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Microbial Decolorization of Reactive Azo Dyes by *Bacillus* spp. ETL-1949 under Anaerobic Condition

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Abstract A bacterial strain Bacillus spp. ETL-1949 with remarkable ability to decolourize the reactive azo dyes such as reactive red, reactive yellow, reactive brilliant red and reactive brilliant blue was isolated from the textile effluent contaminated site. The effluent sample was collected from local textile industry, Ankleshwar, Gujarat, India Static conditions with 10 g/l glucose, pH 9, 37°C, 20% inoculum concentration, 50 mg/l of dye concentration, 3 g/l of NH4NO3, were considered to be the optimum decolourizing conditions. Bacillus spp. ETL-1949 grew well in these optimum conditions, resulting in 82% decolourization extent 7 days of incubation. Phenotypic characterization and phylogenetic analysis of the 16S rRNA sequence indicated that the bacterial strain belonged to the genus Bacillus. UV (Ultra Violet) analysis of bacterial isolate suggested that it exhibited decolourizing activity, rather than inactive surface adsorption. This decolourization extent and facile conditions show the potential for this bacterial strain to be used in the biological treatment of textile effluent or dyes.

Keywords: *Bacillus, decolorization, 16S rRNA, Ankleshwar*

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1. Introduction

The textile industry plays an important role in the world economy as well as in our daily life, but at the same time, it consumes large quantities of water and generates large amount of waste water. The chemical reagents used in textile sector are diverse in chemical composition ranging from inorganic to organic [1]. Azo dyes, aromatic moieties together by azo (-N=N-) chromophores, represent the largest class of dyes used in textile processing and other industries such as cosmetic, food colorants, printing and pharmaceutical industries. As a characteristic of the textile-processing industry, a wide range of structurally diverse dyes are used within short periods in one and the same textile industry and therefore, effluents from textile industry are extremely variable in composition [2]. Dyes bearing effluents are complex, most often nonbiodegradable and exhibit toxicity to both aquatic and non aquatic biota1. Around 10,000 different dyes with an annual production of more than 7×10^5 metric tons worldwide are commercially available [3]. In India an average discharges about 1.5 million litres of dye-contaminated effluent per day, which leads to chronic and acute toxicity to the living things [4]. The coloured wastewater treatment methods based on physical and chemical procedures are effective but suffer from shortcomings such as high expenditure, intensive energy requirements and formation of perilous by products [1,5] whereas, biological degradation of these dyes does not face such problems. Microbial methods have recently received much attention owing to its ease of application, low cost and environmental benignity [6]. In the present study, we focused on the isolation and identification of microorganisms from textile effluents having decolourizing ability for several different dyes. Various parameters have been optimized to achieve maximum dye decolourization for reactive red, reactive yellow, reactive brilliant red and reactive brilliant blue.

2. Material and Methods

2.1. Sample and Dyes Collection

Textile dyes and effluent sample were collected from a dying industry located at Ankleshwar, GIDC. The sample was collected from the effluent disposal site of the industry. The sample was transported to the laboratory without delay and preserved in the refrigerator at 4°C before and after the microbial analysis. The dyes used for this study (reactive red, reactive yellow, reactive brilliant blue and reactive brilliant red) were also procured from the same industry.

2.2. Isolation of Microorganism

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,

Ankleshwar. The sample collected from the textile mill was screened for azo dyes (reactive red, reactive yellow, reactive brilliant red and reactive brilliant blue) 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 (140rpm). After 48h of incubation, 1.0ml. of the culture broth was appropriately diluted and plated on Nutrient Agar containing 20 mg $L^{-1}$of each dye (reactive red, reactive yellow, reactive brilliant red and reactive brilliant blue). 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 each dye (reactive red, reactive yellow, reactive brilliant red and reactive brilliant blue). These isolates were screened for their ability to decolorize different dyes 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 each dye under static conditions. After 24h of incubation, 1ml. of cell suspension was transferred to fresh nutrient broth containing each dye 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 each dye (50 mg L^{-1}). The bacterial isolate which tolerated higher concentration of the Azo dye was isolated by streaking 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 were determined by inoculating the isolate into carbohydrate broth supplemented with respective carbon source. After inoculation the tubes were incubated at 37ºC for 24-48h. Microorganism was isolated from the effluent and soil sample by preparing aliquot (10 ml) dilutions. Nine millilitre of sterile water was placed in test tubes and labeled as 10^{-1} to 10^{-6}, after 1ml of effluent sample was transferred into each tubes using sterile pipette. Then 1ml was taken from both soil and effluent aliquots and plated on dye fermentation agar medium containing reactive red, reactive yellow, reactive brilliant red and reactive brilliant blue respectively and incubated at 37°C for 72 hrs. The culture capable of growth on this medium was isolated and purified by sub-culturing on dye fermentation agar medium [7]. The well grown culture in the medium was taken for further studies.

2.3. Identification of Bacteria using 16S rRNA

The total genomic DNA of ETL-1949 was isolated using Promega WIZARDR Genomic DNA Purification kit. The forward primers, FD1 (5'- AGAGTT TGATCC TGGCTCAG -3') and the reverse primer, RD1 (5'- AAGGAGGTGATCCAGCC -3') were used to amplify the 16S rRNA sequence of ETL-1949. PCR conditions included an initial denaturation for 5 min at 95°C followed by 30 cycles of 1 min at 95°C, 1min at 50°C and 2 min at 72°C. The amplicons were purified and sequenced. The 16S rRNA sequence of ETL-1949 was analyzed using Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov/BLAST).

2.4. Phylogenetic Analyses of ETL-1949 16S rRNA Gene Sequences

Genomic DNA was isolated from the pure culture pellet using consensus primers and partial 16s rRNA genes were amplified by PCR using forward primer (5'–GAGCGGATAACAATTTCACACAGG-3'), reverse primer (5'- CGCCAGGGTTTTCCCAGTCACGAC-3') and internal primer (5'- CAGCAGCCGCGGTAATAC-3'). The amplified 16s rRNA gene was sequenced. The obtained sequence data was aligned and analyzed for identification and finding the closest homology for the isolate. The next closet homo- logy was found with *Pseudomonas* and it was designated as *Pseudomonas spp.* ETL-1949. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 [19]. The evolutionary history was inferred using the Neighbor-Joining method [20]. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxonomy analyzed [21]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxonomy clustered together in the bootstrap test (500 replicates) is shown next to the branches [21]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [22] and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 1420 positions in the final dataset.

2.5. Decolourization Assay

Decolourization studies were followed in static condition by bacterial isolate, such as *Bacillus* spp. ETL-1949 by using various carbon sources, nitrogen sources, and combination of carbon + nitrogen sources at different pH were followed. Four reactive azo dyes were selected for decolourization purpose that is reactive red, reactive yellow, reactive brilliant red and reactive brilliant blue. Carbon sources such as glucose, sucrose, lactose, maltose 10 g/l and nitrogen sources such as ammonium nitrate, ammonium sulphate were used. In addition to this effect of pH at 7, 8 and 9, temperature at 28°C, 37°C and 45°C, effect of dye concentrations of 50, 100, 150 and 200 mg/l and effect of inoculum concentrations (5-20%) were carried out for decolourization studies. It was taken into separate conical flask and microbial inoculum was added to each and incubated for 7 days, by using mineral salt medium (K^2 HPO_4 - 1.6 g, KH_2 PO_4 - 0.2 g, (NH_4) SO_4 - 1.0 g, $MgSO_4.7H_2O$ - 0.2 g, $FeSO_4.7H_2O$ - 0.01 g, NaCl - 0.1, $COCl_2$. $2H_2O$ - 0.002 g, glucose - 3 g, yeast extract - 1.0 g/l, pH 7) [8]. Then results were noted based on

turbidity and Optical Density (OD) value by using following formula.

$$Decolourization\ (\%) = \frac{A_o - A_t}{A_o} \times 100$$

Where: Ao = Absorbance of the dye solution, At = Absorbance of the treated dyes solution at specific time, t.

3. Results and Discussion

3.1. Isolation and Identification of Microorganism

Among the 20 isolated bacterial strains, one strain was selected based on its ability to form high dye decolourization zone on fermentation agar medium. Biochemical test and physiological tests were performed to identify the isolated strain. These strain was gram positive rods, had spores, motile, occur single rod or in chains, yellowish colour colony, and showed positive to Voges-Proskauer test, catalase test and negative to methyl red test, indole test, urease activity and citrate utilization. Then, 1200 bp of 16S rRNA gene of the isolated strain was determined. The nucleotide alignment of the strain showed most phylogenetic similarity to *Bacillus* genus. The isolated strain was 99% similar to *Bacillus* sp. In the present study revealed the findings of *Bacillus* spp. ETL-1949 a bacterial isolate, isolated from textile effluent which was beneficial for the degradation of reactive azo dyes. The morphological and biochemical character of the isolated strain was performed [9]. The isolated strain was identified, belonging to the *Bacillus* sp by 16S rRNA sequence analysis.

3.2. Microbial Decolourization

The development of dye decolourizer *Bacillus* spp. ETL-1949 from the effluent sample collected from contaminated site of textile industry indicates the natural adaptation of this organism to survive in the presence of toxic dyes [10]. Among seven isolated bacteria, *Bacillus* spp. ETL-1949 was screened for their ability to decolourize reactive red, reactive yellow, reactive brilliant red and reactive brilliant blue. This isolate efficiently decolourize 82 % of reactive red, 71 % of reactive yellow, 69 % of reactive brilliant red and 69 % of reactive brilliant blue (50 mg/l) also showed good growth. With dyes reactive brilliant red, reactive brilliant blue however lesser decolourization and growth was noted even on complete incubation. The result inferred from our work involving screening of isolate to decolourize structurally different dyes prompted us to conclude that the bacterial isolate would be better option for decolourization work. In the presence of these four dyes in the mineral salt medium indicated that they were not toxic to the culture. The differential rates of decolourization will all the four dye/organism, providing the different reduction capacity of bacterium and different sensitivity of dyes. The ability of microbial isolates to achieve a high percentage of decolourization on single dyes has been reported in previous studies [11]. In the present study the bacterial isolate have the ability to achieve high percentage decolourization on four reactive azo dyes. The preponderance of biodegration reports are on anareobic treatment probably because earlier reports indicated that azo dyes were resistant to bacterial degradation under aerobic conditions while anaerobic decolourization was perceived to be easy to achieve [12]. In the present study decolourization was performed in static conditions, azo dye acts as artificial electron acceptor and activity is rather nonspecific with respect to the azo compounds involved thus, static condition was preferred in decolourization.

3.3. Evaluation of Optimum Conditions: Effect of pH

The effect of pH (7, 8, and 9) on decolourization with incubation period of four reactive dyes at 50 mg/l concentration of dye with 20% inoculums is shown in Figure 1. The figure shows that the percentage removal of dye increased with increase in incubation period at pH 9. The maximum removal 80%, 73%, 63% and 63.5% dyes were found at pH 9 after 7 days of incubation period. In pH 7 and 8 resulted in decreased percentage removal of dye when compared to pH 9, the optimum pH was found to be 9 for maximum removal of dye. The pH plays great influence in decolourization of four reactive dyes. *Bacillus* spp. ETL-1949 prefers alkaline range of higher decolourization extent. Chen *et al.* reported that the optimum pH was found to be 6-8 for maximum removal of dye. The pH has a major effect on the efficiency of dye decolourization and the optimal pH for colour removal is often between 6.0 and 10.0 for most of the dyes [10]. The pH tolerance of decolourizing bacteria is quite important because reactive azo dyes bind to cotton fibers by addition or substitution mechanisms under alkaline conditions and at high temperature [13].

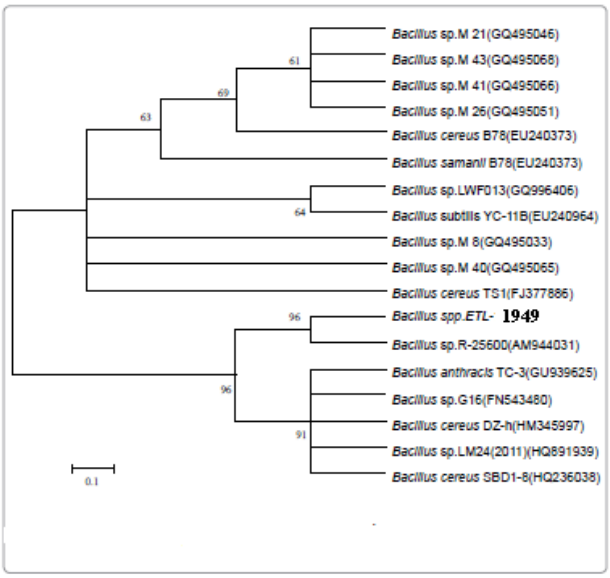

Figure 1. Phylogenetic tree of Bacillus spp. ETL-1949

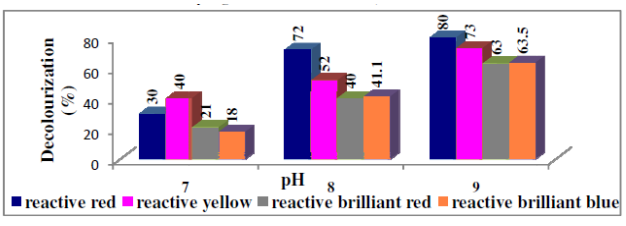

Figure 2. Effect of different pH on Decolorization of reactive dyes by *Bacillus* spp. ETL-1949 (37°C, 50 mg/l, 20 % inoclulum, 7th day of inoculation)

3.4. Effect of Temperature

Figure 3 shows decolourization of dye with incubation period at three different temperatures (28°C, 37°C and 45°C) at 50 mg/l dye concentration and 20% inoculums. It is clear from the figure that percentage removal of dye increased at 37°C and there was no decolourization activity at 28°C, at 45°C there was no activity and cell death occurred slowly. Our results similar to that Ponraj *et al*. they reported that the range of activity on decolourization of orange 3R with 37°C was 78.57%, *Bacillus* sp was found to be the most effective decolourizer17. Ceten Domnoz reported that a suppressed decolourizing activity at 45°C, this might be due to the loss of cell viability or deactivation of the enzymes responsible for decolourization at higher temperature [15].

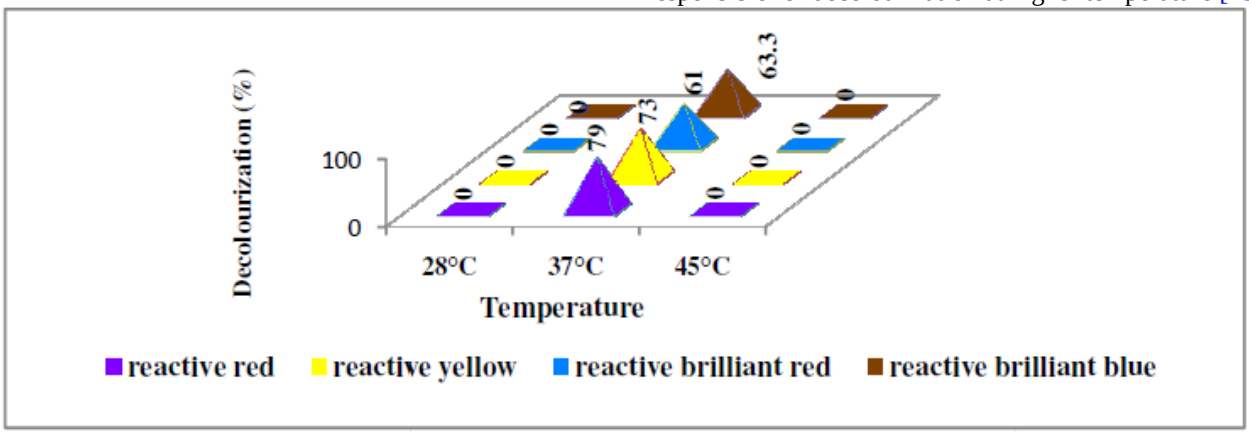

Figure 3. Effect of different temperature on decoloriztion of reactive dyes by *Bacillus* spp. ETL-1949 (Ph 9, 50 mg/l, 20 % inoclulum, 7th day of inoculation)

3.5. Effect of Various Carbon Sources

The effect of glucose, sucrose, lactose and maltose (10 g/l) on decolourization with time performed at 50 mg/l initial concentration of dyes, 20% of inoculum concentration and at 37°C in static condition. The Figure 4 clearly shows that maximum removal of dye 82% for reactive red, 80% for reactive yellow, 79% for reactive brilliant red and 78% reactive brilliant blue was achieved after 7 days of incubation period using glucose as a carbon source. Whereas with co substrate sucrose shows that 71%, 68%, 63% and 62.5% of dye decolourization by reactive red, reactive yellow, reactive brilliant red and reactive brilliant blue respectively. But it was less when compared to glucose used as a carbon source and also figure shows lower decolourization at lactose that was 67%, 62%, 65% and 69%, maltose that was 69%, 62%, 68% and 65% for reactive red, reactive yellow, reactive brilliant red and reactive brilliant blue respectively. The reason for low decolourization at lactose and maltose might be that this carbon sources could not meet the good growth requirements for the bacterial isolate. In the present study it was found that glucose was optimum carbon source for decolourization of reactive red, reactive yellow, reactive brilliant red and reactive brilliant blue dyes. Dyes being deficient in carbon sources the biodegradation of dyes without any extra carbon source is very difficult [16] and therefore, in our study, glucose, sucrose, maltose and lactose were used as carbon source was supplemented in the mineral salt medium. Saraswathi and Balakumar also reported that *Bacillus firmus* and *Bacillus laterosporus* show maximum decolourization of azo dye pigment red 208 in the presence of glucose as a carbon source [17].

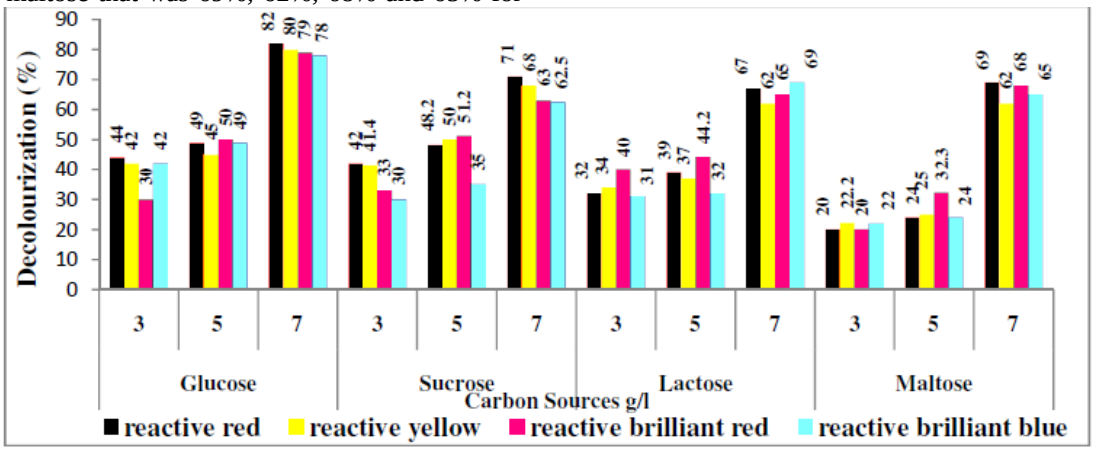

Figure 4. Effect of different carbon source on decolorization of reative dyes by *Bacillus* spp. ETL-1949 (37°C, pH 9, 50 mg/l, 20 % inoclulum, 7th day of inoculation)

3.6. Effect of Inoculum Concentrations

Figure 5 shows the effect of inoculum concentration (5-20%) with incubation period on decolourization of dye at 50 mg/l dye concentration. It is clear from the figure that percentage removal of dye increased with an increase in incubation period at 20% inoculums concentration. At this concentration the dye removal was found to be 85.2%,

73%, 63% and 67% for reactive red, reactive yellow, reactive brilliant red and reactive brilliant blue respectively when compared to 5%, 10% and 15% inoculums concentrations. This is the optimum inoculums concentration. Decolourization activity of *Bacillus* spp. ETL-1949 has high in 20% of inoculum. In previous studies Ponraj *et al*. reported that decolourization activity of *Bacillus* sp has high (86.72%) in 4 % of inoculums [14].

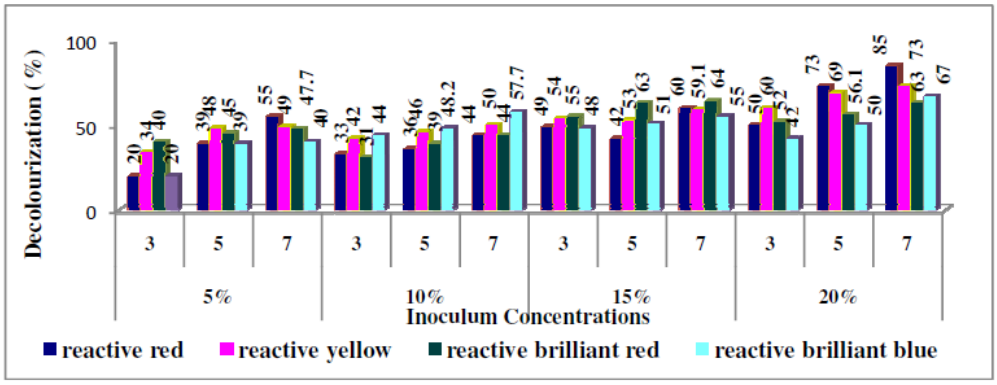

Figure 5. Effect of inoculums concentration on decolorization of reactive dyes *Bacillus* spp. ETL-1949 (37°C, 50 mg/l, pH 9, 7th day incubation)

3.7. Effect of Dye Concentrations

Figure 6 shows the effect of dye concentration ranging from 50-200 mg/l of dye at pH 9, 20% inoculum concentration and at 37°C. It is clear from the figure that percentage removal of dye increased with an increase in incubation period at 50 mg/l of dye concentration. Percentage removal of dye found to be 85%, 86%, 73% and 79% for reactive red, reactive yellow, reactive brilliant red and reactive brilliant blue respectively. Upon increasing the dye concentrations from 50-200 mg/l gradual decrease in both decolourization and growth observed probably due to the toxic effect of dyes or different structures our results similar with Sponza and Isik [18]. Sani and Banerjee also found that dyes were easily decolourized at concentration of 10 mM by *Kurthia* sp.

But colour removal was reduced when dye concentration was increased to 30 mM as the colour reduction capability of the cells was not enough to degrade all the transferred dye through the cell membrane [19].

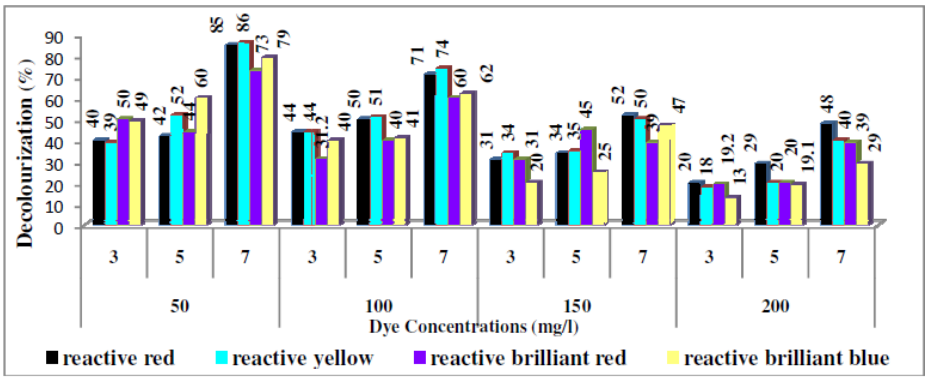

Figure 6. Effect of different dye concentration on decolorization of reactive dyes by *Bacillus* spp. ETL-1949 (37°C, 20% inoculum, pH 9, 7th day incubation)

3.8. Effect of Nitrogen Sources

Figure 7 shows the effect of nitrogen sources (NH_4NO_3 and NH_4SO_3 3 g/l) on decolourization with incubation period at 50 mg/l of dye concentration, 20% inoculum concentration and at 37°C at static conditions. The figure clearly shows that maximum removal of dye 67%, 57%, 66% and 50% for four reactive dyes respectively was achieved after 7 days of incubation period using NH_4NO_3 as a nitrogen source and 56%, 65%, 60% and 51% of decolourization when NH_4SO_3 used as a nitrogen sources. These results indicated that the nitrogen sources were less efficient than carbon source availed by microorganism [7].

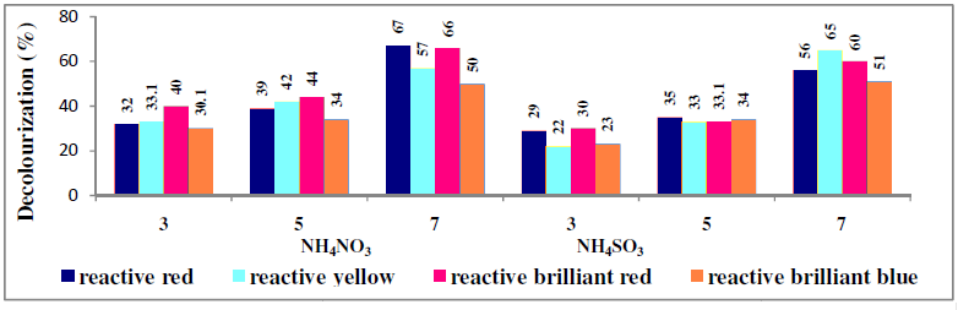

Figure 7. Effect of different nitrogen source on decolorization of reactive dyes by *Bacillus* spp. ETL-1949 (37°C, 50 mg/l, pH 9, 7th day incubation)

3.9. Effect of Nitrogen + Carbon Sources Combination

Effect of nitrogen + carbon sources (NH_4NO_3 + NH_4NO_3+ glucose, glucose+ NH_4SO_3) on decolourization with incubation of four reactive dyes at 50 mg/l dye concentrations with 20% inoculums is shown in Figure 8. The figure clearly shows that the percentage removal of dye increased with increase in incubation time. The maximum removal 68%, 68%, 69% and 52% of dyes were achieved at 7 days of incubation period using NH_4NO_3+glucose and 61%, 59%, 50% and 49% of decolourization was achieved with NH_4NO_3+glucose combinations. The results from the figure showed that the decolourization percentage was higher than the medium contains only nitrogen source and lower than the medium contains only carbon source. The growth of bacteria was not significantly different in the fermentation medium. *Bacillus cereus* grew best in ammonium nitrate/glucose and decolorizes best in ammonium nitrate/sucrose combination 81% textile dyes (reactive red, reactive yellow, reactive brilliant red and reactive brilliant blue) is degradable under static conditions with a concerted effort of bacterium isolated from effluent. Nutrients (carbon and nitrogen sources) and physical parameters (pH, temperature and inoculums size, dye concentration) had significant effect on dye decolourization. *Bacillus* spp. ETL-1949 decolourizes four reactive azo dyes effectively during optimization throughout the study.

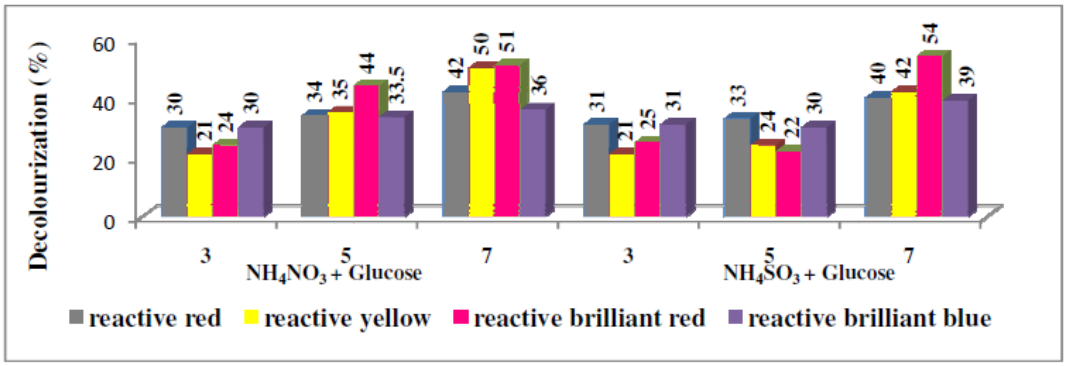

Figure 8. Effect of different nitrogen & carbon source on decolorization of reactive dyes by *Bacillus* spp. ETL-1949 (37°C, 50 mg/l, 20% inoculum, pH 9, 7th day incubation)

4. Conclusion

The present study concludes that dye-degrading microorganism; *Bacillus* spp. ETL-1949 from an effluent contaminated site of textile dyeing industry have potential of decolourization. This observation has established that the bacteria are adaptive in nature and can degrade the dye contaminants. The ability of the strain *Bacillus* spp. ETL-1949 to tolerate, decolorize and degrade reactive azo dyes at high concentration gave it an advantage for treatment of textile industry wastewaters. However, potential of culture needs to be demonstrated for its application in treatment of real dye bearing wastewaters using appropriate decolourization methodology and also anaerobic conditions favours the growth of *Bacillus* spp. ETL-1949 in decolourization broth. This biological method can be promoted to degrade the variety of reactive dyes from the textile industries. The treated textile dyes when disposed to the land has several applications, includes, soil fertility improvement, easy transport of compost, humus rich, very slow release of nutrient and increases in water holding capacity of the soil. This potential strain may be used for the treatment of the dye industrial effluents, may also be applicable for the treatment of sewage and other polluted water.

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