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Decolorisation of Reactive Voilet-1 by Novel Isolate Bacillus Cereus Strain CMGS-4

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Abstract

Microorganisms play an important role in biodegradation of pollutants including recalcitrant azo dyes. Out of 20 bacterial isolates, bacillus cereus strains- CMGS-4 isolated from textile mill effluent, Sholapur, Maharashtra gave excellent result, in the utilizing reactive violet-1 as a sole source of carbon. The strain identified as Bacillus cereus sps (Gene bank accession num-633716), by conventional and 16S rRNA sequencing methods. Optimized the biotic and abiotic parameters for the maximum decolorization of RV-1 by bacterial isolate. Organism decolorized initially added RV-1 up to 200mg/L within 12 hours and could decolorized more than 70% at 800mg/L and 60% when dye concentration increased to 1000mg/L within 24 hours. The isolate had a capacity to decolorize more than 80% in a wide range of pH 7 to10 and temperature (25 to 45° C) however it decolorizes RV-1 better only in 1% salt concentration and also shown decolorization of five structurally different azo reactive dyes in mixed (in equal quantity) within 24 hrs. So it could be a better candidate for the decolorisation of textile effluents containing reactive azo dyes.

Keywords: Decolorisation, Reactive violet-1, *Bacillus cereus*, Reactive azo dyes, Recalcitrant.

Introduction

In recent past the natural colors are replaced by chemically synthesized colors and became essential for rapid developments in the appearance sector. In order to ensure the long life, brightness, different shades, solubility, adsorbility to fiber of colors, dye producers use high molecular weight complex structured aromatic hydrocarbons. Thus prepared dyes are more recalcitrant and remains in nature for longer time as residues and their intermediate which harmful to environment and living things[1]. These dyes are used in Textile, leather, cosmetics, pharmaceuticals, paper, food industries etc[2]. Now in the world there are 10000 different kinds of dyes are available with annual production over $7x10^5$ metric tons. Textile industries are the major consumers of synthetic dyes, on an average textile mill produces 60×10□/m of fabric and discharges approximately 1.5 million liters of effluents per day in India[3]. The textile effluents released from industries are complex hazardous chemicals, which contain chlorine groups, acidic group, heavy metals and other harmful chemicals[4]. The receiving water bodies physicochemical parameters changes drastically, and leads to the drastic effect on the biodiversity[5]. The compounds in effluents may converted into toxic and carcinogenic intermediates by photochemical reactions that may creates health related problems like allergy, skin infections, respiratory diseases, kidney problems to humans[3]. It became a long lasting problem in worldwide, presently various physicochemical treatment methods available but they were very costly, time consuming, leads to production of secondary metabolite and residues which may be toxic and also generate huge sludge. Nowadays biological process gain an importance, microbial degradation is eco friendly, cost effective and leads to the less or no intermediate toxin production[6]. Among microorganisms bacterial decolorisation process is faster, due to its replication cycle and genes arrangement[7]. Various research studies shown bacterial degradation gave promising results on reactive dye degradation[8].

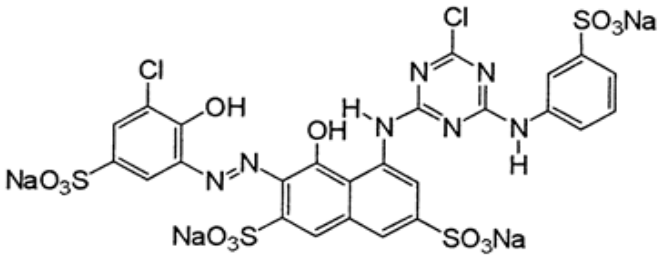
The present investigation conducted to work on essential objectives, isolating wild novel bacterial strain to achieve a good degradation on reactive violet–1 and its identification and characterization. Optimization of biotic and abiotic parameters for dye decolorization process to increase the efficiency of isolate was performed.

Materials and Methods

Sources of sample: Soil and effluent samples were collected from the textile industrial areas of Sholapur Maharashtra. Also soil samples at M.S.K textile mill, Kalaburagi and Soil samples and waste water samples were collected in and surroundings of Kalaburagi city.

Dyes and Chemicals: Six reactive azo dyes (Commercial grade) used in this study were procured from the Colorise and Heena textile industries, Ahmadabad (Gujarat) and Sigma Aldrich U.S.A. The dyes are Reactive red 11, Reactive blue 4, Reactive yellow 86, Reactive navy blue 59, Reactive orange 16 and Reactive violet 1. RV-1 had been selected for the isolation and dye degradation bacteria and optimization study; it is a

polycyclic aromatic azo dye containing two chlorine and four sulphonic groups which leads to the dye more water soluble and recalcitrant.



Structure of reactive violet-1

Media and Reagents: The required media, reagents and solutions were prepared using analytic grade chemicals and following the standard methods in the microbiological lab manuals. The mineral salt medium broth was prepared by adding ingredients (gms/l) of $Na_2HPO_4.2H_2O$ -12.00g, KH_2PO_4 -2.00g, NH_4NO_3 -0.50g, $MgCl_2.6H_2O$ -0.10g, $Ca(NO_3)_2.4H_2O$ - 50.00mg, $FeCl_2.4H_2O$ - 7.50mg, to 1000 ml of distilled water. To this 10 ml of trace elements solution was added and adjusted pH -7.00. Trace element solution prepared by addition of trace elements (g/l) of $FeSO_4.7H_2O$-0.10, $ZnSO_4.7H_2O$- 0.05, $CuSO_4.5H_2O$- 0.02, $CaCl_2.6H_2O$- 0.005, $MnSO_4.H_2O$-0.017 to 1000 ml distilled water.

Screening, Iolation and Identification of Dye Decolorizing Bacteria: Ten percent of the collected samples were homogenized in a 100 ml of sterile normal saline and after settling supernatant was collected and used for inoculating the decolorizing medium (DM) consist of mineral salt medium blended with 50mg/L RV-1. The flasks were inoculated with 20% supernatant of sample homogenized and incubated at 35^o C for 30 days and observed for decrease in color intensity in test flaks compared to control flasks (MD without sample). Flasks showing less than 50% color intensity were selected and once again the 20 ml of cultures were transfer to 100 ml of fresh MD and incubated again and observed for the maximum decolorization in short incubation time and it was measured using spectrophotometer at 540 nm and calculated the percent decolorization. Maximum decolorized flasks were used for isolation of bacteria by streak and spread plate method using MS agar medium containing 50mg/l reactive violet-1. The colonies showing clear zone around them were picked up and cultures on the MS agar plate and observed for the colony characteristics and performed the gram staining. Different types of colonies were selected and subculture on MS agar slants. Each isolates were once again tested for their *decolorization* ability by adding ten ml of overnight culture to 100 ml DM and incubated at 35^0 C till visual decolorization observed. Again maximum decolorized flasks were selected and isolated the bacterial strain and confirm whether decolorization is due to single or mixed bacterial strains. From this pure culture of dye decolorizing bacterial strains were selected and sub cultured on nutrient agar slants and stored in refrigerator with 25% sterile glycerol. And identification, various biochemical tests, and sugar utilization tests were performed.

Identification by 16S rRNA Sequencing: Pure bacterial colonies were selected for the sequencing, sent for the identification, at Royal Life Sciences Pvt. Ltd.Hyderabad, India. Once the organism was identified ,Evolutionary history checked by To see the Phylogenetic position of bacterial isolate using the neighbor-joining method[9]. The optimal tree with the sum of branch length = 0.28801765 is shown. The Phylogenetic tree was linearized assuming equal evolutionary rates in all lineages[10]. The clock calibration to convert distance to time was 0.02 (time/node height). 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[11] and are in the units of the number of base substitutions per site. Codon positions included were 1st + 2nd + 3rd + Noncoding. Evolutionary analyses were conducted in MEGA6 software[12].

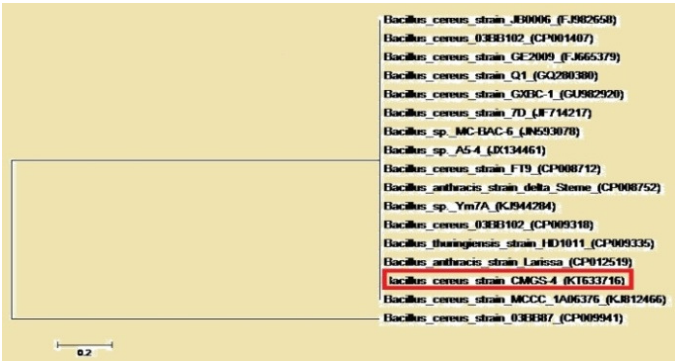


Figure-1
Phylogenetic tree of bacillus cereus CMGS-4

Optimization of various abiotic and biotic factors for maximum dye decolorisation by isolated bacterium: The abiotic factors including pH, temperature and aeration were optimized. Using wide range of pH from 4-12 with an increase of pH values 1 at a time and temperature in the range from 20-50^{0}C with 5^{0}C (interval). For the aeriation flasks were incubated on incubator shaker with a speed of 120 rpm and for static condition flaks were incubated in normal incubator at 35^{o}C. After knowing the optimum pH and temperature bacterial inoculums size was measured by adding 5 to 20% (5, 10, 15, 20) to 100 ml of DM. Then isolate was tested for the tolerance to various salt concentrations by adding 1 to 10% of NaCl with 1% increase to 100 ml of DM.

The effect of additional carbon and nitrogen nutrients on the dye degrading efficiency of isolated bacterium with all optimized conditions was performed by adding 1% of selected organic and inorganic nitrogen and different carbon sources to DM culture. The nutrient source showed maximum percent decolonization of

RV-1 and was more than the control (culture without the additional nutrients) was selected and used for the determination of optimum concentration required for maximum decolorization by isolate. Determination of minimum yeast extract concentration required to optimize the decolorisation efficiency of an isolated bacterium was performed by adding 0.1 to 2% of yeast extract with an intervals of 0.5 %.

Determination of RV-1 decolorizing efficiency of isolate: To determine the ability of the bacterial isolates for their decolorization of reactive violet 1 (RV -1), inoculated 10 % of bacterial inoculum to 100 ml of DM containing 100 mg/L RV-1. The decolorization medium without culture served as control. The flasks were incubated at 35 ^{0}C at static condition, every 4 hours 3 ml of the culture was drawn from each flask and analyzed for the dye present in the supernatant by taking OD at 540 nm. A decrease in the optical density with incubation time period is taken as an indication of decolorization. To confirm the decolorization is due to degradation of dye not due to change in the pH of the medium and adsorption or absorption. The change in the pH of the culture filtrates with HCl or NaOH. The adsorption was tested by dissolving the culture pellet in the methanol. Similarly absorption was performed by analyzing the dye in the cell lysate.

Effect of dye concentrations on decolorization efficiency of an isolate was determined by adding different concentrations of RV-1 starting from 50 mg/l to 1000 mg/l to 100 ml of decolorisation medium and the percent decolorisation was calculated after 24 hours of incubation at 35^{0}C.

Decolorisation procedure: Dye decolorisation in DM supplemented with yeast extract (0.8% w/v) and reactive violet 1 (200 mg/ml) complete decolorisation of dye occurred within 16 hours of duration. Dye degradation was confirmed by the checking optical density at 540 nm for different intervals of time. The percentage of decolorisation was calculated by following equation. It was calculated at regular intervals (0, 2, 4, 8, 12, 16, 20 and 24 hours) of incubation.

Calculation of % decolorization: **percent decolonization=**initial O.D-final O.D X100/initial O.D

Results and Discussion

Screening, isolation and characterization of decolorizing bacteria: A total of twenty isolates were obtained from different samples viz., sewage water, red soil, black soil and compost, garden soil, contaminated soil, textile effluent soil and textile mill effluent. Highest percent decolorization of RV-1 (70%) was noticed in CMGS-4 bacterial strain isolated from textile effluent.

Isolation and Characterization of Reactive Violet-1 decolorizing bacteria: The isolated bacteria were identified by conventional and molecular methods. The colony characteristics CMGS-4 on the nutrient agar plate- medium in size with irregular margin, smooth, opaque appears as grounded glass like. They are positive for Grams staining, non motile big rods arranged in chain with spore in the center of the cell. Results of biochemical tests sugar utilization tests. 16s rRNA sequencing was done.

A-Growth on NA plate **B-**Microscopic image grams staining

Figure-2
Cultural and morphological characteristics of isolate CMGS-4

Table-1
Morphological and biochemical characteristics of *Bacillus cereus* CMGS-4

Tests	**Observation**
A. Colony character Size	 Medium
Shape	Branch
Color	White
B. Morphological Characteristics Grams staining	Blue colour rodes (Positive)
Motility	motile
Cell shape and arrangement of spore	Center spore
C. Carbohydrate Glucose	 Acid production
Sucrose	Acid production
Lactose	Acid production
Mannitol fermentation	+ve
D. IMViC Indole	 - ve
Methyl Red	+ ve
Voges Proskaur	+ ve
Citrate	+ ve
E. Urease production **F.catalase**	+ve +ve
G. Gelatin hydrolysis	+ ve
H.Nitrate reduction	+ve
I Starch hydrolysis	+ve

Table-2
Sugar utilization tests

Tests	Observation
Xylose	-ve
Maltose	+ve
Fructose	+ve
Dextrose	-ve
Galactose	-ve
Raffinose	-ve
Trehalose	+ve
Melibiose	-ve
L-Arabionose	-ve
Mannose	-ve
Inulin	-ve
Sodium gluconate	-ve
Glycerol	-ve
Salicin	-ve
Dulcitol	+ve
Inositol	-ve
Sorbitol	-ve
Adonitol	-ve
Arabitol	-ve
Erythritol	-ve
Alpha-methyl-D-glucoside	-ve
Rhamnose	-ve
Cellobiose	-ve
Melezitose	-ve
Alpha-methyl-d-mannoside	-ve
Xylitol	-ve
ONPG	+ve
Esculin hydrolysis	-ve
d-arabinose	-ve
malonate utilization	-ve
Sorbose	-ve

Decolorization assay for determination of dye decolorizing efficiency isolate *Bacillus cereus*-**CMGS-4:** At various incubation time beginning with 0 hours of incubation time to 24 hours with regular intervals of 4 h of incubation time. The percent decolorization values at different incubation period are depicted in the Figures-3 and 4. Maximum of 93.90 % dye decolorization was achieved by the *Bacillus cereus*-CMGS-4 at an incubation time of 24 h. Further incubation does not shown much increase in the decolorization even after increased incubation time up to 48 hrs. So we observed that this organism can decolorize RV-1 up to 95% but not 100%.

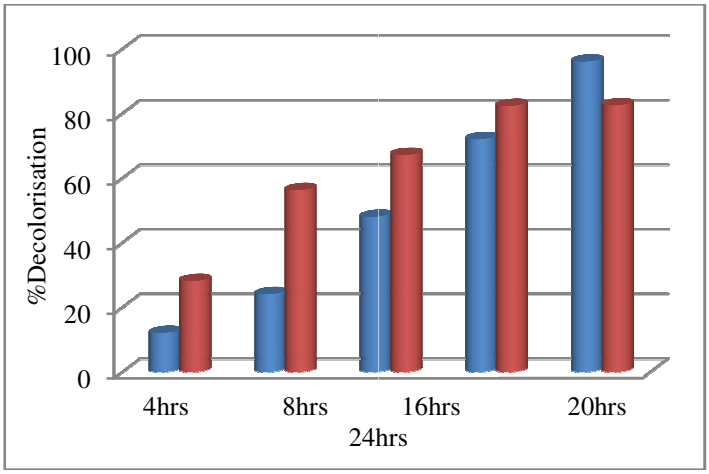


Figure-3
Decolorisation in hours of RV-1 by isolate-CMGS-4

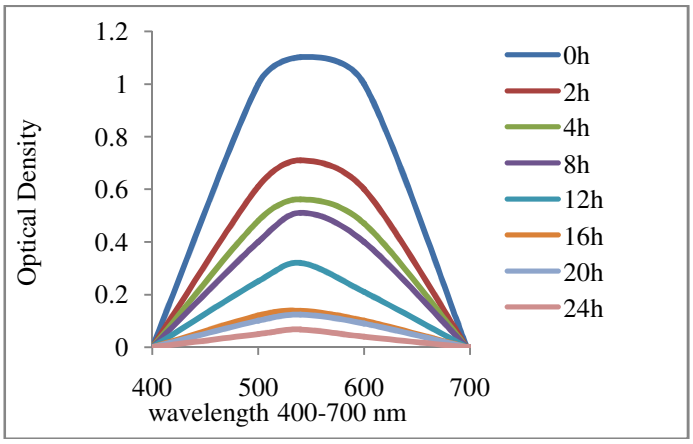


Figure-4
Decolorization of RV-1 by isolate-CMGS-4 through optical density study

Optimization of biotic and abiotic factors for the maximum dye degradation efficiency of *Bacillus cereus*-CMGS-4: The various biotic and a biotic factors are optimized for the maximum decolorization by isolated bacterium CMGS-4 as follows.

Decolorization in the static and shaking conditions: Isolate *Bacillus* cereus-CMGS-4 is more efficiently decolorizes (89.1%) in the static condition compared to shaking condition (22.3%) with the 12 hours of incubation. It reveals that isolate is microaerophilic in nature (Figure-5). Similar with our results, reactive Red 120 by *Bacillus lentus* B1377 the decolorization was very high in static incubation compared to shaking[13]. And in another shown static condition suits for the better decolorisation of RV-5 dye[14].

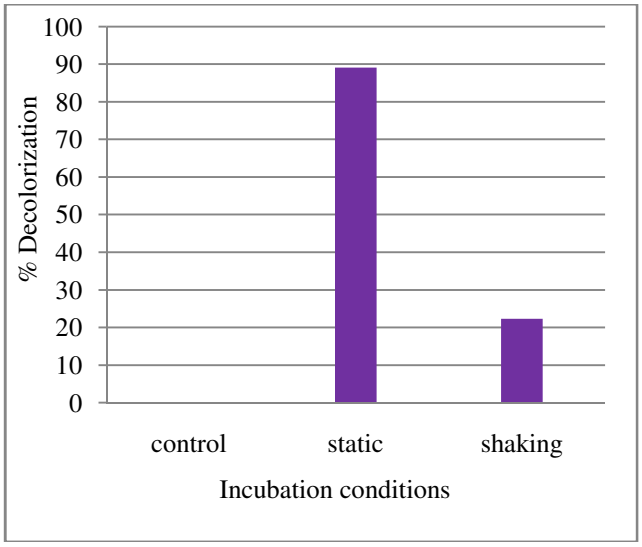


Figure-5
Effect of aeriation on decolorization of RV-1 by isolate *Bacillus cereus*-CMGS-4

Optimization of pH for the isolate *Bacillus cereus*-CMGS-4 **-** The hydrogen ion concentration in the decolorizing medium is considered as major factor. The results are shown in fig.6. Isolate CMGS-4 shown decolorisation in wide range of pH (4-12) and showed maximum decolorization at pH 8(89.5%), which is considered as optimum. However the percent decolorization was above 80% in the range of pH 7 to 10. Overall isolate has shown higher decolorizing activity (more than 60%) in a very wide range of pH (4-12). Similarly in the research article showed maximum decolorization of reactive red 120 by *Bacillus lentus* B1377 in a pH range 7-9 which is comparable with our study however there was no drastic reduction in decolorization by our isolate as with *Bacillus lentus* B1377 when decrease pH below 7 and increased above 10[13]. However our study is in contrast with decolorization ability (above 90%) of RV-5 by *Clostridium bifermentans* in a wide range of pH 6-12 [15]. Many reports on RV-5 decolorization showed narrow pH range compared to ours[14,16]. Generally Halophilic and halotolerant withstand wide range of pH [17]. In contrast that neutral pH would be more favorable for removal of reactive azo dyes and the suitable industrial applications[18].

Optimization of temperature: The decolorization efficiency of this isolate was above 80% in a temperature ranges from 25 to 45^0 C with maximum of 93% at 35^0C and around 70% decolorization was seen at 20^0 C and 50^0C (Figure-7). In Ali's review mentioned that microorganisms degrade synthetic dyes best in the range of 25-37^0 C[18]. Considering overall results it indicates that isolate *Bacillus* cereus-CMGS-4 is a mesophilic and alkalophilic organism and it will be a candidate for decolorization of textile effluents containing reactive azo dyes without neutralizing.

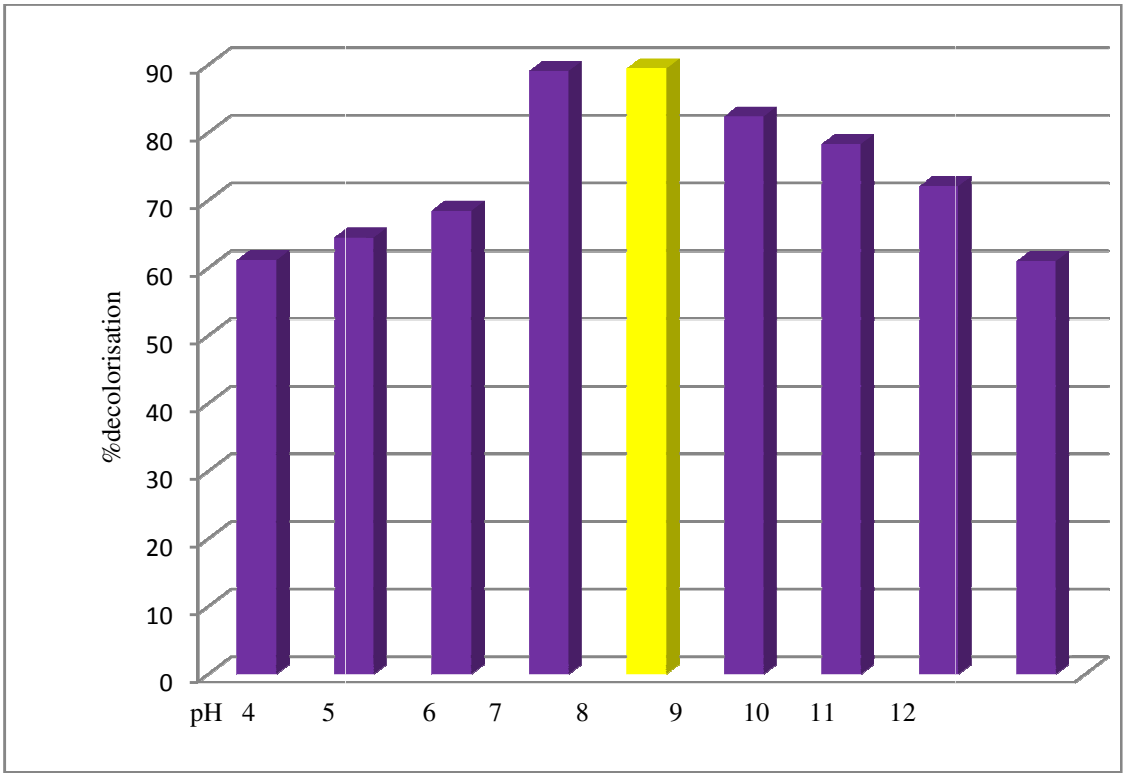


Figure-6
Effect of pH on decolorization of RV-1 by isolate *Bacillus cereus*-CMGS-4

Optimization of inoculums size: Optimization of biological factor (inoculums size) for the effective decolorization of RV-1 by the bacterial isolate *Bacillus cereus*-CMGS-4 was performed with all optimized abiotic parameters and results presented in Figure-8. The decolorization percentage recorded with increased inoculum size from 5 to 20 ml and above that it was almost constant of around 90%. Highest 93% of decolorization was noticed at 10% inoculum size and goes on decreased gradually with increase in inoculum size 15 and 20% and was found to be around 90%, the lowest of 84% of decolorization was seen with 5% inoculum size. Optimum of 10% inoculum size selected for the further studies of decolorization of Reactive Violet 1 by isolate. *Bacillus cereus* CMGS-4 shown better decolorisation in inoculum of 10ml. Concentration of cells is not so important because during azo bond cleavage, the cells compete for electron donors which effect decolorization even at low percentage of inoculum size[19]. In one report low concentration of 5% inoculum of *Micrococcus* sps used to decolorize azo dye Orange MR[20].

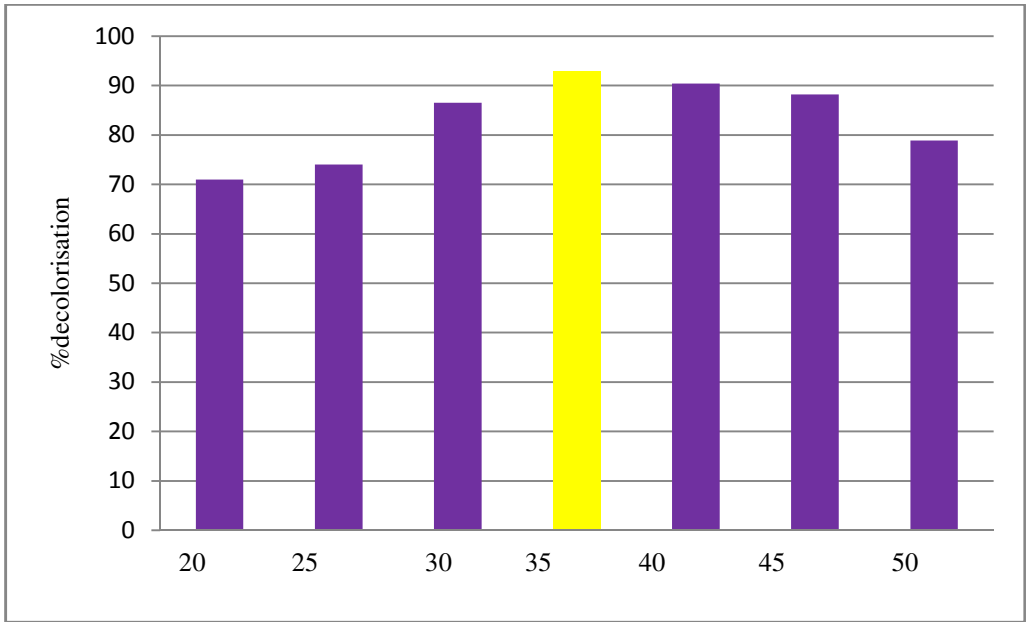


Figure-7
Effect of temperature on decolorization of RV-1 by *Bacillus cereus*-CMGS-4

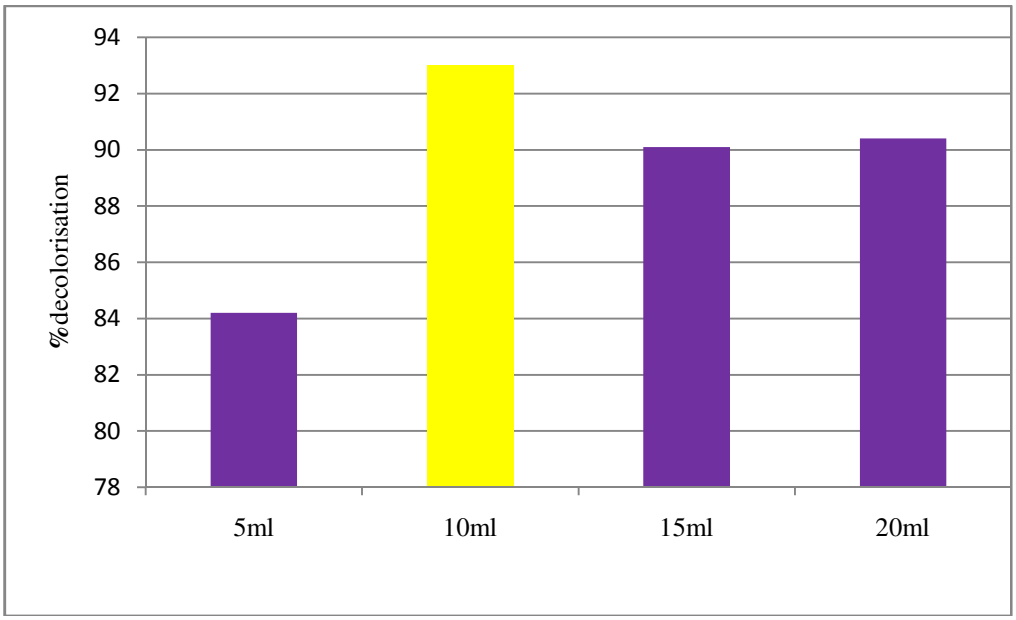


Figure-8
Effect of inoculums size on decolorization of RV-1 by *Bacillus cereus*-CMGS-4

Optimization of salt concentration: Isolate *Bacillus cereus*-CMGS-4 is shown maximum decolorization at 1% NaCl and its dye degrading efficiency went on decreased with increased NaCl concentration and was around 60% at 4% salt (Figure- 9). So, it is not a high salt tolerant strain; similar report has been reported by Oturkar *et al.,* with reactive Red 120 by *Bacillus lentus* B1377[13].

Optimization of Dye concentration: Effect of different initial RV-1 concentrations on the decolorization efficiency of isolate *Bacillus cereus*-CMGS-4 was performed by adding 50, 100, 200, 400, 600, 800 and 1000 mg/L in a decolorization medium and percent decolorization values were calculated at different intervals of incubation. Highest decolorization of 96.30 % of RV -1 observed up to 200 mg/L then gradual fall in percent decolorization with increase in RV- 1 concentration and was around 60% at 1000mg/L (Figure-10). The maximum dye concentration of 200 mg/L of RV-1 decolorized within 24 hrs by isolate CMGS-4. Which is higher than that of earlier reports[14-16]. Jain *et al.,* showed the complete decolorization of initially added 1500mg/L of reactive violet 5 within 42 h by a bacterial consortium SB4[19]. Further high concentration of reactive azo dye inhibits nucleic acid synthesis in microbial cell growth[19]. For the particular organisms removal of reactive azo dye in the decolorization process depends on the dye concentration in the effluent. Maximum of 96% decolorization efficiency of this organism for initial RV-1 concentration was found to be 400 ppm and was decreased 70% at 1000ppm.

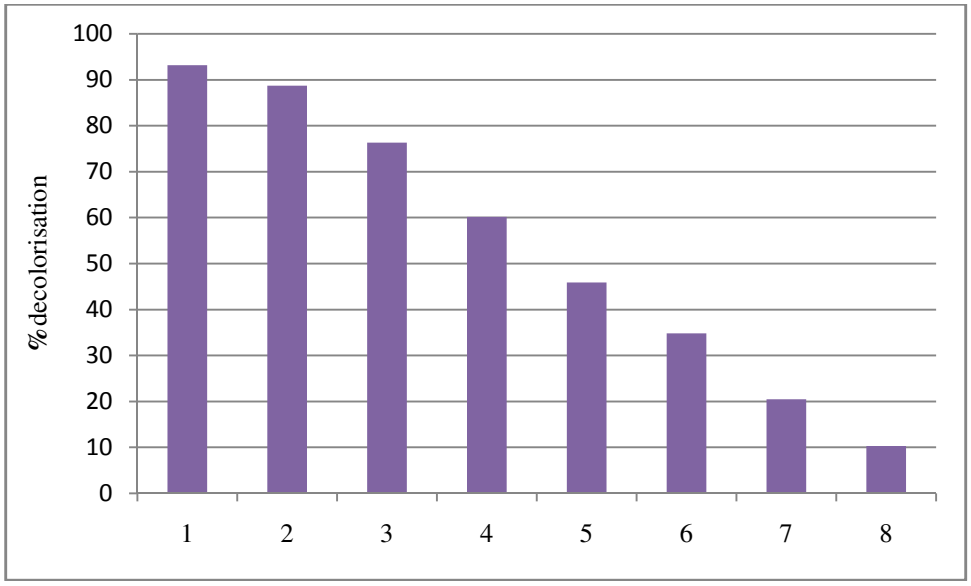


Figure-9
Effect of Salt concentrations on decolorization of RV-1 by *Bacillus cereus* CMGS-4

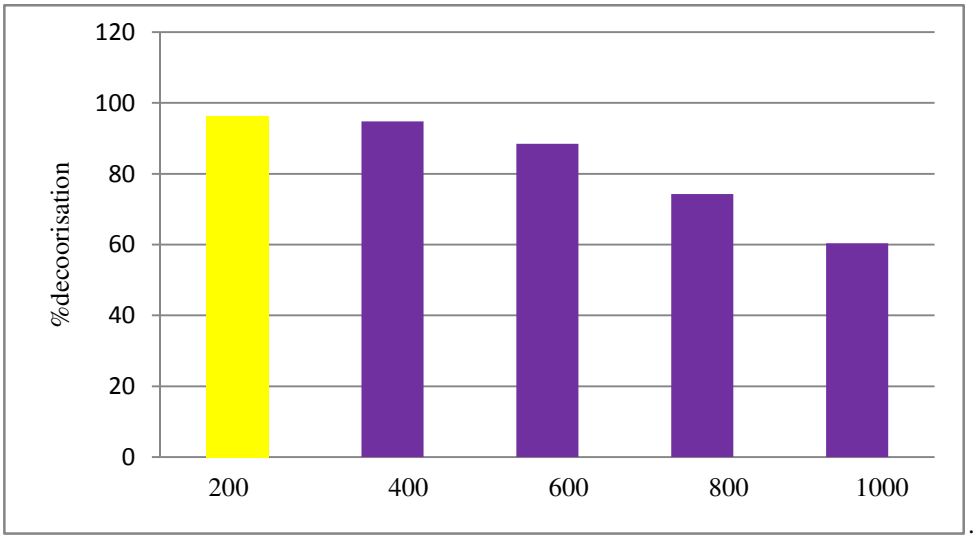


Figure-10
Effect of dye concentrations on maximum decolorization by *Bacillus cereus*-CMGS-4

Decolorization of mixed structurally different kinds of dyes: Isolate *Bacillus cereus*-CMGS-4 shown decolorisation in the mixture of five structurally different dyes. They are reactive red-11, reactive orange-16, Reactive blue 59, reactive yellow -84, and reactive blue-4. Total 5 dyes mixed in equal weights and finally 50 mg/L of concentration was used for decolorization study. Isolate shown decolorisation up to 75% within 24 hours of duration and no increase in the decolorization on further incubation. It shows stagnant decolorisation after 24 hours of time (Figure-11). Researcher revealed that for microorganisms additional concentration may inhibit the replication of organisms. At a certain dye concentration microbes may tolerate[19].

Optimization of additional nutrients for maximum decolorization of *Bacillus* cereus-CMGS-4: Optimization of additional carbon source: Results of optimization of three additional carbon sources glucose, sucrose and starch for effective degradation of RV-1 by *Bacillus* cereus-CMGS-4 is shown in Figure-12. Maximum decolorization of 92.4% was observed with glucose among three carbon sources tested while for sucrose it was only 10% less as compared to starch (30%). Additional carbon sources are not enhancing the efficiency of decolorization of RV-1 by isolate *Bacillus cereus*-CMGS-4. Additional carbon sources are essential for increase in dye degradation efficiency of *Bacillus lentus* BI377[13]. However, in our study there is no much influence on decolorizing efficiency on addition of carbon sources similar reports are reported on RV-5 dye decolorization using different bacterial strains[14-16].

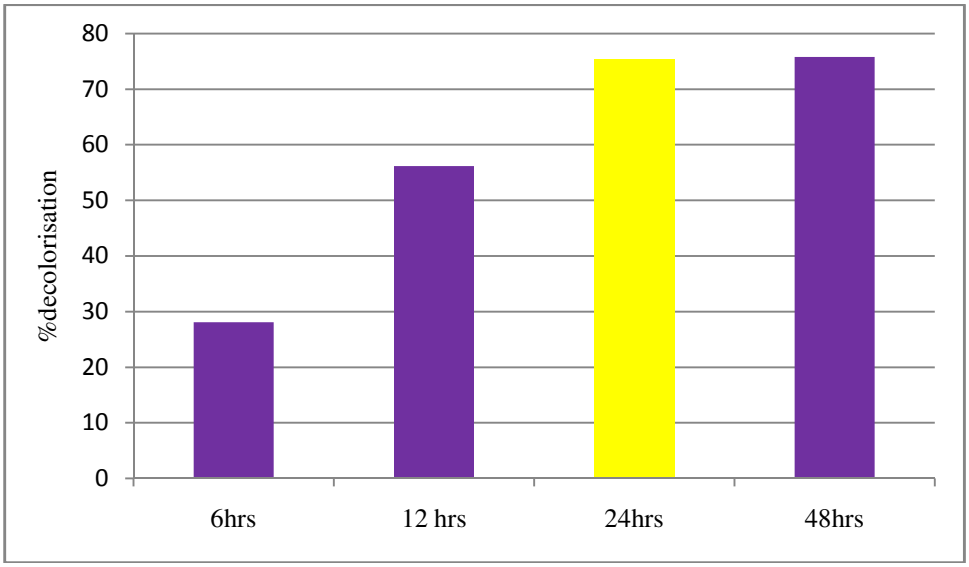


Figure-11
Effect of mixture of dyes on decolorization efficiency of *Bacillus cereus*-CMGS-4

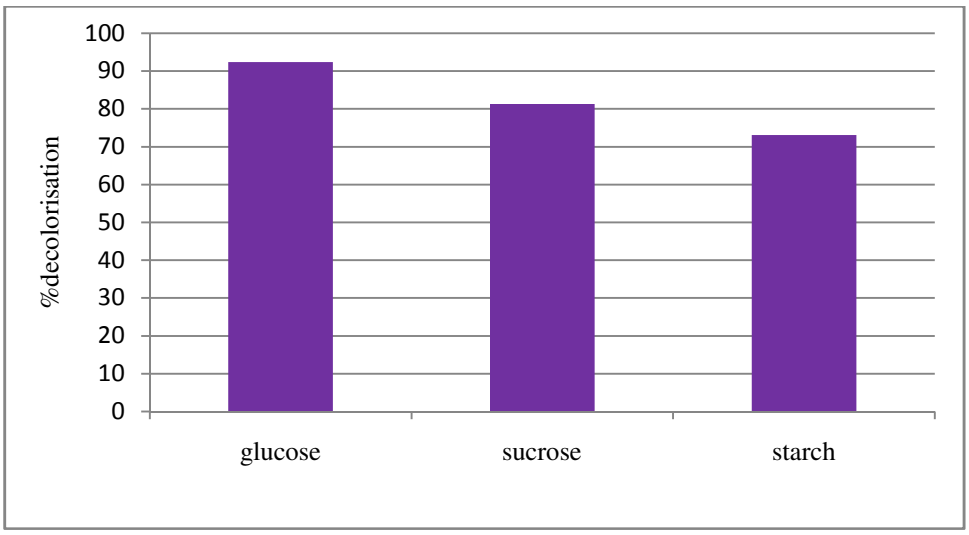


Figure-12
Effect of carbon sources on decolorization of RV-1 by isolate CTSB -16

Optimization of Nitrogen sources: Results of optimization of additional nitrogen sources (1g/L) yeast extract and peptone for effective decolorization of Reactive Violet -1 by isolate CMGS-4 is depicted in Figure-13. Decrease in the incubation time was observed for both nitrogen sources tested. Maximum of 94% decolorization with yeast and 92% with peptone within the 12 hours of incubation was seen.

Optimization of concentration of Yeast extract: The exact concentration of yeast extract was determined for maximum decolorization RV-1 by *Bacillus cereus*-CMGS-4 at different time intervals by adding different concentrations of yeast extract (0.2g/L to 1g/L) in the decolorization medium and results are defected in the Figure-14. The maximum effect on decolorization of RV-1 was seen with 0.8g/L of yeast extract within the 12 h of incubation. Above this concentration there was no much effect on decolorization of RV-1, therefore 1g/L of yeast extract concentration was used as optimal additional nitrogen source for further studies. Then the exact concentration of yeast extract determined for maximum decolorization RV-1 by isolate CTSB -16 at different time intervals by adding different concentrations of yeast extract (0.1g/L to 1g/L) in the decolorization medium and results are defected in the Figure-14. The maximum effect on decolorization of RV-1 was seen with 0.8g/L of yeast extract within the 16 h of incubation. Our results are comparable with the reports were bacterial mixed culture-SB4 on azo reactive violet 5R decolorization[19] and with *Paracoccus sps* GSM2 on RV-5[14]. In other reports reported that yeast extract act as dual source of carbon and nitrogen in decolorization of various dyes by bacteria[21]. Organic nitrogen sources like Yeast extract are considered essential for regeneration of NADH which act as electron donor in azo bond reduction under static incubation[19].

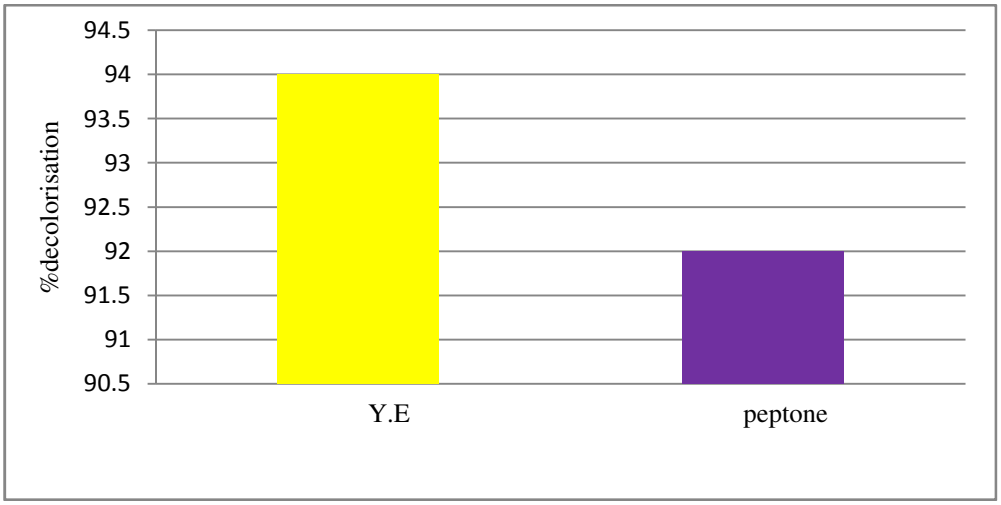


Figure-13
Effect of Nitrogen sources on decolorization of RV-1 *Bacillus cereus*-CMGS-4

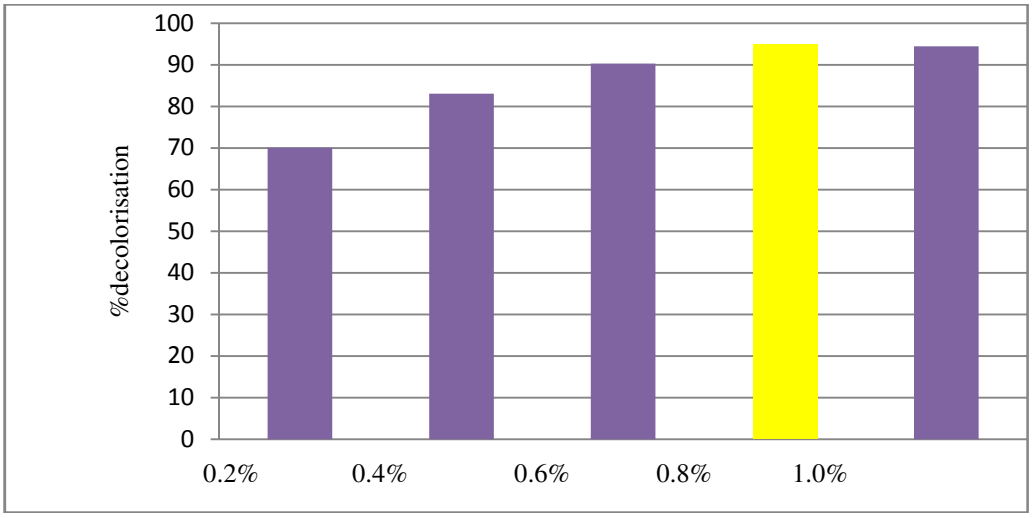


Figure-14
Effect of Yeast extract on decolorization of RV-1 by *Bacillus cereus*-CMGS-4

Conclusion

As *Bacillus cereus*-CMGS-4 was isolated from the textile effluents and utilized the RV-1 up to 70% during primary isolation as a sole source of carbon and energy. Isolate has decolorized RV-1 more effectively in a very wide range of pH values (4 to 12), temperature (25 to 45^0 C) and dye concentration up to 1000 ppm. *Bacillus cereus*-CMGS-4 has capacity to decolorize more efficiently other 5 structurally different reactive azo dyes individually and in combination of all five proves it to be a potential candidate for treatment of textile mill effluents.

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