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Decolorization of Azo dyes and Triphenylmethane dyes using Citrobacter diverses isolated from textile waste

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ABSTRACT

Release of residual dyes into natural water bodies through industrial waste water is considered to be a potential threat to the environment. There are several traditional physio-chemical treatment methods to remove such organic and inorganic dyes, but these are costly and create secondary sludge disposal problems. Bioremediation is offers the best approach to overcome the limitations of traditional methods. Thus, this study was undertaken to isolate bacteria possessing high potential to degrade the different types of dyes. Total 12 different dyes decolorizing bacterial isolates were obtained from textile waste water using enrichment and isolation techniques. Out of these isolates, Citrobacter diverses was found to be the most prominent dyes decolorizing bacteria. It showed 79% to 98% decolorization of MR (Methyl red), MG (Malachite green), CR (Congo red), CV (Crystal violet), CF (Carbol fuchsin) dyes within 72 h. It showed maximum decolorization in presence of optimum growth conditions like 1.5gm/L yeast extract, sucrose and glucose as a carbon source, peptone and NH_4SO_4 as a nitrogen source of medium pH 7.0 at 37°C. This isolate showed highest decolorization in its free cell form as compared to its immobilized form. According to these results, obtained strain of Citrobacter diverses confirms its application in bioremediation process of dyes in In-situ.

Keywords: *Dyes, Bioremediation, Textile waste, Bacteria, Optimum growth conditions*

1. INTRODUCTION

Dyes are colored organic compounds that are used to impart color to various substrates. They have an affinity to the substrate on to which they are applied. The chemical structure of dyes comprises of a conjugated system of double bonds and aromatic rings. On the basis of chemical structure, dyes are classified into Acid dyes, Basic dyes, Reactive dyes, Dispersive dyes, Azo dyes and Anthrquinone dyes [1].

Dyes are highly stable to light, heat, water, detergents, bleach and perspiration due to their resonance and π-conjugated azo bond characteristics. Approximately 10,000 different dyes and pigments are produced commercially worldwide including Methyl red (MR), Congo red (CR), Malachite green (MG), Crystal violet (CV) and Carbol fuchsin (CF). These dyes are used as biological stain for many microscopic analysis of cell biology. There are some disinfectants and antiseptics available constituting these dyes. Some of the chemical industries including textile, paper, pharmaceutical and cosmetic industries also use these dyes. About 10% to 15% of unused dyes come out in the wastewater of these industries. The characteristics of waste water coming out from these industries are totally dependent upon the manufacturing process and dyes which are used for the process (Ghale *et al*., 2014). If such dye containing wastewater is not treated properly before being disposed into water resource, it can be a problematic. It will lead to enormous environment pollution [5].

Dyes containing wastewater alters the color and quality of ecosystem. Dyes layer can block the penetration of sunlight from water surface preventing photosynthesis, thus affect aquatic plants and animals. Dyes are mutagenic and carcinogenic in nature. In humans, they causes diseases like haemorrhage, ulceration of skin, severe irritation of skin, nausea and dermatitis [7].

Dyes cannot be removed from waste water by common effluent treatment methods because of their recalcitrant nature [1]. Various physical and chemical methods such as filtration, coagulation, use of activated carbon and flocculation are effective methods in dyes removal but they are quite expensive. They generate disposal problem of secondary waste. Instead of these traditional methods, Biotreatment offers a cheaper and environmental friendly alternative for dyes removal. The many experiment is going on worldwide in degradation of dyes by biological means [11].

In previous studies, many bacterial strains have been isolated and found to be able to degrade azo and triphenylmethane dyes. It includes *Brevibacterium casei*, *Enterobacter spp.*, *Pseudomonas spp.*, *Morgonella spp.*, *Providencia rettgeri*, *Bacillus spp.* etc [6].

This study evaluated the potential isolated bacterial strain from textile waste in decolorization of MR, CR, CV, CF, and MG dyes under in vitro conditions. The optimization of medium and immobilization effect on dyes decolorization was also checked to achieve maximum decolorization.

2. MATERIAL AND METHODS

2.1 Waste water Sample and Chemical collection

Untreated textile effluent samples were collected from small dyeing industries located in Kalyan- Dombivali MIDC area, Dist.: Thane. The samples were collected in sterile plastic bottles. MR (Methyl red), MG (Malachite green), CR (Congo red), CV (Crystal violet), CF (Carbol fuchsin) dyes, microbiological media and medium ingredients were purchased from Himedia laboratories, Mumbai (MH, India).

2.2 Isolation of dye decolorizing bacteria

The collected samples were enriched by inoculating of sample into 100 ml of Nutrient broth with 0.02 gm/L dyes concentration. The flasks were incubated at R.T. (28°C±2) for 48 h under shaking conditions (140 rpm). Enriched samples were serially diluted upto 10^{-6} dilution. Isolation of bacteria was carried out using spread plate method on nutrient agar plates (0.5% peptone, 0.3% meat extract, 0.5% NaCl, 1.5% Agar and 1% individual dye). On incubation, the colonies, which showed zone of dyes decolorization on agar plate, were selected for further analysis.

2.3 Primary decolorization screening of selected isolates

Decolorization activity of isolated bacteria was determined using 100ml Nutrient broth containing 0.02 gm/L dyes separately. Medium was inoculated with 3 ml of 24 h old culture with 0.6 O.D. at 600nm. Medium without culture was kept as it is for control analysis. Azo dyes (MR, MG and CR) containing flasks were incubated in static condition while Triphenylmethane dyes (CV and CF) containing flasks were incubated in shaking condition with 120 rpm at R. T. (28°C±2). The media was checked for decolorization after every 24 h. After incubation 10ml medium centrifuged at 5000rpm for 15min. and supernatant was removed. Decolorization potential was assessed by measuring the absorbance of supernatant at respective Optical Density (O.D.) of dyes using colorimeter. O. D. taken at respective wavelength is as follows: MR (410nm), MG (620nm), CV (580nm), CF (520nm) and CR (530nm). Medium without dye used as blank for colorimetric readings. Uninoculated dyes containing medium served as control medium. Decolorization Percentage calculated by using following formula:

$$\%\ \boldsymbol{Decolorization} = \frac{\boldsymbol{Initial\ O.D. - Final\ O.D}}{\boldsymbol{Initial O.D.}} \boldsymbol{x}\ \mathbf{100}$$

Where, Initial O. D. is an O. D. of control dye medium and Final O. D. is an O. D. of decolorized medium

2.4 Identification of most prominent isolate

The isolate which showed higher decolorization efficiency was identified using Gram staining, special staining, specific cultural characteristics and various biochemical tests. The bacterial identity was confirmed by comparing biochemical results with the Bergyes manual of bacteriology.

2.5 Optimization of media to achieve highest decolorization by selected isolate

The Decolorization activity of selected isolate was optimized by observing the effect of various carbon sources, Nitrogen sources, dye concentration and yeast extract concentration on the growth of isolate. A working volume of 100ml Mineral salt medium (MSM : KH_2PO_4- 0.5gm, K_2HPO_4 - 0.5gm, $MgSO_4$ - 0.5gm, NH_4SO_4 - 1.0gm, NaCl- 0.5gm, Glucose - 0.5gm, $FeSo_4.2H_2O$- 0.02gm, Yeast extract – 1.0gm, D/W – 1000ml , pH - 7.0) containing 0.02gm/L was used for optimization studies. The MR, MG, CV, CR and CF dyes decolorization activity was optimized separately with 0.02gm/L dye concentration.

2.6 Effect of immobilization

The isolate was immobilized by using 4% (w/v) Sodium alginate and 6% (w/v) Calcium chloride ($CaCl_2$). 50 Ca-alginate beads of selected isolate were inoculated in 100ml MSM medium containing 0.02gm/L dyes (MR, MG, CV, CR and CF) separately. For control analysis, Cell free Ca-alginate beads were added in 100 ml MSM medium containing dyes. Flasks were incubated at R.T. (28°C±2) for 48 h on shaking condition. Dyes decolorization was observed and percentage dye decolorization was calculated.

2.7 Decolorization of Mixture of dyes

An experiment was carried out to study the efficiency of selected isolates to decolorize mixture of dyes (MR, MG, CR, CF and CV). 100ml MSM medium with 1gm/L yeast extract was prepared. The medium was added with all five dyes with 0.02gm/L concentration and sterilized. 3ml 24 h old culture of selected isolate having 0.6 O.D. at 600nm was inoculated in medium and incubated at R.T. (28ºC± 2ºC) in shaking condition. Control flasks medium was kept as it is. The color removal was observed after every 24 h. As decolorization was observed percentage decolorization was calculated.

3. RESULTS AND DISCUSSION

3.1 Isolation of dye decolorizing bacteria

The experiment of Enrichment and isolation of dye degrading bacteria from soil sample resulted in total 12 isolates. All these isolates were with different colony characteristics showing zones of dyes decolorization on cultured Nutrient agar plates. In this study, Nutrient agar medium was found to be suitable for the isolation of dye degrading bacteria as the colonies showed clear zone of dyes decolorization and thus could be easily selected for further analysis. Similarly, Kumar *et al*. (2015) isolated 13 morphologically different dye degrading organisms with zone of clearance from textile effluent samples. A total 18 bacterial cultures were isolated, purified and screened for the degradation of azo dyes from textile effluent and sludge sample by Sethi *et al*. in 2012.

3.2 Dyes decolorization assay of selected isolates

The selected 12 bacterial isolates were tested for decolorization of MR, CR, MG, CF and CV dyes individually. All the selected isolates were able to decolorize Methyl red and Malachite green dyes within 24 h to 48 h. However, 06, 03 and 04 bacterial isolates showed decolorization of Congo red, Crystal violet and Carbol fuchsin respectively. Only one isolate showed decolorization of all five dyes up to 79% to 98% within 72 h. umber of isolates decolorizing CR, CV and CF dyes were found to be very less than the number of MR and MG degrading isolates. This may be because of dyes structure as dyes decolorization is totally dependent on the structure, no. of azo bonds present, functional groups surrounding the azo bond, position of functional groups on benzene ring and molecular weight of the dyes. MR and MG dyes are having lower molecular weight than CR, CF and CV dyes [2]-[3]. During investigation, decolorization was not observed in uninoculated control flasks. This confirms that the isolates decolorized the dyes and decolorizing efficiency was totally dependent on the growth and metabolic activity of the isolate.

3.3 Identification of most prominent isolate

The selected prominent isolate was found as rod shaped Gram negative bacteria producing late pink colonies on Mac-conkeys plate and found as non- heamolytic on SIBA plate. It was Oxidase negative and Catalase positive organism. It showed positive results for glucose, lactose, xylose, Nitrate, motility, Citrate, Methyl red, Indole, Ornithine decarboxylase and delayed positive to Urease, sucrose, maltose test. While negative results were observed for VP, PPA (Phenylalanine deaminase) and lysine decarboxylase test. These results were compared with Bergey's manual and the isolate was identified as *Citrobacter diversus. Citrobacter* genus of bacteria was well known for its xenobiotic compounds degrading ability and has been studied in the past specifically for its dyes degrading ability. Similar to our study, Wang *et al*. (2009), isolated the bacterial culture *Citrobacter spp.* CK3, which showed a strong ability to decolorize various reactive textile dyes, including both azo and anthraquinone dyes. The other *Citrobacter* spp., isolated by Sun-young *et al*. (2002), from soil of an effluent treatment plant of a textile and dyeing industry showed decolorization of MG, CV and MR (100mg/L) up to 80 to 90% within 36h.

3.4 Media optimization

The results of effect of various growth conditions on decolorization activity of *Citrobacter diversus* was mentioned graphically. It showed maximum decolorization of all five dyes in presence of 1.5gm/L yeast extract, sucrose and glucose as a carbon source, peptone and NH_4SO_4 as a nitrogen source of medium pH 7.0 at 37°C. It was observed that yeast extract concentration had great influence on the decolorization activity of *Citrobacter diversus*. It showed the highest decolorization of all five dyes in presence of 1.5gm/L yeast extract. It showed 97.8%, 97.5%, 88.1% 93.8% and 94.1% decolorization of MR, MG, CR, CV and CF dyes at 1.5gm/L yeast extract and it remained same for 2gm/L yeast extract concentration (Fig.1).

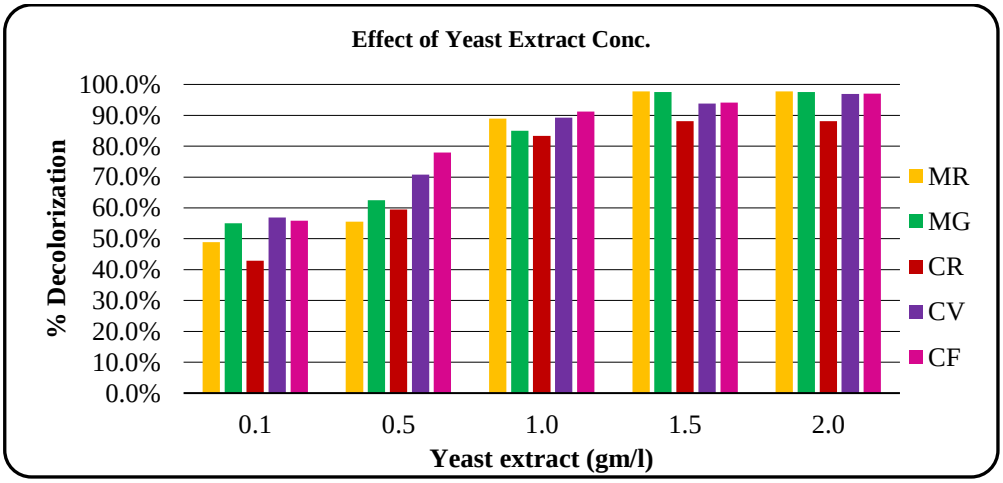

Fig. 1: Effect of yeast extract concentration

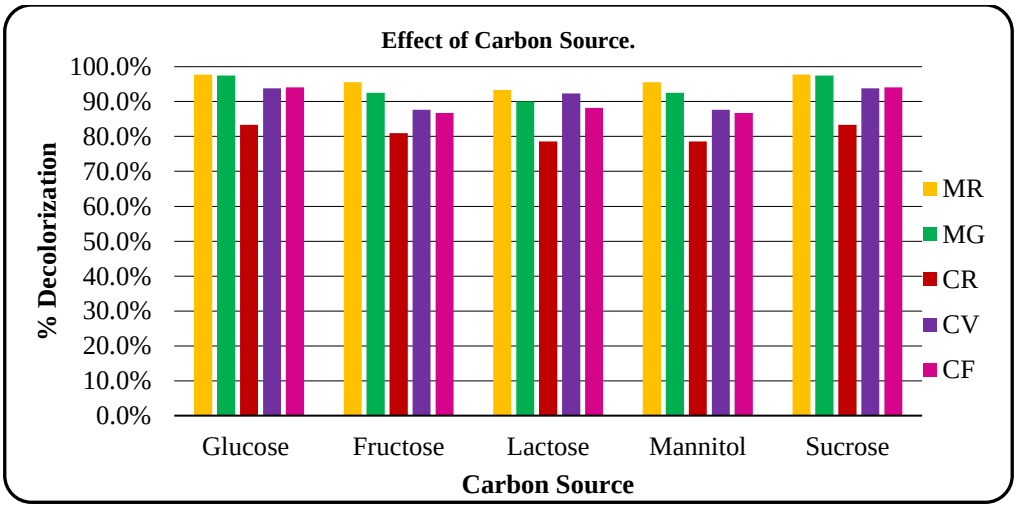

Fig. 2: Effect of Carbon source

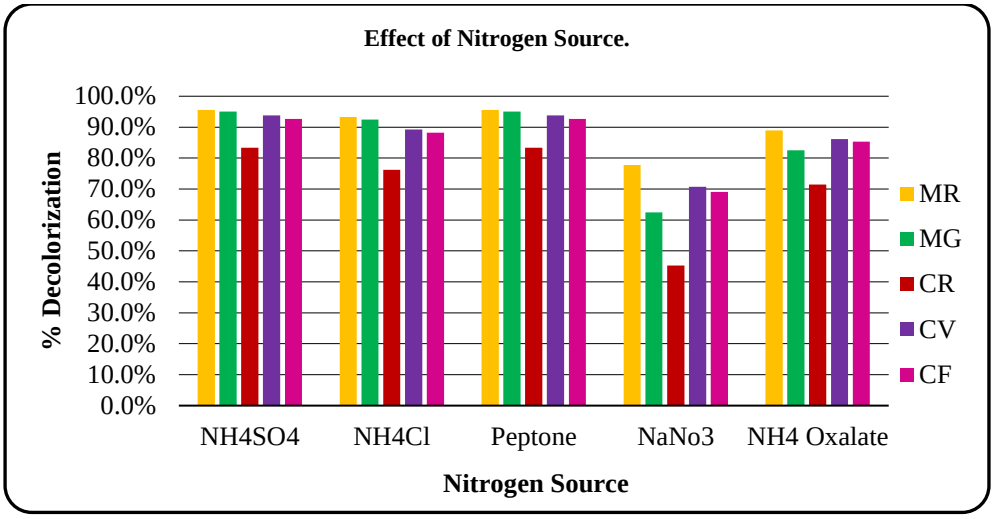

Fig. 3: Effect of Nitrogen source

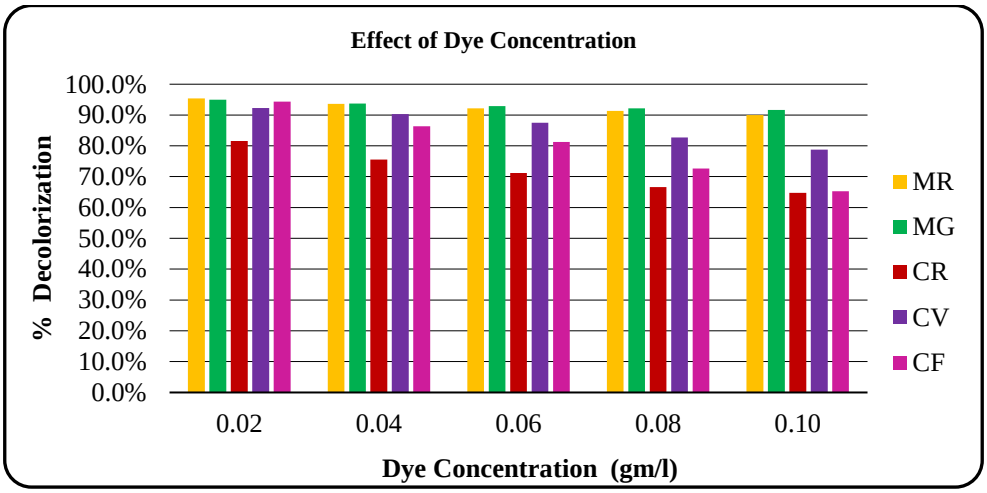

Fig. 4: Effect of Dyes concentration

Citrobacter diversus showed decolorization in presence of all carbon sources. Maximum decolorization, 97.8%, 97.5%, 83.3%, 93.8%, 97.1% of MR, MG, CR, CV and CF respectively was observed in presence of glucose and sucrose as a carbon source (Fig.2). Peptone and NH_4SO_4 as a nitrogen source resulted in maximum decolorization. *Citrobacter diversus* isolate showed 95.6%, 95.0%, 83.3%, 93.8% and 92.6% decolorization of MR, MG, CR, CV and CF in presence of both nitrogen sources (Fig.3). The bacterium was able to decolorize the dyes efficiently in presence of other nitrogen sources also. NH_4Cl resulted in better decolorization efficiency with 93.3% (MR), 92.5% (MG), 76.2% (CR), 89.2% (CV) and 88.2% (CF). The decolorization efficiency decreased with NH_4 Oxalate. Least decolorization was observed with $NaNO_3$.

Dyes concentration affects the decolorization activity of *Citrobacter diversus*. The bacterium could degrade MR and MG dyes up to 0.1gm/L dye concentration efficiently. 0.1gm/L MR and MG dyes decolorized up to 90% and 91%. But in case of CV, CF and CR dyes, as the concentration of dyes increased the percentage decolorization decreased significantly as shown in Fig.4. Bacterium showed 81.6%, 92.3% and 94.3 % decolorization of CR, CV and CF dyes at 0.02gm/L dye concentration. It reduced to 64.7% (CR), 78.7% (CV) and 65.3% (CF) at 0.1gm/L concentration.

***3.5* Effect of immobilization**

Better decolorization activity of isolate was obtained in Free State as compared to immobilized state. The immobilized *Citrobacter diversus* showed drastic reduction in decolorization activity in immobilized form. From Fig.5, Free cells of *Citrobacter diversus* decolorized the MR, MG, CR, CV and CF dyes up to 98% (24h) , 95% (24h), 79% (72h), 85% (72h) and 78% (72h) respectively while its immobilized cells showed only 80.0% (48h), 71.4% (72h), 47.7% (120h), 61.5% (120h) and 57.4% (120h) decolorization respectively. This increased in decolorization time requirement immobilized form. This could be because of loss of "direct" contact in between cells surface and dyes. Solid matrix (Ca-alginate) was present in between them and thus more time was required for adsorption of dyes on cell surface in immobilized form [9].

Also, the matrices used for the immobilization play a major role in decolorization activity of cells. Cheng *et al*. (2012), reported improved Crystal violet decolorization ability of *Burkholderia vietnamiensis* using PAV- kaolin gel beads than the Ca-alginate beads. They stated that kaolin is good adsorbent and PAV is a better porous material than Ca- alginate which adsorbs the dye and transfer on to the cell membrane more quickly than the Ca- alginate and hence it gave fast result of decolorization than the Ca-alginate beads.

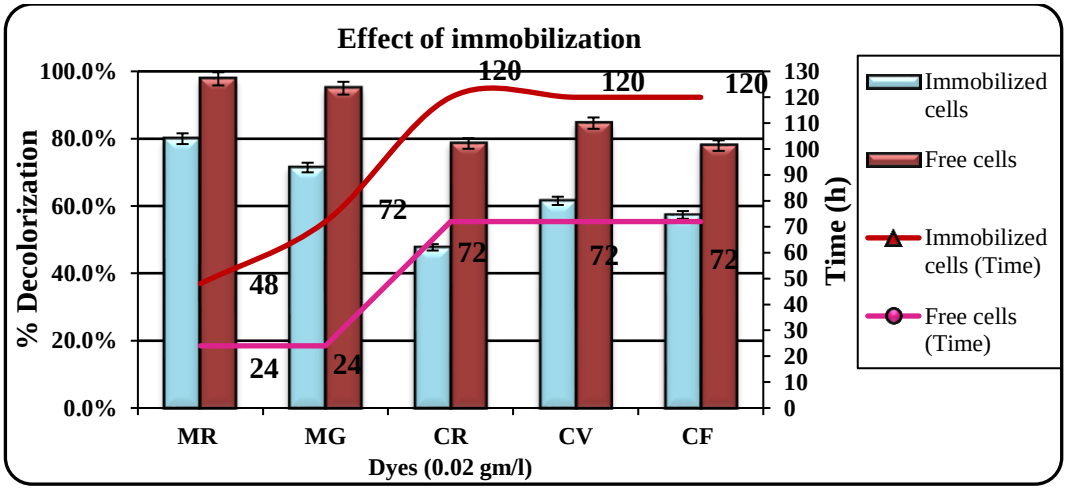

Fig. 5: Effect of Immobilization on decolorization

3.6 Decolorization of Mixture of dyes

Citrobacter diversus exhibited 64% decolorization of mixture of dyes after 96h respectively. It indicates that the respective isolates can withstand the presence of dyes with different structures. The concomitant presence of more than one dye (MR, MG, CR, CV and CF) slightly affects the decolorization activity of *Citrobacter diversus*.

4. CONCLUSION

A textile waste found to be good option for primary screening of dyes degrading bacteria. The biological decolorization of dyes by using *Citrobacter diversus* was highly promising, relatively inexpensive, ecofriendly and less sludge producing. It decolorizes complex chemical compounds into simple compounds in a much safer way. With specific physiochemical factors, selected isolates will grow more rapidly and it may result in more decolorization of dyes. It can be applied in *in situ* textile effluent treatment process to achieve maximum dyes decolorization.

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