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A STUDY ON BACTERIAL DECOLORIZATION OF CRYSTAL VIOLET DYE BY *CLOSTRIDIUM PERFRINGENS, PSEUDOMONAS AERUGINOSA AND PROTEUS VULGARIS*

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ABSTRACT

In the present study bacterial decolorization of Crystal violet dye is reported. The three bacterial species were tested for their decolorization abilities towards Crystal violet at increasing concentrations. The studies of decolorization were performed by maintaining batch cultures of the pure cultures of bacterial species i.e., Pseudomonas aeruginosa, Clostridium perfringens and Proteus vulgaris in organic and inorganic media. The percent of decolorization in general is good in the presence of organic compounds, so in comparison the work was done in the presence of inorganic salts. Pseudomonas aeruginosa is very efficient in decolorization of Crystal violet dye both in organic and inorganic media.

KEY WORDS

Bacterial Species, Crystal violet, Decolorization, Organic, Inorganic.

1. INTRODUCTION

Worldwide over 10,000 different dyes and pigments are used in dyeing and printing industries. The total world colorant production is estimated to be 8, 00,000 tons per year and at least 10% of the used dyestuff enters the environment through wastes [1].Industrial waste water effluent that contains dye is a life threatening problem [2]. Synthetic dyes are chemically diverse, divided into azo, triphenylmethane or heterocyclic and polymeric structures [3]. The triphenylmethane dye crystal violet has seen extensive use in human and veterinary medicine as a biological stain and as a textile dye [4,5,6,7,8,9].Unfortunately, waste water treatment facilities are often unable to completely remove commercial dyestuffs, including triphenylmethane dyes such as crystal violet, from contaminated waste water, thus contributing to the pollution of aqueous habitats [10,11,12,13]. Also triphenylmethane dyes have been found in soil and river sediment as a consequence of improper chemical waste disposal [14]. Primary studies of the biodegradation of triphenylmethane dyes are through decolorization of dyes via reduction reactions. Although several triphenylmethane dyes decolorizing bacteria have been isolated, many other micro-organisms including bacteria and fungi can be employed [15].

In the present study, pure cultures of three species of bacteria were tested for decolorization activity under aerobic conditions in organic and inorganic media at different Dye concentrations and different pH ranging from 2.2-8.0 is reported.

2. MATERIALS AND METHODS

Cultures: Three Bacterial strains (Pseudomonas aeruginosa, Clostridium perfringens and Proteus vulgaris) were obtained from Department of Microbiology Osmania University.

Dye: The Azo dye used was Crystal violet certified stain supplied by sd FiNE-CHEM Limited. It is

commonly used in textile processing and paper industry. The dye is used as a histological stain and in Gram's Method of classifying bacteria. The dye has absorption maxima of 620-670nm with the following chemical structure.

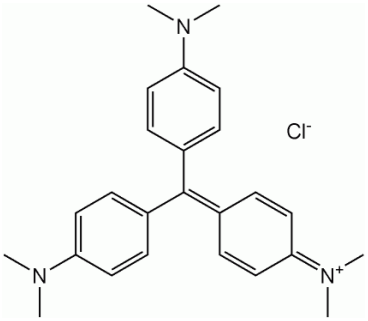


Figure 1: Chemical structure of Crystal violet dye.

2.1 Decolorization in different media's

2.1.1 Media's used

Nutrient Media (Nutrient Agar (N.A)/Nutrient Broth (N.B)): Broth /Agar per liter (Agar-15 gms, Beef Extract-3 gms, Peptone-5 gms, pH-7.2)[16].

Mineral Salt Media (MSM):Broth/Agar per liter(Agar-15 gms, $(NH_4)_2SO_4$-0.5 g/l, KH_2PO_4-2.66 g/l, Na_2HPO_4-4.32 g/l, Glucose 1 g/l) [17].

To the above media's the Crystal violet Dye was added in various concentration and volumes mentioned in Table 1.

Table 1 - To the above media's the Crystal violet Dye was added in various concentration and volumes as shown below.

Dye Concentration	1.25PPM	2.50PPM	3.75PPM	5.00PPM
Dye Volume	0.25ML	0.50ML	0.75ML	1.00ML

For the detection and measurement of decolorization methods used were Plate assay and Broth assay was done.

2.1.2 Plate assay

Nutrient Agar and Mineral Salt Media Agar plates were prepared and all three cultures individually inoculated by primary streaking at the centre of the plate. The plates were incubated for 24 hrs at room temperature in presence of Light to detect the decolorization ability of the isolates.

2.1.3 Broth assay

For the detection of decolorization in Nutrient Broth and Mineral Salt Media Broth 50 ml media taken in 250 ml Erlenmeyer flask in triplicates and 1 ml of 24 hrs culture (3.0×10^9 CFU/ml) was inoculated in liquid medium. The cultures of Pseudomonas aeruginosa, Clostridium perfringens and Proteus vulgaris were incubated for 24 hrs,48 hrs,72 hrs,96 hrs,120 hrs with 2.50 PPM Dye Concentration. In dark and light. The cultures of Proteus vulgaris were also incubated at 24 hrs but with different Dye Concentrations i.e., 1.25 PPM, 2.50 PPM, 3.75 PPM, 5.00 PPM. In light.

2.2 Decolorization at different pH

The decolorization of Crystal violet dye at a Standard 1 mg/ ml concentration was also detected at different pH using Phosphate-Citrate buffer (0.2M solution of $Na_2HPO_4.2H_2O$ + 0.1M Citric Acid Solution) at pH range of 2.2-8.0 [16] by taking 2 ml buffer (different pH + 0.5 ml dye and 2 ml distilled water) O.D values taken at 670 nm but no significant difference detected.

2.3 Colour reduction measurement

Decolorization of dye in agar plates was observed surrounding the bacterial colonies. Compared with the original dye medium as a control for liquid medium, the triplicated samples of the culture broth were analyzed at 670 nm using the supernatant from the culture medium after centrifugation at 8000 rpm in cooling centrifuge for 10 minutes. The culture broths were collected for decolorization measurement after 24 hrs. Colour removal was reported as percent decolorization and expressed as

Decolorization (%) = $(A_0 - A_t) \times 100 / A_o$

Where A_O and A_t are absorbency of the dye solution initially and at time (t) respectively.

3. RESULTS

3.1 Plate assay

The Pure cultures of the isolates were inoculated on the Nutrient Agar plates with different volumes of dye (1 mg / ml concentration) i.e., 0.25 ml, 0.50 ml, 0.75 ml, 1.00 ml. Plates were incubated at temperatures in

light and dark cycle for 24hrs.The Agar assay was done to detect whether the cultures were able to decolorize the dye or not. After incubation, zone of clearance around the colonies was measured, and it was found that after every 24 hrs there was 1-1.5 cm clearance around the colonies in the Nutrient Agar plates kept in light. No zone of clearance was observed in MSM plates kept in dark.

3.2 Broth Assay

The liquid cultures after every 24hrs were collected centrifuged and optical density was analyzed. The Decolorization % was calculated using O.D Values and the graphs were plotted.

Figure 2 – In Nutrient Broth under light and dark state cycle for 24 hrs, with 50 ml Nutrient Broth + 0.5 ml Dye+ 1 ml culture (Biotic conditions).

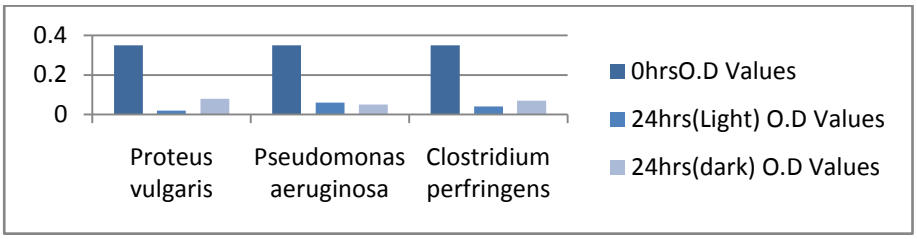


Figure 3 –In Nutrient Broth under light and dark state cycle for 24 hrs with 50 ml Nutrient Broth + 0.5 ml Dye (Abiotic conditions).

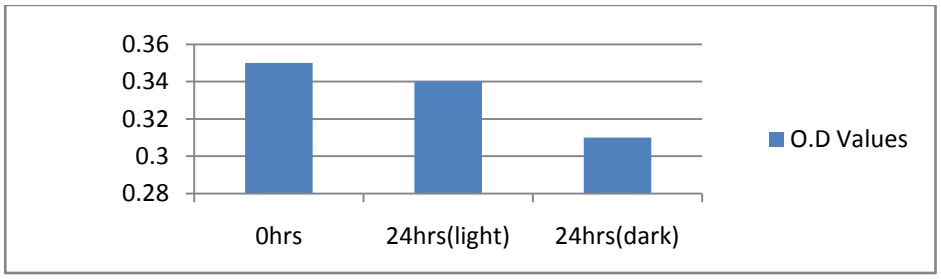


Figure 4 –In Mineral Salt Medium (MSM) under light and dark state cycle for 24 hrs with 50 ml MSM Broth + 0.5 ml Dye+ 1 ml culture (Biotic conditions).

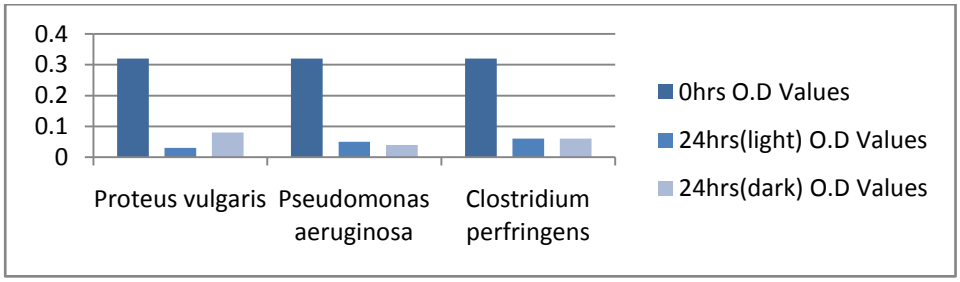


Figure 5 –In Mineral Salt Medium (MSM) under light and dark state cycle for 24 hrs with 50 ml MSM Broth + 0.5 ml Dye (Abiotic conditions).

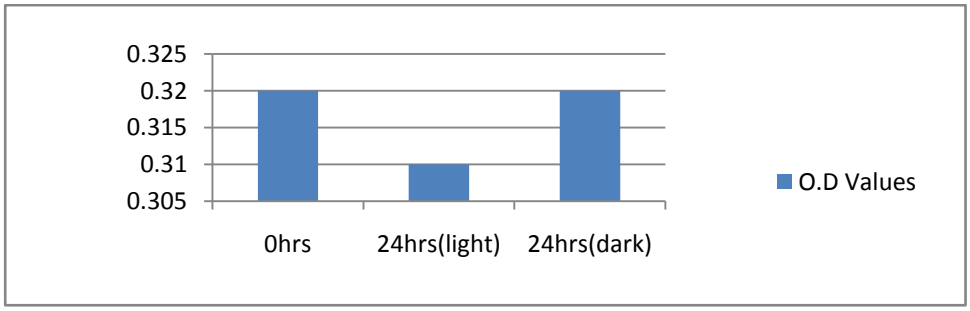


Figure 6 –In Nutrient Broth under light and dark state cycle with 50 ml Nutrient Broth + 0.5 ml Dye+ 1 ml culture (Biotic conditions).At 24 hrs, 48 hrs, 96 hrs, 144 hrs, 168 hrs of Proteus vulgaris.

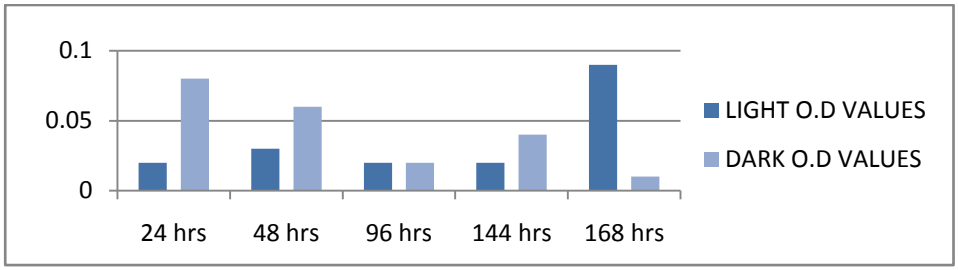


Figure 7 –In Nutrient Broth under light and dark state cycle with 50 ml Nutrient Broth + 0.5 ml Dye+ 1 ml culture (Biotic conditions).At 24 hrs, 48 hrs, 96 hrs, 144 hrs, 168 hrs of Pseudomonas aeruginosa.

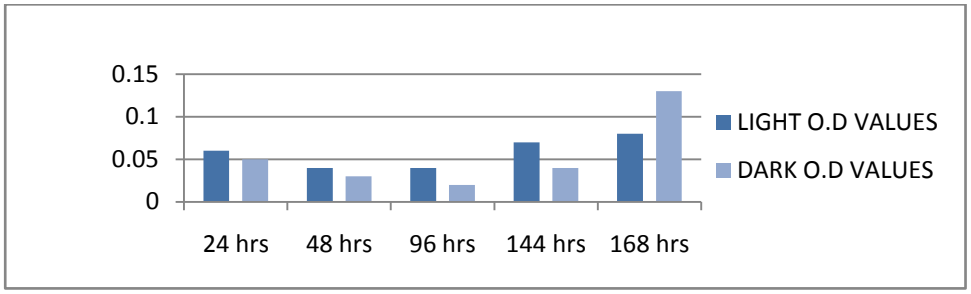


Figure 8 –In Nutrient Broth under light and dark state cycle with 50 ml Nutrient Broth + 0.5 ml Dye+ 1 ml culture (Biotic conditions).At 24 hrs, 48 hrs, 96 hrs, 144 hrs, 168 hrs of Clostridium perfringens.

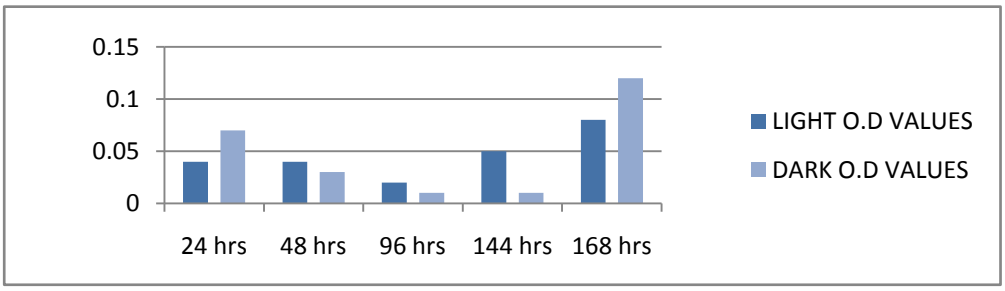


Figure 9 –In MSM Broth under light and dark state cycle for 24 hrs, with 50 ml Nutrient Broth + 0.5 ml Dye + 1 ml culture. (Biotic conditions). At 24 hrs, 48 hrs, 96 hrs, 144 hrs, 168 hrs of Proteus vulgaris.

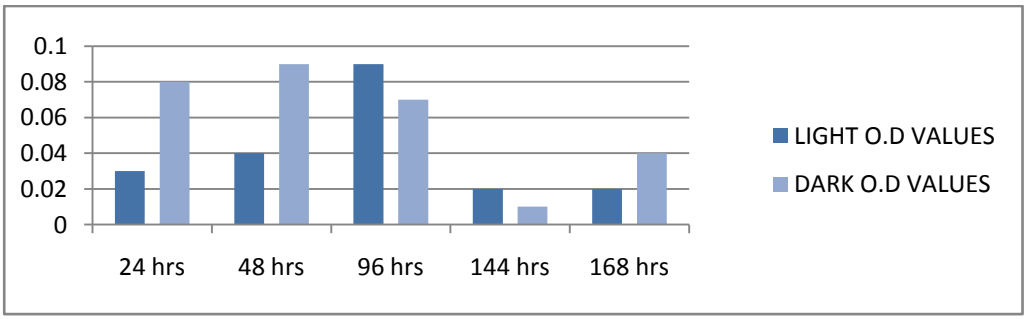


Figure 10 –In MSM Broth under light and dark state cycle for 24 hrs, with 50 ml Nutrient Broth + 0.5 ml Dye + 1 ml culture. (Biotic conditions). At 24 hrs, 48 hrs, 96 hrs, 144 hrs, 168 hrs of Pseudomonas aeruginosa.

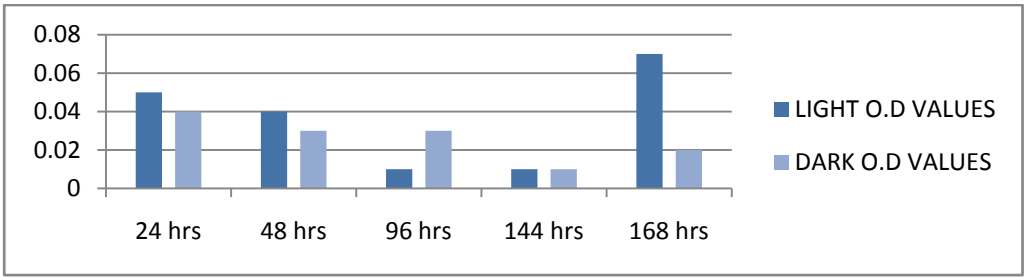


Figure 11 –In MSM Broth under light and dark state cycle for 24 hrs, with 50 ml Nutrient Broth + 0.5 ml Dye + 1 ml culture. (Biotic conditions). At 24 hrs, 48 hrs, 96 hrs, 144 hrs, 168 hrs of Clostridium perfringens.

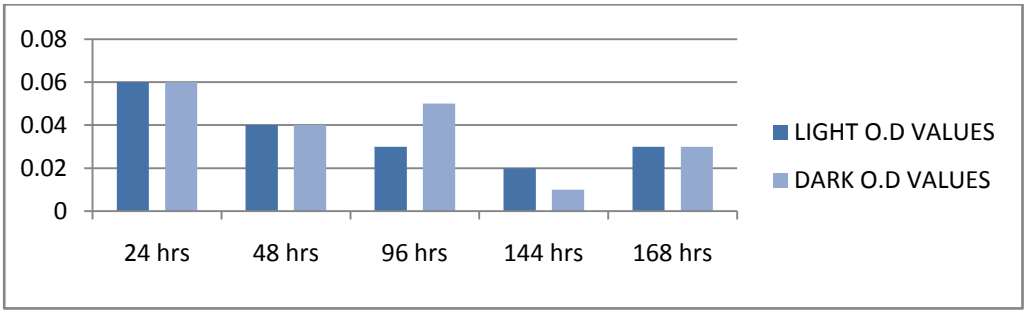


Figure 12 –In MSM Broth under light state. MSM Broth 50 ml + Different Dye Concentrations + 1 ml Culture. (Light cycle for 24 hrs) of Proteus vulgaris.

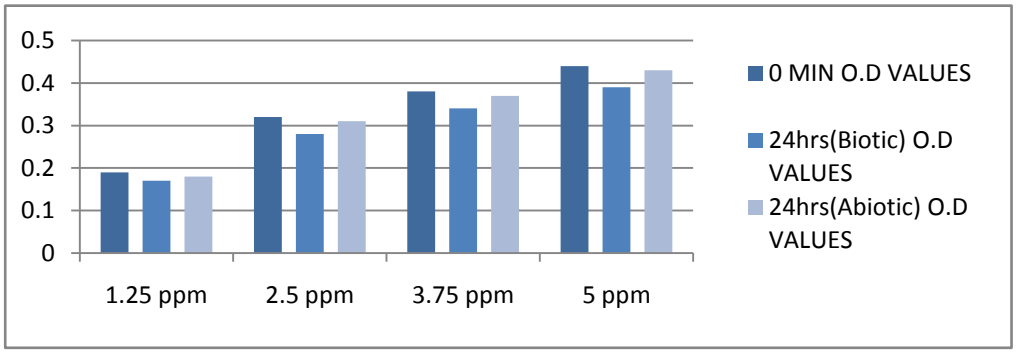


Figure 13 –In Nutrient Broth under light state. Nutrient Broth 50 ml + Different dye Concentrations +1 ml Culture. (Light cycle for 24 hrs)

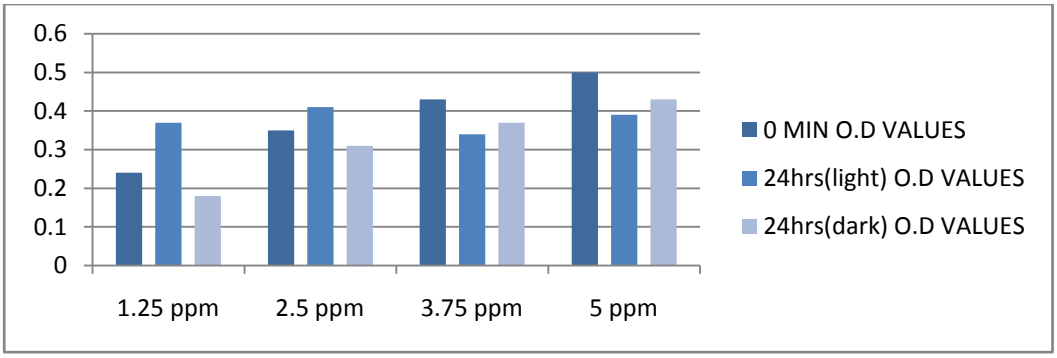
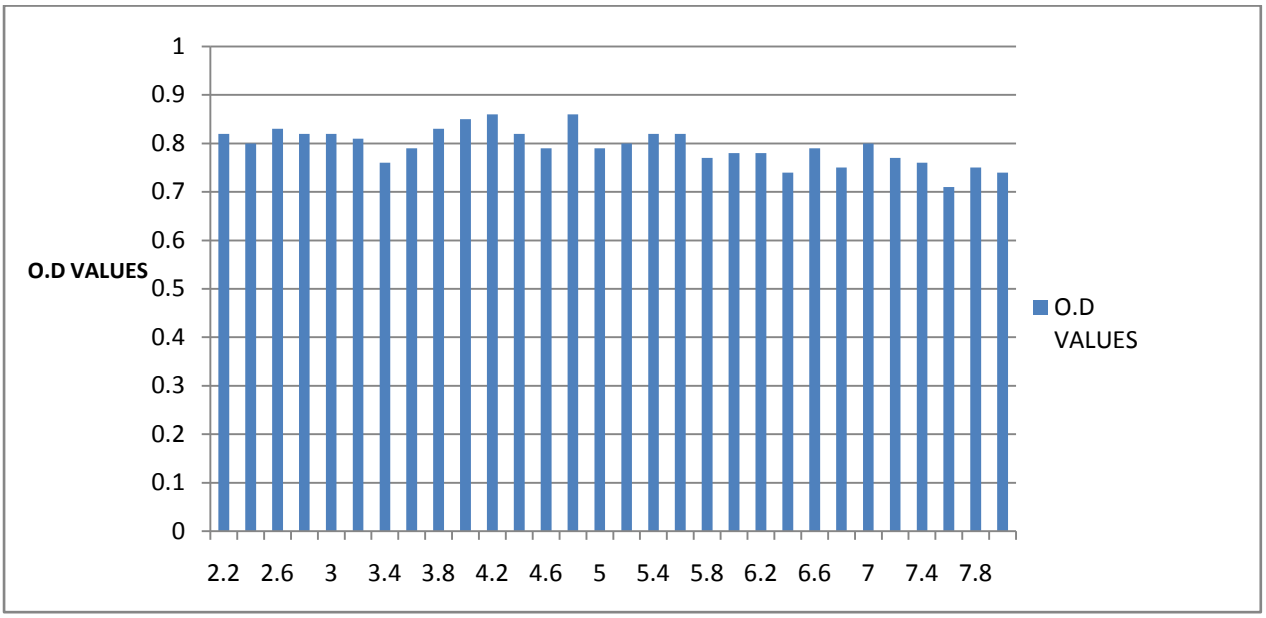


Figure 14 -Decolorization at different pH:



4. DISCUSSION

From the forgoing results of decolorization it may be interpreted that the 3 isolates under study are able to decolorize the Crystal violet dye in Mineral Salt Media (with only inorganic salts) also. Of the three cultures used for the studies, Pseudomonas aeruginosa was found to be more efficient (figure 10), as it is able to decolorize the dye in dark (i.e., 85% in N.B and 87 % in MSM broth), and can withstand a high concentration of 5 PPM, which has not been reported to date. Proteus vulgaris, though able to decolorize Crystal violet with a DC % of 94% - N.B (figure 13) and 90.6% - MSM (figure 12), but it is in presence of light, which means along with the microbial agencies even the physical agent i.e., UV radiation's are responsible for decolorization of the dye.

Both mixed and pure cultures of bacteria have been shown to be able to aerobically degrade and detoxify aromatic amines produced by anaerobic decolorization of Azo dyes [18]. Bacterial decolorization is associated with involvement of various enzymes such as lignin peroxidase, laccase, azoreductase and biotransformation enzymes [19, 20]. Interestingly in recent years several fungi have been reported to posses decolorization potential [21, 22]. [23]El-Naggar et al. (2004) reported the biodegradation of Crystal violet using air bubble bioreactor packed with Pseudomonas aeruginosa. However, most of the studies were carried out with Crystal violet and a few researches have reported the degradation mechanisms or pathways underlying the decolorization of the triphenylmethane dye, with some authors reporting as due to enzyme actions as mentioned above[24].

A number of studies on the degradation of Azo dyes by bacteria and fungi have indicated the involvement of extracellular oxidative enzymes such as, tyrosinase, lignin and manganese peroxidases and laccases [25, 26, 27, 28]. Phenol oxidases, which can be divided into tyrosinases and laccases, are oxido-reductases that can catalyze the oxidation of phenolic and other aromatic compounds without the use of cofactors (L-DOPA and Copper respectively). In cultures which have shown the activity of these enzymes during dye degradation it has been reported that the dye structures can be cleaved symmetrically and asymmetrically [29, 30].

Due to the recalcitrance of Azo dyes to strictly aerobic conditions and the production and accumulation of environmentally deleterious amines under anaerobic conditions a combination of anaerobic decolorization followed by aerobic degradation of the amines to non-toxic products is considered viable biological treatment scheme [31, 32, 33]. Moreover, the high salt content and extremes of pH associated with textile dye effluent have been shown to inhibit Azo

dye degradation by waste water microbial communities indicating that tolerance to these conditions as an important parameter for efficient biotransformation of Azo dye waste streams [34, 35]. [36]Parshetti G.K et al. (2011), reported possible mechanism of the dye decolorization, also analyzed the products of biotransformation of Crystal violet by GC-MS analysis. Five intermediates N,N,N',N" -tetramethylpararosaniline, [N; N-dimethylaminophenyl] [N-methylaminophenyl] benzophenone, 4-methyl amino phenol, N; N-dimethylaminobenzaldehyde, and phenol with a molecular mass of 345, 253, 124, 150 and 95, were deduced with the corresponding mass spectrum at GC retention times of 25.49, 22.34, 19.10, 11.67 and 7.32 min respectively.

5. CONCLUSION

In the light of the above discussion, it is obvious that, Azo dyes rapidly decolorize under inorganic conditions, in view of this, suitable strains of microorganisms have to be identified and characterized to identify the ideal conditions for effective biological treatment of Azo dyes. Further work in this direction is in progress.

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