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ISOLATION, SCREENING AND BIOCHEMICAL CHARACTERIZATION OF BACTERIA CAPABLE OF CRYSTAL VIOLET DYE DECOLORIZATION

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Abstract:

Crystal Violet (CV) is a basic dye which belongs to triphenylmethane dye group. It has been extensively used in human and veterinary medicine as a biological stain and most importantly as a textile dye in textile processing industries but has been reported as a recalcitrant dye molecule. In-vitro studies have revealed that CV acts as a mitotic poison, potent carcinogen, potent clastogene and promotes tumor growth in some species of fish and thus, CV is regarded as a biohazard substance. Due to its adverse effects on human, animals and plants, CV has been listed as hazardous chemical or material. Therefore, the removal of dye from industrial wastewaters for environmental safety is a serious matter of concern. Thus, in this study two bacterial strains SJ4 and SJ5 capable for dye decolorization were isolated from textile wastewater collected from Kanpur industrial area Panki site 3. The isolated bacterial strains SJ4 and SJ5 have shown the maximum decolorization i.e. 90% and 82% of Crystal violet dye (100 mg/L) within 8 hrs of incubation period, respectively in axenic conditions, but in mixed culture condition, 97% decolorization of CV was achieved within 6 hrs of incubation period. Therefore, this study showed that the bacterial strains when combined perform better as compared to single conditions and can be useful for the development of effective bioremediation strategies in the treatment of dye contaminated wastewater.

Key Words: Crystal Violet, Textile Dye, Recalcitrant, Carcinogen, Decolorization& Bioremediation

Introduction:

Synthetic textile dyes are one of the major water and soil pollutants released from different industries into the environments. Among different classes of synthetic dyes used in textile, dyeing, paper, leather, cosmetic, and food industries, triphenylmethane dyes are the largest and most versatile group of dyes that plays a major role in various industrial applications (Ahmad, 2009; Azmi et al., 1998; Nelson and Hites, 1980). Crystal Violet (CV), a triphenylmethane dye, has been extensively used in human and veterinary medicine as a biological stain, as a textile dye in textile processing industries and also used to provide a deep violet color to paints and printing ink. CV is also used as a mutagenic and bacteriostatic agent in medical solutions and antimicrobial agent to prevent the fungal growth in poultry feed. Inspite of its many uses, CV has been reported as a recalcitrant dye molecule that persists in environment for a long period of time and pose toxic effects. It acts as a mitotic poison, potent carcinogen and a potent clastogene promoting tumor growth in some species of fish (Mani and Bharagava, 2016).

Thus, CV is regarded as a biohazard substance. The dye is also found to cause moderate eye irritation, painful sensitization to light, permanent injury to cornea and conjunctiva, since the product contains a cationic dye, which is highly toxic to mammalian cells. Nevertheless, in extreme cases it may lead to respiratory and kidney failures also (Ahmad, 2009; Amini and Younesi, 2009; Azargohar and Dalai, 2005). Triphenylmethane dyes are one of the most widely used dermatological agents. Earlier, CV was widely used for the treatment of pinworms through oral route and in topical applications in humans and domestic animals. It has been shown to be effective in controlling the fungal growth under varying conditions and therefore was added to poultry feed to control the fungal growth exposing the human population directly or indirectly to CV through its extensive medicinal and commercial use (Willian et al., 1978).

Huge quantities of different groups of synthetic dyes such as azo disperse, acidic, basic triphenylmethane etc. is used in textile dyeing processes and significant amount of these dyes enter into the environment along with wastewater (Ahmad, 2009; Raffi et al., 1997). Industries generating dye-containing wastewater include pigment manufacture, textile, printing, dyeing, leather, food and cosmetic industries (Ahmad, 2009; Amini et al., 2008). It is estimated that upto 15% of the total textile dyes used in dying process remains unreacted and are directly lost in the effluents and finally goes into the environment and causes serious environmental problems (Jadhave and Govindwar, 2006; O' Neill et al., 1999).

Thus, it becomes very essential to the wastewaters coming out from various industries should be treated adequately for environmental safety. Although a number of physico-chemical methods are reported effective for the removal of dyes from industrial wastewaters, but require high cost and generate large amount sludge, which acts as a secondary pollutants. On the other hand, biological treatment methods are regarded as cost-effective, simple structural set-up, easy to operate, diverse metabolic pathways, versatility of

microorganisms, wider application and environment friendly and also produce less amount of sludge compared to physico-chemical methods (Pandey et al., 2007; Mendez-Paz et al., 2005; Banat et al., 1996). Therefore, biological methods using bacteria and fungi have been successfully used for the removal of dye from textile wastewaters (Azmi et al., 1998). Thus, this study aims to isolate, screen and characterize the potential bacteria capable for the decolorization of CV dye.

2. Materials and Methods:

2.1 Collection of Dye Wastewater Sample: One of the most industrialized areas and known chemical hub in India is Kanpur and therefore, was chosen for effluent sample collection. Kanpur is located between 26.4670° North, 80.3500° East and covers the area of 3,029 km2 (1,170 m2). The effluent sample was collected from a handloom textile company from the Panki Industrial area of Kanpur city (site 3), India

2.2 Physico-Chemical Analysis of Dye Wastewater: The textile wastewater was collected from the outlet of a handloom industry located in Panki industrial area, Kanpur. The wastewater was collected in a pre-sterilized container (5 liter), brought to laboratory and maintained at 4 °C for further analysis. The parameters such as pH, temperature, color and odour of collected wastewater sample were recorded on the spot. The pH was determined using a pH meter (Merck) and temperature with a laboratory thermometer. The collected wastewater was also analyzed for the presence of crystal violet dye by spectrophotometer (Evolution 201, Australia) at 590 nm. Various other physico-chemical parameters analyzed in laboratory as per the standard methods are BOD, COD, TDS, TSS, EC, phosphate, nitrite, residual chlorine, carbonates, and sulphates (APHA, 2012).

2.3 Chemicals: The textile dye, Crystal violet was purchased from spectrochem, India. Minimal-salt-medium containing 5.22 g K2HPO4 l–1, 4.08 g KH2PO4 l–1, 0.2 g MgSO4 .7H2O l–1, 0.55 g CaCl2 l–1, 0.4 g NH4Cl l–1, agar 15g and supplemented with 100 mg/L of CV. A stock solution of the dye was prepared using de-ionized water and further used for all studies.

2.4 Isolation, Purification and Screening of CV Decolorizing Bacterial Strains:

2.4.1 Isolation and Purification of Bacterial Strains Capable for CV Decolorization: The bacterial colonies were isolated from textile dye effluent sample by serial dilution technique and purified by repeated streaking method on the same culture medium. The morphologically distinct bacterial colonies showing clear zones of dye decolorization around their colonies were selected for further studies.

2.4.2 Screening of CV Decolorizing Bacterial Strains: The isolated bacterial strains having the ability to decolorize CV dye were screened through primary and secondary screening test using modified Cheriaa and Bakhrouf (2009) method on solid MSM-agar-dye media method.

2.4.2.1 Primary Screening Test: The isolated bacterial strains were primarily screened by nutrient enrichment technique on the solid mineral salt medium amended with agar and different concentrations of CV dye. The plates were streaked with isolated bacterial strains and incubated at 37 °C for 48 hours. The bacterial strains that were found capable to grow and tolerate the highest concentration of CV dye were selected for the secondary screening test and the pure culture stocks were preserved on glycerol stock solutions at -20 °C.

2.4.2.2 Secondary Screening Test: The secondary screening test of the primarily screened bacterial strains was carried out at 37 °C by shaking culture method. Eight primarily screened bacterial strains were grown in 250 ml Erlenmeyer flasks containing 100 ml of autoclaved mineral salt medium amended with CV dye at 37 °C for six consecutive days under aerobic conditions. The bacterial strains were screened on the basis of their growth performance and decolorization potential for CV dye assessed spectrophotometrically (Evolution 201, United States) at 600 and 590 nm, respectively.

2.5 Assessment of CV Decolorizing Potential of Isolated Bacterial Strains: The CV decolorizing potential of the isolated bacterial strains were assessed in mineral salt medium amended with 100 ppm of CV dye.

2.5.1 Decolorization Study: The decolorization experiment was carried out in 250 ml Erlenmeyer flasks containing 100 ml of autoclaved MSM broth amended with 100 ppm CV dye and a loop full of overnight grown culture, which was incubated at 37 °C and 100 rpm. The experiment was performed in triplicate and flasks were monitored at regular time intervals by withdrawing samples and centrifuging it at 10,000 rpm for 20 min. The supernatant was used for determining the reduction in color. After completion of experiment, the bacterial culture was collected through centrifugation and the obtained pellet was inoculated in another flask containing media and dye and was further determined whether the cells adsorbed the dye or was degraded by it. A separate set of uninoculated flask was maintained in parallel as control.

The decolorization of CV was monitored in terms of bacterial growth and decrease in color intensity by measuring the absorbance at 600 nm for bacterial growth and at 590 nm for CV decolorization at regular time intervals of 50 minutes. The decolorization of CV was expressed on decrease in absorbance at 590 nm against the initial absorbance at the same wavelength (λmax) using the spectrophotometer (Evolution 201, United States). The percent decolorization was calculated by using the formula.

$$\% \text{ Decolorization} = \frac{\text{Initial OD} - \text{Final OD} \times 100}{\text{Initial OD}}$$

Where, OD = Optical Density at 590 nm

2.6 Morphological and Biochemical Characterization of the Isolated Bacterial Strains: The morphological and biochemical characterization of isolated CV decolorizing bacterial strains were done as per the

Bergey'smanual of determinative bacteriology and Cowan and Steel's manual for the identification of medical bacteria (Barrow and Feltham; 2009; Holt et al., 1994).

2.7 Statistical Analysis: All the experiments were performed in triplicates. For all experiments and determinations, data were entered in a Microsoft® Excel 2007 spreadsheet and means was calculated.

3. Results and Discussion:

3.1 Physico-Chemical Characteristics of Dye Wastewater: The wastewater collected from textile handloom, Kanpur, India was dark purple in color with sharp pungent and fishy smell, which will cause nuisance and decline the esthetic value of environment and receiving water resources. The pH of collected textile wastewater was noticeably below neutral level than that of NEQ standards. High pH reduces fish production as well as slows down the growth of aquatic macrophytes and vice-versa (Argo, 2003). The temperature of wastewater was also above the standards, which is harmful for aquatic life affecting dissolved oxygen concentration and also can persuade the activity of bacteria in water resources. The collected sample also showed higher EC, TDS and TSS than that of NEQS. High values of TDS and EC can cause osmotic stress at the plants root zone making it difficult to absorb water for its growth and thus causing lower crop production when used in irrigation (Mojiri, 2011).

The chemical oxygen demand (COD) and biological oxygen demand (BOD) values were also higher than the permissible limits (Table 1), which is very harmful to the aquatic life. Higher BOD causes bad taste to the drinking water (Singh et al., 1998). Phosphate range was 0.45 mg/l in the sample. Higher value of phosphate leads to eutrophication and oxygen depletion in water bodies and leads to osteoporosis and kidney damage in humans. Nitrite and sulphates content in collected textile wastewater was found to be lower than the standard values. The value of carbonates and residual chlorine of the sample was also higher indicating that the textile wastewater to be highly polluted and harmful.

3.2 Isolated, Purified and Screened CV Decolorizing Bacterial Strains:

3.2.1 Isolated Dye Decolorizing Bacteria: Initially, eight (SJ1-SJ8) bacterial strains were isolated from textile effluent sample collected from the textile effluent disposal site where the chances to get the potent CV decolorizing bacterial strains were maximum. The isolated bacterial strains were streaked successively for several times to get distinct and pure colonies. The bacterial strains were maintained at 4 °C and used for screening test.

3.2.2 Screened CV Decolorizing Bacterial Strains: In primary screening test of eight isolated bacterial strains performed on solid MSM amended with different concentrations of CV dye in plates, four (04) potential bacterial strains capable to tolerate the maximum concentration of CV dye were selected for secondary screening test. Out of which, two (02) bacterial strains were finally selected as the most potent CV decolorizing bacterial strains.

3.2.3 Biochemical Characteristics of Bacterial Strains: Two morphologically different strains SJ4 and SJ5 was isolated and screened for the decolorization of CV dye. The gram-staining test showed both the isolates to be motile, rod-shaped and gram-negative. Biochemical characterization of the isolates revealed it to be positive for indole, catalase, oxidase test and starch hydrolysis test and negative for Voges-Proskauer test, Citrate utilization and H2S production. The isolates individually showed both positive and negative results for methyl red test, Casein hydrolysis, sucrose, lactose and glucose fermentation, respectively. The various morphological and biochemical tests shown by SJ4 and SJ5 are shown in Table 2.

3.3 Assessment of CV Decolorizing Ability of the Screened Bacterial Strains: The potent bacterial strains selected for the decolorization of CV dye grow well in MSM-agar medium amended with 100 ppm CV dye while the growth of screened bacterial strains was suppressed to some extent. The growth pattern and decolorization potential of the isolated bacterial strains are shown in Fig 1.

Out of the two potent bacterial strains, strain SJ4 has shown much decolorization percentage i.e. 90% than strain SJ5 i.e. 82% within 8 hours of the incubation period. However, it was observed that strain SJ4 was more potent than strain SJ5. But, when these strains were mixed to perform on the same concentration, temperature and pH, these decolorized the crystal violet dye upto 97% within 6 hrs of incubation period. The CV decolorizing potential of each strain as well as their mixture was expressed as decrease in absorbance at 590 nm against the initial absorbance at the same wavelength using a spectrophotometer and the pellets obtained were observed to be non-colored indicating that the dye was degraded instead of adsorption by the bacterial cells. Parshetti et al., (2011) have completely decolorized the Crystal violet dye within 8 hr (10 mg/L) at anoxic conditions by using the Agrobacterium radiobacter MTCC 8161. Shah et al., (2013) have also reported the Crystal violet decolorization by Bacillus subtilis ETL-2211 upto 90% under static conditions within 24 hrs of incubation period. Moturi and Singara (2009) have worked on the decolorization of Crystal violet dye by white rot fungus Polyporuselegans, Trametesversicolor, Lenzitesbetulina and soil fungus Mucormucedo. They found maximum decolorization of 78% in 15 days of incubation time by Mucormucedo. Polyporuselegans, Trametesversicolor, Lenzitesbetulina showed 73%, 72 % and 75 % decolorization of crystal violet in 15 days of incubation period, respectively.

4. Conclusion:

The decolorization of industrial wastewaters is a challenging process to both the textile industry and the wastewater treatment. The result of this findings and literature suggest a great potential for bacteria to be used to remove color from dye wastewaters as compared to fungal decolorization. The isolated bacterial strains SJ4 and SJ5 showed the maximum decolorization of 90% and 82% within 8 hrs (100 mg/L) of incubation period at pH 8 and 37°C temperature, respectively. But, the mixture of strains SJ4 and SJ5 have shown the maximum decolorization of 97 % within 6 hrs (100 mg/L) of incubation period at pH 8 and 37 °C temperature. The overall findings also suggest that these two strains were capable of not only decolorizing but also degrading dye individually as well as in mixed conditions which may due to the involvement of enzymes. Therefore, this study concludes about the advancement of the mixed bacterial strains over individuals and thus, can be further applied for the treatment of dye containing industrial wastewaters.

5. Acknowledgement:

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6. Conflict of Interest:

The authors declare that there are no conflicts of interest associated with this work.

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Tables:

Table 1: Physico-chemical characteristics of textile wastewater

S.No	Parameters	Unit	Calculated Value	*NEQS
1.	Color	-	Dark purple	Colorless
2.	Odour	-	Pungent	No odour
3.	Temperature	°C	43.73	40
4.	pH	mg/l	6.35	6-9
5.	TDS	mg/l	3843.00	3500
6.	TSS	mg/l	850	100
7.	BOD	mg/l	364.00	30
8.	COD	mg/l	1562.00	250
9.	EC	ds/m	6.45	-
10.	Phosphate	mg/l	0.45	-
11.	Nitrite	mg/l	0.27	-
12.	Residual Chlorine	mg/l	3.34	1
13.	Carbonates	mg/l	4.3	-
14.	Sulphates	mg/l	0.32	2
15.	Bacterial count	cfu/ml	31×10^5	

Table 2: Morphological and biochemical characteristics of isolated bacterial strains

Characteristics	Bacterial strains	
	SJ4	SJ5
Morphological		
Gram Staining	Gram negative	Gram negative
Shape	Short rods with rounded ends	Rod with rounded ends
Color	Beige(slightly yellowish)	transparent
Surface Texture	Normal	Smooth
Margin	Even	Even
ElevationMotility	FlatMotile	ConvexMotile
Biochemical		
Catalase Test	+ve	+ve
Oxidase Test	+ve	+ve
Indole Test	+ve	+ve
Methyl Red Test	-ve	+ve
VogesProskauer	-ve	-ve
Citrate Utilization Test	-ve	-ve
Casein Hydrolysis	+ve	-ve
Glucose Fermentation	+ve	-ve
Sucrose Fermentation	-ve	+ve
Lactose Fermentation	+ve	+ve
Hydrogen Sulfide Test	-ve	-ve
Starch Hydrolysis	+ve	+ve

Figure:

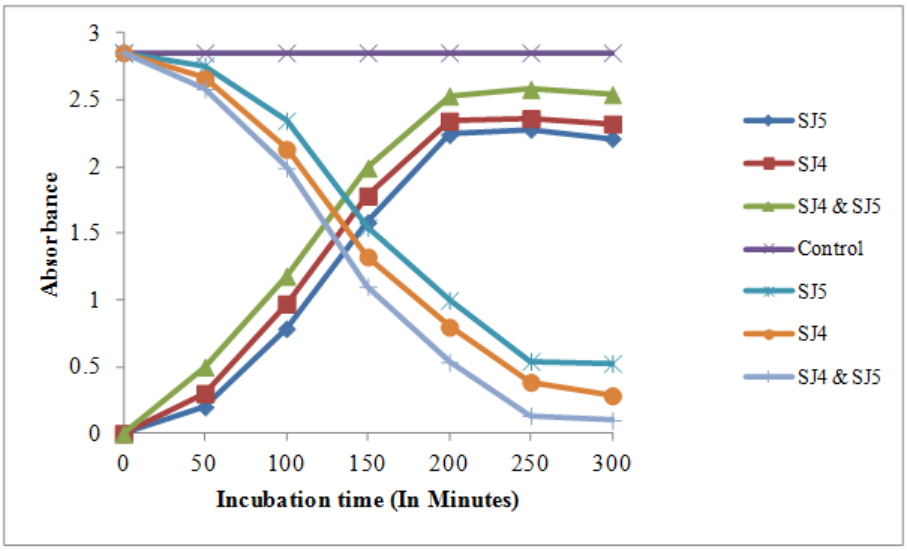

Figure 1: Growth pattern and decolorization of crystal violet dye by isolated bacterial strains SJ4 and SJ5 in axenic and mixed culture conditions.

Legands:

Table 1: Physico-chemical characteristics of textile wastewater

Table 2: Morphological and biochemical characteristics of isolated bacterial strains

Figure 1: Growth pattern and decolorization of crystal violet dye by isolated bacterial strains SJ4 and SJ5 in axenic and mixed culture conditions