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DECOLORIZATION OF DYES DIRECT RED 31 (DR 31); DIRECT BLUE 71 (DB 71) AND DIRECT BLACK 19 (DB 19) BY NEWLY ISOLATED *Bacillus sp.* DM1

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ABSTRACT

In this study, the decolorization potential of novel Bacillus sp. DM1 isolated from textile effluents was evaluated. Decolorization of direct red 31 dye was 89% (± 1.20); direct blue 71 dye was 90% (± 0.84) and direct black 19 dye was 81% (± 0.39) after 24 hours at 100 ppm concentration. It took 48 hours for nearly complete decolorization of red and blue dyes and 72 hours for black dye. Dextrose showed 97% (± 0.91) decolorization of blue dye, sucrose 95% (± 1.11) decolorization of black dye and lactose 98% (± 1.35) decolorization of red dye. Yeast extract showed 94% (± 0.80) and 97% (± 0.43) decolorization of black and red dye respectively, whereas sodium nitrate showed 95% (± 0.73) decolorization of blue dye. pH 7 and temperature 35^{0}C was found optimum for decolorization of the dyes. 7% inoculum size for red and blue dye and 8% for black dye were optimum.

Keywords: *Bacillus sp.*, Bioremediation, Direct black, Direct blue, Direct red, Effluent treatment

Introduction

Due to rapid industrialization, lots of chemicals including dyes are manufactured and used in day to day life **(Moosvi et al., 2005)**. During the dyeing process 2% of these dyes fail to bind to the substrate and are discharged in aqueous effluents **(Ndasi et al., 2011)**. The wastewater from textile when directly released in the surface water without treatment can cause a rapid depletion of dissolved oxygen and lead to a great environmental damage **(Ramasany et al., 2012)**. Synthetic dyes used most commonly in the textile industry for dyeing clothes is the major source of polluting our water resources. Water pollution has been recognized as the major hazard of the modern world and is one of the main cause of death and disease **(Pratiwi et al., 2017)**. Pollution caused by dye effluent is mainly due to durability of the dyes in wastewater **(Jadhav et al., 2007)**. Therefore, the removal of dyes from textile effluents is a major concern for protection of environment. Existing methods for decolorization of dyes in waste water include pH neutralization, membrane filtration, irradiation, ozonation, adsorption on activated carbon, oxidation, reverse osmosis, electrocoagulation, flocculation but these methods are inefficient, expensive and lead to development of waste in the form of sludge which again needs to be disposed off **(Keharia and Madamwar, 2003; Ogugbue and Sawidis, 2011)**. Dyes are difficult to degrade because of their color fastness, synthetic origin and complex aromatic molecular structures which make them stable **(Fu and Viraraghavan, 2001; Anjaneyulu et al., 2005)**. Microbial decolorization and degradation of azo dyes has gained considerable interest of researchers as it is inexpensive, eco-friendly, and produces less amount of sludge **(Parshetti et al., 2006)**. In recent years work has been done to isolate microorganisms capable of decolorizing textile wastewater **(Chen et al., 2003; Coughlin et al., 2002; Gurulakshmi et al., 2008; Joshi and Saxena, 2018; Kalyani et al., 2009; Lalnunhlimi and Krishnaswamy, 2016; Patel et al., 2017; Shah et al., 2014)**. But in most of the studies the rate of decolorization is low and/or the isolates take many days to degrade the dye, for example, *Alcaligenes aquatilis* was found to decolorize 82% Synazol red 6HBN after incubation of 4 days at 37 °C and pH 7 **(Ajaz et al., 2019)**. *Bacillus sp*. and *Pseudomonas sp*. have showed 89% of decolourization of Orange 3R at 144 h followed by 80 and 76% by *Salmonella sp*. and *Klebsiella sp*., respectively **(Ponraj et al., 2011)**. *Pseudomonas aeuroginosa* has been reported to decolorize remazol black B from 85 to 94.4% after 5 days **(Junior et al., 2015)**. The overall aim of this research work was therefore to isolate bacterial strains that show high decolorization potential and to develop a fast, enhanced and cost efficient method to degrade dyes released from the textile processes.

Material and Methods

Selection of dyes

Direct red 31 (DR 31), direct blue 71 (DB 71) and direct black 19 (DB 19) dyes,

widely used for dyeing, were purchased from textile industry. For convenience common name of the dyes used in this study are used. All media and chemicals used in the study were of analytical grade and purchased from Hi-media, India. Medium with dyes was used as control.

Isolation of bacteria from textile dye effluent

One ml of each of the textile effluent samples from Selaqui Industrial area, Dehra Dun were mixed in water blank and was then serially diluted from 10^{-1} to 10^{-9}. All dilutions were plated on nutrient agar media and incubated at 37^0C for 24 hours. Morphologically distinct colonies so obtained were then streaked on nutrient agar medium. Bacterial pure culture was maintained in nutrient agar medium slants and stored at 4°C and was sub cultured every month.

Determination of dye spectrum

Absorption of Red, Blue, Black dyes and textile effluent between wavelengths 400-700 nm were read to determine the wavelengths that showed the maximum absorbance. Decolorization of respective dyes was measured on the basis of decrease in absorbance at the determined wavelength.

Preliminary screening for dye decolorization ability

Liquid culture assay

For liquid culture dye decolorizing experiment, sterile nutrient broth supplemented with 100 ppm dyes was inoculated with bacterial cultures and incubated for 24 hours at 37°C. Extent of dye decolorization was assessed spectrophotometrically and the rate of decolorization was calculated using the following formula as described by **Sani and Banerjee, 1999**

$$\text{Decolorization } (\%) = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100$$

Characterization of bacterial isolate

Characterization was done on the basis of colony morphology in plate, slant and broth. For characterization of cell morphology simple, gram, acid fast, negative and capsule staining was done. Indole production, Methyl Red-Voges Proskauer, Citrate, Hydrogen Sulfide production, Catalase, Oxidase, Amylase, Carbohydrate fermentation, Nitrate tests were performed for biochemical characterization.

Optimization of culture conditions

a. Effect of dye concentration on decolorization

Nutrient media was amended with the selected dye at a concentration of 25 to 800 ppm. After different time intervals, aliquot (1.5 ml) of the culture media was withdrawn, centrifuged and supernatant obtained was used for analysis of % decolorization spectrophotometrically at optimum wavelengths.

b. Effect of time of incubation on dye decolorization

Nutrient media with dye concentration 100 ppm was inoculated with the test organism. The culture was incubated for 96 hours at 37°C. The result of decolorization was observed spectrophotometrically.

c. Effect of pH on dye decolorization

Nutrient media at different pH (3, 4, 5, 6, 7, 8, and 9) with each dye concentration 100 ppm was inoculated with bacterial culture at optimum time of incubation for each dye. The result of decolorization was observed spectrophotometrically.

d. Effect of temperature on dye decolorization

Nutrient media prepared at optimum conditions, supplemented with dye was incubated at different temperatures ranging from 25°C to 65°C. The result of decolorization was observed spectrophotometrically.

e. Effect of carbon and nitrogen sources on dye decolorization

Minimal broth medium (KH_2PO_4 2.0, NaCl 0.5, $MgSO_4$ 0.2, $CaCl_2$ 0.02, $(NH_4)_2SO_4$ 1.0 g l^{-1}) was supplemented with 1% concentration of carbon and nitrogen sources respectively. Peptone, sodium nitrate, yeast extract and ammonium nitrate were used as nitrogen source. Maltose, dextrose, sucrose and lactose were used as carbon source.

f. Effect of inoculum size on dye decolorization

Inoculum size ranging from 1% to 10% was prepared containing media supplemented with bacterial isolate. The result of decolorization was observed spectrophotometrically.

Screening for azoreductase

The bacterial cell pellet was suspended in 0.1 M phosphate buffer (pH 7.2) and 0.5 w/v Triton-X 100 was added. The mixture was incubated at 4^0C for 30 minutes with continuous stirring followed by centrifugation at 10000 rpm for 25 minutes. The supernatant was used as crude enzyme extract for the assay. The azoreductase assay was carried out according to the procedure of **Liger et al., 2004**. The reaction mixture (2 ml) contained 35 µM azo dye, 100 µM NADH, 20 µM FMN, and 2.4 µM of enzyme in 25 mM Tris-HCl (pH 7.5). The reaction was run at 30°C, and the decrease in absorbance at optimal wavelength for each azo dye tested as a substrate was monitored: direct red 31 dye at 520 nm, direct blue 71 dye at 625 nm and direct black 19 dye at 620 nm. Total protein was estimated using the standard Lowry's method **(Lowry et al., 1951)**. One-unit enzyme activity was calculated as one µmole of dye reduced per minute. Specific enzyme activity was calculated as unit per mg.

Statistical analysis

All experiments were performed in triplicates. Results are reported as mean ± S.E (Standard Error Mean).

Results and Discussion

Isolation and screening of bacterial strains

A total of 13 morphologically distinct strains isolated from effluent samples were screened for decolorization of the

dyes. Three showed good decolorization activity but eventually one isolate showing maximum activity for all the three dyes was selected for further experimentation. The selected isolate was characterized morphologically in agar plate, broth and slant followed by biochemical characterization. This novel *Bacillus sp*. DM1 showed 89% (± 1.20) decolorization of direct red 31 (DR 31) dye; 90% (± 0.84) for direct blue 71 (DB 71) dye and 81% (± 0.39) for direct black 19 (DB 19) dye after 24 hours incubation at 100 ppm dye concentration. The absorption maxima of the direct red 31 dye was found to be 520 nm, direct blue 71 dye 625 nm, direct black 19 dye 620 nm and textile effluent 410 nm. Preliminary studies showed the necessity for optimizing process parameters and therefore parameters such as pH, temperature, time of incubation, dye concentration and inoculum size were optimized. Nutritional parameters such as carbon and nitrogen sources were also optimized.

Optimization of culture conditions

Textile dye degradation using the isolated test organism was optimized under different conditions and parameters.

a. Effect of dye concentration on % decolorization

The decolorization of all three dyes was studied at different dye concentrations ranging from 25 ppm to 800 ppm. Maximum decolorization was observed at 100 ppm (Figure 1).

b. Effect of time of incubation on % dye decolorization

The decolorization efficiency of *Bacillus sp*. recorded up to 96 hours of incubation showed maximum decolorization at 48 hours for red (94% ± 0.95) and blue dye (96% ± 0.46). Thereafter increasing incubation time showed little change in decolorization efficiency. Black dye was 97% ± 0.17 decolorized after 72 hours of incubation (Figure 2).

c. Effect of pH on % dye decolorization

The isolate was able to decolorize the dyes over a wide range of pH. However, maximum decolorization was recorded at pH 7 for all the three dyes (Figure 3).

d. Effect of temperature on % dye decolorization

Optimal temperature for decolorization of all dyes was 35^0C. Decolorization rate dropped sharply as the temperature increased from 35^0C to 65^0C (Figure 4).

e. Effect of carbon sources on % dye decolorization

Among the sources studied dextrose showed 97% (± 0.91) decolorization of blue dye, sucrose showed 95% (± 1.11) decolorization of black dye and lactose showed 98% (± 1.35) decolorization of red dye (Figure 5).

f. Effect of nitrogen sources on % dye decolorization

Among the sources studied yeast extract showed maximum decolorization of black and red dye with 94% (± 0.80) and 97 % (± 0.43) respectively, whereas sodium nitrate showed 95% (± 0.73) decolorization of blue dye (Figure 6).

g. Effect of inoculum size on % dye decolorization

With inoculum size ranging from 1-10%, it was observed that red and blue dye showed 95% (± 0.42) and 96% (± 0.26)

decolorization, respectively, in 7% inoculum size, whereas black dye showed 98% (± 1.33) decolorization in 8% inoculum size (Figure 7).

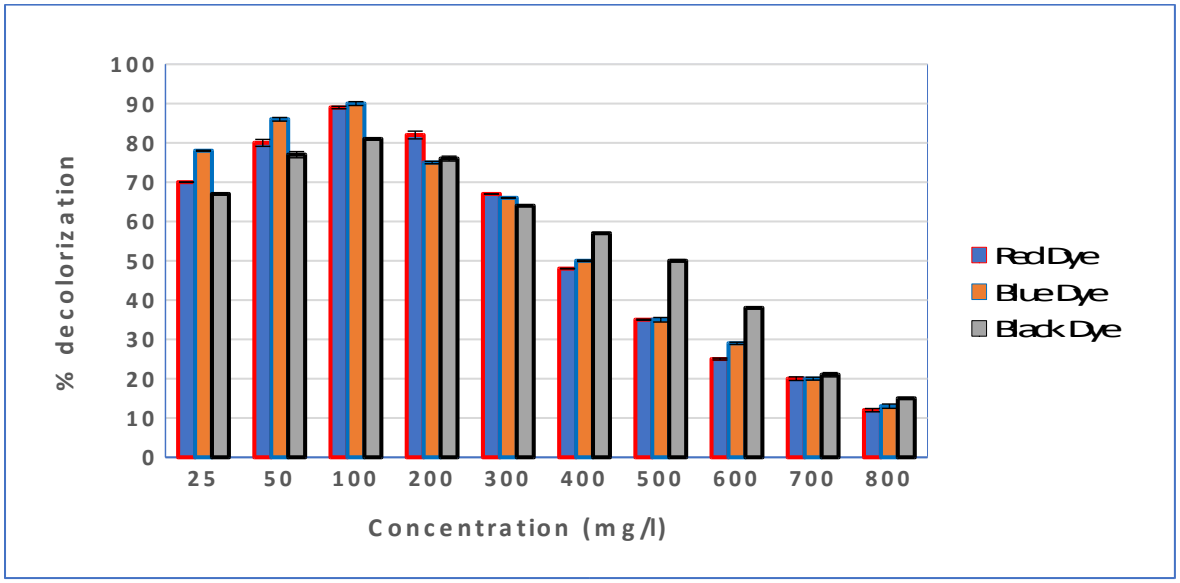

Figure 1: Effect of dye concentration on % decolorization

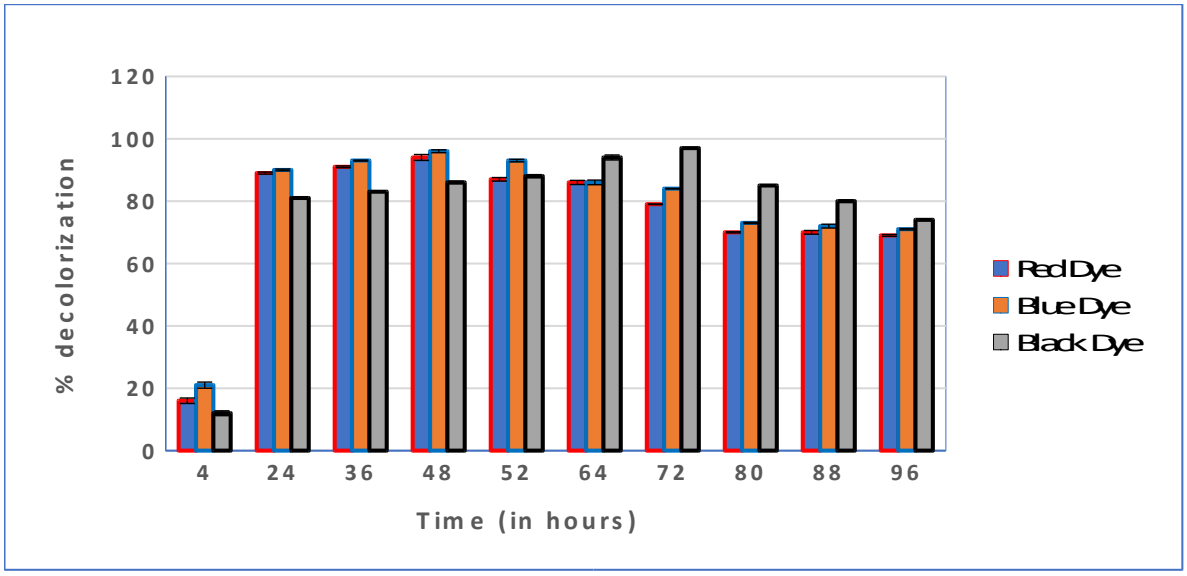

Figure 2: Effect of time of incubation on % dye decolorization

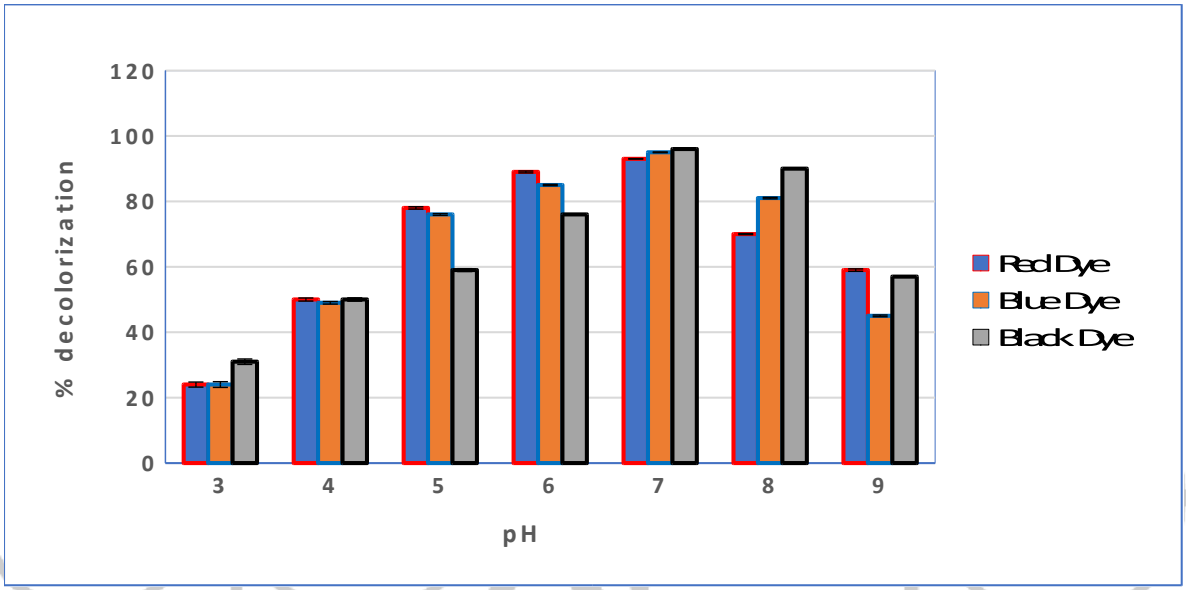

Figure 3: Effect of pH on % dye decolorization

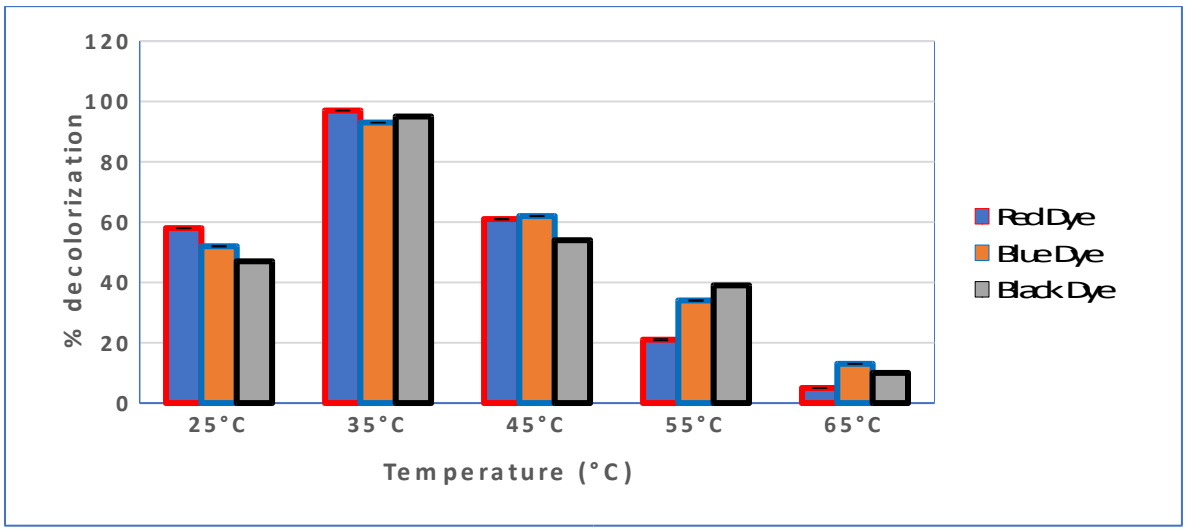

Figure 4: Effect of temperature on % dye decolorization

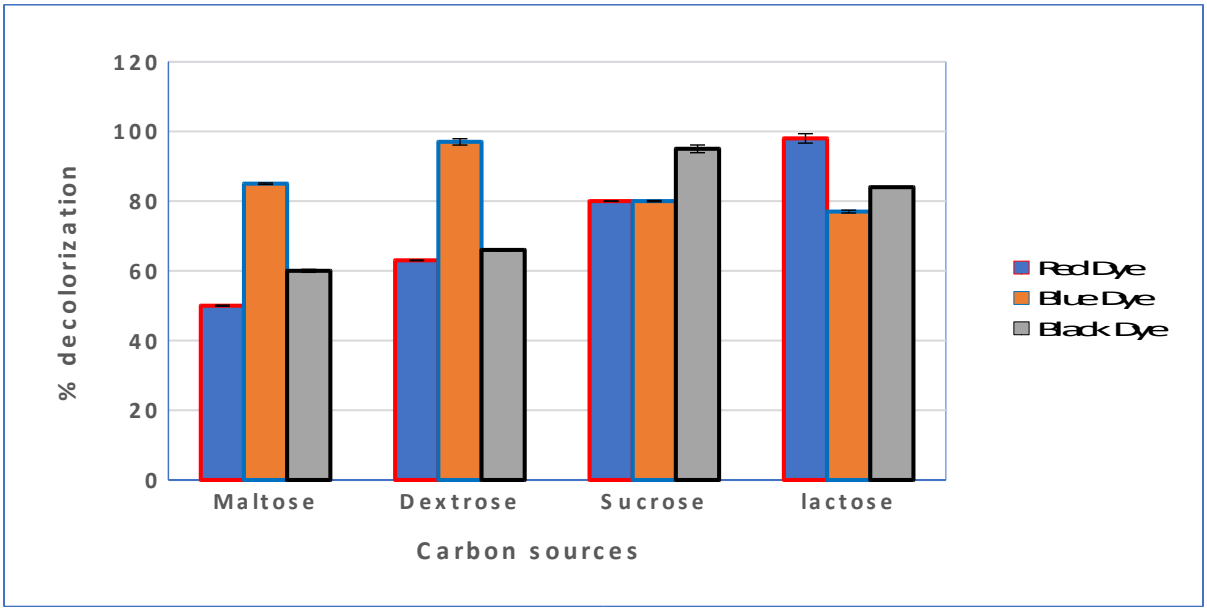

Figure 5: Effect of carbon sources on % dye decolorization

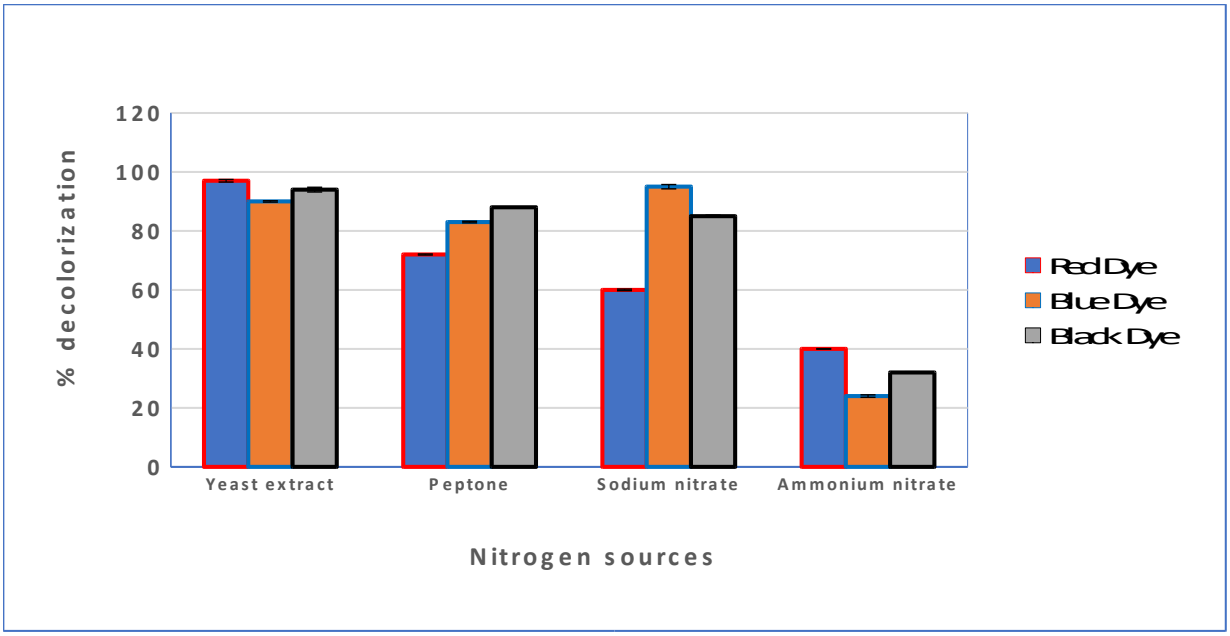

Figure 6: Effect of nitrogen sources on % dye decolorization

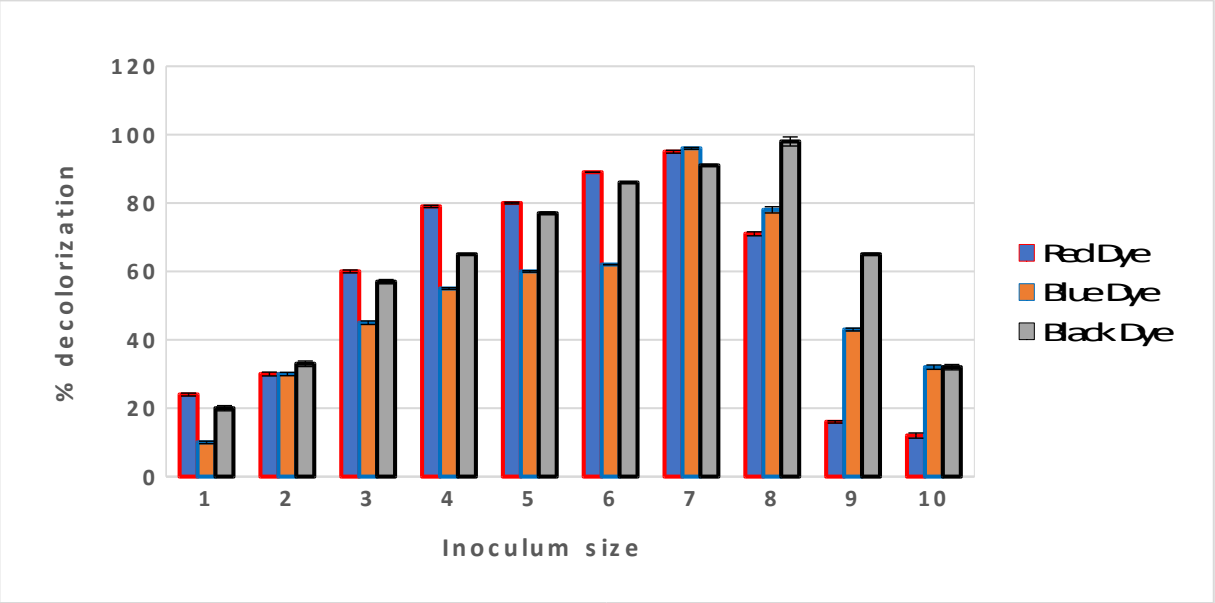

Figure 7: Effect of inoculum size on % dye decolorization

Calculation of enzyme activity

Azoreductases are flavoenzymes involved in the biotransformation and detoxification of azo dyes. The isolate showed total enzyme activity 155.32 U (± 0.01) and specific enzyme activity 8.08 U/mg (blue dye); 153.18 U (± 0.04) and 7.97 U/mg (red dye); 148.72 U (± 0.07) and 7.74 U/mg (black dye). The result is similar to a study carried out by **Saini et al., 2018** where *Bacillus flexus* showed total enzyme activity of 154.9 U and specific activity of 6.811 U mg^{-1}.

Discussion

Our isolate showed a better decolorization efficiency as compared to the other dye decolorization studies conducted. **Junnarkar et al., 2006** selected a novel bacterial consortium that exhibited 90% decolorization ability within 35 h. Maximum rate of decolorization was observed at pH 7.0, incubation temperature of 37^0C at 200 mg l^{-1} concentration of Direct Red 81 dye when starch (0.6 g l^{-1}) and casein (0.9 g l^{-1}) were supplemented in the medium. **Kumar et al., 2009** reported 98% decolorization at 25 ppm concentration of dye Remazol Black B after 18 hours of incubation at pH between 7-8, temperature 30°C at 10% (v/v) inoculum and 1% (w/v) glucose concentration. *Pseudomonas sp*. and *Bacillus sp*. isolated from the textile effluent showed maximum Orange 3R dye decolorization

of 89% at the end of 144 h under optimum conditions with *Bacillus sp*. being the most efficient decolorizer **(Ponraj et al., 2011)**. A bacterial consortium exhibited 97% decolorization in Direct Blue 151 whereas 95% in Direct Red 31 within 72 hours at pH 9.5, incubation temperature 36℃ at 200 mg/l dye concentration with medium supplemented with sucrose and yeast extract **(Lalnunhlimi and Krishnaswamy, 2016)**.

Conclusion

The isolated *Bacillus sp*. showed enormous potential to decolorize all the three textile dyes studied even at high concentration of the dyes and could be effectively utilized for treatment of dye contaminated textile effluents before discharge into environment. The decolorization efficiency of this versatile strain was found to be enhanced by varying pH, temperature, inoculum size, carbon and nitrogen sources. In further work, genetic makeup of the isolated *Bacillus sp*. can be identified which might further be helpful in enhancing the dye decolorization efficiency of the isolated strain.

Declarations

Conflict of Interest: None

Authors' contributions: The 1st author contributed to the concept, work plan, analysis of data and writing of manuscript. 2nd author did the survey, sample collection and lab work. Both authors read and approved the final manuscript.

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