CHAPTER 2

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2. REVIEW OF LITERATURE

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2.1. DYES

Highly colored substances, known logically enough as colorants, can be used to impart color to an infinite variety of materials described technically as substrates. Colorants can be subdivided (Aspland, J. R., 1980) into dyes, which are soluble in medium in which they are applied, and pigments which are insoluble in the application medium.

Dye may be defined as a colored substances which when applied to fibers give it a permanent color; resistant to action of light, water, and soap. There are two important conditions for a colored compound to act as dye.

(a) Presence of chromophore

These are the groups which are responsible for producing a color to a dye because they are capable of absorbing light in the near ultraviolet region. Some important chromophores' are: \(-N=O\), \(-NO_2\), \(-N=N\), \(-C=O\), \(-C=S\), \(-ON\), (CH=CH)_n. The compounds bearing chromophores are known as chromogens.

(b) Presence of auxochromes

Dye should be attached to the fibers by means of stable chemical bonds. These chemical bonds are formed by some groups which may be either acidic or basic in nature. Such groups are known as auxochromes, some are; \(-OH\), \(-COOH\), \(-SO_3H\) (acidic), \(-NH_2\), \(-NHR\), \(-NR_2\) (basic). A chromogen without auxochrome can never act as a dye.

2.1.1. CI Constitution number

This is based on the chemical structure of the dye, which contains chromophores (color-producing groups) like azo, anthraquinone, azine, oxazine, thiazine, stilbene etc.
A range of numbers is reserved for each chemical class and a five-digit number is assigned to each dye belonging to that class (Shenai, 1994). The chemical structure class and the corresponding range of CI constitution numbers are given in Table 1. The numbering starts from 10,000 and the first 300 numbers are reserved for nitroso dyes. Azo, anthraquinone, triphenylmethane and quinoline dyes are allocated 26000, 15000, 3000, and 1000 numbers, respectively. In each group the numbering is not continuous but with a difference of four between two dyes -- 10,000, 10,005, 10,010, 10,015 ..., based on variation in the position (ortho, meta, para etc.) of substituent groups in the dye molecule and variation in the type of the substituent (methyl, ethyl, methoxy, cyano, amino, sulphonic acid, hydroxy, carboxy, chloro, bromo, etc.). If, in future, a dyestuff of slightly varied structure is found out; the intermediate number (such as 17,001) will be assigned to it. Thus four dyes can be incorporated in the numbering system. If more than four dyes are found out, they too can be assigned constitution numbers such as 17001:1.

2.1.2. Classification of dyes

Dyes may be classified according to either by the chemical structure or by their usage or application method. The most appropriate system for the classification of dyes is by chemical structure, which has many advantages. It readily identifies dyes as belonging to a group that has characteristic properties and there are manageable number of chemical groups. The usage classification is advantageous to consider the classification of dyes by use and method of application before considering chemical structures in detail because of dye nomenclature and jargon that arises from this system. So, on the basis of use or application method, dyes can be classified as:
Table 1: Classification of dyes based on chemical constitution and the corresponding range of CI Constitution numbers

<table>
<thead>
<tr>
<th>Chemical class</th>
<th>CI Constitution number range</th>
<th>Total number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitroso</td>
<td>10,000-10,299</td>
<td>300</td>
</tr>
<tr>
<td>Nitro</td>
<td>10,300-10,999</td>
<td>700</td>
</tr>
<tr>
<td>Azo</td>
<td>11,000-36,999</td>
<td></td>
</tr>
<tr>
<td>(a) Monoazo</td>
<td>11,000-19,999</td>
<td>26,000</td>
</tr>
<tr>
<td>(b) Diazo</td>
<td>20,000-29,999</td>
<td>9,000</td>
</tr>
<tr>
<td>(c) Triazo</td>
<td>30,000-34,999</td>
<td>10,000</td>
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<tr>
<td>(d) Polyazo</td>
<td>35,000-36,999</td>
<td>5,000</td>
</tr>
<tr>
<td>Azoic</td>
<td>37,000-39,999</td>
<td>2,000</td>
</tr>
<tr>
<td>Stilbene</td>
<td>40,000-40,799</td>
<td>1,000</td>
</tr>
<tr>
<td>Carotenoid</td>
<td>40,800-40,999</td>
<td>800</td>
</tr>
<tr>
<td>Diphenylmethane</td>
<td>41,000-41,999</td>
<td>200</td>
</tr>
<tr>
<td>Triphenylmethane</td>
<td>42,000-44,999</td>
<td>1,000</td>
</tr>
<tr>
<td>Xanthane</td>
<td>45,000-45,999</td>
<td>1,000</td>
</tr>
<tr>
<td>Acridine</td>
<td>46,000-46,999</td>
<td>1,000</td>
</tr>
<tr>
<td>Quinoline</td>
<td>47,000-47,999</td>
<td>1,000</td>
</tr>
<tr>
<td>Methane/Polymethine</td>
<td>48,000-48,999</td>
<td>1,000</td>
</tr>
<tr>
<td>Thiazole</td>
<td>49,000-49,999</td>
<td>1,000</td>
</tr>
<tr>
<td>Indamine/Indophenol</td>
<td>49,400-49,999</td>
<td>600</td>
</tr>
<tr>
<td>Azine</td>
<td>50,000-50,999</td>
<td>1,000</td>
</tr>
<tr>
<td>Oxazine</td>
<td>51,000-51,999</td>
<td>1,000</td>
</tr>
<tr>
<td>Thiazone</td>
<td>52,000-52,999</td>
<td>1,000</td>
</tr>
<tr>
<td>Sulphur</td>
<td>53,000-54,999</td>
<td>2,000</td>
</tr>
<tr>
<td>Lactone</td>
<td>55,000-55,999</td>
<td>1,000</td>
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<tr>
<td>Aminoketone</td>
<td>56,000-56,999</td>
<td>1,000</td>
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<tr>
<td>Hydroxyketone</td>
<td>57,000-57,999</td>
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<tr>
<td>Anthraquinone</td>
<td>58,000-72,999</td>
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</tr>
<tr>
<td>Indigoid</td>
<td>73,000-73,999</td>
<td>15,000</td>
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<tr>
<td>Phthalocyanine</td>
<td>74,000-74,999</td>
<td>1,000</td>
</tr>
<tr>
<td>Natural organic coloring matters</td>
<td>75,000-75,999</td>
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<tr>
<td>Oxidation bases</td>
<td>76,000-76,999</td>
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<tr>
<td>Inorganic coloring matters</td>
<td>77,000-77,999</td>
<td>1,000</td>
</tr>
</tbody>
</table>
2.1.2.1. Reactive dyes

These dyes form a covalent bond with the fiber, usually cotton although they are used to a smaller extent with wool and nylon. It shows extremely high washfastness property by relatively simple dyeing methods. The marked advantages of reactive dyes over direct dyes are that their chemical structures are much simpler, their absorption spectra show narrower absorption band and the dyeing are brighter. The principle chemical classes of reactive dyes are azo, triphendioxazine, phthalocyanine, formazan and anthraquinone.

2.1.2.2. Direct dyes

These water soluble anionic dyes, when dyed from aqueous solution in the presence of electrolytes, are substantive to (i.e. have high affinity for) cellulosic fibers. The principal use is the dyeing of cotton, regenerated cellulose paper, leather, and to a lesser extent nylon. Most of the dyes in this class are azo compounds with some stilbenes, phthalocyanines and oxazines. After-treatments, frequently given to the dyed material to improve washfastness properties, include chelation with salts of metals (usually copper or chromium), and treatment with formaldehyde or a cationic dye-complexing resin.

2.1.2.3. Vat dyes

These water insoluble dyes are applied mainly to cellulosic fibers as soluble leuco-salts after reduction in an alkaline bath, usually with sodium hydrosulfite. Following exhaustion onto the fiber, the leuco forms are reoxidized to the insoluble keto forms and after treatment, usually by soaping, redevelops the crystal structure. The principal chemical classes of vat dyes are anthraquinone and indigoid.
2.1.2.4. Sulfur dyes

These dyes are applied to cotton from an alkaline-reducing bath with sodium sulfide as the reducing agent. Numerically, this is a relatively smaller group. This group of dyes shows good washfastness property.

2.1.2.5. Disperse dyes

These are substantially water-insoluble nonionic dyes for application to hydrophobic fibers from aqueous dispersion. They are used predominantly on polyester and to a lesser extent on nylon, cellulose, cellulose acetate and acrylic fibers. Thermal transfer printing, in which disperse dyes are printed onto paper and subsequently transferred to the fiber by a dry-heat process, represents a niche market for the selected members of this class.

2.1.2.6. Solvent dyes

These water insoluble dyes are devoid of polar solubilizing groups such as sulfonic acid, carboxylic acid, or quaternary ammonium salts. They are used for coloring plastics, gasoline, oils, and waxes. These dyes are predominantly azo and anthraquinone, but phthalocyanines and triphenylmethane dyes are also used.

2.1.2.7. Acid dyes

These water-soluble anionic dyes are applied to nylon, wool, silk, and modified acrylics. They are also used to some extent for paper, leather, food, and cosmetics. All original members of this class had one or more sulfonic or carboxylic acid groups in their molecule. Chemically, the acid dyes consist of azo (including preformed metal complexes), anthraquinone, and triphenylmethane compounds with a few azine, xanthene, ketone imine, nitro, nitroso, and quinophthalone compounds.
2.1.2.8. Basic dyes

These water-soluble cationic dyes are applied to paper, polyacrylonitrile (e.g. Dralon), modified nylons, and modified polyesters. Their original use was for silk, wool, and tannin-mordanted cotton when brightness of the shade was more important than fastness to light and washing. Basic dyes are water-soluble and yield colored cations in solution. For this reason, they are frequently referred to as cationic dyes. The principal chemical classes are diazahemicyanine, triphenylmethane, cyanine, hemicyanine, thiazine, oxazine, and acridine. Some basic dyes show biological activity and are used in medicine as antiseptic.

2.1.2.9. Natural dyes

Natural dyes comprise those colorants (dyes and pigments) that are obtained from animal or vegetable matter without chemical processing. They are mainly mordant dyes, although some vat, solvent, pigment, direct, and acid types are also known. Of the 92 natural dyes listed in color index, chemical structures of 67 dyes are known. Many dyes have more than one compound and some dyes have identical structure. Natural dyes were classified into two groups, namely substantive and adjective dyes. According to this classification, the dyes such as indigo orchil, turmeric etc. which dye the fibers directly are classified as substantive dyes. While adjective dyes only dye material mordanted with a metallic salt, or with addition of the metallic salt to the dyebath, examples of such dyes are log wood, madder, cochneal, fustic etc. Generally the adjective dyes are slightly colored and when used alone they give poor dyeing. Subsequently, dyes were classified as direct dyes and mordant dyes. The direct dyes may further be classified as direct dyes for cotton, direct dyes for wool and silk: acid
dyes and basic dyes. The direct dyes for cotton dye all natural textile fibers; the acid dyes are mostly only applicable to wool and silk; while the basic dyes have a direct affinity for wool and silk, and tannic acid treated cotton. The mordant dyes can equally be well fixed on the animal as well as vegetable fibers, therefore, they are not further divided into subgroups.

2.1.3. Triphenylmethane dyes

In triphenylmethane dyes, a central carbon atom is bonded to two benzene rings and one p-quinoid group (chromophore). The auxochromes are -NH₂, -NR₂ and -OH. These dyes have brilliant colors but not very fast to light or washing. Triphenylmethane dyes are extensively used in dyeing textile, cotton, wool, and as human and veterinary medicine. This group of dye is also used in paper printing and ink manufacturing. Some important members (Fig. 1) of this class of dyes are:

2.1.3.1. Malachite Green (Basic Green)

It has a deep blue green color which resembles that of copper or malachite. Although the color fades in light, malachite green is used in direct dyeing of wool, silk, and cotton after mordanting. It is prepared by condensing benzaldehyde with N,N-dimethylaniline in the presence of concentrated H₂SO₄. The leuco base (colorless), so formed, is oxidized with HCl to get the dye.

2.1.3.2. Crystal Violet (Basic Violet 3)

It has bronyz-green crystal which dissolve in water to give deep violet color. 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 POCl₃ or COCl₃.
Fig. 1. Structure of various triphenylmethane dyes.
2.1.3.3. Magenta

It is also called fuchsin or rosaniline. It has p-quinoid chromophore. Its crystals have green metallic luster. It dissolves in water to give a deep red solution. Wool and silk are directly dyed by magenta and cotton after mordanting. The dye is prepared by oxidizing an equimolar mixture of aniline, o-toluidine and p-toluidine with nitrobenzene. The leuco base is treated with HCl to generate the dye.

2.1.3.4. Brilliant Green (Basic Green 1)

It has minute, glistening, and golden crystals. It is soluble in water and alcohol and give green color on dissolution. Color of the solution changes from yellow to green at pH 0.0 to 2.6. This dye is used in dyeing silk, wool, leather, jute, and cotton. It is also used in manufacturing of green ink, as biological stain, and indicator. It can be used as human and veterinary antiseptic for external and internal (oral) use.

2.1.3.5. Brilliant Blue FCF (Food Blue 2)

It is reddish-violet powder or granule with a metallic luster and fairly soluble in water and alcohol. This dye is practically insoluble in vegetable oils. It forms pale amber solution in concentrated \( \text{H}_2\text{SO}_4 \) which first changes to yellow then to greenish blue on further dilution. This dye is approved by FDA for use in food, drugs and cosmetics excluding use in eye area. Brilliant Blue is used in textile dyeing, as biological stain, as wood stain, and also as an indicator.

2.1.3.6. Bromocresol Green

It is prepared by adding bromine to a suspension of m-cresolsulfonaphthalein in glacial acetic acid. The slightly yellow crystals are sparingly soluble in water but readily soluble in alcohol, ether and ethyl acetate. It is fairly soluble in benzene and very
sensitive to alkalis. Bromocresol Green is mainly used as pH indicator. Color of the solution changes from yellow to blue-green at pH 3.8 to 5.4.

2.1.3.7. Bromocresol Purple

It is prepared by treating o-cresol red with bromine in glacial acetic acid. Its minute, slightly yellow crystals are practically insoluble in water but readily soluble in alcohol and dilute alkalis. It is generally used as pH indicator. Color of the solution changes from yellow to purple at pH 5.2 to 6.8.

2.1.3.8. Cresol Red

Its reddish-brown crystalline powder can be recrystallized from glacial acetic acid. It is soluble in alcohol, water, dilute acid (yellow solution), and dilute alkalis (purple solution). It is used as pH indicator and the color with pH varies from yellow (pH 7.2) to red (pH 8.8) to orange and amber (pH 2-3).

2.1.3.9. Acid Violet 7 B

It is also known as Acid Violet 25 and prepared by the sulfonation of the condensation product from p-dimethylaminobenzoyl chloride with n-ethyl diphenylamine. Its violet powder is insoluble in ether but soluble in water and alcohol with deep blue color and violet tinge. It is used as dye, in inks, and stains.

2.2. TOXICITY OF TRIPHENYL METHANE DYES

The first case of toxicity of Gentian Violet (an impure form of Crystal Violet) and its fate in animal body was reported by Churchman and Herz (1913). They did a series of seventy five experiments on dogs and rabbits to observe the bactericidal property possessed by Gentian Violet. For the intravenous injections of Gentian Violet, rabbits were used. The dye in varying concentrations was injected into the ear vein. The
cornea, conjunctiva and mucous membranes of both mouth and lips immediately became deep blue. The results of intravenous injections were of two kinds. In one group of animals, the dye seemed to be absolutely without any effect. They usually sat still for a short time after the experiment and then resumed their normal habits. The experiment proved conclusively that large amount of Gentian Violet in the circulation may be borne by rabbits without any apparent harm what so ever. In the other group of experiments, however, the animals died within a minute or so after injection. In these cases the rabbits went into violent convulsions as soon as the injection was completed and died rapidly often in opisthotonus. The fate of the dye in the blood was studied by means of divided blood plates made from animals which had previously received intravenous injection of Gentian Violet. These experiments showed conclusively that blood withdrawn from animals, so injected, possesses the selective bactericidal power of the dye, but that this power disappears completely in about two hours. It was thought that this loss of bactericidal power is due to disappearance of the dyes which might be due to a chemical reaction between blood itself and Gentian Violet. With these sets of experiments, it is evident that Gentian Violet injected intravenously into rabbits disappears from the blood in a short time, and that there is no similar loss of selective bactericidal power when the dye is simply allowed to remain in contact with blood in vitro.

They have made a number of observations on the effect of dye when applied directly to the living tissues. On painting the tongue of dogs and rabbits with strong solution of dye, frozen sections showed that penetration had occurred through the thickness of the mucosa down to muscularis. Similar observations were made on the bladder, the mucosa of which is readily stained by injecting the dye into that viscera through the
urethra. On the tongue it seems to be without any irritant effect; however in the bladder, there was a good deal of inflammatory reactions when very strong solutions were used.

2.2.1. Cytogenetic toxicity

The cytogenetic toxicity of Gentian Violet and Crystal Violet in Chinese hamster ovary (CHO) cells has been studied (Au et al., 1978) in vitro by analyzing (1) squash preparations from direct fixation for recording mitotic anomalies and (2) air-dried preparations for recording metaphase chromosome aberrations. The first series of experiments were conducted on CHO cells with Gentian Violet dissolved in the growth medium at concentrations 1 and 10 µg/mL. Cultures treated for a longer period (8 h) or with a higher dosage (10 µg/ml) showed a significant accumulation of abnormal metaphases as evidenced by the elevated values of mitotic index and metaphase/anaphase ratio. Thus Gentian Violet may also be a mitotic poison. The frequency of anaphase abnormalities (chromatin bridges, lagging chromosomes, chromosome fragments, and sticky chromosomes) increased as the dose and the duration of treatment was increased. The disturbance in orderly karyokinesis is evidenced by the increase of cells with multinucleation. Both Gentian Violet and Crystal Violet, induced rather severe damage to chromosomes. Out of these two dyes, Gentian Violet was distinctly less toxic than other. The Crystal Violet sample caused mitotic inhibition even at 5 µg/mL. These results demonstrate that these dyes cause severe cytogenetic toxicity in cultured cell line (mitotic poison as well as clastogen) and the cytotoxicity of these dyes was very high. Moreover, these dyes induced a high frequency of chromosome breakage in a number of cell types, indicating that their effects are not limited to one cell line. Whether Gentian Violet and Crystal Violet are
also toxic to the chromosomes of cells in vivo remains to be verified, but these preliminary results from in vitro studies are sufficiently alarming to regard Gentian Violet and Crystal Violet as biohazardous substances. Adding such compounds to animal feed should not be permitted unless sufficient data on in vivo system prove that they are harmless.

To further check the cytotoxic nature of Gentian Violet and Crystal Violet, genetic toxicity of Gentian Violet was studied (Au et al., 1979) with the Ames and Rozenkranz bacterial assays as well as the cytogenetic assays (CHO cell in vitro in the presence of rat-liver S-9 fractions, the chicken embryo, and mouse bone marrow cell in vivo). Gentian Violet was found to be toxic but not mutagenic in the Ames assay. The doses of Gentian Violet (0.1-50 µg/mL) were added to the bacterial culture and it was observed that when Gentian Violet was present without the S-9 mixture, the dye was bactericidal at doses of 10 µg and higher. When S-9 mixture was present with Gentian Violet, the purple color of the dye was converted to pink and it lost its toxicity. Slight toxicity was evidenced under a dissecting microscope by a decrease in the number of background micro colonies. In Rosenkranz assay, Gentian Violet was added to the disc of the bacterial cultures at concentrations of 1, 10, 25 and 100 µg/plate with or without the simultaneous addition of S-9 mixture to the overlay agar. The inhibition of growth of the wild type and mutant *E. coli* showed a dose response relationship with Gentian Violet treatment. The growth inhibition of the mutant cells was consistently higher than that of wild type. Although the inhibitory activity of the Gentian Violet was reduced by S-9 in both the wild-types and the mutant cultures, it was not completely eliminated. The direct-acting mutagens inhibited growth much more in the mutant than in that of the wild type. Chinese hamster ovary cultures (CHO) were
treated with 5, 10, and 20 µg/mL of Gentian Violet with or without the presence of S-9 mixture, fraction or cofactors required for chromosome breakage assay. The presence of S-9 mixture in the above assay did not induce any increase in the chromosome damage as compared to the untreated control. The damage induced by 5 µg/mL Gentian Violet was reduced to the control level in the presence of S-9 mixture and close to the control level in the presence of S-9 fraction. When the concentration of Gentian Violet was increased to 10 µg/mL without the proportional increase in S-9 mixture, induction of chromosome damage was observed again. When the concentration was increased to 20 µg/mL, chromosomes were so badly damaged that the enumeration of the amount of damage was impossible even though S-9 was present.

In chicken embryo and mouse bone marrow in vivo systems, chromosome damage was not observed. Probably Gentian Violet was inactivated by the organisms when the concentration is relatively low. However, Gentian Violet is ingested as a feed by fowls and domestic animals. Thus a toxic effect on the epithelia of the gastrointestinal system may still exist since the dye will be metabolized only after it reaches the liver. These dyes have been found to be toxic to experimental animals (Churchman and Herz, 1913; Cutlip and Mouluk, 1967; Horsfield et al., 1976; Rosenkranz and Carr, 1971) and cell cultures (Norrby and Mobacken, 1972). Crystal Violet was also found to cause reduced RNA and protein synthesis and decreased oxygen consumption in rabbit granulation tissue (Mobacken et al., 1974).

2.2.2. Toxicity in terms of inhibition of growth

Dye industries use the activated sludge process to treat effluent. However, triphenylmethane dyes are toxic to the microbes and lessen their purifying actions. Dye
industries very frequently change the kind of dyes, so it is important for the treatment process by the acclimated microbes to maintain their adaptation to different kinds of dyes. The oxygen uptake rates of microbes, acclimatized through continuous culture in a medium containing dyes were obtained for the same type and different kinds of coexisting dyes, and the influence of these dyes was investigated by Ogawa et al. (1981). The inhibition level of the unacclimatized microbes, with Methyl Violet 2B, increases as the dye concentration increases. Compared to other classes of dyes, triphenylmethane dye Methyl Violet 2B shows a greater growth inhibitive ability. This coincides with the previous reports showing the relationship of respiratory inhibition: Basic dye > acid dye ≡ direct dye ≡ disperse dye. The acclimatized microbes showed a negative inhibition at low dye concentration and a positive one at a high concentration. This tendency was remarkable for triphenylmethane dyes like Crystal Violet and Methyl Violet 2B.

The respiratory inhibition of the microbes acclimatized to Methyl Violet 2B was measured and the acclimatized microbes showed the lowest inhibited, but inhibition was higher for the other class of dyes like Acid Orange 1. It was thought that the microbes acquired tolerance only for that class of dyes which is used for the acclimatization and fail to display any adaptability for different class of dyes. A report published by Inouye and Honda (1971), where the toxicity of waste water to acclimatized sludge was examined, described the presence of a mixture of various kinds of organic and inorganic compounds in the sludge. The inhibition of microbial growth by certain basic dyes was reported by Ogawa et al. (1988). They used Bacillus subtilis as a test organism and determined the effect of basic dyes (including Crystal Violet and Methyl Violet) on the growth rate and nucleic acid content of the cells.
They observed that both, the mean growth rate of the cell population at the logarithmic phase and the cell concentration at the stationary phase decrease, with the addition of dyes. It is also reported that triphenylmethane dyes (e.g. Crystal Violet and Methyl violet) strongly inhibited cell growth. The elucidation of inhibition mechanism by the dyes on chemical composition of cells was needed. For this purpose, they determined the nucleic acid content of the cells, harvested at both logarithmic and the stationary phase. RNA content decreased with the increasing concentration of Methyl Violet, this tendency was more remarkable in the logarithmic phase cells than the stationary phase cells, corresponding to difference in the cellular physiological activity at each phase.

2.2.3. Effect of dye on DNA and RNA content

It is known in the case of bacterial population in activated sludge that the growth rate at the logarithmic phase increases in proportion to cellular RNA content (Kaneko and Nanbe, 1973). So a similar relationship in between cell growth inhibition and of RNA synthesis in the cells was predicted. To confirm this, the ratio of decrement of RNA content to increment in the concentration of Methyl Violet was determined. The ratio indicated that the growth inhibition strongly depends on the rate of RNA synthesis. The content ratios of nucleic acid, [RNA]/[DNA], decreased with increasing dye concentration. Thus, dyes act more preferentially to lower protein synthesis than inhibit cell division. Due to the inhibitive action, cell shape varied. Cell growing under ordinary culture conditions appeared as small rods and those in the presence of dyes, as filaments. Successive batch cultures containing Methyl Violet was carried out to elucidate the acclimatization effect of the dye on the cells. The cells were inoculated successively at 12 h intervals and harvested to determine the nucleic acid content in each batch culture. It was found that DNA content increased a little with batch
number, and RNA content somewhat. The physiological activity of the cells was shown to be restored partially by acclimatization.

So, it is established that growth inhibition by triphenylmethane dyes (Crystal Violet and Methyl Violet) results from lowering the rate of nucleic acid synthesis (Ogawa et al., 1988) but the adsorption isotherms of basic dyes to cells and effects of dyes on melting temperature of DNA were measured and a correlation between these values and cell growth inhibition was determined (Ogawa et al., 1989). It was noted that dye inhibit DNA synthesis by stabilizing the double helix and by inhibiting enzyme activities. From microbes (Bacillus subtilis) cultivated in systems with and without dye, DNA was isolated. DNA of former indicated the higher melting temperature ($T_m$) than that of the latter. However, in the process of isolating DNA, a part of dye desorbed and therefore $T_m$ corresponding to the incubation conditions was not assayable. Thus using calf thymus DNA, changes of $T_m$ upon addition of dye were measured as indirect means. It was found that the value of $T_m$ increased with the dye concentration. The same phenomenon was also observed with acridine dyes such as proflavine. It has been envisaged that dye intercalate between the base pairs of DNA thus stabilizing the double helix (Gersch and Jordan, 1965). So, it can be considered that dyes increase the $T_m$ of DNA and inhibit the separation of two strands, which could be the factor responsible for the retarded growth of cells. Finally, it can be concluded that the stabilization of DNA double helix is related closely to cell growth inhibition.
2.3. ANTIMICROBIAL ACTION OF TRIPHENYLMETHANE DYES

2.3.1. Anti-bacterial action

Crystal Violet is reported to have an antibacterial action against *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus fecalis*, and *Bacillus subtilis* (Adams, 1967). Crystal Violet at a concentration of $1 \times 10^{-6}$ to $6 \times 10^{-6}$ M was used. The effect of dye measured as minimum inhibitory concentration in terms of retardation of growth, increased as the pH rose from 6 to 8. Of the four strains tested, *E. coli* was found to be most resistant to dye. The mode of action of the Crystal Violet is the formation of an un-ionized complex of bacteria with dye. Gram -ve organisms, such as *E. coli*, have high isoelectric point and contain less acidic components than Gram +ve bacteria which usually have lower isoelectric point, so the former combine with Crystal Violet less readily and are thus more resistant to dye. It was also observed that the negative charge on bacteria increased as the pH of the medium increased and the organism became more sensitive to dye.

In an interesting experiment, Shimada and Shimahara (1987) found the effect of alternating current (A. C.) exposure on the resistivity of resting *E. coli* B cells to Crystal Violet and other basic dyes. Phosphate buffer suspensions of resting *E. coli* B cells at pH 7.0 were anaerobically exposed to alternating current of 50 Hz (current density $600 \pm 60$ mA/cm$^2$) at a temperature of $34 \pm 3^\circ$C. The minimum inhibitory concentrations for eight basic dyes: Crystal Violet, Malachite Green, Brilliant Green, Magenta, Methylene Blue, Toluidine Blue, Safranin, and Acriflavine for exposed cells were decreased to about half of the values of those for unexposed ones when both types of cells were grown in the minimal medium containing one of the dyes. These
results suggested that alternating current exposure may serve as an agent which renders *E. coli* cells susceptible to the basic dyes.

### 2.3.2. Anti-fungal action

Triphenylmethane dyes have been shown to be effective in controlling fungal growth under varying conditions. Crystal Violet has also been added to poultry feed to control fungus (Migazawa, 1956; Kingsland and Anderson, 1976; Cross and Hughes, 1976). Ferenczy *et al.* (1987) used various antifungal compounds including Crystal Violet to select fungal protoplast fusion hybrids. Antifungal compounds, which irreversibly inactivate the important enzymes, were screened. The compounds may cause cell death but cell reactivation occurs after transfer of functional enzymes via protoplast fusion (enzyme transfusion). By selecting the conditions, only fusion hybrids yield colonies on plates containing those antifungal compounds. Protoplast of the *Aspergillus nidulans* strain 1156 and 1157 were tested with various concentrations of antifungal compounds for different time periods. The compounds were removed and the protoplasts were checked for integrity, inactivation, and reactivation by fusion. The compounds found to be most effective were Crystal Violet, N-ethylmaleimide, 8-hydroxy quinolinem, Malachite Green etc.

*Volvariella volvacea*, the straw mushroom has long been considered as primary homothallic species. Ultraviolet irradiation of basidiospores and mycelial fragments was used (Li and Chang, 1991) to induce Crystal Violet and Malachite Green resistant mutants. Cross-resistance to Crystal Violet and Malachite Green occurred between both classes of mutants. All wild type strains of the fungus were inhibited by 3 µg/mL Crystal Violet or by 0.5 µg/mL Malachite Green. These are the minimum inhibitory concentration (MIC) values of the two dyes. Basidiospores failed to germinate on 2
μg/mL Crystal Violet or on 0.06 μg/mL Malachite Green supplemented to potato dextrose agar (PDA). This clearly shows that Malachite Green acts as better antifungal agent than Crystal Violet for *Volvariella volvacea*.

2.3.3. Anti-yeast action

Of 44 representative isolates of yeast, isolated from the poultry upper digestive tract (Lin et al., 1989) from 40 clinical thrush cases in Taiwan during 1985-87, 39 (89%) isolates were classified as *Candida albicans* and fifteen commercial antifungal drugs, incorporated individually in Sabourand’s dextrose agar by serial two fold dilutions, were tested for their inhibitory effects against the 44 isolates. The MIC$_{50}$ of these drugs in increasing order was less than or equal to 2 ppm GU-11, 6 ppm Crystal Violet, < 16 ppm amphotericin B, 16 ppm hyamine 1622, 25 ppm econazole, 35 ppm chlorohydroxy quinoline, 40 ppm nystatin, 64 ppm miconazole, 747 ppm Malachite Green, 1550 ppm benzoic acid, 1536 ppm copper sulfate, 3144 ppm monoprop, 4951 ppm Mold Zap > 16384 ppm propionic acid and > 16384 ppm sodium propionate.

2.3.4. Anti-viral action

A strain of genus *Aquareo virus*, which belongs to the family Reoviridae, has been detected in a large number of fish and Shell fish (Hetrick et al., 1992). The susceptibility of turbot aquareovirus (TRV) to five commercially available disinfectants including Malachite Green was examined (Rivas et al., 1994). Malachite Green at a concentration of 2, 5, and 10 mg/L was used to test the ability to inactivate TRV. Malachite Green (1 to 2 mg/L) was used as a bath or flush solution for 1 h (Poupard, 1978). Results showed that a low reduction (90%, corresponding to 1 log unit) in TRV titers occurred after treatment of viral suspensions with 5 mg/L Malachite Green for up to 24 h at 15°C. No reduction in the titer was observed when lower
concentrations of Malachite Green were tested for the same length of time. This finding is in agreement with those reported for the virucidal activity of this agent against other fish viruses, confirming that such treatment does not eliminate the viral agents from the aquatic environment (Amend and Pietsch, 1972; Frerichs, 1990).

2.4. MUTAGENICITY OF TRIPHENYLMETHANE DYES

The majority of the synthetic food colors are of the mono-azo or triphenylmethane types. Thirty-seven azo, xanthene, and triphenylmethane dyes, including FD and C colors, approved for use in USA and a number of other delisted food colors were tested (Brown et al., 1978) in the Salmonella/microsome system. They tried to assess the mutagenic and possibly carcinogenic potential of certified food colors and a variety of related chemicals in short terms in vitro tests. Various triphenylmethane dyes used in this study are Brilliant Blue FCF, Benzyl Violet 4B, Bromophenol Blue, Bromocresol Green etc. and it was found that all these dyes are non mutagenic for the Salmonella/microsome test.

Several (22) arylmethane dyes (including Methyl Violet, Crystal Violet, Benzyl Violet, Cresol Red, Magenta, and Brilliant Green etc.), which have been used as food colors, commercial dyes, laboratory stains and pH indicator, were tested (Bonin et al., 1981) in the Salmonella/mammalian microsome mutagenecity assay by using five standard tester strains of Salmonella typhimurium TA98, TA100, TA1535, TA1537, and TA1538. Most of these compounds gave weak mutagenic responses with Salmonella and were positive only with narrow dose range. Food colors like Benzyl Violet, Light Green SF, and Brilliant Green showed weak mutagenic property and detected throughout in frameshift mutation detector strains TA98, IA1538 or TA 1537 and
always in the presence of the S-9 rat liver preparations. All three laboratory and commercial dyes, Basic Fuchsin (Magenta), Crystal Violet, and Methyl Violet 2B were also mutagenic. With respect to the mutagenic dyes, it is apparent that genotoxicity was only seen within narrow dose range; at higher or lower chemical concentrations no effect was seen. Basic Fuchsin (Magenta) behaved like the food dyes in inducing mutations specifically in strain TA98 and TA1538. Although there was some evidence for direct acting mutagenesis in TA98, S-9 clearly enhanced the genotoxicity of Basic Fuchsin. In direct contrast, Crystal Violet and Methyl Violet 2B were both mutagenic in strain TA1535, at low chemical concentrations, and in the absence of metabolic activation. Mutagenicity responses were negative for indicator dyes like Cresol Red, Bromocresol Purple, Bromocresol Green, and Bromophenol Blue.

So, previously the reports related to mutagenicity and toxicity of the Crystal Violet had indicated that it was negative in Bacillus subtilis rec assay (Kada et al., 1972), negative in the Ames assay with Salmonella typhimurium strains TA1535, TA1537, TA98 and TA100 (Au et al., 1979) and clastogen in 6 different mammalian cell lines (Au et al., 1978). Crystal Violet was found to be marginal positive for mutagenicity test using S. typhimurium although no clear dose/response relationship was observed (Bonin et al., 1981). Since all the strains used by both Au et al. (1979) and Bonin et al. (1981) carried rfa mutations, they are exceptionally sensitive to the toxic effect of Crystal Violet and hence would not appear to be the most suitable strains with which to evaluate the mutagenicity of Crystal Violet except of very low level. This may be particularly important if, as seems likely, the DNA damaging effect of Crystal Violet can be masked by lethality resulting from its non-DNA-mediated toxic effects. So, due to these reasons Thomas and MacPhee (1984) used two tester strains of S.
typhimurium TA1535 and DG1669. They also constructed a DNA-repair-proficient strain of *E. coli* which does not carry any cell wall defects and is highly sensitive strain for detecting mutagen. They observed that in strain TA1535, no significant mutagenic effects of Crystal Violet were observed at the relatively low doses 0.025-0.5 µg/plate, although the compound was clearly detoxified significantly when the rat liver S-9 mixture was present. By contrast, the strain DG1669 clearly showed that Crystal Violet is a mutagen causing frameshift mutations in repair-proficient bacteria. Mutagenicity was apparent with both, with and without metabolic activation, although the response was much more obvious on the plates containing S-9 mixture; presumably, this was because detoxification (which was very obviously occurring) was allowing more mutant colonies to be recovered. At dose 75-100 µg/plate, when no S-9 was present, the Crystal Violet concentration was sufficient to kill a high proportion of the cells and hence significantly lower mutant number was observed. At the corresponding doses in the presence of S-9, Crystal Violet was detoxified to such an extent that the mutant yield was similar to that observed with the lower, much less toxic, Crystal Violet concentration. They also used the various batches of Crystal Violet with varied purity (between 94 to 98%) to see if the purity or source of material affect the result; this was found that it did not appreciably affect the yield of mutants obtained. So, it can be concluded that the strain DG1663 clearly showed that Crystal Violet is a direct-acting frameshift mutagen which appears to be made even more potent as a mutagen in the presence of mammalian enzymes.

Mutagenicity of certain basic dyes including Crystal Violet was also determined by Ogawa *et al.* (1989). They used the strains of *Bacillus subtilis* H17rec' and M45rec'
for mutagenicity studies and cultivated each strain in the presence of a dye at 37°C for 8 h using liquid medium. Each cell suspension collected at a definite volume was cultivated on agar in petri plate, and mutagenicity was evaluated in comparison of both colony counts. The degree of mutagenicity R, was expressed as

\[ R = \frac{(A - B)}{A} \]

where A and B represent respectively a dye concentration when each cell count of rec+ and rec' strains are reduced to half of that without any dye. They found the mutagenicity occurred stronger with Crystal Violet and weaker with Acridine Orange NS.

2.5. PERMEABILITY AND UPTAKE OF CRYSTAL VIOLET BY DIFFERENT MICROORGANISMS

Gram -ve bacteria are generally more tolerant to antibacterial agents than Gram +ve bacteria. This tolerance has been attributed to a penetration barrier outside the cytoplasmic membrane (Hamilton-Miller, 1966; Smith, 1963). The existence of a barrier has been revealed by various chemical treatments of the cell and by the isolation of mutants. Various types of mutants were constructed to find out the permeability of Crystal Violet and its uptake mechanism by cells. Wild type strains of *E. coli* K12 adsorb Crystal Violet to the cell surface, but the dye is not transported into cytoplasm. Envelope mutants were constructed to understand the uptake of Crystal Violet (Gustafsson *et al.*, 1973). It is known that sterols are important component of biological membranes influencing membrane functions such as transport and activity of membrane bound enzymes (Demel and Dekruyff, 1976). Sterol mutants of *S. cerevisiae* were used (Bard *et al.*, 1978) to investigate the effects of altered sterol
composition on membrane permeability. These mutants were derived from the wild-type strain, *S. cerevisiae* A184D and none of the mutants require exogenous sterol for growth. To determine whether the altered sterol composition of these mutants results in altered membrane permeability, the uptake of Crystal Violet dye was measured with a final concentration of 5 μg/mL.

2.5.1. Uptake of Crystal Violet

The uptake of Crystal Violet was followed (Gustafsson *et al.*, 1973) in strain D21 and in a number of envelope mutants of *E. coli*. At 0°C, all strains instantaneously took up 20% of the 10 μg Crystal Violet added per milliliter, after which no further uptake was recorded. At 37°C, parent strain D21 showed the same uptake as that at 0°C. The envelope mutants also showed the same instantaneous uptake at 37°C as at 0°C. However at the higher temperature, the mutant exhibited a continued gradual uptake of the dye until plateau value was reached. The rate of uptake differed markedly among the envelope mutants. During these conditions, there was no change in the viable count of the wild type strain D21, whereas at least 90% of the envA cells were rapidly killed within 5 min. The other strains showed a slower and less pronounced killing. The initial rate of uptake as well as the amount of Crystal Violet found in the cytoplasm increases with the increase in the concentration of dye in the medium. The rate of transport of the dye into the cytoplasm is much lower for mutant cells at stationary phase than in exponentially growing cells. The rate of uptake into the cytoplasm increases with increasing deficiency of carbohydrate in the lipopolysaccharide. However, other components are also responsible for the barrier since an envA mutant which is not altered in the lipopolysaccharide carbohydrates show an extremely rapid uptake of dye.
Guymon and Sparling (1975) worked on antibiotic-resistant and sensitive mutants of *Neisseria gonorrhoeae* and demonstrated that Crystal Violet uptake was the result of both cell envelope adsorption and cell membrane permeability (about half of the Crystal Violet taken up by the cells was found in the cytoplasm). The amount of Crystal Violet removed from broth suspension by cells at 0 and 37°C was measured for various wild-type, antibiotic-resistant, and hypersensitive strains. All strains removed approximately equal quantities of dye at 0°C. This value range from 15 to 25% of the total dye in the solution. At 37°C, however, the amount of dye absorbed by the strains varied. The naturally occurring and ultra violet-induced multiple drug resistant (MDR) mutants removed little or no dye in excess of the absorbed at 0°C (i.e. 15 to 25%), whereas wild-type sensitive strains absorbed approximately 35 to 45% of the dye available and several antibiotic-hypersensitive (*env*) mutants removed 50 to 75% of the dye from the solution. They calculated this as the difference in percentage of dye removed at 37 and 0°C, respectively.

In two of the best studied examples, outer membrane mutants of *E. coli* (Gustafsson *et al.*, 1973) and *Salmonella minnesota* (Alexanian *et al.*, 1974) with increased sensitivity to antibiotics were also more permeable to Crystal Violet. In *Salmonella*, the extent of alteration of lipopolysaccharide (LPS) was directly correlated with the total amount of the Crystal Violet absorbed. In *E. coli*, alteration of LPS structure were best correlated with initial rates of the Crystal Violet uptake, although total uptake differed in several mutants. In *Neisseria genorrhoeae*, initial rate of Crystal Violet uptake was extremely rapid and no appreciable difference between wild type and *env*-2 mutant FA 47 was seen.
In sterol mutants of *S. cerevisiae* (Bard et al., 1978), at 0°C, there was virtually no uptake of Crystal Violet by the wild type strain A184D and only 2 and 3% uptake by *erg2* and *erg3*, respectively. At 30°C, all strains showed significant Crystal Violet uptake. At 35°C, *erg6* and *erg6/2* showed no increase in the dye uptake relative to that observed at 30°C and only small increment was obtained for *erg2*, *erg3*, and A184 D. The variation in dye uptake in the wild-type and mutant strains was as a result of change in membrane sterol composition. They interpreted these differences as alterations in membrane permeability rather than cell wall alterations. It is significant that a change in membrane composition due to altered membrane sterols also leads to an increased sensitivity to cations.

### 2.5.2. Model for uptake of Crystal Violet

Gustofsson *et al.*, (1973) proposed a model for the uptake of Crystal Violet. According to them, (i) first there is a strong binding of Crystal Violet, presumably, to negative groups on the surface of the bacteria. (ii) when these sites are saturated, more Crystal Violet molecules bind to the surface, but these molecules are less tightly bound to the cells. Hence, the firmly and the loosely bound molecules are located outside the penetration barrier. (iii) In the envelope mutants, Crystal Violet molecules are allowed to pass the barrier. They believe that this passage is through simple diffusion and it results in an increase in the number of Crystal Violet molecules at the outside of the cytoplasmic membrane. (iv) The Crystal Violet molecules present outside the cytoplasmic membrane are then transported in to the cytoplasm through the membrane by an unknown process. (v) In the cytoplasm, the majority of the Crystal Violet molecules bind to the ribosomes. This binding leads to a reduction in the concentration of Crystal Violet in solution in cytoplasm. The explanation for the toxicity of the
Crystal Violet is a cation that binds rapidly to negatively charged particles such as ribosomes. The ribosomes to which the dye is bound function less efficiently in protein synthesis.

2.6. OCCUPATIONAL HEALTH PROBLEMS CAUSED BY DYSES

There is a risk of exposure to chemicals because dye manufacturing is generally a batch process with no or limited automation and large manual handling is involved (Desai, 1993). Thus there is an interaction between chemicals, man, and occupational health problems.

2.6.1. Irritation, sensitization, and pigmentory disorder

These effects are mainly observed on the skin. The condition is called Dermatitis. The irritants act by direct action on normal skin. The common irritants act by combining with some protein molecules in the skin. Sensitizer means that there are no changes at the first contact of the chemical but they occur after 7-10 days on the site of contact or elsewhere. This is a condition of hypersensitivity. For some group of chemicals, the skin changes are mainly of pigmentory nature. The change occurs following prolonged contact of the compound to skin together with the exposure to sunlight. This results in hyper pigmentation in the affected area.

2.6.2. Effect on blood and haemopoetic system

Some dye intermediates like benzene produce changes in the various components of blood either after short exposure or on prolonged exposure. The effects seen after prolonged exposure are reduction in the number of red blood cells and haemoglobin content of the blood, causing anaemia. The toxic effects may cause bone-marrow depression resulting in the reduction in the number of all types of cells of the blood.
This condition is called aplastic anaemia. The reduction of platelets will cause haemorrhagic disease of various organs. The prolonged absorption of benzene can produce leukaemia in few cases.

2.6.3. Nephrotoxic and hepatotoxic effects

There are certain chemicals related to dye-stuff industries which can cause injury to the kidney. This may be transient following an acute exposure. There are others, which produce irreversible kidney damage following an exposure over some years. The toxic effects can be detected by regular urine examination. The urine picture will show the presence of albumin, RBC, and WBC. The injury to the liver cells occurs following absorption of certain chemicals. The offending chemical causes the disturbance of the liver function. The symptoms may be mild loss of appetite to severe jaundice.

2.6.4. Cyanosis

Cyanosis means the deprivation of the availability of oxygen to the tissues. This effect will be evident by a peculiar blue discoloration of the skin and mucous membrane. In dye-stuff industry, certain chemicals cause "methaemoglobinemia" by conversion of hemoglobin to methaemoglobin. The condition is also called anilism. This name itself is derived from aniline, which is a well known cyanosing agent. The affected person feels weakness, headache, and giddiness after a mild exposure. Serious poisoning leads to collapse, loss of consciousness, and even death. The cyanosing compounds mainly enter the body through the skin.

2.6.5. Pulmonary effect

The effect on the lungs commonly seen in the dye-stuff industry is following exposure to noxious gases. They may have direct irritant effect on the lungs or systemic toxic effect after their absorption from the lungs. In case of nitrous fumes and phosgene,
there may be delayed pulmonary effects also. HCN and H₂S have systemic effects which may cause fatality if not treated in time. The dust like nature of dye may cause asthma like condition arising due to its exposure.

2.6.6. Carcinogen

The greatest tragedy of dye-stuff industry has been the occurrence of occupation tumors of urinary bladder (papilloma). They are usually malignant. There is a long latent period (20 to 25 years) between the first exposure and ultimate development of tumor. The early diagnosis influences prognosis.

2.7. METHODS OF DYE-STUFF EFFLUENT TREATMENT

Various effluent treatment methods from dye-stuff industries may be classified broadly into three main category: physical, chemical, and biological (Table 2). There are three stages of treatment namely primary, secondary, and tertiary treatment processes, which differ mainly by the number of operations performed on the waste streams. Primary treatment processes of dye waste include equalization, neutralization, and possibly disinfection. Primary stages are mainly physical and include screening, sedimentation, floatation, and flocculation. The objective is to remove debris, undissolved chemicals, and particulate matter. Secondary stages are used to reduce the organic load. Tertiary stages are important because they serve as a polishing of the effluent treatment. These methods are adsorption, ion exchange, chemical oxidation, reverse osmosis, electrochemical etc. (Reife, 1993).
Table 2: Various methods for the treatment of dye waste water (Reife, 1993)

<table>
<thead>
<tr>
<th>Physical</th>
<th>Chemical</th>
<th>Biological</th>
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<tbody>
<tr>
<td>Adsorption</td>
<td>Neutralization</td>
<td>Stabilization ponds</td>
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<tr>
<td>Sedimentation</td>
<td>Reduction</td>
<td>Aerated lagoons</td>
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<tr>
<td>Floatation</td>
<td>Oxidation</td>
<td>Trickling filters</td>
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<tr>
<td>Flocculation</td>
<td>Electrolysis</td>
<td>Activated sludge</td>
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<tr>
<td>Coagulation</td>
<td>Ion exchange</td>
<td>Anaerobic digestion</td>
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<tr>
<td>Foam fractionation</td>
<td>Wet-air oxidation</td>
<td>Bioaugmentation</td>
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<tr>
<td>Polymer flocculation</td>
<td>Ozonization</td>
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<td>Reverse osmosis/Ultrafiltration</td>
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<td>Ionization radiation</td>
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<td>Incineration</td>
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2.7.1. Physical methods

2.7.1.1. Adsorption

Adsorption is an effective means of lowering the concentration of dissolved organics in effluent. Activated carbon is the most widely used and effective adsorbent for dyes. Commercial activated carbon can be prepared from lignite and bituminous coal, wood, pulpmill residue, and coconut shell and have a surface area ranging from 500-1400 m$^2$/g. Investigations have been undertaken to evaluate the cheap alternative materials as potential adsorbents for dyes using activated carbon as a reference. These include peat, corn stalks, chitin, carbonized wool, bagasse pith, bentonite, calcium metasilicate, organosilicon, clays, activated alumina, pig and human hair, meat, bone meal, wheat and rice bran, and turkey feathers. Activated sludge which is discussed under biological treatment, has been found to show behavior similar to that of activated carbon in the adsorption of acid, direct, reactive, disperse, and basic dyes. The adsorbability of dyes by activated sludge is mainly dependent on dye properties, molecular structure, type, number, and position of the substituents in the dye molecule. Adsorption is increased by the presence of hydroxy, nitro, and azo groups. On the other hand, adsorption is decreased by sulphonic acid group.

2.7.1.2. Sedimentation

This is the traditional method of treating wastewater in lagoons and uses the force of gravity to remove the settleable solids. These solids are separated out as a watery sludge which is removed mechanically.

2.7.1.3. Floatation

Floatation is used to remove suspended solids from wastes and for the separation and concentration of sludge. The waste flow is pressurized in the presence of sufficient air.
to approach saturation. When the pressurized air-liquid mixture is released to atmospheric pressure in the floatation unit, minute air bubbles are formed. As they rise in the liquor, the sludge flocs and suspended solids are floated to the surface where the air-solid mixture can be skimmed off.

2.7.1.4. Flocculation

Flocculation uses chemical precipitation to affect separation. It is used to increase the rate of sedimentation and floatation. The resulting material known as floc can be removed by filtration, sedimentation, or floatation.

2.7.1.5. Polymer flocculation

Polymer flocculation plays an important role in the removal of dyes from the effluent. Polymer flocculation is the aggregation or the coagulation and subsequent removal of the dye using a suitable synthetic non-ionic, cationic, or anionic polymer. Basic dyes naturally can not be decolorized by cationic polymer flocculants. Using Armour’s cationic polymer Armeen C and carbon adsorption the total organic carbon (TOC) of four azo dye effluents were reduced to 80-96.3% and showed no detectable dye in the effluent.

2.7.1.6. Coagulation

Coagulation results from lowering the zeta potential at the particle surface to permit closer approach, followed by association of the particles to form large flocculated agglomerates. Coagulation is an economical and feasible method of treating dye waste, especially in the removal of color. The main inorganic coagulant used is lime. Other coagulants used are calcium hydroxide, calcium sulfate, magnesium hydroxide, magnesium sulfate, ferric chloride, ferrous sulfate, aluminium sulfate (alum), and a
combination of these inorganic salts. The ferric salts, although commonly used as coagulants, has the disadvantages of being difficult to handle.

2.7.1.7. Foam fractionation

This method is based on the phenomenon that surface active solutes collect at gas-liquid interphase. The results were 86-96% color removal from a brown disperse dye solution and 75% color removal from a textile mill waste water. But the chemical costs make this method relatively expensive.

2.7.1.8. Reverse osmosis and ultrafiltration

Reverse osmosis (or hyperfiltration) and ultrafiltration are pressure driven membrane processes that have become well established in pollution control. There is no sharp distinction between the two: both processes remove solutes from the solution. Whereas ultrafiltration usually implies the separation of macromolecules from relatively low molecular weight solvent, reverse osmosis normally refers to the separation of the solute and solvent molecules within the same order of magnitude of molecular weight. Cellulose acetate, the earliest reverse osmosis membrane is still being widely used, whereas polysulfone is the most practical membrane material in the ultrafiltration applications.

The earliest reverse osmosis and ultrafiltration units were based on flat membrane sheets in an arrangement similar to that of a plate and frame filter press. Since then, more efficient membrane configurations, i.e. tubular, spiral wound, and hollow fiber have emerged. Another type of membrane is the dynamic membrane, formed dynamically by coating a selective membrane layer on a fine porous support. Advantages of these membranes are high water flux, generation and regeneration in situ, ability to withstand elevated temperatures and corrosive feeds and relatively low
capital and operating costs. Reverse osmosis and ultrafiltration are very effective for the removal of all types of color from dye house discharge, decolorization in the order of 95-100% is readily obtained. However, clogging of the membranes with concentrated dyes after prolonged use is a problem. About 10-25% of the original volume of the waste water treated becomes concentrate which if it cannot be reused has to be disposed off by incineration. Although the high capital costs and limited life of membranes are disadvantages of hyperfiltration and ultrafiltration, the continuing increase in the cost of dyes, auxiliary chemicals, water, energy, and demanding regulations governing waste water discharge make recovery and reuse by these systems more economically attractive.

2.7.1.9. Ionizing radiation

Gamma irradiation has been used to decolorize dye-house waste water. Most of the dyes showing resistance oxidation or reduction can be degraded by ionizing radiation. The rate of reaction is controlled by the radiation dose and the availability of oxygen in the solution. For optimum efficiency, the radiation should be totally adsorbed by the organic waste. One approach to this is the adsorption onto activated charcoal before exposure to gamma radiation.

2.7.1.10. Incineration

Although incineration or thermal treatment of waste has met opposition in USA because of fear of air emission of toxic pollutants and concentration of heavy metals in the resulting ash, Europe and Japan readily use this method of waste disposal. Its modification described that dyeing waste water treated with Fenton’s reagent (hydrogen peroxide plus ferrous sulfate) is allowed to coagulate the dye and the resulting sludge is then incinerated and iron recovered from ash is then recycled.
2.7.2. Chemical methods

2.7.2.1. Neutralization

The choice of a reagent for pH adjustment depends on the cost, ease, effectiveness, and availability. The three principal reagents for neutralization of acid wastes are sodium hydroxide, sodium carbonate, and hydrated calcium hydroxide. The waste water of many textile mills are alkaline and the two most commonly employed reagents for pH adjustment are sulfuric and hydrochloric acids. Another reagent gaining wide acceptance for neutralizing alkaline waste water in textile production is the carbon dioxide. The use of carbon dioxide is safe, effective, and more economical than sulfuric or hydrochloric acids for neutralization. When CO₂ is dissolved in an aqueous solution, H₂CO₃ (carbonic acid), HCO₃⁻ (bicarbonate) and CO₃²⁻ (carbonate) buffer system is formed.

2.7.2.2. Reduction

Many dyes, particularly azo dyes, are susceptible to destructive reduction. The reducing agents that can be used are sodium hydrosulfite, thiourea dioxide, sodium borohydride, zinc sulfoxylate, and ferrous ions. The reduction of dyes, and especially reactive dyes into their respective amines is a well known analytical technique. For example, using zinc hydrosulfite for the decolorization of dyed paper stock resulted in 90% color reduction for azo direct dyes. Thiourea dioxide as well as sodium hydrosulfite are mainly used in the dyeing industry as reducing agents in vat dyeing. Thiourea dioxide can also be used as a reducing agent to decolorize a number of direct dyes for dyeing paper.
2.7.2.3. Oxidation

Oxidation is one of the main chemical methods to treat and decompose dyes in waste water. The oxidizing agents used are chlorine, bleach, ozone, hydrogen peroxide, Fenton's reagent, and potassium permanganate. Chlorination was found to be the most suitable and effective method for decolorizing and reducing the COD of waste dye-baths containing azo dyes. Reactive and acid dyes were readily destroyed by chlorination but direct and disperse dyes reacted slowly. Although, chlorination is cheaper than ozonization, the possible formation of chlorination compounds such as dioxin and its environmental impact cannot be overlooked. For color removal, ozonization has achieved the greatest practical importance. Ozonization in combination with treatments such as coagulation, flocculation, carbon adsorption, UV-irradiation, and gamma radiation significantly and successfully remove dye wastes and reduce cost.

The reaction between ozone and organics does not depend on the ozone concentration, but rather on the concentration of the decomposed products of ozone. The free radicals and ions formed by ozone degradation are the chief reacting species with organics. Generally, the final products of ozonolysis are benzene, chlorobenzene, phenol, nitrogen, phthalic anhydride, and phthalide.

Uncatalyzed hydrogen peroxide is used commercially to destroy pollutants. Without activation, however, hydrogen peroxide will not destroy the more difficult to oxidize pollutants. Hydrogen peroxide can be activated to form hydroxyl radicals, OH*, which can destroy these organics. This increased ability to destroy organics is related to the substantially higher oxidation potential of the hydroxyl radical (2.80 V) as compared to that of hydrogen peroxide (1.78 V). The method of generating hydroxyl radicals is by adding soluble iron salts to an acid solution of hydrogen peroxide (Fenton's reagent).
Hydrogen peroxide can also be activated by ultra violet radiation or ozone.

2.7.2.4. Wet air oxidation

Wet air oxidation refers to the aqueous phase oxidation of organic and inorganic materials at elevated temperatures and pressures. Oxidation takes place through a family to related oxidation and hydrolysis reactions at temperatures of 175-320°C and a pressure of 2-20 Mpa (300-3000 psi). The enhanced solubility of oxygen in aqueous solution at elevated temperature provides a strong driving force for oxidation. The source of oxygen is compressed air or high pressure pure oxygen.

The wet air oxidation process can treat a wide variety of oxidizable materials. The primary products of oxidation are carbon dioxide and water. Sulfur is oxidized to sulfate which remains in the aqueous phase. Organic nitrogen is converted primarily to ammonia. No sulfur or nitrogen oxides are formed. Metals are generally converted to their higher oxidation states and remain in the aqueous phase as dissolved or suspended solids. Halogens also stay in the aqueous phase. The gas discharged from a wet air oxidation unit consists mainly of spent air and carbon dioxide and is essentially free of any air polluting constituent. So, the wet air oxidation offers an alternative to conventional incineration for the destruction and detoxification of dilute hazardous and toxic waste waters. A 98% removal efficiency of dye-house effluent has been observed by wet air oxidation.
2.7.2.5. Electrolysis

Electrolytic precipitation of the concentrated dye wastes by reduction in the cathode space of an electrolytic bath had been tried, but extremely long contact times were required. An electrochemical process was developed for removal of several classes of dyes including disperse, reactive, acid, and textile auxiliaries. The dyes are converted to insoluble complexes that are floated to the surface of the water and later removed from it. In the treatment process, waste water is circulated between the two iron electrodes. An electric current is passed between the electrodes, slowly removing iron from the anode, the reaction releases ferrous and hydroxide ions, thus:

\[
\text{Anode (oxidation)} \quad \text{Fe} \rightarrow \text{Fe}^{2+} + 2e^- \\
\text{Cathode (reduction)} \quad 2\text{H}_2\text{O} + 2e^- \rightarrow \text{H}_2 + 2\text{OH}^- \\
\text{Overall (reaction)} \quad \text{Fe} + 2\text{H}_2\text{O} + \text{electrical energy} \rightarrow \text{Fe(OH)}_2 + \text{H}_2
\]

The ferrous ions that dissolve from the anode combine with the hydroxide ions produced at the cathode to give an iron hydroxide precipitate. The active surface of ferrous hydroxide can adsorb a number of organic compounds as well as heavy metals from the waste water passing through the cell. The iron hydroxide and adsorbed substances are then removed by flocculation and filtration. The separation process was enhanced by the addition of a small quantity of an anionic polymer.

2.7.2.6. Ion exchange

Ion exchange treatment entails elution of waste water through a suitable resin until the available sites for ion exchange become fully occupied and the contaminated ions appear in the outflow. Treatment is then stopped. The bed is back washed and then regenerated using an appropriate acidic and basic solution. A further washing is given to remove excess regenerant and the bed is ready for the next treatment cycle.
Although highly effective for eliminating dissolved contaminants of known constitution, like toxic metal ions, this technique is clearly unsuitable for large volume multicomponent effluents.

2.7.3. Biological methods

There are two methods of biological treatment, aerobic and anaerobic. The aerobic system use free oxygen dissolved in the waste water to convert wastes in the presence of microorganisms to more microorganisms, energy required for their existence and carbon dioxide. The anaerobic process occurs in the absence of free oxygen and converts the waste to methane and carbon dioxide, generally in deep tank or basins and can produce odor problems when sulfides or sulfates are present in the waste water.

2.7.3.1. Aerobic processes

The four most common aerobic biological treatment processes are stabilization ponds, aerated lagoons, trickling filters, and activated sludge.

2.7.3.1.1. Stabilization ponds

Waste stabilization ponds are a low cost and low technology process, but highly efficient method of dye waste water treatment (Mara and Pearson, 1986). Stabilization ponds have a water depth of 1-2 m and oxygen is supplied by surface entrainment or by algae. The BOD loading must be low and the detention time is 5-25 d. There are three main types of ponds and they are usually arranged in sequence for waste water treatment process. Anaerobic pond is usually 2 to 5 m deep and receives raw waste water. Due to high BOD loading, these ponds are completely devoid of dissolved oxygen. Facultative ponds are 1 to 2 m deep. They have a lower anaerobic zone and an upper aerobic zone where oxygen for bacterial metabolism is largely provided by the photosynthetic activity of algae. Maturation ponds are 1 to 2 m deep in which the
facultative pond effluent is treated further. Also, there is some additional removal of BOD.

2.7.3.1.2. Aerated lagoons

Aerated lagoons are 2-5 m liquid depth depending on the aeration system and detention times are 2-10 d. They are mainly used because of their efficiency in removing BOD from textile effluents.

2.7.3.1.3. Trickling filters

Trickling filters are cylindrical tanks packed either with stone or with synthetic medium. The effluent flow onto the filter media by means of a rotating arm that distributes the waste load uniformly over the circular bed. The effluent trickles through the filter and over a slime of bacteria that adheres to the filter media. As bacteria die, they fall off the filter and are removed from the effluent throughout the secondary settling stage. The removal efficiencies depend on the type of media used, the organic loading ratio, the ratio of raw waste to recycle waste water, and operating temperature.

2.7.3.1.4. Activated sludge

Of the aerobic biological treatment methods available, the activated sludge process and its various modifications are the most popular. The activated sludge process relies on microorganisms in suspension to oxidize soluble and colloidal organics with molecular oxygen. Many variables affect the performance of the system including pH, temperature, dissolved oxygen concentration, detention time, nutrients, and the presence of toxic components in the raw waste stream. Although the activated sludge process is capable of providing high BOD, COD, TOC, and TSS removal, it is less effective for color removal. Since synthetic dyes used by the textile industries are
specially formulated to resist breakdown under oxidizing conditions, most dyes are resistant to biological degradation. The color removal in the activated sludge process is primarily due to adsorption of the dyes by sludge to the extent of 40-80% or more depending on the individual dye-stuffs and treatment conditions. Complete removal or decolorization can only be achieved by combination with other treatment processes.

Adding powdered activated carbon to the activated sludge process can be beneficial, i.e., more uniform operation and uniform effluent quality, improved BOD, COD and TOC removal, better removal of phosphorus and nitrogen, less tendency of foaming in the aerator. The mechanism by which the pollutant removal is increased with powdered activated carbon addition is not fully understood. The assumption is that the activated carbon aids by direct adsorption of pollutants and by providing a more favorable environment for the microorganisms to propagate. Soluble organic carbon (SOC) and color removal was enhanced by the addition of powdered activated carbon to an activated sludge system, generally in direct relation to the steady-state concentration of powdered activated carbon in the reactor.

2.7.3.2. Anaerobic digestion

Following biological treatment, the sludge containing adsorbed dyes may be digested under anaerobic conditions. Anaerobic digestion treatment of reactive dye manufacturing waste water in a multisectional bioreactor removed 88% color and produced an effluent suitable for further biological treatment in conventional system. The addition of excess activated sludge to waste water storage tank at a pretreatment stage decrease COD and BOD by 48.2 and 59%, respectively.
2.7.3.3. Bioaugmentation

Bioaugmentation is obtaining maximum biodegradability by addition of non-indigenous microbial cultures in order to have optimum bacterial biomass. This offers flexibility in dealing with the problem of bacterial acclimatization, toxicity of compounds, and restart of the system. Biodegradation of various azo and triphenylmethane dyes and dye effluents were observed using specific strains of bacteria or fungi (Sani et al., 1998).

2.8. TREATMENT OF EFFLUENT FROM TEXTILE AND DYE-STUFF MANUFACTURING INDUSTRIES

Chemical processes are extensively used for the decolorization of effluent from different dye manufacturing and dyeing industries. These effluents generally do not undergo aerobic biodegradation during sewage treatment process. Due to the presence of the dyes in the effluent, an assessment of their bioaccumulation in fish is a requirement under certain environmental chemical legislation. Under the Japanese “chemical substances control act (1974),” the compounds which are not readily biodegradable must pass a bioaccumulation test in fish (Kubota, 1979). The fish is an appropriate test animal because of its position in the food chain, also because, especially in some communities, it form an important constituent of the human diet. The partition coefficient in n-octanol/water is a useful indicator of the bioaccumulation tendency of dye-stuffs (Anlinker et al., 1981).

Matsui et al. (1984) reported the ozonization of Malachite Green, a model compound of triphenylmethane dyes. Reactions of dyes with ozone were studied from the treatment point of view. Such reactions are looked at in terms of reduction of
chemical oxygen demand (COD) and the decolorization. However, the ozonization mechanism has scarcely been touched. Reaction involving triphenylmethane dyes have been discussed (Porter and Spears, 1970) regarding photofading. The photo reaction of Malachite Green in water has been reported to give 4-(dimethylamino) benzophenone, the carbinal base of Malachite Green and p-(dimethylamino) phenol. The photoreaction of Crystal Violet has been reported to give p-(dimethylamino) phenol and 4,4' - bis (dimethylamino) benzophenone, the leuco and demethylated derivative of Crystal Violet (Kuramoto and Kito, 1982; Nakamura and Hida, 1982). Textile and dye-stuff industrial wastes having different dyes are generally treated by physico-chemical methods. Various properties of dye house waste water is given in Table 3 (Kothandaraman et al., 1976). Various treatment methods reported in literature such as adsorption, chemical precipitation and flocculation, oxidation by chlorine, hydrogen peroxide, and ozone, electrochemical treatment, and ion pair extraction. But none of them has been found to be very suitable as they produce a large quantity of sludge. All these methods possess significant differences in color removal result, volume capability, operating speeds, and cost (Cooper, 1995). For example, activated charcoal is extremely effective for removing color, but is incapable of treating large effluent volume, operates at slow speeds and has high capital cost. Membrane technology, ozone treatment, coagulation, and flocculation permit good color removal in large effluent volume. Membrane technology is fast, but the capital cost for implementing this technology is high. Ozone treatment operates at moderate speeds and also require high capital investment, coagulation and flocculation techniques operate at moderate to fast speeds and require a somewhat lower capital investment. But the biological methods are having many advantages such as possibility
Table 3: Characteristics of dye house waste water (Kothandaraman et al., 1976)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Maximum (mg/L)</th>
<th>Minimum (mg/L)</th>
<th>Average (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkalinity</td>
<td>3160</td>
<td>340</td>
<td>1375</td>
</tr>
<tr>
<td>Total solids</td>
<td>7130</td>
<td>700</td>
<td>3030</td>
</tr>
<tr>
<td>Total dissolve solid (TDS)</td>
<td>6180</td>
<td>680</td>
<td>2720</td>
</tr>
<tr>
<td>Suspended solid (SS)</td>
<td>950</td>
<td>20</td>
<td>310</td>
</tr>
<tr>
<td>Biochemical oxygen demand (BOD)</td>
<td>850</td>
<td>20</td>
<td>220</td>
</tr>
<tr>
<td>Chemical oxygen demand (COD)</td>
<td>1910</td>
<td>55</td>
<td>850</td>
</tr>
</tbody>
</table>
of degradation of dye molecules to carbon dioxide and water, formation of less sludge and they are environment friendly. Ogawa et al. in 1981 reported the use of activated sludge system for the treatment of dye wastes. Dye industries use the activated sludge processes to treat effluent. However, some dyes are toxic to the microbes and lessen their purification function. They have also reported the elimination of Methyl Violet and Crystal Violet up to 80-100% by continuous culture of activated sludge.

The fate of Congo Red, Orange II and Crystal Violet was examined (Idaka et al., 1985) in the activated sludge which was previously acclimatized with the medium and dyes. They also studied the effect of dye concentration on the acclimatized sludge. They observed that with increase in dye concentration, the color loss was decreased.

The inhibition of growth and respiration of activated sludge by various dyes were studied (Ogawa et al., 1978) and also the inhibitory characteristics of dyes were examined. These tests were carried out on unacclimatized sludge. They also used (Ogawa et al., 1981) sludge acclimatized to different triphenylmethane dyes and having adaptability to different concentrations of dyes.

Eighty seven dye-stuffs have been tested in short term aerobic biodegradation tests (Pagga and Brown, 1986) to investigate whether some dye-stuffs might be susceptible to aerobic biodegradation and, if so, to what extent this occurs? For this work, the dye-stuffs chosen were typical commercial products and bacterial inocula were from effluent treatment plant. As in the static tests, the criteria for biodegradation were both decolorization at the absorption maxima and dissolved organic carbon (DOC) elimination. Their results confirmed that dye-stuffs are most unlikely to show any significant biodegradation in such tests (Figs. 2 and 3). With many dye-stuffs a substantial color removal was observed which may be attributed to the elimination of
Fig. 2. Elimination of color from textile and dye-stuff industrial waste

Fig. 3. Elimination of dissolved organic carbon (DOC) from textile and dye-stuff industrial waste
dyes by adsorption. In some cases DOC removal did not correlate with the color removal and this is attributed to the presence of non-colored organic components in the dye-stuffs.

Generally the dye-stuff waste water were effectively degraded by the continuous submerged cultures of microbes. These methods had, however, a shortcoming that it was difficult to separate the microbes from the effluent due to the low rate of sedimentation. Ogawa and Yatome (1990) treated the dye waste water by a rotating biological contactor with disc on which *Pseudomonas cepacia* 13NA was immobilized with κ-carrageenan gel. They found that dye decolorization activity was stable for long time. The COD value reduced to one third at 10 h retention time, while at 20 h retention time, 4.5 times reduction in COD value was observed. Two fungi, *Myrothecium sp.* and *Ganoderma sp.* were used as microbial agents for the decolorization of dye waste water (Mou *et al.*, 1991). For decolorization, cells were grown for 7 to 14 d. Afterwards, dye decolorization tests were performed and the mixtures were allowed to stand for 2 d. Dye molecule adsorption onto cell surface appears to be a quick process and generally gets completed within an hour. They found that this process is not specific and acid, basic, direct, reactive, and disperse dyes could all be cleared out of solution using same approach. There is no specific nutrient requirement and is insensitive to high salt concentration or wide variations in pH. The degradation of azo dyes by algae was evaluated (Jinqi and Houtian, 1992) and they found that certain algae can degrade a number of azo dyes to some extent (50-100%). The algae used were *Chlorella pyrenoidosa*, *C. vulgaris*, and *Osillatoria temuis*. The reduction rate of azo dyes appears to be related to the molecular structure of the dyes and species of algae used. They found that azoreductase of the algae is responsible for
degrading azo dyes into aromatic amines by breaking the azo linkage, which are then subjected to further metabolism by algae. The highest decolorizing activity is observed in the culture fluid which is free of inorganic carbon and nitrogen. This suggests that algae can utilize dye as its sole source of carbon and nitrogen and the process of azo compound degradation is related to physiological metabolism of algae. Apart from algae, various actinomycetes strains have also been identified which decolorize effluent containing different types of reactive dyes (Zhou and Zimmerman, 1993). Adsorption of the anthraquinone, phthalocyanine, and azo dyes to the cells of some of the strains resulted in the decolorization of the effluent, but no degradation of the dye was observed. All of the dyes which have been removed from the medium could be released from the cells after their solubilization. The amount of dye adsorbed on the cells was determined by washing the cells several times with water, methanol, ethanol, and acetone. The cells were then suspended in 4 N NaOH and disintegrated by ultrasoincation. After centrifugation, the absorbance of the solubilized dye in the supernatant was measured. In contrast, effluent containing an azo-copper complex and a formazan copper complex dye were almost completely decolorized by several of the strains without adsorption to the cells.

A white rot fungus *Phanerochaete chrysosporium* was used to decolorize an artificial textile effluent (Kirby *et al.*, 1995). This fungus decolorized 6 out of 9 synthetic textile dyes in the presence of glucose. Three textile dyes were decolorized in the absence of a primary carbon source, glucose. These results suggest that whilst a primary carbon source, such as glucose, is essential for extensive dye decolorization, certain dyes may be metabolized as sole carbon and energy sources by *P. chrysosporium*. In the presence of an artificial textile effluent (0.5 g/L) dye decolorization commenced at day
5 and was completed by 7 d. It is found that the effluent decolorization shows both a significant decrease in color intensity and a shift towards the UV-region. Previously it has been suggested that some decolorization can result from dye binding to fungal mycelium (Cripps et al., 1990). This phenomenon accounts for only a small percentage of color removal observed in these experiments. However, the role of lignin peroxidase in these cases was not very clear.

Nigam and Marchant (1995) used the textile dye-decolorizing facultative anaerobic bacterium PDW to develop biofilm on abundantly and cheaply available substrates. The log phase culture was used for biofilm formation. The materials tested were able to build up biofilm in 3 d. This biofilm system was compared for its decolorization efficiency in packed bed system. The author took two main criteria into account to select the best material, faster decolorization and intactness of biofilm. It was observed that biofilm system on mineral kissiris, α-biofixation material, and gravel did not lose cells whereas the effluent was turbid in other systems, showing lots of cells. A faster decolorization was achieved with films on foam, vermiculite, and nylon web but their biofilm was not sufficiently stable for long term use. The microbial consortium PDW was capable of rapid decolorization of effluent from the textile industry under anaerobic conditions (Nigam et al., 1996b). Decolorization of textile dyes by PDW was shown to be dependent upon the presence of an additional carbon and energy source. PDW rapidly decolorized 8 out of 9 textile dyes by at least 67% within 24 h of incubation at 26°C in the presence of yeast extract (5 g/L), however, no decolorization was observed when the dyes were present as sole source of carbon and energy. It was observed that PDW was capable of dye decolorization while utilizing cheap and readily available carbon sources such as lactose, starch, and distillery waste. PDW removed
76% of color from textile plant effluent after 3 d. Using the same microbial consortium, a simple and practical biological process for the decolorization of colored effluent from a textile company is described (Nigam et al., 1996a). A number of aerobic and anaerobic cultures capable of decolorizing dyes in textile effluent samples were isolated after prolonged enrichment of cultures from textile dye effluent samples. It was observed that the availability of a supplementary carbon source seems to be necessary for faster growth and decolorization. The extent of decolorization was less in effluent dye mixture sample as compared to the individual dye component. These decolorization processes were permanent with no color change upon exposure to air. This microbial treatment process is simple, fast, and economical for the decolorization of dyes and may have potential applications for the biotreatment of colored effluent from textile and dye industries.

2.9. FATE OF DYES

In 1980, approximately 111,000 tons of synthetic organic dye-stuffs were produced in the United States alone. In addition, another 13,000 tons were imported. The largest consumers of these dyes is the textile industry accounting for two-thirds of the market. Recent estimates indicate that 12% of the synthetic textile dyes used yearly are lost to waste streams during dye-stuff manufacturing and textile processing operations. Approximately 20% of these losses enter the environment through effluent from waste water treatment plants. With few exceptions, the normal use of organic colorants poses few problems in terms of acute ecological effects. On the other hand, certain dye-stuffs exhibit toxic effects towards microbial populations and can be toxic and/or carcinogenic to animals. Also the possible contamination of drinking water supplies is
of concern because certain classes of dyes are known to be enzymatically degraded in the human digestive system, producing carcinogenic substances. U. S. Environmental Protection Agency (USEPA) and Ecological and Toxicological Association of the Dye-stuff Manufacturing Industry (ETAD) jointly carried out the work on the fate of the dyes in the environment (Reife, 1993).

Fate studies on eighteen dyes were done by the Water Engineering Research Laboratory of USEPA. The objective was to determine the partitioning of the water soluble azo dyes in the activated sludge process (ASP). Specific azo dyes were spiked at 1 and 5 mg/L to pilot scale treatment systems with both liquid and samples collected. Samples were analyzed by high pressure liquid chromatography (HPLC) and a UV-VIS detector. Mass balance calculations were made to determine the amount of dye in waste activated sludge (WAS) and in the activated sludge effluent (ASE). Of the eighteen dyes studied, eleven compounds passed through the ASP substantially untreated, four were significantly adsorbed into the WAS and three were apparently biodegraded. A study on the degradation of two dyes, Disperse Orange 5 and Disperse Red 5 showed reduction of the azo linkages to aromatic amines and further dealkylation to p-phenylenediamine. The study of Direct Red 28 in a sediment water system indicated that the amount of recovered benzimide accounted for only 2-5% of lost dye. For Reactive Blue 19, its reactive form, the vinyl sulphone, was found in the effluent of a textile mill and a waste water treatment plant. The hydrolysis products of the vinylsulfone was detected only in the effluent of the textile mill. Dye-stuffs in general, and azo dyes in particular, are likely to undergo substantial primary biodegradation in an anaerobic environment through reductive cleavage of azo bonds to aromatic amines. Lipophilic aromatic primary amines are aerobically degraded, but
depending on their precise structure, some sulfonated aromatic amines may not be biodegradable.

2.10. BIOLOGICAL DECOLORIZATION OF TRIPHENYLMETHANE DYES

2.10.1. Degradation by bacteria

The organisms reported to decolorize various triphenylmethane dyes are given in Table 4 (Azmi et al., 1998). There are few reports on the biodegradation of triphenylmethane dyes by bacteria. In 1981, Yatome et al. reported the biodegradation of triphenylmethane dyes by Pseudomonas pseudomallei 13 NA. At this time, the information related to the biodegradation of dyes other than azo-dyes was insignificant. Four triphenylmethane dyes such as Basic Fuchsin, Methyl Violet, Crystal Violet, and Victoria Blue were used for biodegradation studies. They demonstrated that the decolorizability of triphenylmethane dyes was inferior to that of azo dyes. They also showed that Methyl Violet and Crystal Violet were appreciably decolorized, while Basic Fuchsin and Victoria Blue were not decolorized under experimental conditions. To determine whether decolorizability was dependent on the chemical structure of the dyes, they attempted to correlate the decolorizability 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 of triphenylmethane dyes had no appreciable correlation with the molecular weight. In general, the decolorization of the dyes by P. pseudomallei is not related to their molecular weights and the octanol-water partition coefficients of the dyes. It was also observed that the half decolorization time for both, Methyl Violet and Crystal Violet at a dye concentration of 1x10⁻⁵ M

52
Table 4. Microorganisms reported for the decolorization of triphenylmethane types of dyes (Azmi et al., 1998)

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BACTERIA</strong></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Yatome et al., 1991</td>
</tr>
<tr>
<td><em>Corynebacterium sp.</em></td>
<td>Akad. Wiss. DDR, 1991</td>
</tr>
<tr>
<td><em>Mycobacterium sp.</em></td>
<td>Roth et al., 1992; Akad. Wiss. DDR, 1991</td>
</tr>
<tr>
<td><em>Pseudomonas pseudomallei</em></td>
<td>Yatome et al., 1981</td>
</tr>
<tr>
<td><em>Rhodococcus sp.</em></td>
<td>Roth et al., 1992</td>
</tr>
<tr>
<td><strong>ACTINOMYCETES</strong></td>
<td></td>
</tr>
<tr>
<td><em>Nocardia sp</em></td>
<td>Roth et al., 1992</td>
</tr>
<tr>
<td><em>Nocardia corallina</em></td>
<td>Yatome et al., 1991; Yatome et al., 1993</td>
</tr>
<tr>
<td><em>Nocardia globerula</em></td>
<td>Yatome et al., 1991; Yatome et al., 1993</td>
</tr>
<tr>
<td><strong>YEASTS</strong></td>
<td></td>
</tr>
<tr>
<td><em>Rhodotorula rubra</em></td>
<td>Kwasniewska, 1985</td>
</tr>
<tr>
<td><em>Rhodotorula sp.</em></td>
<td>Kwasniewska, 1985</td>
</tr>
<tr>
<td><strong>FUNGI</strong></td>
<td></td>
</tr>
<tr>
<td><em>Coriolus versicolor</em></td>
<td>Yesilda, 1995; Knapp et al., 1995</td>
</tr>
<tr>
<td><em>Cyathus bulleri</em></td>
<td>Vasdev et al., 1995</td>
</tr>
<tr>
<td><em>Cyathus stercoreus</em></td>
<td>Vasdev et al., 1995</td>
</tr>
<tr>
<td><em>Cyathus striatus</em></td>
<td>Vasdev et al., 1995</td>
</tr>
<tr>
<td><em>Fumalia trogii</em></td>
<td>Yesilda, 1995</td>
</tr>
<tr>
<td><em>Laetiporus sulphureus</em></td>
<td>Yesilda, 1995</td>
</tr>
<tr>
<td><em>Phanerochaete chrysosporium</em></td>
<td>Yesilda, 1995; Knapp et al., 1995; Das et al., 1995; Olikka, et al., 1993</td>
</tr>
<tr>
<td><em>Piptoporus betulinus</em></td>
<td>Knapp et al., 1995</td>
</tr>
<tr>
<td><em>Pleurotus ostreatus</em></td>
<td>Knapp et al., 1995</td>
</tr>
</tbody>
</table>
was 54 h. The viable cell count from the incubation mixture with Crystal Violet was found to be a function of incubation time. Under the experimental conditions, the viable cell count was constant up to 100-140 h. Viable cell count did not decrease during the incubation period. The ultraviolet (UV) spectrum of the reaction products of Crystal Violet with intact cells extracted in n-butanol showed the degradation of Crystal Violet. The shift of the peak and increase in absorbance were indicative that the reaction products were different from the original substrate.

In 1991, Yatome et al. reported the degradation of Crystal Violet by *Bacillus subtilis* IFO 13719. Besides Crystal Violet, two other triphenylmethane dyes (Pararosaniline and Victoria Blue) were degraded with the growing cells of *B. subtilis* IFO 13719. With low cell growth, Crystal Violet was remarkably decolorized by *B. subtilis* after 8 h. The compound was completely decolorized in 24 h when the cell growth was higher. They observed that at a very low level of dye concentration (below 7x10^-6 mol/L), the decolorization of Crystal Violet occurred. The growth of the bacterium was completely inhibited at a dye concentration of 1.5-2.0x10^-5 mol/L. Other dyes like Basic Auramine O, Basic Fuchsin, and Victoria Blue were decolorized in the culture of *B. subtilis* while cell growth was poor. They also tried other bacteria for decolorization studies. *E. coli*, although grew remarkably, did not decolorize Crystal Violet. Two other cultures, *Pseudomonas cepacia* and *Pseudomonas cruciviae*, also showed similar results. Biodegradation of two non-permitted dyes Malachite Green and Rhodamine B was reported by Singh et al. in 1994. Degradation studies were carried out with rat caecal microflora. Maximum degradation of Malachite Green (98.1%) was observed within 2-3 h but remained untreated thereafter up to 24 h of incubation. Malachite Green showed faster degradation having an ID50 value of 10 min. One fluorescent
metabolite of rf value 0.09 was obtained with biotransformed Malachite Green. The solvent system used for paper chromatography was isopropanol: ammonia (25%): water (7:2:1). Sattler et al. (1991) isolated various oleophilic strains which are Gram +ve, non sporogenic bacteria, belonging to the group of coryneforms. All these strains grow on a broad range of substrates. Out of 30 strains, 6 strains were able to degrade triphenylmethane dyes like Crystal Violet and Malachite Green. Roth et al. (1992) isolated twenty one hydrophobic oleophilic bacterial strains which showed decolorization activity, the most active strain was Mycobacterium. Crystal Violet and Malachite Green dyes were attacked by all active strains. Cells immobilized on the glass beads showed similar activity. Cell growth was inhibited when dyes were present but resumed when the dyes were transformed.

A German patent (Akad. Wiss. DDR., 1991) states that a new process by which the xenobiotic dyes of triphenylmethane series are degraded using Corynebacterium and Mycobacterium sp. The process is rapid and simple and may be carried out over a wide range of temperature (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. For decolorization, Mycobacterium sp. was pre-cultured at 32°C in a mineral salt medium containing 1% methanol as sole energy and carbon source. After 72 h, cells were harvested and suspended in tap water at a cell concentration of $10^{10}$/mL. An aqueous solution (10.5 mL) of Malachite Green (20 mg/mL) was inoculated with 1.5 mL cell suspension at 24°C. The dye concentration was reduced to 53% of the initial value within 2 h and at 32°C, the solution was completely decolorized after 22 h, the pH remained constant at 6.8, and the number of cells did not increase.
2.10.2. Degradation by actinomycetes

The first report of biodegradation of triphenylmethane dyes by actinomycetes was published by Yatome et al. in 1991. With two actinomycetes, Nocardia corallina and N. globerula, the decolorization of Crystal Violet was observed. They also showed that decolorization activity of actinomycetes is intracellular as there was no activity in the culture filtrate. The dyes were completely decolorized in 24 h. They also detected a degradation product of Crystal Violet digestion as Michler's Ketone (MK) by N. globerula.

In 1993, another report was published by Yatome et al. regarding the degradation of triphenylmethane dyes by actinomycetes. They have shown that Crystal Violet was degraded to Michler's Ketone by Nocardia corallina. However, N. corallina could not decolorize Auramine O, a typical diphenylmethane dye. N. corallina also decolorized Methyl Violet, Ethyl Violet, Basic Fuchsin and Victoria Blue to a much greater extent but Crystal Violet was the compound most remarkably decolorized. The decolorization rate was dependent upon the initial concentration of N. corallina in the medium. The decolorization rate was also dependent upon the growth phase of the precultured cells. The rates at early log phase (10-20 h incubation), mid log phase (20-30 h incubation) and late log phase (30-40 h incubation) were 10.6, 4.4 and 2.2 nmol/mg/min, respectively. The decolorization of Crystal Violet was observed only at a low concentration (5 μmol/L), whereas, the cell growth was completely inhibited at 7 μmol/L. The decolorization rates of Crystal Violet in LB, Bennett, and Minimal medium were 10.6, 6.9 and 2.8 nmol/mg/min, respectively. With cell homogenates and extracellular extracts of N. corallina, the decolorization of Crystal Violet was not observed even after prolonged incubation. The decolorization activity was also not
observed in washed cells of *N. corallina*, when the cells were incubated in buffer but the activity regained when the cells were incubated in LB medium. Product of biodegradation was Michler's Ketone.

2.10.3. Degradation by yeast

Few yeast strains like *Rhodotorula* and *Candida* have been extensively used in the degradation of aromatic compounds, but the utilization of aromatic compounds by yeasts was limited and slow (Chain, 1968). However, certain soil yeasts, isolated from habitats around paper mills or gas works, were reported to be capable of rapid and vigorous growth on phenols, cresols or phenol derivatives (Neujahr and Varga, 1970). Biodegradation of Crystal Violet by oxidative red yeasts is reported by Kwasniewska (1985). He described the role of yeasts or yeast like fungi in the removal of environmental contaminants. He showed that oxidative yeasts such as *Rhodotorula rubra* and *Rhodotorula sp.* were capable of degrading Crystal Violet in the liquid broth. The growth media contains (g/L) glucose 10; peptone 10; ox-bile 15 and Crystal Violet 0.01. No antibiotics were added in the media. The final concentration of Crystal Violet in the growth media was at the level of 10 ppm. Crystal Violet degradation was measured in terms of primary degradation; by following the disappearance of Crystal Violet from the flask broth. After four d of incubation, the absorbance of the supernatant at 600 nm became unmeasurable, indicating the complete biodegradation of Crystal Violet by both the oxidative yeasts. In order to test the adsorption of the dye by the cells, the cells after biodegradation, were sonicated in 70% (v/v) ethanol and the extract did not show any absorbance at 600 nm. It was also noticed that the fermentative yeast *S. cerevisiae* did not degrade Crystal Violet in the liquid medium even after prolonged incubation of 30 days. There may be little decrease in optical
density due to the adsorption of the dye to the cells. The inability of the fermentative yeast *S. cerevisiae* to biodegrade Crystal Violet was apparently not due to any toxic effect of the dye. The yeast culture was found to grow equally well in both the control and in the test flasks in terms of cell dry weight and cell morphology as seen in phase contrast microscope. There was a linear degradation of Crystal Violet by the two oxidative red yeasts between 2nd and 4th d of incubation indicating the presence of an enzyme system for the degradation of Crystal Violet.

### 2.10.4. Degradation by fungi

An overview on the degradation of xenobiotics by white-rot fungi *Phanerochaete chrysosporium* was presented by Hugson in 1991. The pollutants checked include DDT, chlorinated anilines, pentachlorophenols, azo dyes, triphenylmethane dyes etc. The ligninolytic system of the white rot-fungi such as *P. chrysosporium* lends itself to the degradation of xenobiotics. In 1988 Bumpus and Brock reported the biodegradation of Crystal Violet in ligninolytic (nitrogen limiting) culture of white rot fungus *P. chrysosporium*. In the culture broth, the Crystal Violet disappeared with the appearance of its three products by the sequential N-demethylation of the parent compound. Demethylation of Crystal Violet was also carried out with H$_2$O$_2$ generating system in the extracellular fluid. The purified ligninase also catalyzed N-demethylation of Crystal Violet. This proved that the lignin degrading system is responsible for the biodegradation of Crystal Violet. Besides Crystal Violet, other triphenylmethane dyes were also degraded by this fungus. They also reported that the nonligninolytic culture of *P. chrysosporium* could also degrade the triphenylmethane dyes. This clearly suggested that in addition to lignin degrading system, another mechanism exists in this fungus which also degrades the dye. Adsorption of dye to
fungal mycelium may account for decolorization, but it is only 22% of the total
decolorization observed. Six different triphenylmethane dyes were degraded by
ligninolytic culture of *P. chrysosporium*. Involvement of the lignin degrading system
was confirmed by the results in which the purified lignin peroxidase was shown to
decolorize these dyes. Brilliant Green, Malachite Green, and Ethyl Violet contain N-
alkyl groups, therefore, the initial oxidation of all these dyes proceeds via N-
demethylation in a manner similar to that of Crystal Violet. Pararosaniline, Cresol Red
and Bromophenol Blue contain no alkyl groups, thus, oxidation of these dyes occurs
by a mechanism different from that observed for Crystal Violet. This clearly indicates
that the lignin degrading system of *P. chrysosporium* is relatively non-specific and this
non-specific nature is due to the presence of lignin peroxidase in the organism. The
initial oxidation of a wide variety of organic compounds is catalyzed by lignin
peroxidases (Haemmerli *et al*., 1986; Hammel *et al*., 1986; Paszczynski *et al*., 1986).
These enzymes have been shown to be able to catalyze a wide variety of reactions
including benzylic oxidation, phenol dimerization, carbon-carbon bond cleavage,
Decolorization of three triphenylmethane dyes (Crystal Violet, Bromophenol Blue, and
Malachite Green) by three bird's nest fungi - *Cyathus bulleri*, *C. stercoreus*, and *C.
striatus* was reported by Vasdev *et al*. (1995). Among the three organisms, *C. bulleri*
was found to be the most efficient in decolorization. The authors also reported the
decolorization activity in extracellular fluid of the culture filtrate. The rate of
Bromophenol Blue decolorization was very fast as compared to other dyes. This may
be due to the less complex structure of Bromophenol Blue compared to other two dyes
tested. They obtained laccase and ligninase activity in *C. bulleri* and also observed the
laccase activity to be on the higher side than that of ligninase during the dye
decolorization period. Under nitrogen sufficient conditions and without addition of
H$_2$O$_2$, the culture filtrate of *C. bulleri* was able to decolorize three triphenylmethane
dyes namely Crystal Violet, Malachite Green, and Bromophenol Blue in a relatively
simple medium. They reported the decolorization of Crystal Violet by ultrafiltered and
dialed extracellular culture filtrate from *Cyathus Bulleri*. This could be due to the
presence of active laccase in ultrafiltered and dialed extracellular fluid. The faster
dye decolorization by whole cultures of fungus (96-100% in 4 d), as compared to that
by extracellular fluid (90% in 10 d) could be the result of the combined activity of
some cell-associated and extracellular ligninolytic enzyme. *C. bulleri* has been found to
be capable of decolorizing the Crystal Violet up to 90 μM, where as *P. chrysosporium*
has been shown (Bumpus and Brock, 1988) to decolorize the dyes to a much lesser
extent (12.3 μM). In 1995, Yesilada reported the decolorization of Crystal Violet by
different fungi. He used three white-rot fungi such as *Coriolus versicolor, Funalia
trogii*, and *Phanerochaete chrysosporium* and one brown-rot fungus *Laetiporus sulphureus*. He also reported the oxidation of Crystal Violet by commercial horseradish
peroxidase. Significant rate of oxidation was observed only when H$_2$O$_2$ was present.
Without H$_2$O$_2$ the enzyme had no effect on the decolorization of dye, thus suggesting
that a H$_2$O$_2$-dependent enzyme is probably involved in the oxidation of dye.
Knapp *et al.* (1995) reported the decolorization of several dyes by a group of wood-
rotting fungi. Fruiting bodies of basidiomycetes fungi growing on the rotting wood
were used for the decolorization experiment. Brilliant Green and Crystal Violet were
completely decolorized by white-rot fungi. The ability of white-rot fungi to degrade a
diverse array of xenobiotic compounds (Field *et al.*, 1993) is often attributed to the use
in the wide range of dye waste treatment. Das et al. (1995) studied the Crystal Violet decolorization in a column bioreactor using *Phanerochaete chrysosporium*. The decolorization was carried out in a glass column bioreactor (31 cm x 5 cm) with 8-tier stainless steel inoculum 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 is there in 82.4 h in the recycled medium as compared to 64% in shake flasks in 17 d. With glucose, the peak decolorization is reached in about 3 d as compared to 4-6 d with sucrose. Ollikka et al. (1993) purified the various isoenzymes of lignin peroxidase (LiP) from *P. chrysosporium* and studied the decolorization efficiencies for several dyes including two triphenylmethane dyes (Bromophenol Blue and Methyl Green) by crude lignin peroxidase and by three purified isoenzymes. The three isoenzymes purified were LiP 4.65, LiP 4.15 and LiP 3.85. However, Ollikka et al. (1993) also reported that the various isoenzymes of lignin peroxidase LiP 4.65 (H₂), LiP 4.15 (H₃), and LiP 3.85 (H₄) are different from each other with respect to their N-terminal amino acid sequence and degree of glycosylation. The specific activities of LiP 4.65, LiP 4.15, and LiP 3.85 were 26, 39, and 31 U/mg respectively. For decolorization of dyes, isoenzymes were purified by preparative isoelectric focussing.

In the presence of 2 mM veratryl alcohol, the crude lignin peroxidase was able to partially decolorize the dyes. The best decolorization was obtained for Bromophenol Blue (93%). The dyes were then studied with purified isoenzymes, LiP 4.65, LiP 4.15, and LiP 3.85, of lignin peroxidase for decolorization. The dye decolorization in the presence of 2 mM veratryl alcohol with the isoenzymes was similar to that found
with the crude lignin peroxidase. The decolorization ability of the purified isoenzymes was greatly decreased when veratryl alcohol was not present in the reaction mixture suggesting 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 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 increased. In the presence of 1 mM veratryl alcohol, the crude lignin peroxidase was able to decolorize Methyl Green almost completely (initial concentration 29 μM). The effect of other enzymes present in the crude enzyme extract on decolorization can not be excluded, but the effect of manganese peroxidase should be minor because the assay conditions for lignin peroxidase do not favor the enzymatic activity of manganese peroxidase.

2.11. BIODEGRADATION PRODUCTS

Since, the researchers had given main emphasis on the decolorization studies, very few reports are available on the biodegradation products or intermediates of triphenylmethane dyes. Yatome et al. (1981) studied the degradation of Crystal Violet with *Pseudomonas pseudomallei* and demonstrated the reaction products of Crystal Violet and Methyl Violet by thin layer chromatography. The dichloromethane extract of the products of Crystal Violet digestion, using methanol and dichloromethane (3:97, v/v) as a developing solvent, produced a spot not for Crystal Violet (Rf = 0.1), but for some unknown product (Rf = 0.6). The results of the above experiments indicated the probability of biodegradation of triphenylmethane dyes. Roth et al. (1992) were not
able to identify the biodegradation products or intermediates of Crystal Violet digestion. They concluded that the decolorization of Crystal Violet and Malachite Green by *Mycobacterium* sp. was irreversible, probably due to the degradation and formation of leuco-derivatives and then their conversion to unidentified fluorescent and UV absorbing intermediates. Yatome *et al.* (1991) reported that the major degradation product of Crystal Violet by growing cells of *Bacillus subtilis* IFO 13719 was 4,4′ bis dimethylamino benzophenone (Michler's Ketone, MK). Same product was again obtained by Yatome *et al.* (1993) with *Nocardia corallina*. The biodegradation product was identified by Thin Layer Chromatography (TLC) and Gas Chromatography-Mass Spectrometry (GC-MS). The product was stable since it was not entirely degraded within 24-48 h by *N. corallina* (Fig. 4).

Bumpus and Brock (1988) reported the biodegradation and mineralization of Crystal Violet by white-rot fungi. Crystal Violet disappearance, as well as the metabolite formation was monitored by High-Performance Liquid Chromatography (HPLC). They used methylene chloride extract for HPLC analysis. It was observed that the initial degradation starts with the sequential demethylation of Crystal Violet. Three degradation products were identified as N,N,N',N''-penta-, N,N,N',N''-tetra-, and N,N,N',N''- trimethylpararosaniline. Biodegradation appears to continue beyond this as they obtained two additional unidentified colored Crystal Violet metabolites during HPLC analysis. But, finally Crystal Violet was degraded to a colorless product.
Fig. 4. Degradation of Crystal Violet by *N. corallina*