorization value of 80%.Whereas it was 55% and 50% at pH 6 and 9.The percentage decolorization decreased markedly at pH 5 (12%) due to acidic conditions (Figure 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 (95%) and least percentage decolorization was at Room Temperature (30°C) (27%). At 37°C there was 85% decolorization noted followed by 70%, 45% and 20% at 40°C, 45°C and 50°C respectively at the end of 24h incubation (Figure 2). No specific decolorization was observed in shaking conditions (120 rpm).

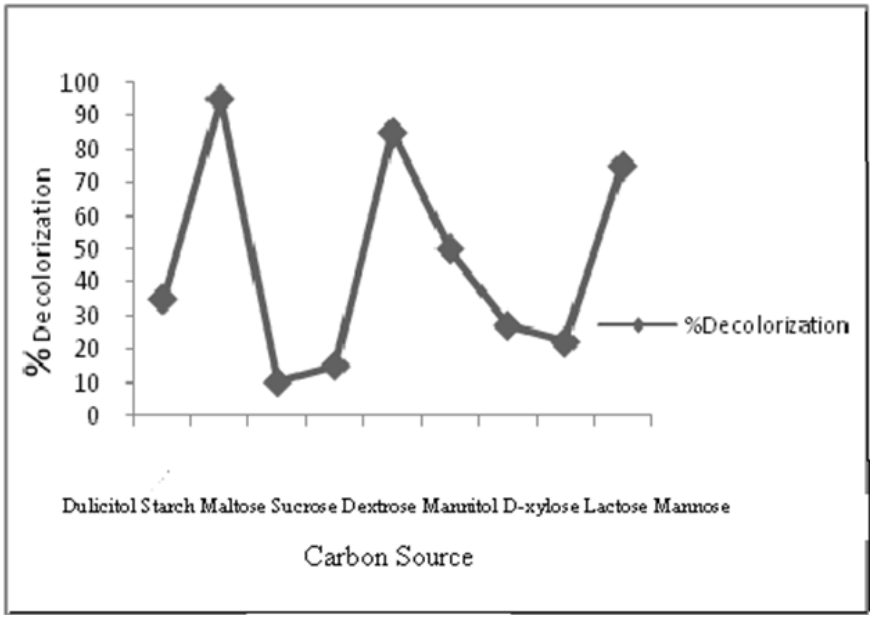

Figure 3. Effect of carbon source on degradation of Remazol Black B

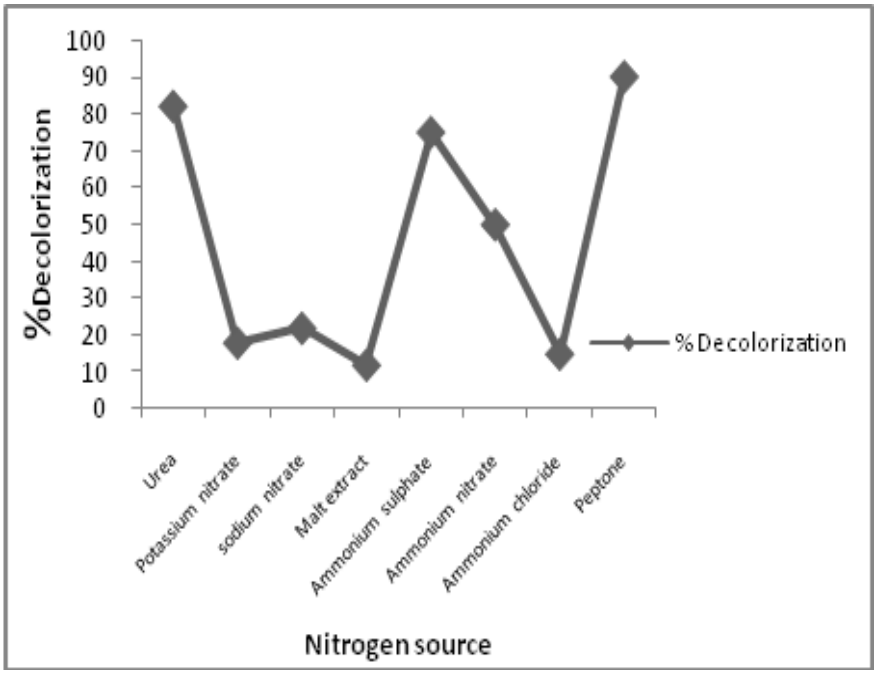

Figure 4. Effect of Nitrogen source on degradation of Remazol Black B

3.4. Effect of Different Carbon and Nitrogen Sources on Crystal Violet Decolorization

Results of Remazol Black B decolorization by with different Carbon (Figure 3) and Nitrogen sources (Figure 4) are depicted. Starch resulted in highest decolorization efficiency with 95% followed by Dextrose (85%) and mannose (75%) at the end of 24h incubation period. The decolorization efficiency decreased with mannitol (50%), dulcitol (35%), d-xylose (27%), lactose (22%) and sucrose (15%). Least decolorization was observed with maltose (10%). Maximum decolorization with nitrogen sources was achieved with Peptone (90%) and least was with Malt extract (12%). Urea and Ammonium sulphate exhibited

good decolorization with 82% and 75%. The decolorization efficiency decreased markedly with Ammonium nitrate (50%), Sodium nitrate (22%), Potassium nitrate (18%) and Ammonium chloride (15%).

4. 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 Ankleshwar Textile Industries, Ankleshwar, Gujarat, India 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. 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 were 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 dying process are the main causes of pollutants which include caustic soda, hypochlorite and peroxides. The isolation of different microorganisms from the effluent sample collected from the Ankleshwar Textile Industries, Gujarat, India 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 spp.* with strong decolorizing ability was isolated from Textile effluent to decolorize the textile Azo dye Remazol Black B (250 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. The percentage decolorization of Remazol Black B by *Bacillus spp.* strain under static conditions was 95% 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 Remazol Black B 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 Remazol Black B 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).

5. 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 Remazol Black B in this study was isolated from the textile dye industry waste effluent. The bacterial strain *Bacillus spp.* showed decolorizing activity through a degradation mechanism. 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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