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Original Research Article

Eco-Friendly Bioremediation of a Triphenylmethane Dye by Textile Effluent Adapted Bacterial Strain vp-64

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A B S T R A C T

Environmental biotechnology is constantly expanding its efforts in the biological treatment of colored textile effluents, which is an environmental friendly and low-cost alternative to physico-chemical decomposition processes. In the present study, effluent samples were collected from various textile and dyeing industries (S_1, S_2 and S_3) located in and around Arni, Tiruvannamalai District, Tamilnadu, India and were exploited for the screening and isolation of bacterial strains that were capable of decolorizing the textile dye, Crystal Violet. Physico-chemical properties of the effluent samples were analyzed. Six bacterial strains, CV-P1 – CV-P6 capable of decolorizing Crystal Violet were screened and isolated from various effluent samples. Out of which, CV-P3 isolate *(Enterobacter* sp. Strain VP-64) exhibited maximum decolorization efficiency of 90% within 24 h of incubation. FTIR spectrum of 24 h extracted metabolites showed significant change in the positions of peaks, when compared to control dye spectrum, indicating the biodegradation of Crystal Violet.

Introduction

Textile industry is providing one of the most basic needs of the people and maintains sustained growth for improving quality of life. The Indian textile industry has been undergoing a rapid transformation and is in the process of integrating with the World textile trade and industry. The contribution of textile industries to the Indian economy is manifested in terms of its contribution to the industrial production, employment generation and foreign exchange earnings. It contributes 20% of industrial production, 9% of excise collections, 18% of employment in the industrial sector, nearly 20% to the country's total export earning and 4% to the gross domestic product. The first synthetic dye, Mauevin was manufactured in the year 1856, since then, more than 1,00,000 new synthetic dyes have been generated (Asad *et al*., 2007). These dyes were used in different industries, with an annual consumption of about 0.7 million tons worldwide (Chen *et al*., 2003; Saratale *et al*., 2006). Synthetic dyes were extensively used in many fields of upto date technology, for example in various branches of the textile industry (Nigam *et al*., 1996), in leather tanning industry, in paper production (Ivanov *et al*., 1996), in food technology (Slampova *et al*., 2001), in agricultural research (Cook and Linden, 1997), in light harvesting arrays, and in photo-electrochemical cells (Wrobel *et al*., 2001). Moreover, synthetic dyes have been employed for the control of efficacy of sewage and wastewater treatment, for the determination of specific surface area of activated sludge for ground water tracing (Field *et al*., 1995).

The chemical classes of dyes employed more frequently on industrial scale are the azo, anthraquinone, sulfur, indigo, triphenylmethyl and phthalocyanine derivatives (Forgacs *et al*., 2004; Parshetti *et al*., 2006). However, it has to be emphasized that the overwhelming majority of the synthetic dyes currently used in the industry are azo derivatives (Chen *et al*., 2003). Considering both the volume generated and the effluent composition, the textile industry wastewater is rated as the most polluting source among all industrial sectors. Textile industry wastewater has been categorized into four types; (a) dispersible waste, (b) hard-to-treat waste, (c) high-volume waste, (d) hazardous and toxic wastes. The disposal of these wastes into receiving water causes damage to the environment. Dyes may significantly affect the photosynthetic activity in aquatic life because of reduced light penetration and may also be toxic to some aquatic life due to the presence of aromatics, metals, chlorides etc, (Daneshwar *et al*., 2007). In addition to their visual effect and adverse impact in terms of chemical oxygen demand (COD), many synthetic dyes show their toxic, carcinogenic and genotoxic effects (Pearce *et al*., 2003). Crystal Violet, a triphenylmethane dye that has been used extensively in human and veterinary medicine as biological stain has been shown to inhibit glutathione S-transferases from rat liver. Crystal Violet has also been suggested to be responsible for the promotion of tumor growth in some species of fish. It exhibited pronounced photo toxicity towards L1210 leukemia cells but comparatively small toxic effects toward normal hematopoietic cells (Indig *et al*., 2000).

Traditional wastewater treatment technologies have proven to be markedly ineffective for handling wastewater of synthetic textile dyes because of the chemical stability of these pollutants (Nigam *et al*., 1996). Government legislation is becoming more and more stringent, especially in the more developed/developing countries, regarding the removal of dyes from industrial effluents. Enforcement of this law will continue to ensure that textile and other dye utilizing industries treat their dye-containing effluent to the required standards (Robinson *et al.,* 2001). Implementation of different physico-chemical techniques including coagulation/ flocculation, membrane filtration, ultrasonic mineralization, precipitation, floatation, adsorption, ion exchange, ion pair extraction, electrolysis, advanced oxidation process (chlorination, bleaching, ozonation, Fenton's oxidation and photo catalytic oxidation) and chemical reduction have inherent drawbacks of being economically unfeasible (more energy consumption and chemical uses), unable to remove the recalcitrant azo dyes and/or their organic metabolites completely, generating a significant amount of sludge that may cause secondary pollution problems (Anjaneyulu *et al*., 2005; Vijayanand and Hemapriya., 2013). The microbial decolorization and degradation of synthetic dyes has been of considerable interest since it is inexpensive, eco-friendly and produces a less amount of sludge (Kalyani *et al*., 2009; Saratale *et al*., 2009). The effectiveness of microbial decolorization depends on the adaptability and the activity of selected microorganisms capable of degrading synthetic dyes. Although numerous microorganisms can decolorize dyes, only a few are able to mineralize these compounds into CO_2 and H_2O. Bacterial strains selected by adaptation from textile effluents have been shown to decolorize textile dyes (Saratale *et al*., 2009; Hemapriya *et al*., 2010).In view of the potential applications of biodecolorization processes in wastewater treatment, the present investigation emphasis on the eco-friendly approach - the bioremediation of Crystal Violet, a triphenylmethane dye by a textile effluent adapted bacterial strain under aerobic conditions.

Materials and Methods

Sampling Sites

The sampling area was the textile industries and dyeing units located in and around Arni, Tiruvannamalai District, Tamil Nadu, India. The effluent samples from both textile industries and dyeing units were characterized by its dark color and extreme turbidity.

Sample Collection

Sampling in this study took place during a transient period (Jan – Feb, 2014). Samples were collected at the surface and at various depths (0.1, 0.2, 0.3M) from each site and were placed in sterile polythene bags to prevent direct contact with air and transported to the laboratory in an ice box for further physico-chemical analysis, dye decolorization and degradation assays. The Physico-chemical properties of the effluent samples such as TS, TDS, TSS, BOD, COD, pH and color were analyzed (APHA, 1980).

Triphenylmethane Dye Used

The commonly used textile dye, Crystal Violet used in this study was procured from a local textile dyeing unit. Stock solution was prepared by dissolving 1 g of Crystal Violet in 100 ml distilled water. The dye solution was sterilized by membrane filtration (Millipore Millex ® - GS, 0.22 Mm filter unit), since dyes may be unstable to moist-heat sterilization. All the chemicals used in this study were of the highest purity available and of an analytical grade.

Isolation and Screening of Bacterial Strains Decolorizing Crystal Violet

Approximately 100 effluent samples were collected from the three different sites (S_1, S_2, and S3). The samples were serially diluted and spread over basal nutrient agar medium (composition gl^{-1}: peptone, 5.0; beef extract, 3.0; NaCl, 5.0) containing 50 ppm of Crystal Violet. pH was adjusted to 7.0 before autoclaving and incubated at 37°C for 5 days. Colonies surrounded by halo (decolorized) zones were picked and streaked on nutrient agar plates containing Crystal Violet (Hemapriya *et al*., 2013). The plates were re-incubated at 37°C for 3 days to confirm their abilities to decolorize Crystal Violet. Different colonies of dye decolorizing bacteria were picked and re-streaked several times to obtain pure cultures. The pure cultures were maintained on dye-containing nutrient agar slants at 4°C.

Decolorization Assay

A loopful of bacterial culture was inoculated in Erlenmeyer flask containing 100 ml of nutrient broth and incubated at 150 rpm at 37°C for 24 h. Then, 1 ml of 24 h old culture of CV-P3 strain was inoculated in 100 ml of nutrient broth containing 50 ppm of Crystal

Violet and re-incubated at 37°C till complete decolorization occurs. Suitable control without any inoculum was also run along with experimental flasks. 1.0 ml of sample was withdrawn every 12 h and centrifuged at 10,000 rpm for 15 min. Decolorization extent was determined by measuring the absorbance of the culture supernatant at 590 nm using UV-visible spectrophotometer (Hitachi U 2800), according to Hemapriya *et al*. (2010).

Decolorization efficiency (%) = Dye (i) - Dye (r) / Dye (i) × 100

Where, Dye (i) refers to the initial dye concentration and
Dye (r) refers to the residual dye concentration.

Bacterial Strain and Culture Conditions

Bacterial strain that showed maximum decolorization percentage on Crystal Violet was aerobically cultured in nutrient broth containing 50 ppm of Crystal Violet. The pH was adjusted to 7.0. For frequent use, the culture was maintained by transfer to a fresh medium at 24 h intervals. When required for prolonged periods, it was maintained by sub-culturing once every 7 days on slants, prepared by solidifying the above mentioned medium with 2.0 (w/v) agar.

Analysis of Biodegraded samples by FTIR

The biodegraded Crystal Violet was characterized by Fourier Transform Infra Red (FTIR) spectroscopy (Perkin-Elmer, Spectrum one). The analysis results were compared with the control dye. The FTIR analysis was done in the mid IR region (400-4000 cm^{-1}) with 16 scan speed. The samples were mixed with spectroscopically pure KBr in the ratio (5:95). The pellets were fixed in sample holder and then analyzed (Saratale *et al*., 2009).

Results and Discussion

Increasing industrialization and urbanization results in the discharge of waste to the environment, which in turn creates more pollution. The discharge of toxic effluents from various industries adversely affects the water resources, soil fertility, aquatic organisms and ecosystem integrity (Puvaneswari *et al.,* 2006). The textile industry is one of the greatest generators of liquid effluent pollutants; Improper textile dye disposal in aqueous ecosystems leads to the reduction in sunlight penetration and depicts acute toxic effects on aquatic flora and fauna, causing severe environmental problems worldwide (Vandevivere *et al*., 1998). The microbial decolorization and degradation of textile dyes has been of considerable interest since it is inexpensive, eco-friendly and produces a less amount of sludge (Saratale *et al*., 2009).

Dye Stuff Used

The dye stuff used in this study was Crystal Violet with color index number 23850 (www.sigmaaldrich.com). The absorption maximum of this dye was 590 nm. They are widely used in textile, leather and printing industries. The structure of Crystal Violet is shown below

Physico-Chemical Analysis of Effluent Samples

The average temperature at the sampling sites was around 35°C at day time. The physico-chemical characteristics of the effluent samples were shown in the Table 1. The pH values of the effluent samples were found to be alkaline. Total solids of S_2 and S_3 samples were found to be lower than the S_1 sample. The highest TSS content was encountered in S_3 sample. TDS content was almost same in both S_1 and S_3 samples.

BOD value of S_1 sample was found to be higher than the S_2 and S_3 samples. However, the COD value was maximum in case of S_2 sample. The effluent samples collected from S_1, S_2 and S_3 sites were found to be dark blue, blackish blue and dark brown respectively.

Isolation and Screening of Bacterial Strains Decolorizing Crystal Violet

The results shown in Table 2 revealed that 6 bacterial isolates, designated as CV-P1 to CV-P6 were found to be effective in decolorizing Crystal Violet. Morphological and the cultural characteristics of the isolates were tabulated in Table 2. They were isolated from 3 different locations (S_1, S_2, and S_3). Out of the 6 bacterial isolates that showed more than 50% decolorization ability on Crystal Violet, CV-P3 was found to be the superior strain with the highest decolorization efficiency of about 90% and was selected for further studies. Based on the 16S r DNA analysis, the isolate was identified as *Enterobacter* sp. Strain VP-64.

Effect of Incubation Time

Incubation time played a substantial role in maximizing the decolorization of Crystal Violet by *Enterobacter* sp. Strain VP-64. Dye decolorization by the isolate was found to be growth dependent, since considerable dye decolorization was noticed in the fermentation broth as soon as the bacteria entered the late exponential phase (~16 h) and the activity reached the maximum level in stationary phase (~24 h) as shown in Fig.1. Hence the optimum cultural conditions for elevating bacterial biomass and dye decolorization in shake flasks were carried out after 24 h of incubation. In contrast, *Citrobacter* sp. CK_3 decolorized an azo dye only after 120 h of incubation (Wang *et al*., 2009) and *Galactomyces geotrichum* MTCC 1360 showed optimum decolorization within 18 h of incubation (Jadhav *et al*., 2009).

Effect of Dye Concentration

The influence of different dye concentrations (200-1000 ppm) were investigated on decolorization ability of CV-P3. The result showed in Fig.2 has revealed that the decolorization rate was maximum at 200 ppm. As the dye concentration increased in the culture medium, a decline in color removal was attained. At high concentration (1000 ppm), Crystal Violet greatly suppressed the decolorization ability of the isolate CV-P3. Reduction in the decolorization rates might be attributed to the toxicity of dye to bacterial cells through the inhibition of metabolic activity (Sumathi and Manju, 2000), saturation of the bacterial cells with dye products (Sponza and Isik, 2004), inactivation of transport system of the dye or the blockage of active sites of azoreductase enzymes by the dye molecules (Vijaykumar *et al*., 2007). It has also been reported that dyes are inhibitors of the nucleic acid synthesis (Chen *et al*., 2003; Asad *et al*., 2007).

Analysis of Biodegraded samples by FTIR FTIR Analysis of Decolorized Sample

Decolorization of dyes may take place either by adsorption (Aravindhan *et al*., 2007) or degradation (Kumar *et al*., 2007). In the case of adsorption, dyes are only adsorbed onto the surface of bacterial cells, whereas new compounds come into being when dyes are degraded by bacterial enzymes during the degradation process (Wang *et al*., 2009). To disclose the possible mechanism of dye decolorization, the products of biotransformation were analyzed by FTIR. Comparison of FTIR spectrum of control dye with extracted metabolites after complete decolorization clearly indicated the biodegradation of Crystal Violet by Cv-P3 isolate .Peaks in the control dye spectrum represented the deformation of C-H at 675 cm^{-1}, CH stretching at 2949.16 cm^{-1}, 2922.16 cm-1 and 2864.29 cm^{-1}. The stretching vibrations at 1112.93 cm^{-1}, 1049.28 cm^{-1} and 1031.92 cm^{-1} showed C-O stretching at 1643.35 cm^{-1} it showed N-H bending (Fig.3.6). The FTIR spectrum of CV-P3 isolate extracted metabolites showed significant change in position of peaks when compared to control dye spectrum. A new peak at 3238 cm^{-1} showing O-H stretch, 1641.42 cm^{-1} representing N-H bending. The stretching vibrations at 1400.32 cm^{-1} and 1111.00 cm^{-1} showed C-C stretching and C-N stretching respectively (Fig.3 and 4).

Fig.1 Decolorization of crystal violet by CV-P3 isolate

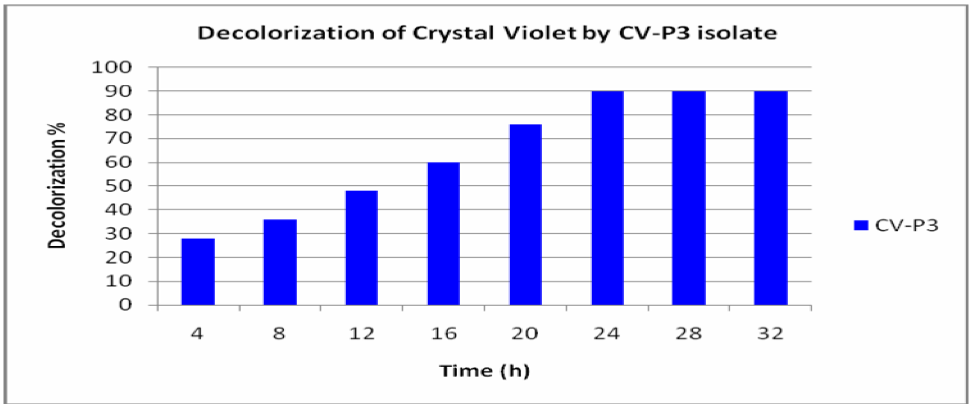

Fig.2 Effect of dye concentration on crystal violet decolorization

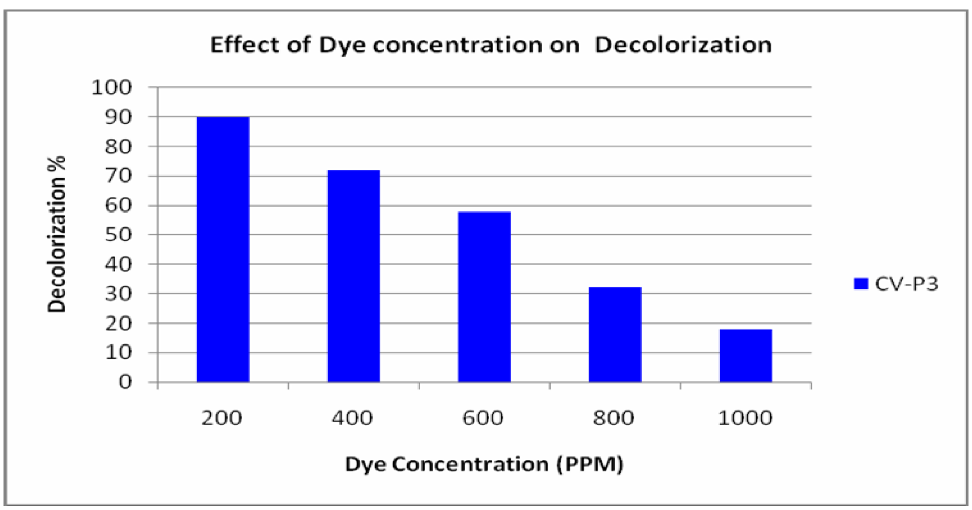

Fig.3 FT-IR spectrum of control (Parental Crystal Violet)

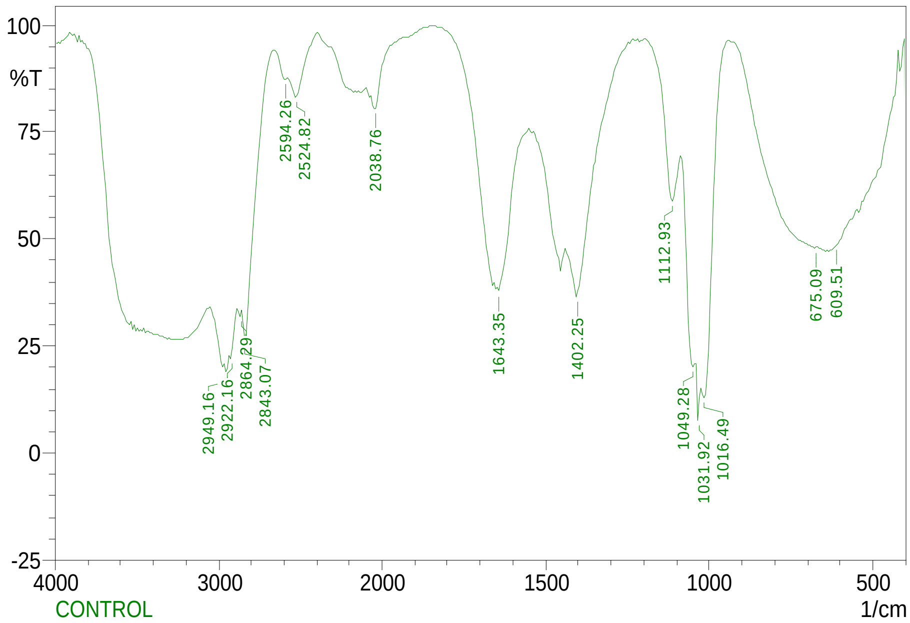

Fig.4 FT-IR spectrum of decolorized crystal violet

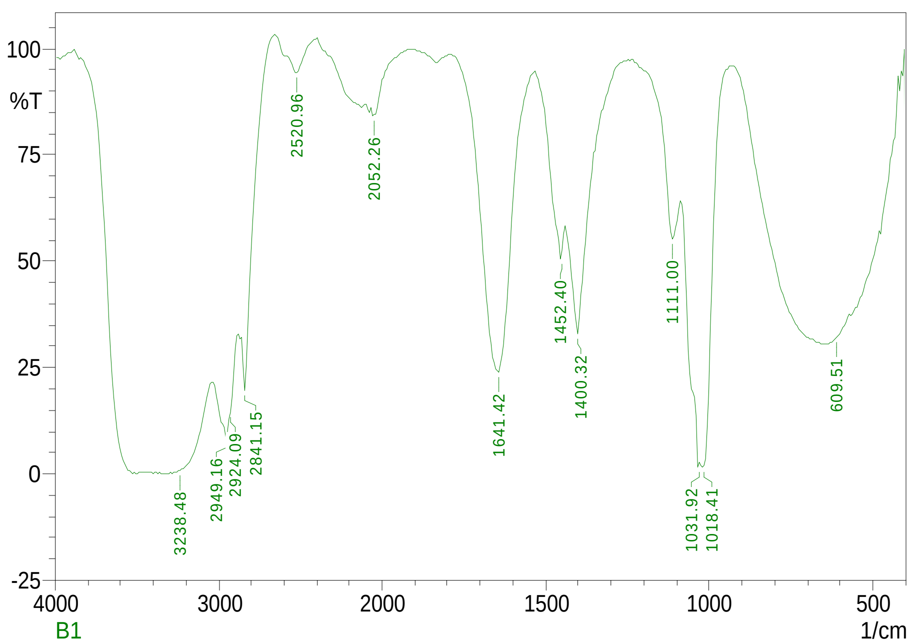

Table.1 Physico-chemical characteristics of textile effluent

Sl. No	Parameters	Tap Water	S_1 Site	S_2 Site	S_3 Site
1	Total Dissolved Solids (mg/l)	500	1768	1517	1712
2	Total Suspended Solids(mg/l)	80	624	629	894
3	Chemical Oxygen Demand (mg/l)	6	1246	1772	1368
4	Biological Oxygen Demand (mg/l)	5	310	287	296
5	pH	7.2	8.5	9.0	10.0
6	Color	Colorless	Dark Blue	Blackish Blue	Dark Brown

Table.2 Decolorization Efficiency of the isolates (CV-P1 to P6)

Sl. No	Isolates	Sample Collection Site	Time taken for Maximum Decolorization	Decolorization Efficiency
1	CV-P1	S1	60 h	64.66 %
2	CV-P2	S3	48 h	60.64 %
3	CV-P3	S1	24h	90.00 %
4	CV-P4	S2	48 h	53.13 %
5	CV-P5	S3	60 h	51.13 %
6	CV-P6	S2	36 h	52.71 %

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