CHAPTER 1

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1.1 INTRODUCTION

There are numerous environmental problems due to the xenobiotic compounds that resist mineralization in the conventional biological waste water system. First synthetic dye was reported in 1856. There are more than 40,000 dyes and pigments with some 7000 different chemical structures, out of which more than 3500 dyes are of practical use. Consumer of dyes are textile, tannery, paper and pulp, paint, electroplating and leather processing industries. Dyes are also used as additives in petroleum products. Based on the chemical structure of the chromophoric groups, the synthetic dyes are classified as azo dyes, nitroso dyes, triphenylmethane dyes, xanthane dyes and anthraquinone dyes (1).

Triphenylmethane dyes (ethyl violet, methyl green, crystal violet, malachite green, brilliant green, methylrosaniline, basic fuchsin, victoria blue etc.) are extensively used in textile industries. The terrible growth in textile dyeing and dye-stuff manufacturing industries have resulted in an increase in the volume and complexity in the wastewater discharge to the environment. In India around 30,000 tons of dyes are produced annually which is around 5% of the world production. It is estimated that 10-15% of the dyes are lost in the effluent during dyeing process. The pollution by dye wastewater is becoming increasingly serious. Some of the triphenylmethane dyes are used as dermatological agents, the best among them is gentian violet which is a mutagen, a mitotic poison and clastogen. It has also been used for oral consumption for pinworms and topical application in human and in domestic animals and has been shown to be effective in controlling fungal growth under varying conditions (2-3). The use of color is indispensable in the human life. Therefore, discharge of the effluent containing triphenylmethane dyes in the ecosystem is unavoidable. Thus human
population is directly or indirectly exposed to dyes through their commercial use. So, there is a need to search a suitable technology applicable practically in degrading chromogenic dyes at a large scale and to find out the routes of their conversion into some beneficial products before these are discharged into the aquatic or terrestrial ecosystem.

Dyes have low biodegradability and greatly influence natural ecosystem because of their toxicity and carcinogenicity. To depollute the dye wastewater, methods include adsorption, chemical precipitation and flocculation, oxidation via chlorine, peroxide, electrolysis and ozone treatment, reduction, electrochemical treatment and ion pair extraction have been employed, but none of them have been found to be very suitable as they produce a huge amount of sludge. Strict regulations that control the discharge of colored effluent from dye house are increasing the use of color removal technologies and leading to the development of new effluent treatment system. Conventional color removal techniques comprise of four primary categories: activated charcoal, membrane technology, ozone treatment and coagulation/flocculation. These techniques possess significant differences in color removal results, volume capability, operating speeds and capital costs. Activated charcoal, for example, is extremely effective for removing color, but is capable of treating only small effluent volume, operates at slow speeds and has high capital costs. Membrane technology, ozone treatment and coagulation/flocculation permit good color removal in large effluent volume. Membrane technology is fast, but the capital costs for implementing this technology are high. Ozone treatment operates at moderate speed and also require a high capital investment. Coagulation/flocculation techniques operate at moderate to fast speed and require a somewhat lower capital investment. New color removal technologies include the use of biological color absorbers, inorganic absorbers, and system based on
electrolysis. The biological methods have many advantages over chemical or physical methods such as possibility of degradation of dye molecules to carbon dioxide and water, formation of less sludge in addition to being environment friendly. Keeping the above facts in mind the present study was envisaged. The objectives of the present study are (a) screening for the organisms which can degrade/decolorize triphenylmethane group of dyes (b) optimization of physico-chemical conditions and medium components for the maximum decolorizing activity (c) Optimization of biotransformation conditions of triphenylmethane dyes in stirred tank reactor (d) identification and characterization of biotransformed product/s (e) Assessment of toxicity untreated and treated triphenylmethane dyes.

1.2 REVIEW OF STATUS OF RESEARCH AND DEVELOPMENT IN THE TEXTILE AND DYE-STUFF INDUSTRIAL EFFLUENT

Traditionally dye effluents are treated using physical methods like screening, equalization, dilution, froth floatation, sedimentation, filtration and gamma-irradiation. Color reduction by physical process was found to be negligible. Chemical and physico-chemical processes include flocculation, coagulation, clarification, chemical precipitation, oxidation, ion-exchange, reverse osmosis, ultrafiltration and adsorption. In 1972, Rutledge (4) reported that (i) industrial wastes should be characterized in terms of BOD, COD, TOC, total solids, fixed solids, volatile solids, nitrogen compounds, phosphorus, selected heavy metals, pH and color to determine the composition and to indicate nutrients to be added for improvement of biological treatment, (ii) aerobic microorganisms may be used successfully to treat certain textile dye wastes following specialized acclimatization, (iii) extended retention time of treatment of certain textile
dye wastes may decrease the efficiency of an activated sludge treatment system and 
(iv) certain dyes are treated more efficiently based on color and organic carbon 
removal, in an activated sludge system as a composite than as separate or segregated 
dyes. Frahne and Lohmueller (5) reported that the water soluble dyes can often be 
removed by adsorption to activated carbon. Kulla and Meyer (3) first time reported two 
microorganisms which were capable of breaking down two azoic compounds and one 
of these microorganism was found to consume dye-stuff as sole carbon and nitrogen 
source. 

Ogawa et al. (7-8) isolated azo dye assimilating bacteria Pseudomonas 
pseudomallei 13NA and Pseudomonas cepacia 13NA and tried to improve the existing 
biological treatments for waste dye liquor. Yatome et al. (9) found that starved intact 
cells of Pseudomonas pseudomallei degrade the dyes efficiently in comparison to fresh 
intact cells. In the biological treatment of industrial effluent, the captor captivated sludge 
system was introduced in the treatment of effluent in those industries where vegetable 
dyes form the main constituent (10). Halliday and Beszedits (11) described the various 
methods for decolorizing textile mill waste water such as chemical coagulation, 
biological oxidation and granular activated carbon adsorption. Reverse osmosis and 
ultrafiltration were also discussed. Waste water generated by textile finishing processes 
was highly variable in quantity and quality (12). Costs were evaluated to optimize the 
water treatment process and water recycling was determined to be the most cost-
effective control. Textile dye waste was treated in a simple electrochemical cell (13). 
The chemistry and costs of this unique process were discussed with reference to 
actual pilot studies at textile mills. Tincher and Dickson (14) found that sludge from 
biological treatment plant strongly absorbed dyes and dye degraded products. Active
sediment, heavily populated with microorganisms degraded industrial chemicals into simple inorganic compounds (15). The Hong Kong Institute of Biotechnology has reported the developed technology for the biodegradable treatment of indigo dyes and other dyes commonly used in the textile industry (16, 17). The process was based on the microbes capable of degrading indigo dyes and its carmine derivative, yielding non-toxic degradation products. In 1991, Carriere et al. (18) reported the decolorization of dye house waste effluent by ozone. Influence of pH and temperature on the rate of color removal and ozone consumption was discussed.

Rajagopal (19) discussed the economically viable and low-cost techniques for treating effluent from dye houses. The methods that were described include physical, physico-chemical, chemical and biological processes. Some of the physical processes were filtration, dilution and gamma irradiation. Physico-chemical processes for waste water treatment include adsorption, flocculation, reverse osmosis, ion exchange, coagulation and clarification. Chemical treatments such as oxidation, reduction and chlorination were often used. Nalco Chemical Company (20) used bioaugmentation for waste water treatment from textile industry. Bioaugmentation provided improvement of BOD removal, solids settling, hydraulic/organic capacity and nitrification, tolerance to toxic materials and oxygen transfer, preferential degradation of recalcitrant compounds, filamentous control, reductions in odor, oil reduction and grease and rapid system start-up seeding. Toxicity reduction and improvement in biodegradability of aromatic compounds by chemical oxidation was reported by Bowers et al. (21). They found that H$_2$O$_2$ catalized oxidation of tested organic compounds with high rate in comparison to ozone and potassium permanganate. These oxidized organic chemicals were found more responsive to biological treatment. Eckenfelder (22) also reported that chemical
Review of literature

oxidation helps in converting the non-biodegradable chemicals to biodegradable chemicals and lowering the toxicity level. Granular activated-carbon adsorption, air stripping, stream stripping and biological treatment were found to be very effective for removing volatile organic compounds from contaminated water (23). Granular activated carbon can remove up to 99% of organic compounds in water. Packed air stripping towers effectively remove 80-90% of dissolved volatile organic compounds. Stream stripping was observed more effective than air stripping for compounds with low volatilities.

Biological processes required more time, but they destroy the pollutants by converting them to less-toxic compounds rather than just concentrating the pollutants. Methods are developed to monitor wastewater for the presence of sulfonated azo dyes and their degradation products (24). They found that anaerobic treatment had little effect, but aerobic treatment oxidized most of them to other forms. Steiner (25) employed hydrogen peroxide and Fenton's reagent to remove contaminants from various textile waste effluent that contained low levels of dye stuffs, metallic ions and dissolved organic material. Treatment of Fenton's reagent with hydrogen peroxide or ferrous oxide were applied to dye house waste effluent and found 90% reduction in COD. Bohn (26) introduced biofiltration for decontaminating gases from textile waste effluent and tested with success in Europe and Japan. In 1992, the Institute for Textil- and Verfahrenstechnik Denkendorf, in cooperation with a private company, has developed a pilot effluent treatment system that cleans wastewater with high dye stuff concentration and allows the water to be reused by the plant (27). The in-plant system was comprised of four units: carbon-biological treatment, fluid-bed adsorption, precipitation/flocculation and fixed-bed filtration. A concept of single-tank system for the biological treatment of industrial wastewater was introduced at the 1992 International
Congress on Technology and Technology Exchange in Paris (28). Wastewater was pumped into the bioreactor after heavy metals were removed. Microorganisms metabolized carbon-based substances and the water was discharged into the municipal sewer system for further treatment. The system was optimal for waste water with low concentration of hazardous substances. Hicks and Caplan (29) showed that in-situ bioremediation destroyed organic contamination on-site, accelerates cleanup time, lowers remediation costs, reduces future liability and causes minimal disruption to operations at the contamination site. Bioremediation uses biological methods to destroy or reduce concentrations of organic wastes at contaminated sites, such as oil tanker spills, land based chemical spills and leaking petroleum and chemical storage tanks. The operational costs of current bioremediation systems, including land treatment, bioventing, bioreactors and bioslurrying are compared by Jespersen et al. (30).

In general, two processes are used for the treatment of textile effluent either separately or in combination of precipitation and biological treatment (31). Precipitation include physico-chemical flocculation and biological treatment involved mixing of effluent with activated carbon and subsequent microbial degradation. Parker (32) reported a new waste water treatment technology for removing color from dye house effluent. In addition to color removal, the catalytic oxidation of effluent also removes toxic substances, deodorizes and reduce the COD of effluent that contain soluble organic compounds. The catalytic oxidation of effluent can be adapted to individual customer needs. Oxidation has also successfully treated effluent contaminated with phenol, nitrophenol, chlorophenol and creosote. Laszlo (33) reported the removal of acid dyes from textile waste water using biomass. They found that the quatemized lignocellulose had great potential treatment for acidic dye containing effluent. Achwal
(34) used Fenton’s reagent (hydrogen peroxide with ferrous sulfate) for treatment of reactive dyeing waste. Fenton’s reagent destroyed the chromophore in the dye, which decolorizes the effluent and significantly lowers the chemical oxygen demand. Langley and Leist (35) reported that ozone, a powerful oxidizing agent play important role to decolorize dyeing and finishing effluent and dramatically improves the biodegradability of textile mill waste water. They also presented data for the treatment of various dye house waste and finishing plants in the southeastern United States, in which ozonation was employed to treat aqueous mixture of direct, disperse, cationic, reactive and sulfur dyes, as well as settling agents and detergents.

Indian researchers (36) developed a styrene based polymer membrane for the recovery of auxiliary chemicals and water discharged by domestic dyeing facilities. They found that the membranes are very effective for the separation and recovery of effluent and represent a viable alternative to more costly biological treatment. Waste minimization was found to be an effective waste management strategy for textile dyeing operations. Waste minimization and waste reduction decrease waste liability and minimize the costs associated with waste collection, handling and treatment (37). Japanese researchers (38) demonstrated the decolorization of reactive dyes and dyeing waste water by microorganisms under anaerobic conditions. The mechanism of decolorization was probably the reductive cleavage of the azo bond. The decolorization rate increased in the presence of organic nutrients, such as desizing waste water. German researchers (39) reported the decolorization of dye house waste water by ion-pair extraction. Ion-pair extraction with long chain aliphatic amines in an acidic medium was an effective process to treat waste water that contain anionic compounds. It is applicable to the selective removal of dyes that contain anionic groups, which include acid, direct, metal complex and reactive dyes. The major advantages of this method are
the simplicity to the liquid-liquid phase separation and the low energy consumption. Lanstar High force (40, 41) developed the COE (catalytic oxidation of effluent) process for removing color, toxic material and odors from dyehouse effluents. COE employs hydrogen peroxide as an oxidant in a catalyzed reaction. Fenton's reagent, which is commonly used to catalyze hydrogen peroxide oxidation, can be dangerous if volatile organic compounds are present and it creates an unwanted sludge. The new catalyst process (COE) possessed distinct advantages when compared with other treatment alternatives such as ozone treatment, adsorption processes, flocculation, membrane systems and biological treatments. Cooper (42) discussed advantages and disadvantages of all conventional color removal including: activated charcoal, membrane technology, ozone treatment and coagulation/flocculation. An et al. (43) reported the biological treatment of dye waste water using an anaerobic-oxic system. They treated three dye solutions namely, acid yellow 17, basic blue 3 and basic red 2 in an upflow anaerobic sludge blanket reactor followed by a semi-continuous aerobic activated sludge tank.

1.3 CONVENTIONAL TREATMENT METHODS: ADVANTAGES AND DISADVANTAGES

The methods for treatment of dye waste water include physical, chemical, physico-chemical and biological processes. Some of the physical processes are filtration and separation, dilution and gamma irradiation. Physical methods are mainly used for the primary treatment of dye waste water. Physico-chemical processes for dye waste water treatment include adsorption, flocculation, ion-pair extraction, reverse osmosis, ion-exchange, coagulation (by alum, ferric chloride, ferrous ammonium sulfate, lime,
natural polyelectrolite like tamarind, seed extract etc.) and clarification. Chemical treatments are oxidation, reduction and chlorination. The literatures cited above reveal that generally the processes used for the treatment of textile and dye-stuff industrial effluent are in combination of physical, chemical, physico-chemical and biological treatment processes. Activated carbon charcoal, anhydrous sodium silicate, mineral, rice husk, teak wood bark, cotton waste, hair coal, bentonite, fly ash, ground nut shell powder, red soil, bauxite, gypsum and clays were used for color removal from textile waste water. Use of powdered activated carbon showed good color removal from dye waste water. Activated charcoal, is very effective for removing color, but it is capable of treating only small effluent volume, operates at slow speeds and has high capital costs. Industries in general, do not use membrane processes such as ultrafiltration, reverse osmosis and nanofiltration on a large scale. Membrane technology is fast, but the capital costs for implementing this technology are high. Coagulation and flocculation techniques operate at moderate to fast speeds and require a somewhat lower capital investment. Chemical process such as oxidation by strong oxidizing agents (ozone, hydrogen peroxide, potassium permanganate) is very effective for treatment of dye waste water. In general, oxidation reduces color and toxicity, but in the presence of resorcinol, vanillin and salicylic acid in waste water, it exhibits an increase in toxicity. However, the oxidized organic chemicals are much more amenable to biological treatment. Ozone treatment operates at moderate speeds and also require a high capital investment. Catalytic oxidation of effluent (COE) was successfully applied to waste water treatment. The process remove color, toxic materials and odors from dye house effluents. The COE process also reduces the level of COD. The COE process is a hydrogen peroxide oxidation reaction that uses a unique catalyst. Fenton's reagent, which is commonly used to catalyze hydrogen peroxide oxidation, can be dangerous if
volatile organic compounds are present and it creates an unwanted sludge. The COE process is more economical than such alternatives as ozone treatment, absorption, flocculation techniques and membrane system.

All methods for treatment of dye waste water cited above are not fault less individually. These methods possess significant differences in color removal results, volume capability operating speeds and capitals costs. Many synthetic dyes are resistant to conventional waste water treatment processes. The biological methods have many advantages over physical and chemical treatments such as possibility of degradation of dye molecules to carbon dioxide and water, formation of less sludge in addition to being environmental friendly. Biological methods are the alternatives as they are simple and low in cost.

1.4 TRIPHENYLMETHANE DYES

1.4.1 Triphenylmethane dyes and their application

Triphenylmethane dyes are the xenobiotic compounds and can not be degraded as a sole source of carbon and energy (44). The structures of different triphenylmethane dyes are shown in Figure 1 which indicates that side chains are different in different dyes. In triphenylmethane dyes, a central carbon atom is bonded to two benzene rings and one p-quinoid group (the chromophore). The auxochromes are, -NH$_2$, -NR$_2$ and -OH. Triphenylmethane dyes have brilliant colors but not very fast to light or washing. Some important members of this class are crystal violet, magenta, malachite green, ethyl violet, pararosaniline, methyl violet, methyl green, brilliant green and victoria blue. Malachite green (basic green 4) has a deep-blue green color which resembles that of the copper or malachite (hence its name). Although the color fades in light, malachite
Crystal violet: \( R_1, R_2, R_3 = (\text{CH}_3)_2, R_4 = \text{H} \)
Magenta: \( R_1, R_2, R_3 = \text{H}_2, R_4 = \text{CH}_3 \)
Malachite green: \( R_1, R_2 = (\text{CH}_3)_2, R_4 = \text{H} \)
Brilliant green: \( R_1, R_2 = (\text{C}_2\text{H}_5)_2, R_4 = \text{H} \)
Ethyl violet: \( R_1, R_2, R_3 = (\text{C}_2\text{H}_5)_2, R_4 = \text{H} \)
Pararosaniline: \( R_1, R_2, R_3 = \text{H}_2, R_4 = \text{H} \)
Methyl violet: \( R_1, R_2 = (\text{CH}_3)_2, R_2 = \text{HCH}_3, R_4 = \text{H} \)

![Figure 1 Structures of triphenylmethane dyes](image-url)
green is used for direct dyeing of wool and silk and cotton after mordanting. Malachite green is prepared by condensing benzaldehyde (1 mole) with N, N-dimethylaniline (2 moles) in the presence of concentrated H$_2$SO$_4$. The leuco base (colorless), so formed, oxidized with lead oxide and then treated with hydrochloric acid to get dye. Crystal violet has bronzy-green crystals which dissolve in water to give deep-violet solution. Crystal violet dyes wool and silk directly and cotton after mordanting. It is prepared by heating Michler’s ketone with N, N-dimethylaniline in the presence of POCI$_3$ or COCl$_2$. Rosaniline is also called fuchsin or magenta. It has a p-quinoid chromophore. Its crystal have a green metallic luster. It dissolves in water to give a deep-red solution. The solution is decolorized by sulphur dioxide and the colorless solution is known as Schiff’s reagent which gives a pink color with aldehyde and not with ketones. Rosaniline dyes wool and silk directly and cotton after mordanting. The dye is prepared by oxidizing an equimolecular mixture of aniline, o-toluidine and p-toluidine with nitrobenzene and treatment of the leucobase with hydrochloric acid.

Triphenylmethane dyes are used as dermatological agent, the best among them is gentian violet, which has also been used for oral consumption for pinworms and topical application in human and in domestic animals and has shown to be effective in controlling fungal growth under varying conditions (2,3). Triphenylmethane dyes with glutaraldehyde are used for fixation and staining of lipids (45). Triphenylmethane dyes (crystal violet, carbol fushsin and malachite green) are also used for counting microorganisms directly on membrane filter (46). This technique is a modification of classical procedures for counting microorganisms (Bacillus subtilis and Sphaerotilus natans) directly on membrane filter. Recently crystal violet, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disufonate (WST-1) and neutral red were
used to assay the cell (HeLa cells) viability and in vitro cytotoxicity by calculating IC\textsubscript{50} values (47). Triphenylmethane dyes are used extensively in textile dyeing and dye-stuff manufacturing industries. It is also used as a biological stain and in paper printing. Malachite green, magenta, crystal violet, pararosanilene, ethyl violet and brilliant green are used for direct dyeing of wool and silk and cotton after mordanting.

1.4.2 Hazardous informations about triphenylmethane dyes

In 1913, Churchman and Herz found out the toxicity of gentian violet and its fate in the animal body (48). To determine the toxicity of this substance and its fate in the animal body, they have done numerous experiments on dogs and rabbits. For the study, the dye in varying concentrations was injected into rabbit ear vein. The cornea, conjunctiva and mucous membranes of both mouth and lips immediately became a deep blue. The animal in some instances remained perfectly passive during the injection, but more often they became restive after a portion of the dye had entered in the vein, their struggles suggested that the material was irritating. This symptom of irritation was present in some cases and absent in others. In another group of experiments, the animals died within a minute or so after the injection. They concluded that gentian violet injected intravenously into rabbits disappears from the blood in a short time and that there was no similar loss of selective bactericidal power when the dye was simply allowed to remain in contact with blood in vitro. When they applied gentian violet (concentrated solution) on tongue and bladder of dogs and rabbits, they found that there was no irritant effect on the tongue, however, in the bladder, there was a good deal of inflammatory reaction. In 1967, Adams (49) reported the antibacterial action of crystal violet against \textit{E. coli, Staphylococcus aureus, Streptococcus faecalis} and \textit{Bacillus subtilis}. He found that out of the four species, \textit{E. coli} was most resistant to the
dye, the resistances of the other organisms were similar, which supported that the action of crystal violet is due to the formation of an unionized complex of bacteria with dye. Gram-negative organisms, such as *E. coli* have high isoelectric points and contain less acidic components than Gram-positive bacteria which usually have lower isoelectric points, the former combine with crystal violet less readily and were more resistant to the dye. Many reports (50-52) showed that the toxicity of triphenylmethane dyes to the experimental animals and cell cultures (53) was found to cause reduced RNA and protein synthetic activity and oxygen consumption in rabbit granulation tissue (54). In 1973, Ballantyne *et al.* reported that crystal violet caused the eye damage (55). Mobacken and Zederfeldt reported the influence of a cationic triphenylmethane dye on granulation tissue growth in vivo (56). The action of a series of triphenylmethane dyes on the membranes of two different cell types, yeast cells and erythrocytes, have been investigated (57). They found that the action of the dyes on yeast cells resembles the action of other positively charged bactericides. Pararosaniline and crystal violet were much more active than malachite green and brilliant green on yeast cells, whereas the reverse sequence of activities applied in erythrocytes. The carbinol form of the dyes played an important role as regard to dye interaction with erythrocytes. Transition of the dye into the carbinol form in water is extremely slow, but is greatly accelerated in the presence of an organic phase, at least for malachite green and brilliant green, but not for crystal violet and pararosaniline. This explained the different action of the two categories of dyes on erythrocytes. Experiments with pure carbinol also supported the hypothesis.

Eriksson and Mabacken (58) also reported the direct cytotoxic effect of crystal violet. The testing system consisted of vital microscopy of the cheek pouch of the
hamster and microangiography of rabbit's ear. They found that the early microcirculatory disturbances caused by a dilute aqueous solution of crystal violet were transient, whereas considerable tissue toxicity was shown in the long-term experimental model. Moats and Maddox (59) reported the effect of pH on the antimicrobial activity of crystal violet, ethyl violet, brilliant green and malachite green. They tested dyes as inhibitors of *Salmonella anatum*, *Enterobacter aerogenes*, *Staphylococcus aureus* and *Bacillus cereus* over the pH range 5.0-9.0. They found that inhibition of the Gram negative type (*Salmonella anatum* and *Enterobacter aerogenes*) was markedly affected by the pH of the medium. These organisms tolerated concentration of crystal violet and ethyl violet about 100-fold higher at pH 5.0 than at pH 9.0. Above pH 7.0 brilliant green and malachite green were precipitated as their respective carbinols and lost their inhibitory properties with these two organisms. *Staphylococcus aureus* and *Bacillus cereus* were more sensitive to dyes and results were less affected by pH. The carbinol forms of malachite green and brilliant green were nearly as inhibitory to these organisms as the ionized forms. In 1978, Au et al. (60) reported the cytogenetic effect of gentian violet (impure form of crystal violet) and its related compound, crystal violet on various cell cultures (CHO, HeLA, human lymphocyte, peromyscus, L cells, and Indian Muntjac) in vitro. They found that at 5 ppm level, the peromyscus cells exhibited complete mitotic inhibition. Effect of different brands of gentian violet and crystal violet on chromosome breakage of CHO cells were also reported. The crystal violet sample from Sigma Chemical Company caused mitotic inhibition at 5 ppm level. The data presented by William et al. performed that the crystal violet and gentian violet are biohazardous substances. These compounds are mitotic poison as well as clastogen. The effect of bactericidal agent, streptomycin was the same for both cultures. The
results evidenced that the gentian violet can be inactivated by the liver detoxification system. It is potentially hazardous to cells that are exposed to the dye directly (e.g. skin epithelium and cell lining of gastrointestinal tract). Graffi (61) reported the effect of triphenylmethane dyes (bromophenol blue, bromocresol green and iodophenol blue) on mouse tumors. Combes and Haveland-Smith (62) reviewed the genotoxicity of the major dye-stuffs used in foods, drugs and cosmetics. Genotoxicity has been discussed with reference to structural chemistry, levels of exposure, absorption and metabolism and to epidemiological information. Genotoxicity activity may be due to the presence of certain functional groups, notably nitro-and amino-substitutents which are metabolized to ultimate electrophiles that may be stabilized by electronic interaction with aryl rings. They suggested that human cancer hazard may exist where significant quantities of these dye samples are handled. Such risks from exposures to other colors and the possibility of human germ-line mutation induction by dye-stuffs can not be meaningfully assessed.

Smith and Squires (63) reported that the dyes (crystal violet and methyl violet 2B) inhibit [3H]flunitrazepam binding to brain specific receptors in the rat with 50% inhibition in the 1 to 100 μM in cerebellum) and inhibited more potently in cerebellum. Thomas and MacPhee (64) described a DNA-repair-proficient strain of *E. coli* which does not carry any cell wall defects and is also a highly sensitive strain for detecting mutagens. They described the results of mutagenicity tests with five separate samples of crystal violet using this strain (DG 1669) both in the presence and absence of mammalian metabolic enzymes. Clemmensen *et al.* (65) reported the toxicity of malachite green. They found that the oral LD₅₀ for malachite green oxalate was 275 mg/kg in rats while the approximate lethal dose for NMRI mice was 50 mg/kg. No systemic effects were
seen after dermal application of 2,000 kg/kg. In accord with human experience malachite green was irritating to mucous membranes, but no effect were seen on intact skin nor was it shown to be sensitizing. It was found to be a mutagen in Salmonella/microcosm test after metabolic activation but without clastogenic activity when tested at maximally tolerated levels in mice in the micronucleus test.

Littlefield et al. found that the gentian violet appears to be carcinogen in mice at several different organ sites (66). A life span dosing study of gentian violet in the diet of 720 males and 720 females of B6C3F1 mice (C57BL/6XC3H) at dose levels of 0, 100, 300 and 600 ppm was done to determine its toxicity and carcinogenicity. Shimada and Shimahara (67) found out the effect of alternating current exposure on the resistivity of resting E. coli B cells to crystal violet and other basic dyes (malachite green, brilliant green, fuchsin, methylene blue, toluidine blue, safranin and acriflavine). They concluded that alternating current exposure may serve as an agent which renders E. coli cells susceptible to basic dyes. A chronic feeding study was carried out in the F1a generation of dosed Fischer 344 rats of both sexes with gentian violet (68). Ogawa et al. (69) reported the growth inhibition of Bacillus subtilis by basic dyes. They found that the content ratio of nucleic acid, [RNA]/[DNA], decreased with increasing dye concentration and so dyes act more preferentially to lower protein synthesis than inhibit cell division. Gentian violet was mutagenic in Salmonella typhimurium tester strain TA97 and TA104, but there was a little mutagenic activity detected in strains TA98 and TA100 (70). They pointed out that gentian violet is a mutagen in bacteria; however, since similar exposure conditions produced weak mutagenic activity in mammalian cells, gentian violet may be carcinogenic by virtue of its clastogenic activity. Evaluation of toxic and mutagenic effects of victory blue (C.I. 44040), methyl violet (C.I. 42535).
and brilliant green (C.I. 42040) were reported by using haploid strain 15B-114 of Saccharomyces cerevisiae (71). They found that the dyes could increase the frequency of appearance of nuclear point mutations and cytoplasmic mutations of respiratory deficiency.

In 1990, Dacampo and Moreno reviewed the metabolism and mode of action of gentian violet (72). Andrews et al. reported the photodeactivation of ethyl violet which is an indicator dye added to Sodasorb to indicate impending exhaustion of the absorbant (73). Basic triphenylmethane dyes (ethyl violet, methyl green and malachite green) inhibited class alpha glutathione S-transferases from rat liver (74). All the triphenylmethane dyes were bleached to varying degrees by glutathione. Malachite green was found to be highly cytotoxic to mammalian cells and also acts as a liver tumour-enhancing agent (75). Rao studied the effect of malachite green on DNA synthesis in primary cultures of normal adult rat hepatocytes maintained under fully defined conditions. He suggested that cytotoxic and mitotic inhibitory properties of malachite green possibly play an important role during tumour promotion. Pholarographic reduction of some triphenylmethane dyes in strictly anhydrous solution was reported in the absence and presence of alpha-lipoic acid (76). Recently Wronska-Nofer et al. (77) reported the enhancement of genotoxic effect of Acid Green 16 (triaryl methane dye) by ethanol, caused by induction of cytochrome P-4502E1 monoxygenase (mice) resulting in an increased bioactivation of the dye. Glanville and Clark (78) isolated the human glutathione S-transferases of the Alpha-, Mu- and Pi classes by expression in E. coli. Human glutathione S-transferase A1-1, was inhibited by malachite green (10 \mu m), but not inhibited by either crystal violet or ethyl violet up to 50 \mu m.
1.4.3 Biological decolorization of triphenylmethane dyes

All the literature cited above reveal that the triphenylmethane dyes have been used in medicine for almost 100 years as an antiseptic for external use, as an antihelminthic agent and more recently, as a blood additive to prevent transmission of chagas' disease. Gentian violet is a mutagen, a mitotic poison and a clastogen. The carcinogenic effect of gentian violet in rodents has been reported recently. Triphenylmethane dyes have been recognized as animal and human carcinogens. Triphenylmethane dyes have an inherent affinity for protein and are thus applicable directly to wool, silk, jute, etc. As they have no affinity for pure cellulose or regenerated cellulose they can be applied to cotton and rayon only after these fibers are mordanted with tannic acid or tartaric acid or synthetic organic mordants. The textile industry is one of the oldest and largest in the country. There are about 700 integrated cotton textile mills (1000 million Kg) of all varieties of cotton and cotton synthetic blended fabrics. The main source of waste water from a textile mill are from the designing kiering, bleaching, rinsing, mecering, dyeing and printing sections. Volume of the waste water varies considerably from mill to mill and lies in the range of 35 to 520 litre/kg cloth. The water is generally colored, high in BOD, COD and suspended solids and highly alkaline and have a fairly high temperature. Dyeing water amounts to 15 to 20% of the total wastewater. Therefore, discharge of the effluent in the ecosystem is unavoidable and leads to ecological imbalance. To depollute the dye waste water physical, chemical, physico-chemical methods have been employed. All the methods possess significant differences in color removal results, volume capability, operating speeds and capital costs.
Biological decolorization of triphenylmethane dyes are widely reported in the literature. Yatome et al. (79) reported the degradation of triphenylmethane dye (Basic violet 3) by *Pseudomonas pseudomallei* 13NA intact cells without identifying the degradation products. They demonstrated the decolorization of azo dyes, triphenylmethane dyes, anthraquinone dyes, oxazine dyes, acridine dyes, xanthene dyes, thiazine dyes and methine dyes by the intact cells of *Pseudomonas pseudomallei* 13NA. They found that the decolorization of triphenylmethane dyes was inferior to that of azo dyes. Crystal violet and methyl violet were appreciably decolorized, but pararosanilin and victoria blue were not decolorized under the same experimental conditions. The half-decolorization values of methyl violet and crystal violet at the concentrations of 20.4 ppm by 1 g dry weight per ml intact *Pseudomonas pseudomallei* 13NA cells were 44 and 61 h, respectively. To determine whether decolorization was dependent on the chemical structure of the dyes, they attempted to correlate the decolorization of dyes with molecular weight and the octanol-water partition coefficient. The octanol-water partition coefficient of an organic compound gives an indication of the permeability of the organic compound through the cell membrane. The half-decolorization time for triphenylmethane dyes had no appreciable correlation with the molecular weight. Ogawa et al. (80) suggested that in the practical treatment of waste water by microbes (activated sludge), the mixing of different kinds of dyes was the cause of the decline of purification capability. They found that the rate of elimination of triphenylmethane dyes (methyl violet and crystal violet) was 80-100%. They used acclimatized sludges for many dyes including crystal violet and methyl violet and observed the respiratory inhibition by these dyes. The degree of inhibition was obtained by measuring the oxygen uptake rate with and without dyes. It was observed
that the inhibition level of the unacclimatized microbes by methyl violet increased with the dye concentration (0-10^2 M). The acclimatized microbes showed negative inhibition up to 10^3 M methyl violet concentration and a positive inhibition above 10^3 M, while crystal violet did not show any inhibition up to 10^4 M and inhibition increased at higher concentration. In general the acclimatized microbes showed negative inhibition at low level of dye concentration and positive inhibition at high concentration. McDonald et al. (81) reported the reduction of gentian violet to leucogentian violet by human, rat and chicken intestinal microflora. They showed that the major portion of the metabolite was often bound to the cells (up to 87% of metabolite produced by human microflora).

Kwasniewska demonstrated that oxidative red yeast Rhodotorulae sp. and Rhodotorulae rubra had a high biodegradation potential against the crystal violet (82). He grew the yeast with 10 ppm crystal violet and found 99% decolorization after 4 d of incubation. He showed the degradation of crystal violet by taking visible spectra at different time interval. In order to test the absorbance of dyes by the cells, the cells after biodegradation were sonicated in 70% (v/v) ethanol. The extract did not show any absorbance at 600 nm. It was observed that the fermentative yeast S. cerevisiae did not degrade crystal violet in the liquid medium even after a prolonged incubation of 30 d. Bumpus and Brock showed that triphenylmethane dyes (cresol red, pararosaniline, bromophenol blue, ethyl violet, malachite green and brilliant green) undergo extensive biodegradation in ligninolytic culture of Phanerochaete chrysosporium (83). They showed that in the 6 d old culture broth, the crystal violet disappeared with the appearance of its three metabolites by the sequential N-demethylation of the parent compound. The purified ligninase also catalyzed N-demethylation of crystal violet. It proved that the lignin-degrading system is responsible for the biodegradation of crystal...
violet. They also reported that the non-ligninolytic culture of *P. chrysosporium* could also degrade the triphenylmethane dyes. This clearly suggested that in addition to the lignin-degrading system, another mechanism exists in this fungus which also degrades the dyes. Other triphenylmethane dyes were also degraded by ligninolytic culture of *P. chrysosporium* as assayed by monitoring decolorization at their respective absorption maxima. Involvement of the lignin-degrading system was confirmed by results in which the purified lignin peroxidase was shown to decolorize these dyes. Brilliant green, malachite green and ethyl violet all contain N-alkyl groups. Therefore, the initial oxidation of these compounds may proceed via N-dealkylation in a manner similar to that shown for crystal violet. However, pararosaniline, cresol red and bromophenol blue contain no N-alkyl group. Thus, oxidation of these dyes occurs by mechanism clearly different from that observed for crystal violet. These findings are consistent with the fact that the lignin degrading system of *P. chrysosporium* is relatively nonspecific and that this nonspecific nature is due, at least in part, to the lignin peroxidases, which catalyze the initial oxidation of a wide variety of organic compounds (84-86).

Yatome *et al.* reported the degradation of triphenylmethane dyes (pararosaniline, crystal violet and victoria blue) by the buffer washed cells of *Bacillus subtilis* IFO 13719, *Nocardia globerula* IFO 13510, *Pseudomonas cepacia* IFO 14595, *Pseudomonas cruciviae* IFO 12047, *Nocardia corallina* IAM 12121, *Escherichia coli* IAM 1264 and *Pseudomonas stutzeri* IAM12097 (87). Complete decolorization of crystal violet by *Bacillus subtilis* was observed after 24 h. They extracted the product with benzene and purified over extrelute-20 column. To identify the degradation product of crystal violet they used gas chromatograph-mass spectrophotometer and identified as 4,4-bis(dimethylamino)benzophenone (Michler's ketone; MK). The
decolorization of crystal violet was similarly observed with *N. corallina* and *N. globerula*. Crystal violet was not decolorized with *E. Coli, P. cepacia, P. cruciviae* and *P. stutzeri*. Pararosaniline and victoria blue were also decolorized by *Bacillus subtilis*. They did not report the metabolites of pararosaniline and victoria blue. *Phanerochaete chrysosporium* was found to degrade triphenylmethane dyes with other xenobiotic compounds such as DDT, chlorinated phenoxyacetates, chlorinated anilines, pentachlorophenol, chlorinated alicyclic insecticides, biphenyls, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, chlorinated dioxins, 2,4,6-trichlorobenzoic acid and azo dyes (88). The ligninolytic system of white rot fungi, *P. chrysosporium* was found to be mainly responsible for degradation of the xenobiotic compounds.

In 1991 a new process was reported for degradation of triphenylmethane dyes by *Corynebacterium* and *Mycobacterium sp.* (89). The process was very simple and rapid and may be performed over a wide range of temperatures (15-40°C) over 1-12 h. No additional substrate supplements are needed. Both crystal violet and malachite green are removed from waste water or soil extracts. Sattler *et al.* (90) reported the degradation of gentian violet and malachite green by oleophilic bacteria which were screened from the different places contaminated with mineral oil, such as airfields, tank surroundings and road surfaces. They found no strain showing amylase activity but all exhibited catalase activity (EC 1.11.1.6) and most had lipase (EC- 3.1.1.3) activity. A few showed cellulase (EC 3.2.1.4) and some showed protease activity. The isolated organisms have the promise for bioremediation processes. Roth *et al.* (91) demonstrated the behavior of the forty hydrophobic oleophilic bacterial strains towards the triphenylmethane dyes (crystal violet and malachite green). They showed that out of the forty strains (*Mycobacterium, Nocardia, Rhodococcus, Escherichia,*)
Pseudomonas and Serratia), twenty one strains had positive decolorizing activity. All the active strains (Mycobacterium, Nocardia and Rhodococcus) attacked both dyes. Decolorization was found to be irreversible, due to degradation and the formation of leuco-derivatives and then to unidentified fluorescent and UV-absorbing intermediates. In another report, Yatome et al. (92) showed the degradation of crystal violet by Nocardia Corallina IAM 12121. The degradation product of crystal violet was identified as MK by gas chromatography-mass spectrometry. The MK was not further metabolized by N. corallina IAM 12121. Nocardia corallina was found to be decolorized methyl violet, ethyl violet, pararosaniline and victoria blue. The decolorization rate was dependent upon the initial concentration of N. corallina in the medium and the growth phase of precultured cells. They also showed that the decolorization of crystal violet was not observed even after prolonged incubation with cell homogenates and extracellular extract of N. corallina.

Ollikka et al. (93) showed that the different lignin peroxidase isoenzymes (LiP 4.65, LiP 4.15 and LiP 3.85) from P. chrysosporium BKM 1767 may have different specificity and efficiencies towards dyes. They purified the three major lignin peroxidase enzymes from carbon-limited cultures of P. chrysosporium and studied decolorization of several dyes. They found that decolorization of bromophenol blue and methyl green by crude lignin peroxidase in the presence of 2 mM veratryl alcohol was 93 and 88%, respectively. The decolorization of methyl green by LiP 4.65, LiP 4.15 and LiP 3.85 in the presence of 2 mM veratryl alcohol was 95, 69 and 89%, respectively. They observed that the decolorization of methyl green by crude lignin peroxidase, Lip 4.15, Lip 4.65 and Lip 3.85 was 93, 5.6 and 11%, respectively. This suggested that veratryl alcohol acts as a mediator in the reaction but the omission of...
veratryl alcohol from the reaction mixture had almost no effect on the ability of the crude lignin peroxidase to decolorize the dyes. This may be due to the fact that the fungus itself synthesizes veratryl alcohol as a product of secondary metabolism. With the increase in the veratryl alcohol concentration, the rate of decolorization of methyl green is increased. Singh et al. (94) reported the biodegradation of malachite green and rhodamine B by rat caecal extract. They showed that the rat caecal contents metabolized malachite green to a unknown fluorescent compound. This suggested that malachite green is biotransformed to different metabolite by rat caecal microflora which may help in delineating the mechanism of carcinogenic potential of this dye. They did not identify the metabolite of malachite green.

Das et al. (95) studied the crystal violet decolorization in a column bioreactor using *P. chrysosprium* NCIM 1197. The decolorization was performed in a glass column bioreactor with an eight tier stainless steel column holder through which the dye containing medium was recirculated by a peristaltic pump. Crystal violet was passed through the column at a concentration of 0.002% with a recycling rate of 20 ml/min at 30°C. The data revealed that almost 92% decolorization was there in 82.4 h in recycled medium as compared to 64% in shake flasks in 17 d. With glucose as a carbon source, the peak decolorization reached in about 3 d as compared to 4-6 d with sucrose. Decolorization of three triphenylmethane dyes (crystal violet, bromophenol blue and malachite green) by three birds nest fungi, *Cyathus bulleri*, *Cyathus sterocoreus* and *Cyathus striatus* was reported by Vasdev et al. (96). Maximum decolorization was achieved with *C. bulleri* (92.6%) as compared with *C. sterocoreus* (84.7%) and *C. striatus* (75.5%) at 96 h after the addition of dyes. The higher dye decolorization by *C. bulleri* was attributed to its higher laccase activity compared to *C.*
striatus and C. stercoreous. Cyathus bulleri culture filtrate was able to decolorize these dyes under nitrogen sufficient condition and without addition of H$_2$O$_2$, in a relatively simple medium. The faster decolorization by whole culture of Cyathus bulleri (96-100% in 4 d) compared to extracellular fluid (90% in 10 days), could be the result of the combined activity of some cell-associated and extracellular ligninolytic enzyme. Cyathus bulleri has been found to be capable of decolorizing the crystal violet up to 36.72 ppm whereas P. chrysosporium has been shown to decolorize the dyes to a much lesser extent (5 ppm). In 1995, Yesilada (97) reported the decolorization of crystal violet (5 ppm) by the 6 d old cultures of Coriolus versicolor, Funalia trogii, Phanerochaete chrysosporium and Lactiporus sulphureus ME446 92, 82, 62 and 86%, respectively, over 3 d incubation. Yesilada also reported the oxidation of crystal violet by commercial horseradish peroxidase. A significant rate of oxidation was observed only when H$_2$O$_2$ was present.

Knapp et al. (98) reported the decolorization of crystal violet and brilliant green by seven wood rotting fungi (P. chrysosporium 586, Piptoporus betulinus, Pleurotus ostreatus, Coriolus versicolor 556, Coriolus versicolor and two unidentified isolates). They found that Phanerochaete chrysosporium 586 was among the least effective of the isolates. All the isolates decolorized some dyes and all dyes were decolorized to some extent. The result presented by Knapp et al. were often dramatic and clearly showed the potential for the use of wood rotting fungi in the degradation and decolorization of dye-stuffs. Processes involving white rot fungi could be proposed for the treatment of effluent from the wood pulping industry and they may be useful in treatment of effluent containing dyestuffs. In 1997 Shin et al. (99) reported the decolorization of crystal violet and malachite green by remazol brilliant blue R (RBBR)
decolorizing peroxidase produced from *Pleurotus ostreatus*. They purified the RBBR decolorization peroxidase by ammonium sulphate fractionation, sephacryl S-200 HR chromatography, DEAE sepharose CL-6B chromatography and mono Q chromatography and showed the ability of the enzyme to catalyze oxidation of crystal violet and malachite green. They found that the relative activities of crystal violet and malachite green with respect to RBBR were 246.6 and 27.6%, respectively. They also found that the addition of veratryl alcohol with purified enzyme did not influence the decolorization of dyes. It clearly suggested that the mode of action of RBBR decolorizing peroxidase is different from that of lignin peroxidase of *Phanerochaete chrysosporium*. Henderson *et al.* (100) reported the reduction of malachite to leucomalachite green by intestinal microfloras from human, rat, mouse and monkey fecal. They identified the metabolite of malachite green by HPLC-retention time in reverse phase system and mass spectral in comparison with authentic compound. They concluded that the enzymatic reduction of malachite green to leucomalachite green by intestinal microflora could play a critical role in metabolic activation to a potential carcinogen. Recently Sani *et al.* reported the decolorization of crystal violet, malachite green and magenta with other industrial textile dyes (red HE-8B and navy blue HE-2R) and dye effluent by the growing cells of *Phanerochaete chrysosporium* MTCC 787 in static as well as in shake flasks (101). They grew the organism in fermenter for four days and the whole broth was used for decolorization experiment. The decolorization of dyes and effluent was found to be more in shake culture in comparison to static culture. A significant color removal (greater then 90%) of dyes and effluent was observed. They concluded that *P. chrysosporium* would be a potent organism to depollute the industrial waste water containing various dyes.
1.4.4 Metabolism of triphenylmethane dyes: biodegradation pathways

Metabolism of triphenylmethane dyes as reported in literature is shown in Figure 2. Yatome et al. (79) showed the degradation of crystal violet by intact cells of *Pseudomonas pseudomallei* 13NA. They showed the possibility of degradation of crystal violet by detecting the degradation products through thin layer chromatography. They did not identify the structures of degradation products of crystal violet. Ogawa et al. (80) also showed the degradation products of methyl violet by thin layer chromatography. They also did not identify the structure of methyl violet metabolites which were obtained after the treatment of methyl violet by activated sludge. McDonald and Cerniglia (81) showed that gentian violet was reduced to leucogentian violet by human, rat and chicken intestinal microflora under anaerobic condition. They identified the metabolite leucogentian violet by HPLC-retention time in a reverse phase system and by mass spectral in comparison with authentic compound. Bumpus and Brock (83) reported the biodegradation of crystal violet by white rot fungus *P. chryosporium*. The metabolites were N,N,N',N',N"-pentamethylpararosaniline, N,N,N',N"-tetramethylpararosaniline and N,N',N"-trimethylpararosaniline. Yatome et al. (96) reported the degradation of crystal violet by the growing cells of *Bacillus subtilis* IFO 13719. The degradation product of crystal violet was extracted with benzene and identified as 4, 4'-bis(dimethylamino)benzophenone (Michler's ketone; MK) by gas chromatograph-mass spectrometer. Crystal violet was degraded by growing cells of *Nocardia corallina* IAM 12121 (92). They also showed that the degradation product of crystal violet was Michler's ketone. It was not further metabolized by *Nocardia corallion* IAM 12121. Henderson et al. (100) reported the reduction of malachite green to leucomalachite green by intestinal microfloras from human, rat, mouse and monkey.
Figure 2  Metabolism of triphenylmethane dyes
fecal. They identified the metabolite of malachite green by HPLC-retention time in reverse phase system and by mass spectral in comparison with authentic compound.

1.4.5 Toxicity of biodegraded metabolites

All the literatures cited above reveal that there are very few reports on the biodegradation products or intermediates of triphenylmethane dyes. Out of different triphenylmethane dyes, the biodegradation products or intermediates of crystal violet and malachite green are reported in literature (83, 87, 92, 100). Crystal violet biodegradation products are leucogentian violet, 4,4'-bis(dimethylamino)benzophenone, N,N,N',N',N''-pentamethylpararosaniline, N,N,N',N''-tetramethylpararosaniline and N,N',N''-trimethylpararosaniline. All these biodegradation products or intermediates of crystal violet are toxic and carcinogenic in nature (66, 68). Michler's ketone [4,4'-bis(dimethylamino)benzophenone] is an irritant, mutagen and cancer suspected agent. Only one report is available on the biotransformation of malachite green to leucomalachite green by intestinal bacteria (100). Leucomalachite green, similar in structure of leucogentian violet is a potent carcinogen.