Microbial decolorization of triphenylmethane dyes

Thesis

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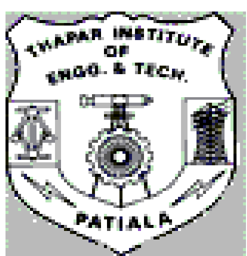
M.Sc in Biotechnology

By

Ms. Beenish Khan
Roll No. 3010107



Department of Biotechnology &Environmental Sciences

Thapar Institute of Engineering and Technology
(Deemed University)
Patiala – 147004
Punjab

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Candidate's Declaration

I hereby declare that the work which is being presented in the dissertation entitled "Microbial Decolorisation of Triphenylmethane Dyes" in partial fulfillment of the requirement for the award of the degree of Master of Science In Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar Institute of Engineering and Technology, Patiala, Punjab in an authentic record of my own work during a period of five months from January 2003 to May 2003, under the supervision of Dr. Dinesh Goyal, Assistant Professor Department of Biotechnology and Environmental Sciences, Thapar Institute of Engineering and Technology.

Patiala **Beenish Khan**
Date: May 15, 2003

This is to certify that the above statement made by the candidate is correct and true to the best of my knowledge.

Dr. Dinesh Goyal
Supervisor
DBTES, TIET
Patiala

Dr. Sunil Khanna
Professor and Head
DBTES, TIET
Patiala

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Date: May 15, 2003 **Beenish Khan**
Place: Patiala

Abstract

Two aerobic bacterial isolates C1A and C2B from dye contaminated soils showed maximum decolorization of crystal violet (20 ppm) and malachite green (100 ppm). In minimal media 68 % decolorization of crystal violet and 89 % decolorization of malachite green was observed by C1A. Malachite green was the compound most remarkably decolorized than crystal violet. Dye decolorization occurred by the dead cells indicating that decolorization is largely due to adsorption to the extent of nearly 60 %. Growth of bacteria in minimal media and increase in viable cell count showed that dye could be utilized by the bacterial systems under conditions of starvation accounting for 30 - 40 % decolorization. Dye decolorization by the immobilized bacterial systems was more than 80 %. Among different solid wastes used as adsorbents the decolorization of dye was in the order of activated charcoal >fly ash > mycelial waste. C1A may be a promising bacterium to depollute the effluent containing triphenylmethane dyes which also could decolorize synthetic effluent containing a mixture of crystal violet and malachite green.

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CHAPTER I

Introduction

The control of water pollution is becoming increasingly important these days. Release of dyes into environment constitutes only a small portion of water pollution. Government legislation is forcing textile industries to treat their waste effluent. Currently, removal of dyes from effluents is by the physico-chemical means. Such methods are often very costly and though the dyes are removed but accumulation of concentrated sludge creates a disposal problem. There is a need to find alternative methods of treatment that are effective in removing dyes from large volumes of effluents and are low cost such as biological or a combination systems.

Textile industries consume large volumes of water and chemicals for wet processing of textiles. The chemical reagents used are very diverse in chemical composition ranging from inorganic compounds to polymers and organic products (Mishra and Tripathy, 1993; Banat *et al.*, 1996; Juang *et al.,* (1996). The presence of very low concentrations of dyes in effluent is highly visible and undesirable (Nigam *et al.,* 2000). There are more than 100,000 commercially available dyes with over 700,000 ton of dyestuff produced annually (Meyer, 1981; Zollinger, 1987). Synthetic dyes are chemically diverse divided into azo, triphenylmethane or heterocylic / polymeric structures (Gregory, 1997). They are used extensively in the biochemical, foodstuff, plastic and textile industries; where it is estimated that 10-14% of the dye is lost in the effluents during the dyeing process (Vaidya and Datye, 1992**).** Synthetic dyes share a common feature in that they are not readily biodegradable and when discharged into the environment they are therefore persistent and many of them are also toxic (Michaels and Lewis, 1985). Many dyes are difficult to decolorize due to their chemical structure and synthetic origin. Decolorization of textile dye effluent does not occur when treated aerobically by municipal sewerage systems (Willmott *et al*., 1998).

Dyes are coloring pigment that imparts color to the substrate when they are in solution form. Technically dyes are distinguished from the intermediates based on the presence of auxochrome

the group that allows the basic unit to attach and impart color to the substrate. Dyes are derived synthetically from raw materials like hydrocarbons, benzene, toluene, naphthalene and anthracene using coal tar obtained from distillation of coal. Both organic and inorganic materials are needed to make dyes and intermediates. The raw material sequence for making dyes is petroleum ---- hydrocarbons ----intermediates ----dyes. Dyes are retained in substrates by physical absorption, metal complex formation or by the formulation of covalent chemical bonds and they obtain their color due to electronic transitions between various molecular orbital where intensity of the color is determined by the probability of transitions.

Triphenylmethane Dyes

Triphenylmethane dyes are those dyes in which a central carbon atom is bonded to two benzene rings and one *p*-quinoid group (chromophore). The auxochromes are - NH_2, NR_2 and –OH. These dyes have brilliant colors but are not very fast to light and washing. Triphenylmethane dyes are used extensively in the textile industries for dyeing of nylon, polyacrylon nitrile, modified nylon, wool, silk and cotton (Gregory, 1993). Some of the triphenylmethane dyes are used in medicine as biological stains, in paper and leather industry; for coloring plastics, in gasoline, paper and leather industries are the major consumer of azo and triphenylmethane dyes. Food and cosmetic industries also use different types of dyes. Triphenylmethane dyes are also some of the widely used dermatological agents. It is known that a large number of dyes have not been tested for their mutagenic, carcinogenic and toxic potential. The triphenylmethne dye crystal violet has seen extensive use in human and veterinary medicine; as a biological stain and as a textile dye. Wastewater treatment facilities are however unable to completely remove commercial dyestuff including triphenylmethane dyes such as crystal violet, from contaminated wastewater, thus contributing to pollution of aquatic habitats.

Triphenylmethane dyes have been found in soil and river sediments as a consequence of improper chemical waste disposal e.g. malachite green has been found in sediments from the Buffalo river a tributary of Lake Erie was shown to be carcinogenic (U.S. Environmental Protection Agency, Genetox' Clastogen, 1986) and crystal violet has been shown to be a potent clastogen. This class of chemicals are reported to be responsible for the promotion of tumor growth in some species of fish. The conventional wastewater treatment systems are unable to

remove recalcitrant dyes from the effluents. Some triphenylmethane dyes have been found in soil due to improper waste disposal. In order to minimize the possible damage to humans and environment arising from the production and application of colorants; an International Association "Ecological and Toxicological association of Dyestuff Manufacturing Industry" (ETAD) was established in 1974. Biological processes are getting more attention as they are cost effective, environment friendly and do not produce large amount of sludge.

The present study was carried out to study the extent of decolorization of crystal violet and malachite green by aerobic bacterial systems isolated from dye contaminated soils.

CHAPTER 2

Review of literature

Dyes

Dye is a substance (generally an organic compound), which is used for imparting permanent color to textiles - silk, wool and other substances.

Natural dyes occur in nature e.g. Indigo (a blue dye), alizarin (a red dye).

Synthetic dyes are man-made dyes e.g. malachite green (a bluish green dye), azo dye, aniline yellow, orange 1 etc.

A colored substance can act as a dye only if it can be fixed to the material being dyed. At the same time it should be resistant to the action of light, water and soap.
There are two important conditions for a colored compound to act as a 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 ultra violet region. Some important chromophores are: - N = O, -N = N-, - C = N, (CH = CH). The compounds bearing chomophores are known as chromogens.(b)

(b) Presence of auxochromes

Dye should be attached to the fibers by means of stable chemical bonds. Some groups form these chemical bonds, which may either be acidic or basic in nature. Such groups are known as auxochromes, some are; - OH. – COOH, - SOH (acidic), NH, NHR, NR (basic). A chromogen without auxochrome can never act as a dye.

Dyes are classified:

(1) According to their chemical composition

(2) According to their mode of application

Table 1. Classification of dyes based on chemical composition (chromophores)

Class of dye	Chromophore present	Example
Nitro dyes	Nitro group $-N(=O)\rightarrow O$	(a) Martuis yellow (b) Naphtol yellow
Nitroso dyes	Nitroso group -- N = O	fast green O
Azo dyes	Azo group --N= N--	(a) Aniline yellow (b) Orange - 1 (c) Methyl orange (d) Congo red
Triphenyl methyl dyes	Triphenyl methyl group	Malachite green
Phthalein dyes	Phthalein group	(a) Phenolhaleinpht (b) Mercurochrome
Indigoid dyes		Indigo
Anthroquinone dyes		Alizarin

Global scenario of dye production

Dyes and pigments constitute the largest segment with a production of 1.3 million tones and market value of 16 billion U.S $. The total size of the world market for dyes, pigment and intermediates is estimated at around 23 billion U.S $ in the year 1999. In terms of market share European countries are the largest producers. This is because they have concentrated on the specialty products. Dystar, the joint venture between Hoechst and Bayer AG, is the largest producer of dyestuff with 15% market share in the world market. Globally, reactive dyes account for around 25% while disperse dyes account for 20% of total dyes production. The two dyes have a dominant share in all the regions of the world. On the other hand market for Direct, Vat dyes and other has remained more or less stagnant.

Asia leads dyestuff production both in terms of volumes and value, with a 44 percent share of the global dyestuff production; followed by U.S with 24% and Europe with about 22%. In the Asian region, China, Korea and Taiwan are strong players in disperse dyes while India leads in the production of reactive dyes. India is a strong player in reactive dyes is due to account of easy availability of intermediates like vinyl sulphone in the country.The Asian region has seen the biggest growth in textile production followed by North America, Latin America and Western Europe. This indicates the shift in the production of global textile industry towards Asia and consequently largest production of textile dyestuff in Asia. The United States market was affected due to closure of plants and shifting of production bases to Mexico and Latin America to take advantage of the NAFTA arrangement. On the other hand European producers shifted to Asia region to take advantage of high growth rates in this region.

Present scenario of dye production and consumption in India

The production capacity for dyestuff in India stood at around 80,000 tones in 1999-2000 (compared to the global capacity of 12.0 – 13.0 million tones). India accounts for around 5% of the global output. Currently there are about 40 – 50 units in the organized sector after taking into account the closure of many units in this sector due to stricter pollution control laws. The organized sector accounts for around 55,000 tones of the total production. Thus organized sector contributes 65% while small-scale sector contributes 35% in overall dyestuff production in the

country. In terms of location nearly 90% of the units are located in Gujarat and Maharashtra. Domestic companies dominate the Indian dyestuff market accounting for nearly 30% while the share of the multinationals has increased from 13-15% in 1992 –93 to 18- 20% in 1998-99.

The Problem

10,000 dyes and pigments are produced annually worldwide amounting to $7x10^5$ tones which are hazardous and pose serious environmental problems. It is estimated that 10-15% of the dye is lost n the effluent during the dying process.

The recent high profile of color pollution is mainly the result of increasing public awareness and expectations of the environment; coinciding with rising levels of color discharges. One of the more pressing environmental problems that have been facing the textile industry is the removal of the color from dye bath effluent prior to discharge to local sewerage treatment facilities or adjoining watercourses. Considerable efforts have been made on developing suitable treatment systems for these effluents. Only biotechnological solutions can offer complete destruction of the dyestuff with a co-reduction in the biological oxygen demand (BOD) and chemical oxygen demand (COD) (Wilmott *et al*., 1998).

Wastewaters originating from reactive dye processes have created a particular problem because the dyes can exhibit low levels of fixation with the fiber. The brightly colored unfixed dyes are highly water-soluble and are not removed by conventional treatment systems. This is particularly noticible as the human eye can detect reactive dyes at a concentration as low as 0.005 mg/l in clear waters. Discoloration of textile dye effluent does not occur when treated aerobically by municipal sewerage systems (Wilmot *et al*, 1998). Though the formation in 1974 of the Ecological and Toxicological Association of the Dye stuff Manufacturing Industry (ETAD), aims were established to minimize environmental damage, protect users and consumers and to cooperate fully with Govt. and public concerns over the toxicological impact of their products (Aniker, 1979). Over 90% of some 4000 dyes tested in an ETAD survey had LD50 values greater than $2x10^3$ mg /kg. The highest rates of toxicity were found amongst basic and diazo direct dyes (Shore, 1996).

Government legislation is becoming more and more stringent, especially in the more developed countries; regarding the removal of dyes from industrial effluents. Environment Policy in UK, since September 1997 has stated that zero synthetic chemicals should be released into the marine environment. Enforcement of this law will continue to ensure that textile industries treat their dye containing effluent to the required standard. European Community (EC) regulations are also becoming more stringent (Neill *et al*; 1999).

Table 2. The characteristics of the dye house waste water

Characteristics	Maximum (mg/l)	Minimum (mg/l)	Average (mg/l)
pH	11	7.3	---
Alkalinity	3,160	340	1,357
Total solids	7,130	700	3,030
TDS	6,180	680	2,720
SS	950	20	310
BOD	850	20	220
COD	1,910	55	850

Toxicity of Triphenylmethane Dyes

Besides the problem of color there is concern that some azo dyes either are toxic or carcinogenic compounds. There are no universally useful methods available for the treatment of dye wastes probably because of the complex and very varied chemical structures of these compounds and few of the currently used biological treatment methods can be successfully employed.

Gentian violet and crystal violet may b e regarded as biohazardous substances.

Crystal Violet - May cause cancer
Severe eye irritant
Harmful by inhalation, ingestion and through

Toxicity Data:

ORL – RAT LD_{450}, 420 mg/kg

IPR – RAT LD_{50}, 8900 mg/kg

Churchman and Herz (1913) reported first case of toxicity of Gentian violet (an impure form of Crystal violet) and its fate in animal body. They did a series of seventy-five experiments on dogs and rabbits to observe the bactericidal property observed by Gentian violet. For the intravenous infection of Gentian violet rabbits were used. The dye in varying concentrations was ejected into the ear vein. The cornea, conjunctiva, mucous membranes of both mouth and lips immediately became blue. It is evident that Gentian violet injected intravenously into rabbits disappears from the blood in a short time and there is no similar loss selective bactericidal power. When the dye is simply allowed to remain in contact with the blood in vitro. On painting the towns of dog with strong solution of the dye frozen section showed that penetration occurred through the thickness of mucosa down to muscularis. Similar observations were made on bladder. On the tongue it seems without irritant effect, however in the bladder it was a good deal of inflammatory reactions where strong solutions were used.

Cytogenetic Toxicity

The cytogenetic toxicity of Crystal violet and Gentian violet in Chinese Hamster ovary cells was studied (Au et al., 1978). Cultures treated for a longest period of time (8 h) or with a higher dosage (10μg/ml) showed a significant accumulation of abnormal metaphases thus they might be mitotic poison. These results demonstrate that these dyes cause severe cytogenic toxicity in cultured cell time (mitotic poison as well as clastogen). Moreover these dyes induce a high frequency of chromosomal breakage in number of cell types, indicating that their defects are not limite4d top one cell line. Under anaerobic conditions bacteria reduce azo leading to the formation of acryl amine derivatives (Cerniglia et al., 1982; Chung et al, 1992), which may be methane, dyes have found, have been toxic to experimental animals (Churchman and Herze. 1913; Cultip and Momluk 1967; Horsefield et al 1976) and cell cultures (Norby and Mogbackel; 1972). Crystal violet was also found to cause reduced RNA and Protein synthesis and decreased oxygen consumption in rabbit granulation tissue (Mogbackel et al 1974). Nelson and Hites in 1980 reported the deposition of Crystal violet and Malachite green and Aniline in the sediments and water of Buffalo river, New York, USA thus posing a threat to aquatic environment and human population. From a study of Black et al. it was shown that theses dyes have mutagenic and carcinogenic properties. Theses chemicals have been suggested to be responsible for the promotion of tumor growth in several bottom feeding species of fish (Diachenko, 1979).

Mutagenic nature of trpihenylmethane dyes

The majority of the synthetic food colors have mono or triphenylmethane types. Triphenylmethane dyes include FD colors and C colors approved for use in USA and a number of delisted food crops were tested (Brown et al; 1978) in the *Salmonella* / Micro some system. They tried to access the mutagenicity and possible carcinogenic potential of certified food colors and a variety of related in short term in invitro tests. All these dyes are nonmutagenic for the *Salmonella* / Microcosm tests. Several dyes used as food colors, commercial dyes, laboratory stains and pH indicators were tested (Bonin *et al.,* 1981) in the *Salmonella* / Mammalian microcosm and mutagenicity was assayed using five standard starter strains of *Salmonella typhymurium* TA 98, TA 100, TA 1535 and TA 1538. Most of the compounds gave weak mutagenic responses with *Salmonella* and were positive with only a narrow dose range. Food colors like Benzyl violet and Brilliant green showed weak mutagenic property and detected throughout in frame shift mutation detector strains TA98, TA 1537 and always in the presence of S – 9 rat liver preparations. All the laboratory and commercial dyes, Basic fuschian, Crystal violet and Methyl violet 2-B were also mutagenic. Mutagenicity of certain basic dyes were determined by Ogawa *et al*., 1989. They use the strains of Bacillus subtilis H17rec+ and M45rec+ for mutagenicity studies and cultivated each strain in the presence of a dye 37°C for 8 hours using liquid medium. Each cell suspension collected at a definite volume was collected on agar in a petri dish and mutagenicity was evaluated in expressed as $R = (A – B)/A$, where A and B represent respectively a dye concentration when each cell count of rec+ and rec+ strain are reduced to half of that without any dye.

Toxicity in terms of inhibition of growth

Dye industries use the activated sludge process to treat effluent. However TPM dyes are toxic to the microbes and lessen their purifying action. Dye industries very frequently change the kind of dyes it is important for the treatment process by the acclimatization microbes to maintain their adaptation to different kind 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 type of co-existing dyes and the influence of these dyes were investigated by Ogawa *et al*., (1981). The inhibition level of the unacclimatized microbes with dye increases as the dye

concentration increases. The acclimatized microbe showed a negative inhibition at low dye concentration. And a positive one at high concentration. It was thought that the microbes acquired tolerance only for that class of dye, which is used for the acclimatization and failed to display any adaptability fo0r different classes of dyes.

Effect of dye on DNA and RNA content

It is known in the case of bacterial population in the activated sludge that the growth rate at the logarithmic phase increases in the proportion to the cellular RNA content (Kaneke and Nambe, 1973). So a similar relationship in between cell growth and RNA synthesis was predicted. The ratio indicated that the growth inhibition strongly depends on the rate of RNA synthesis. The content ratios of the nucleic acids (RNA/DNA); decreased with the increasing dye concentration. These dyes act more preferentially to lower protein synthesis that inhibits cell division. Due to the inhibitive action; cell shape varied, cells growing under ordinary conditions appeared as small rods and those in the presence of dyes, as filaments. Ogawa et al, 1989 noted that dye inhibits DNA synthesis by stabilizing the double helix and by inhibiting the enzyme activities.

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 are reduction in the numbers of the red blood cells and hemoglobin content of the blood-causing anaemia. The toxic effect may cause bone- marrow depression, epileptic anemia and hemorrhagic diseases of the various organs and leukemia in few cases.

Nephrotoxic and Hepatotoxic effects

There are certain chemicals related to the dyestuff industries, which can cause injury to the kidney. This may be the transient following an acute exposure. The injury to the liver cells occurs following adsorption to certain chemicals. The offending chemicals cause the disturbance of the liver function.

Cyanosis

Certain dyestuff chemicals forms methaemoglobin by conversion of haemoglobin to metahemoglobin also called anilism. The cyanising compounds mainly enter our body through skin leading to deprivation of the availability of oxygen.

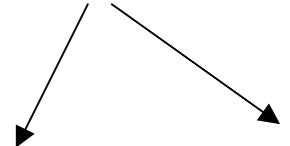
Carcinogen

The greatest tragedy of dyestuff industry has been the occurrence of occupation tumors of urinary bladder (Pappiloma). They are usually malignant. There is a long latent period (20 to 25 years) between the first exposure and ultimate development of tumor, e.g. malachite green and its reduced form leucomalachite green may persist in edible fish tissues for extended period of time. Therefore there are human concern about bioaccumulation of malachite green and leucomalachite green in terrestrial and aquatic system.

Solution

Dyes (industrial waste) → Decolorization
Degradation

1. Decolorization.



Adsorption   Changing the chromophoric group to the non- chromophoric group

2. Degradation → breakdown of the substrate molecule (dye) the biological process.

Presently most of the processes used for the treatment of dye wastewaters are chemical processes, physical or physico-chemical processes which aer generally expensive and of limited applicability. All these methods possess significant differences in color removal, volume, capability, operating speeds and capital costs. Nowadays Biological treatment methods are getting more attention since it is cheap and offer best alternative with proper analysis and environmental control. Almost all wastewaters can be treated by the use of a number of naturally occurring microorganisms such as bacteria and fungi.

The color removal concentration in the textile industries varies between 1100-1300 mg/L. During coloration more than 15% of the dyes is wasted which enters the effluents. These effluents a source of environmental contamination characterized by high levels of chemical and biochemical (COD, BOD) oxygen demand, suspended matter solids, toxic and hazardous materials. The BOD of from dye waters typically varies between 200-3000/ LSS and a pH of 4 – 12.

Various effluent treatment methods from dyestuff industries, classified in to three main categories involve three basic stages of treatment namely primary, secondary, tertiary. Primary treatment processes of dye wastewater include equalization neutralization and possible disinfections. Primary stages are mainly physical and include screening, sedimentation, floatation and flocculation. The objective is removal of debris, undissolved chemicals and particulate matter. In the Secondary stage the organic load is reduced. Tertiary method involves adsorption, ion exchange chemical exudation, reverse osmosis etc (Reffie, 1993).

Activated charcoal

This is the most commonly used method of dye removal by adsorption (Nasser and El-Geundi, 1991) and is very effective for adsorbing cationic, mordant, and acid dyes and to a slightly lesser extent, dispersed, direct, vat, pigment and reactive dyes (Raghavacharya, 1997 Rao *et al.*, 1994). Performance is dependent on the type of carbon used and the characteristics of the wastewater. Removal rates can be improved by using massive doses, although regeneration or reuse results in a steep reduction in performance, and

efficiency of dye removal become unpredictable and dependent on massive doses of carbon. Activated carbon, like many other dye removal treatments, is well suited for one particular waste system and ineffective in another. Activated carbon is expensive. The carbon also has to be reactivated otherwise disposal of the concentrates has to be considered. Reactivation results in 10 – 15% loss of he sorbent.

Flyash

The fly ash concentration increases the adsorption rates of the mixture due to increases in the surface area available for the adsorption. The adsorption by fly ash and coal in different concentrations showed a good adsorption capacity towards metal containing dyes (Gupta *et al*., 1991).

Biological systems

87 dyestuffs were tested in short-term aerobic biodegradation tests (Paggga and Brown, 1986) to investigate whether some dyes might be susceptible to aerobic biodegradation and, if so, to what extent this occurs? For this work the dyestuff chosen were typical commercial products and bacteria inoculated were from effluent treatment plant. As in the static test, the criteria for biodegradation were both decolorization at the absorption maximum and dissolved organic carbon elimination. Their results confirmed that dyestuffs are most unlikely to show any significant biodegradation in such tests.

Table 3. Methods (Physical/Chemical) available for the treatment of dye waste water and their advantages and disadvantages are summarized as follows (Robinson *et al*., 2001).

Physical and Chemical methods	Advantages	Disadvantages
Fentons reagent	Effective decolorization of both soluble and insoluble dyes	Sludge generation
Ozonation	Applied in gaseous state; no alteration of volume	Short half-life (20 min)
Photochemical	No sludge production	Formation of by- products
NaOCl	Initiates and accelerates azo-bond cleavage	Release of aromatic amines
Activated carbon	Good removal of wide variety of dyes	Very expensive
Peat	Good adsorbent due to cellular structure	Specific surface areas are lower than activated carbon
Membrane filtration	Removes all dye types	Concentrated sludge formation and very expensive
Ion exchange	Regeneration; no adsorbent loss	Not effective for all dye types
Irradiation	Effective oxidation at lab scale	Requires a lot of dissolved oxygen
Silica gel	Effective for basic dye removal	Side reactions prevent commercial application
Electro kinetic coagulation	Economically feasible	High sludge production
Wood chips	Good sorption capacity for acid dyes	Requires long retention times
Electrochemical destruction	Breakdown compounds are non – hazardous	High cost of electricity

Decolorization by bacteria

Many organisms are reported to decolorize various triphenlymethane and azo dyes. There are a few reports on the biodegradation of theses dyes by bacteria. In 1981 Yatome *et al*., reported the biodegradation of triphenylmethane dyes by ***Pseudomonas*** *pseudomallei 13NA*. In general the decolorization of the dyes is not related to their molecular weights and the octanol- water coefficients of the dyes. Yatome *et al.,* (1991) again reported the degradation of Crystal violet, Pararosaniline and Victoria growing cells of *B.subtilis* IFO 13719.

Biodegradation of triphenylmethane dyes by bacteria, fungi and yeasts (Azmi *et al*., 1998). They showed the advantages of using biological processes for degradation of dye molecules to carbon dioxide and water and with concomitant formation of less sludge and being eco-friendly.

Decolorization of triphenylmethane dyes and textile and dyestuff effluent by *Kurthia sp.* (Sani *et al.,* 1999). They screened a number of soil and water samples and isolated the *Kurthia species* on the basis of rapid dye decolorization. Under aerobic conditions 98% of the color was removed intracellular by this strain. A number of dyes such as crystal violet, malachite green. Ethyl violet etc was used for the studies. After the decolorization of most of the dyes, viable cell concentration of *Kurthia* sp. reduced significantly. Compared to crystal violet higher concentrations of malachite green was decolorized by the same amount of cell mass which may be due to the difference in the structure of both the dyes.

Decolorization by Actinomycetes

The first report of decolorization of triphenylmethane dyes (Yatome, 1991) by two Actinomycetes, *Nocardia corallina* and *N. globerulla* showed that decolorization activity is intracellular as there was no activity in the culture filtrate. The dyes were completely decolorized in 24 hours. They also detected degradation product of crystal violet digestion as Michler's Ketone (MK) by *N. globerulla*. The decolorization activity was also not observed in the washed cells of *N. corallina*, when the cells were incubated in

buffer but the activity regained when the cells were incubated in LB medium alongwith the product of biodegradation with Michler's ketone.

Decolorization by Yeast

Yeast strains like *Rhodotorulla* 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 1986). However, certain soil yeasts, isolated from habitats around paper mills or gas works were reported to be capable of rapid an vigorous growth on phenols, cresols or phenol derivatives (Neujhar and Varga, 1970) Kwasniewska reported that oxidative red yeasts such as *Rhodotorulla rubra* and *R.sp* were capable of degrading certain triphenylmethane dyes in the liquid broth.

Decolorization by fungi

White – rot fungi are those organisms that are able to degrade lignin, the structural polymer found in woody plants (Barr and Aust, 1994). The most widely studied white-rot fungus is *Phanerochaete chrysosporium*. This fungus is capable of degrading dioxins, polychlorinated biphenyls (PCBs) and other chloro-organics (Chao and Lee, 1994; Reddy, 1995). Davis *et al*., (1993) showed the potential of using *P. sordida* to treat creosote-contaminated soil. Kirby (1999) has shown that *P. chrysosporium* had the ability to decolorize artificial textile effluent by 99 % within 7 days.

White rot fungi are able to degrade dyes using enzymes, such as lignin peroxidases (LiP), manganese dependent peroxidases (MnP). Other enzymes used for this purpose include H_2O_2 producing enzymes such as glucose-1-oxidase and glucose-2-oxidase, along with laccase, and a phenol oxidase enzyme (Archibald and Roy, 1992; Thurston, 1994; Schliephake and Lonergan, 1996; Kirby, 1999). These are the same enzymes used for the lignin degradation. Other fungi such as *Hirschioporus larincinus, Inonotus hispidus, Phlebia tremallosa and Cariolus versicolor* have also been shown to decolorize dye-containing effluent (Banat *et al*., 1996;Kirby, 1999).

Decoloration of triphenylmethane dyes (Crystal violet, Bromophenol blue and Malachite green) by three birds nest fungi – *Cyathus bulleri, C. stercoreus, C. striatus* was reported by Vasdev *et al*., 1995. Among the three organisms, *C. buleri* was found to be the most efficient in decolorization. They obtained ligninase and laccase activity in ***Cythus bulleri*** and also observed the activity on the higher side than the ligninase activity during decolorization period. The ability of white – rot fungi to degrade a diverse array of xenobiotic compounds (Field *et al.,* 1993) is often attributed for use in wide range of dye waste treatments. Das *et al*., (1995) studied the crystal violet decolorization using *P. chrysosporium* in a column bioreactor. Olika *et al.*, 1993 purified iso-enzymes of lignin peroxidases (LiP) from ***P.chrysosporium*** and studied the decoloroization efficiencies for several dyes including azo and triphenylmethane dyes by crude lignin peroxidases and three purified iso enzymes. Isoenzymes purified were LiP 4.65, LiP 4.15, LiP3.85. The specific activities were 26, 39 and 31 U/mg respectively.

CHAPTER 3

Materials and methods

1. Growth and maintenance of bacterial strains

Two bacterial consortia C1 and C2 and four pure bacterial cultures (C1A, C1B, C2A and C2B) already isolated from the dye-contaminated soils were maintained on nutrient agar plates and in nutrient broth. Growth of the bacteria in minimal media MM 2 (KH_2PO_4 – 3g/L, K_2HPO_4 – 6g/L, $(NH_4)2SO_4$ – 1g/L, $MgSO_4$. 7H20 – 0.2g/L, Glucose – 4g/L, Ph – 7.0) with and without dye was studied. The media was autoclaved at 121°C for 15 min. at 15-psi pressure; glucose was filter sterilized and then was added to the media.

2. Dyes

Two different dyes used were Crystal violet and Malachite green, which were procured from Hi-Media. The properties of the dyes are given in Table 3. Stock solutions of 5000 ppm were prepared by dissolving the dye in distilled water and were filter sterilized and kept at 4°C. Dye at different concentrations (20 ppm, 50 ppm, 100 ppm, 200 ppm) were used to study the their effect on bacterial growth and adsorption after adding to the culture media. Absorbance of Malachite green and Crystal violet was studied by Spectrophotometer at their respective wavelengths 595 nm (Malachite green) and 585 nm (Crystal violet).

Table 4. The following table gives the properties of the dyes

Property	Crystal violet	Malachite green
Solubility in water	1.68%	Highly soluble
Solubility in ethanol	13.78%	Highly soluble
Absorption maximum	584 – 595	595 – 614
Color	Blue violet	Green
Empirical formula	$C_{25}H_{30}N_3Cl$	$C_{23}H_{25}N_2Cl$
Formula weight	407.966	365

3.Assay of decolorization activity

The bacterial strains were grown on nutrient agar plates and were streaked on plates containing dyes [crystal violet (20 ppm), malachite green (100 ppm)] in MM2 media. The plates contained MM2 – Carbon, MM2 – Nitrogen, MM2 – Nitrogen – Carbon. Decolorization of the dye was visually observed for the extent of zone clearing on the plates.

The extent of dye decolorization by the microbial cultures in broth (nutrient broth and MM2 broth) was determined by spectrophotometer at the maximum absorbance of the respective dyes in the cell free extracts. The percentage of dye decolorization by the cells was done using the modified method of Yatome *et al*., (1991).

Cultures (C1A and C2B) were grown in 50 ml of nutrient broth for overnight at 37 C and 80 rpm to an OD of 1.00 at 600 nm (C1A-2.01×10^5 cfu/ml, C2B – 3.31×10^5 cfu/ml). The cultures were centrifuged at 10,000 rpm for 10 min. and washed twice with sterile saline (0.85%) and resuspended in 10 ml of saline solution. 0.1ml of the inoculum was added to the broth containing dye and incubated at 37°C, 85 – 110 rpm for 24 hr. The supernatant was collected after centrifugation for absorbance measurement at respective wavelengths. The percentage decolorization was calculated as follows:

$$\%\ \text{age Decolorization} = \frac{\text{Initial O.D} - \text{Final O.D}}{\text{Initial O.D}}$$

4. Dye decolorization by heat-killed cells

50 ml grown cultures (C1A, O.D = 1.007, C2B, O.D = 1.006) were divided into equal halves, centrifuged at 10,000 rpm for 10 min., washed twice with saline (0.85%) and resuspended in 10 ml saline. The 0.1 ml of cell suspension of C1A and C2B was added to the tubes containing 5 ml of Nutrient broth (one set) and MM2 – C – N (second set). The tubes were then autoclaved at 121°C and 15-psi pressure for 15 min. The dyes were then added to the heat killed cell suspension and incubated at 37°C, 80 rpm for 24 hr. The assay of decolorization was studied as above.

5. Impact of contact time on dye decolorization

The viable and the heat killed cells of C1A and C2B were studied for the impact of contact time on the extent of dye decolorization. Cells of the overnight grown cultures were harvested, washed twice with saline (0.85%) to remove any adhered media particle and resuspended in 0.1 M of sodium phosphate buffer (12 g/l of Na_2HPO4 and 17.8 g/l of NaH_2PO_4). The cell suspension was divided equally into two sets and one was used as viable cells and the second set was autoclaved. Viable and heat killed cells were equally resuspended in phosphate buffer (pH– 7.0) containing dye and incubated at 37° C and were centrifuged at hourly intervals for measurement of absorbance of the supernatant at respective wavelengths.

6.Optimization of biomass concentration for maximum dye decolorization

Overnight grown cultures of C1A and C2B (of known density and cfu/ml) were washed twice with saline and resuspended in 15 ml of saline. Different concentrations (by taking different volumes of cell suspension) were added to the tubes containing 5 ml of Nutrient broth (one set) and 5 ml of MM2 – Carbon – Nitrogen (second set) containing dye and were incubated at 37°C, 80 rpm for overnight and assayed for the extent of dye decolorization.

7. Dye decolorization by mixed cultures

Cells from over night grown cultures of C1A and C2B (of known density and cfu/ml) were harvested and washed twice with saline (0.85%) and resuspended in 5 ml of saline. Tubes containing 5 ml of Nutrient broth (one set) and MM2 – Carbon – Nitrogen (second set) and dye were inoculated with 800 µl of each C1A and C2B, incubated for overnight and assayed for dye decolorization. Similarly decolorization of mixed dyes (Crystal violet: 20 ppm + Malachite green: 100 ppm) was also studied by mixed bacterial cultures of CIA and C2A

9. Effect of dye on viable cell count of bacteria (C1A and C2B)

Effect of dye crystal violet (20 ppm) and malachite green (100 ppm) on viable cell count was studied by growing bacterial culture in nutrient broth, MM2 and in MM2 – Carbon – Nitrogen. The growth of bacteria grown in nutrient broth and MM2 (complete) were analyzed by taking absorbance at 600 nm at hourly intervals. Whereas in minimal media without carbon and nitrogen (MM2 – Carbon – Nitrogen) the growth or viable cell count was measured by estimating cfu/ml at hourly intervals. The cells were harvested, washed with saline, serially diluted and plated on agar plates and incubated at 37°C for 24 hr and observed for colony forming units.

10.Swarm plate assay

Swarm plate assay (Parales *et al*., 2000) was carried out to check the motility of the bacteria towards the carbon and nitrogen source of the dye. Swarm plates were prepared using minimal media MM2 without carbon and nitrogen source and the agar concentration was optimized with 0.15%, 0.25% and 0.35% of agar (Hi Media) concentration. Plates of 0.25 % strength were used and overnight grown culture of C1A (1.5 ml) was centrifuged and was given two saline washes. The culture was starved for 15 min. after which 20 µl of the inoculum was loaded in the plates containing 5% of the dye in minimal media and 100 µl of TTC. For the control glucose (0.02-0.05 %) was added.

11. Studies with immobilized bacterial cells (C1A)

Immobilization of bacterial cells was done using 6% sodium alginate and 2% Calcium chloride (from Hi Media). 5 ml of well-grown bacterial culture was mixed with 50 ml of Sodium Alginate and was added drop by drop in cold calcium chloride solution kept in ice. The alginate beads were collected and washed with sterile water and kept at 4° C till further use. Immobilization was also done without bacterial culture and both the type of immobilized beads (10 g wet weight) were used for studying dye decolorization in batch conditions.

12.Batch studies on decolorization of dye by solid wastes

Using solid wastes such as fly ash, activated charcoal, mycelial waste along with immobilized bacterial cells, batch decolorization studies of crystal violet and malachite green was carried out. To 40 ml of the dye solution (crystal violet 20 ppm and malachite green 100 ppm) 10 g each of fly ash, activated charcoal and mycelial waste was added separately. The dye decolorization was then studied by taking the samples at hourly intervals, centrifuged and the supernatant was assayed for the extent of decolorization.

CHAPTER 4

Results and Discussion

Decolorization of dyes by growing cells

Aerobic bacterial isolates (C1, C1A, C1B, C2, C2B) from the dye-contaminated soils were found capable of growing in media containing dyes. They were checked for the extent of dye decolorization [crystal violet (20 ppm) and malachite green (100 ppm)] on solid media plates (nutrient agar) as well as in liquid media (nutrient broth and minimal media with and without carbon and nitrogen sources). C1A and C2B gave maximum decolorization among all the five isolates. Visual decolorization indicated that decolorization was higher in case of nutrient broth as compared to minimal media. C1A and C2B were studied further for dye decolorization. In nutrient broth decolorization of crystal violet (20 ppm) was 80 % (Table 6, Fig 2) and for malachite green it was 96 % (Table 6, Fig 3) where as in minimal media without carbon and nitrogen sources decolorization was 68 % (Table 6, Fig 3) for 20 ppm crystal violet and 89 % (Table 6, Fig 3) for 100 ppm malachite green by the isolate C1A. However for C2B the decolorization was 61 % (Table 7, Fig 4) for crystal violet (20 ppm) and 95 % (Table 7, Fig 4) for malachite green in nutrient broth and 50 % (Table 7, Fig 4) for crystal violet (20 ppm) and 89 % (Table 7, Fig 4) for malachite green in minimal media containing no carbon and nitrogen.

In case of crystal violet maximum decolorization (96.3%) has been achieved at 29.38 ppm of crystal violet by fungal system *Cyathus bulleri* (Vasdev *et al*.,1995) whereas with bacterial system *Pseudomonas pseudumallei* 13 NA and *Rhodotorulae rubra*, maximum of 20.4 and 10 ppm of crystal violet were decolorized effectively to the extent of 96 and 99 % respectively (Yatome *et al.*,1981 ; Kwasniewska, 1985). As compared to crystal violet higher concentration of malachite green was decolorized by the same amount of cell mass, which may be due to the difference in the structure of

both the dyes. The rate of decolorization of malachite green by *Phanerochaete chrysosporium* was found to be higher than crystal violet (Bumpus *et al.,* 1988). Since malachite green has two dimethyl groups in two side chains where as crystal violet is having three dimethyl groups in three side chains which may be the reason that decolorization of more substituted triphenylmethane dyes took longer time (Sani and Banerjee, 1999). Moreover malachite green has greater solubility than crystal violet (Table 4). C1A and C2B were able to decolorize dye effectively at lower concentrations. Yatome *et al* (1993) had earlier reported that the growth of cells was completed inhibited at higher concentrations of crystal violet.

Decolorization of dyes by dead cells

Dye decolorization was studied by the heat-killed cells of C1A and C2B and compared with that of live cells. Heat killed cells of C1A decolorized 20 ppm of crystal violet by 65 % (Table 8 Fig 4) 100 ppm of malachite green by 79 % (Table 8 Fig 5). Similar trend was observed for C2B. Both the strains do not exhibit extracellular decolorizing activity. Only intact cells were responsible for decolorization of the dyes. Decolorization by heat killed cells indicates that dye decolorization to the extent of 60 % is primarily due to adsorption However nearly 40 % of the dye decolorization can be assumed to be due to degradation by the bacterial system which was confirmed by studying the growth kinetics of the bacterial cultures in minimal media containing no nitrogen or carbon source except for the dye. Growth patterns of C1A and C2B as observed in minimal media containing no nitrogen and carbon source [MM2 – C – N + (dye: 20 ppm crystal violet and 100 ppm malachite green)]. The colony forming units or the viable cell count increased at hourly intervals and showed exponential growth between 5 – 8 hr after prolonged lag phase. Viable cell count increased in the presence of crystal violet and malachite green separately. Malachite green was better utilized by bacterial cells and was maximally decolorized than crystal violet.

The bacterial isolate C1A could sense and showed motility towards crystal violet (0.1 ppm, 0.5 ppm, 1.0 ppm, 5 ppm ,10 ppm) on semi solid agar plates containing

minimal media without carbon and nitrogen source. The mobility could be due to chemotaxis, which gives the motile bacteria an advantage of being able to locate compounds such as carbon or nitrogen that can support their growth. Chemotactic cells may be especially efficient at sensing and swimming towards chemicals that are present at point sources, for example adsorbed to soil particles in ground water or within soil moving pollutant fumes. In this way chemotactic bacteria can over come mass transfer limitations that impede the bioremediation processes (Parales *et al*., 2000)

These results indicates that the bacteria could utilize the dye as the carbon and nitrogen source for its survival and multiplication and also shows that the dye is acting as the sole source of carbon and nitrogen and is being metabolized by the bacterial systems.

Decolorization of dyes by resting cells

Decolorization of the dyes by resting cells of C1A and C2B (crystal violet : 20 ppm and malachite green:100 ppm) was studied to optimize of cell density and contact time to obtain maximum decolorization. It was observed that decolorization increased with the increase in cell mass concentration only up to a certain level, beyond which there was no increase in percentage decolorization even at higher cell concentration. In the initial one hour contact time decolorization was rapid to the extent of 35-40 % and increased up to 50-65 % in 4 hr (Table 14).

Dye decolorization by mixed cultures (C1A and C2B) was however almost same as with individual cultures. However 88 % decolorization of mixed dye system was observed by mixed cultures (C1A and C2B). Indicating that both the organisms are capable of decolorizing a mixture of dye solution, since effluent mainly contains a mixture of different dyes.

Decolorization by immobilized bacterial system

The immobilization of the bacterial system C1A was carried out using sodium alginate and calcium chloride. Calcium alginate beads without the bacterial cells were also prepared, which worked as the control. As sodium alginate is a polymer so some amount of dye was found to adsorb on the beads but was less than the immobilized bacterial system. It was seen that 82 % crystal violet (Table 15) and 86 % malachite green (Table 15) was decolorized by the immobilized C1A bacterial system.

Batch studies were carried out using the solid wastes such as fly ash, activated charcoal and mycelial waste. Maximum decolorization was attained in case of activated charcoal followed by fly ash and then mycelial waste (Table 15, 16).

onclusions

1. Two aerobic bacterial isolates C1A and C2B from dye contaminated soils showed maximum decolorization of crystal violet (20 ppm) and malachite green (100 ppm) among all the isolates from dye contaminated soils.
2. Malachite green (100 ppm) was the compound most remarkably decolorized than crystal violet.
3. Dye decolorization occurred by the dead cells indicating that the decolorization is largely due to adsorption, which can be to the extent of nearly 60 %.
4. Growth of bacteria in minimal media and increase in viable cell count showed that dye could be utilized by the bacterial systems under conditions of starvation which accounts for decolorization to the extent of 30 - 40 %.
5. Dye decolorization by the immobilized bacterial systems was also more than 80 %
6. Among different solid wastes used as adsorbents the decolorization of dye was in the order of Activated charcoal >Fly ash > Mycelial waste.
7. C1A may be a promising bacterium to depollute the effluent containing triphenylmethane dyes which also could decolorize synthetic mixture effluent containing crystal violet and malachite green.

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