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**DEGRADATION OF SYNTHETIC DYES IN
TEXTILE EFFLUENT USING BACTERIA AND
FUNGI**

A Project Report

Submitted by

Akila A (0710204002)

Jothi P (0710204014)

Khyati R Patel (0710204019)

Lalitha Kumar (0710204020)

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**KUMARAGURU COLLEGE OF TECHNOLOGY
COIMBATORE 641049**

BONAFIDE CERTIFICATE

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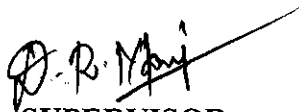
Akila. A (0710204002)

Jothi. P (0710204014)

Khyati. R .Patel (0710204019)

Lalitha Kumar (0710204020)

who carried out the project work under my supervision.



SUPERVISOR

Mr. D.R. Manimaran

Assistant Professor

Department of Biotechnology

Kumaraguru College of Technology

Coimbatore-641049



HEAD OF THE DEPARTMENT

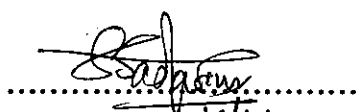
Dr. S. Sadasivam

DEAN (BioTechnology)

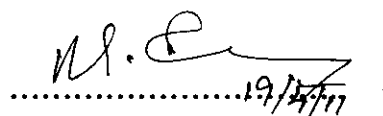
Department of Biotechnology

Kumaraguru College of Technology

Coimbatore-641049



Internal Examiner



External Examiner

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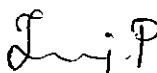
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[AKILA. A]


[JOTHI. P]


[KHYATI. R. PATEL]


[LALITHA KUMAR]

Abstract

ABSTRACT

Liquid-liquid extraction using chloroform was carried out at 30°C to remove the hazardous dye, malachite green present in simulated textile effluent. Different parameters such as pH, temperature, equilibrium time, dye concentration and volume of extractant on the extraction efficiency were studied. pH had no profound effect on the extraction of dyes from aqueous phase. Single-stage solvent extraction was sufficient to extract the dye completely at lower concentration (from 0.004 ppm to 0.016 ppm) while multi-stage extraction method completely extracted the dyes at higher concentrations (from 0.04 ppm to 0.12 ppm). Among the various ratios of dye to extractant used, 1:3 (v/v) ratio was suitable for all the dye concentrations (0.004 ppm to 0.12 ppm). Solvent extraction of dyes by this method avoids the use of stripping agents and other chemicals for maximum dye extraction. A simple distillation was done to recover the solvent and the solvent was recycled. Recovered chloroform was also found to be as effective as actual chloroform for extraction. Degradation of malachite green, bromophenol blue and amaranth by *Pleurotus eous* was proved by spectrophotometric analysis. *Bacillus licheniformis* also successfully degraded amaranth dye. Laccase enzyme secreted by the bacterium and fungus is believed to be responsible for the degradation of dye molecules.

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Introduction

1. INTRODUCTION

Colours give delightful pleasure to eyesight but at the same time they may act as serious pollutants when their origin is dyes and dyestuffs. Textile industries have been using dyes intensively because of their ease and cost effectiveness in synthesis most widely used in textile, rubber, and enamel, plastic, cosmetic and many other industries (Chaube *et al.*, 2010).

1.1 DYES

The dye is a coloured substance that has an affinity to the substrate to which it is being applied. A dye is used to impart color to materials of which it becomes an integral part. An aromatic ring structure coupled with a side chain is usually required for resonance and thus to impart color (Resonance structures that cause displacement or appearance of absorption bands in the visible spectrum of light are responsible for color.) Dyes are compounds that absorb light with wavelengths in the visible range, i.e., 400 to 700 nm (Marias *et al.*, 1976, Zee *et al.*, 2002). The major structure element responsible for light absorption in dye molecules is the chromophore group, i.e. a delocalised electron system with conjugated double bonds. The absorption of UV/Vis radiation by an organic molecule is associated with electronic transitions between molecular orbitals. The energy of the absorbed radiation is given by:

$$\Delta E = E_1 - E_0 = h\nu = hc / \lambda$$

where

E_0 is the energy corresponding to the fundamental state of the molecule (J)

E_1 is the excited state energy (J), h is the Planck's constant (6.626×10^{-34} Js)

ν is the electromagnetic radiation frequency (Hz),

c is the light velocity (3×10^8 m.s⁻¹)

λ is the wavelength (nm).

The more extended the electronic delocalisation, the lower is the transition energy and the higher is the wavelength. To allow delocalization of the electrons double bonds must alternate with single bonds. In the case of synthetic dyes, delocalization is also promoted by benzene or naphthalene rings (Gomes *et al.*, 2001). Correlation of chemical structure with colour has been accomplished in the synthesis

of dye using a chromogen-chromophore with auxochrome. Chromogen is the aromatic structure containing benzene, naphthalene, or anthracene rings. A chromophore group is a colour giver and is represented by the following radicals, which form a basis for the chemical classification of dyes when coupled with the chromogen:

- azo ($-\text{N}=\text{N}-$);
- carbonyl ($=\text{C}=\text{O}$);
- carbon($=\text{C}=\text{C}=-$);
- carbon-nitrogen ($>\text{C}=\text{NH}$ or $-\text{CH}=\text{N}-$);
- nitroso ($-\text{NO}$ or $\text{N}-\text{OH}$);
- nitro ($-\text{NO}_2$ or $=\text{NO}-\text{OH}$);
- sulfur ($>\text{C}=\text{S}$, and other carbon-sulfur groups).

The chromogen-chromophore structure is often not sufficient to impart solubility and cause adherence of dye to fiber. The auxochromes are electron withdrawing or electron donating substituents that cause or intensify the colour of the chromophore by altering the overall energy of the electron system. The auxochrome or bonding affinity groups are: hydroxyl and derivatives, $-\text{OH}$, $-\text{OR}$; amino and derivatives, $-\text{NH}_2$, $-\text{NHR}$, $-\text{NHR}_2$; sulphonic, $-\text{SO}_3\text{H}$; carboxylic, $-\text{COOH}$; and sulphide, $-\text{SR}$ (Marias *et al.*, 1976, Zee *et al.*, 2002). These auxochromes are important in the use classification of dyes.

According to the Colour Index dyes can be classified on the basis of colour and application method. Various attractive forces have the potential of binding dyes to fibres, and often more than one type of chemical bonding can operate with the same dye-fibre combination. The dominant force depends on the chemical character of the fibre and the chemical groups in the dye molecule. The types of bonds established between the dye and the fibre, by increasing relative strength of the bond, can be: Van der Waals, hydrogen, ionic or covalent (Ingamels *et al.*, 1993, Guaratini and Zanoni 2000, Rocha Gomes *et al.*, 2001).

Type of dye	Characteristics	Substrates
Acid	When in solution are negatively charged; bind to the cationic NH_3^+ groups present in fibres	Nylon, wool, polyamide, silk, modified acryl, paper, inks and leather
Reactive	Form covalent bonds with OH-, NH- or SH- groups	Cotton, wool, silk and nylon
Metal complex	Strong complexes of one metal ion (usually chromium, copper, cobalt or nickel) and one or two dye molecules (acid or reactive)	Silk, wool and polyamide
Direct	Large molecules bound by Van der Waals forces to the fibre	Cellulose fibres, cotton, viscose, paper, leather and nylon
Basic	Cationic compounds that bind to the acid groups of the fibre	Synthetic fibres, paper and inks
Mordant	Require the addition of a chemical that combines with the dye and the fibre, like tannic acid, alum, chrome alum, and other salts of aluminium, chromium, copper, iron, potassium, and tin	Wool, leather, silk, paper, modified cellulose fibres and anodised aluminium
Disperse	Scarcely soluble dyes that penetrate the fibre through fibres swelling	Polyester, polyamide, acetate, acrylic and plastics
Pigment	Insoluble, non-ionic compounds or insoluble salts that retain their crystalline or particulate structure throughout their application	Paints, inks, plastics and textiles
Vat	Insoluble coloured dyes which on reduction give soluble colourless forms (leuco form) with affinity for the fibre; on exposure to air are reoxidised	Cellulose fibres, cotton, viscose and wool

Azoic and Ingrain	Insoluble products of a reaction between a coupling component and a diazotised aromatic amine that occurs in the fibre	Cotton, viscose, cellulose acetate and polyester
Sulphur	Complex polymeric aromatics with heterocyclic S-containing rings	Cellulose fibres, cotton and viscose
Solvent	Non ionic dyes that dissolve the substrate to which they bind	Plastics, gasoline, varnish, lacquer, stains, inks, oils, waxes and fats
Fluorescence brighteners	Mask the yellowish tint of natural fibres	Soaps and detergents, all fibres, oils, paints and plastics
Food	Non-toxic and not used as textile dyes	Food
Natural	Obtained mainly from plants	Food, cotton, wool, silk, polyester, polyamide and polyacrylonitrile

Table 1.1. Application of categories of dyes (adapted from O'Neill *et al.* 1999 and Rocha Gomes 2001)

Reactive dyes, including many structurally different dyes, are extensively used in the textile industry because of their wide variety of colour shades, high wet fastness profiles, ease of application, brilliant colours, and minimal energy consumption. The three most common groups are azo, anthraquinone and phthalocyanine dyes (Axelsson *et al.*, 2006), most of which are toxic and carcinogenic (Acuner and Dilek, 2004). Disposal of these dyes into the environment causes serious damage, since they may significantly affect the photosynthetic activity of hydrophytes by reducing light penetration (Aksu *et al.*, 2007) and also they may be toxic to some aquatic organisms due to their breakdown products (Hao *et al.*, 2000).

Dyes are synthesized in a reactor, filtered, dried, and blended with other additives to produce the final product. The synthesis step involves reactions such as sulfonation, halogenation, amination, diazotization, and coupling, followed by separation processes that may include distillation, precipitation, and crystallization. In general, organic compounds such as naphthalene are reacted with an acid or an alkali along with an intermediate (such as a nitrating or a sulfonating compound) and a solvent to form a dye mixture. The dye is then separated from the mixture and purified. On completion of the manufacture of actual colour, finishing operations, including drying, grinding, and standardization, are performed; these are important for maintaining consistent product quality.

1.2 NATURAL DYESTUFFS

Dyestuffs and dyeing are as old as textiles themselves. Nature provides a wealth of plants which will yield their colour for the purpose of dyeing, many having been used since antiquity. Dyeing from natural sources is the oldest way of colouring textiles. Natural dyes can give subtle soft colours through to the very brightest of colour to yarn and fabric. The first colours used to dye fabrics were obtained from animal and vegetable sources. Cochineal pink from a South American beetle, yellow from flowers such as saffron and marigolds, sepia brown was obtained from cuttlefish, green from mosses and lichens, and browns and blacks from logwood bark. Colouring fabrics with natural dyes produces amazing colour combinations, depending upon the amount of natural product, fibre being dyed, concentration of the dye, time spent in the dye bath, and mordant used. Almost anything from nature can be gathered to produce colour e.g. flower heads, twigs, stems, roots, berries, fruit, herbs and vegetables. Many varieties of colours can be obtained from such natural dyes, but these colours are not very colourfast. They fade in sunlight and wash out. Because they are difficult to collect, they are also uneconomical for dyeing large amounts of fabric. A mordant allows dyes to be taken up readily, evenly and permanently by fibres, and are usually placed in the dye bath before dyeing takes place. The most common chemicals used as a mordant are alum, chrome, iron and tin. Almost any organic material will produce a colour when boiled in a dye-bath, but only certain plants will yield a colour that will act as a dye. The plants given in Table 1.2 are a selection of plants that have stood the test of time, and are used widely and traditionally by natural dyers.

Common Name	Latin Name	Parts Used	General Colours	Suggested Mordants
Alder	<i>Alnus spp</i>	Bark	Yellow/ brown/ black	Alum, iron. Copper sulphate
Alkanet	<i>Anchusa tinctoria</i>	Root	Grey	Alum, cream of tartar
Apple	<i>Malus spp</i>	Bark	Yellow	Alum
Blackberry	<i>Rubus spp</i>	Berries	Pink	Alum, tin
Lackwillow	<i>Salix negra</i>	Bark	Red, brown	Iron
Bloodroot	<i>Sanguinaria canadensis</i>	Roots	Red	Alum, tin
Buckthorn	<i>Rhamnus cathartica</i>	Twigs, berries, bark	Yellow, brown	Alum, cream of tartar, tin, iron
Cherry (wild)	<i>Prunus spp</i>	Bark	Pink, yellow, brown	Alum
Dahlia	<i>Dahlia spp</i>	Petals	Yellow bronze	Alum
Dog's mercury	<i>Mercurialis perennis</i>	Whole plant	Yellow	Alum
Dyer's broom	<i>Genista tinctoria</i>	Flowering tops	Yellow	Alum
Elder	<i>Sambucus negra</i>	Leaves, bark,	Yellow, grey	Iron, alum
Eucalyptus	<i>Eucalyptus</i>	Leaves	Deep gold, grey	
Fustic	<i>Chlorophoria tinctoria</i>	Wood shavings	Yellow	
Groundnut	<i>Arachis hypogea</i>	Kernel skins	Purple, brown, pink	Copper sulphate, alum
Henna	<i>Lawsonia inermis</i>	Leaves	Gold	
Hypogymnia lichen	<i>Hypogymnia psychodes</i>	Whole lichen	Gold, brown	
Indigo	<i>Indigofera</i>	Leaves	Blue	Not required

Ivy	<i>Hedera helix</i>	Berries	Yellow, green	Alum, tin
Madder	<i>Rubia tinctora</i>	Whole plant	Orange, red	Alum, tin
Maple	<i>Acer spp</i>	Bark	Tan	Copper sulphate
Marigold	<i>Calendula spp</i>	Whole plant, flower heads	Yellow	Alum
Nettles	<i>Urtica dioica</i>	Leaves	Beige, yellowy greens	Alum, copper
Onion	<i>Allium cepa</i>	Skins	Yellow, orange	Alum
Oak	<i>Quercus spp</i>	Inner bark	Gold, brown	Alum
Ochrolech-ina lichen	<i>Ochrolech-ina parella</i>	Whole lichen	Orange, red (when fermented in urine then boiled)	Alum
Privet	<i>Ligustrum vulgare</i>	Leaves, berries	Yellow, green, red, purple	Alum, tin
Ragwort	<i>Senecio</i>	Flowers	Deep yellow	
Safflower	<i>Carthamus tinctoria</i>	Petals	Yellow, red	Alum
Sloe-Blackthorn	<i>Prunus spinosa</i>	Sloe berries, bark	Red, pink, brown	Alum
Tea	<i>Camelia sinensis</i>	Leaves	Beige	
Turmeric	<i>Circuma longa</i>	Root	Yellow	
Wild mangosteen	<i>Diospyros peregrina</i>	Fruit	Grey, pink	
Weld (wild mignonette)	<i>Reseda luteula</i>	Whole plant	Olive green	Alum, cream of tartar
Woad	<i>Isatis tinctoria</i>	Whole plant	Blue	Lime

Table 1.2. Selection of natural plants that have been widely used by natural dyers

1.2.1 ADVANTAGES OF NATURAL DYES

Though all natural dyes are not 100% safe they are less toxic than their synthetic counterparts. Many of the natural dyes like turmeric, annatto and saffron are permitted as food additives. Many natural dyes have pharmacological effects and possible health benefits.

- They are obtained from renewable sources.
- Natural dyes cause no disposal problems, as they are biodegradable.
- Practically no or mild reactions are involved in their preparation.
- They are unsophisticated and harmonized with nature.
- Natural dyes are cost effective
- It is possible to obtain a full range of colours using various mordants.

1.2.2 DISADVANTAGES OF NATURAL DYES

- Availability
- Colour yield
- Complexity of textile dyeing process
- Reproducibility of shade

Besides these there are other technical drawbacks of natural dyes. These are:-

- Limited number of suitable dyes
- Great difficulty in blending dyes
- Non-standardized
- Inadequate degree of fixation
- Inadequate fastness properties except few exceptions
- Water pollution by heavy metals and large amounts of organic substances.

1.3 DYEING PROCESS:

Dyeing is an ancient art which predates written records. It was practiced during the Bronze age in Europe. Primitive dyeing techniques included sticking plants to fabric or rubbing crushed pigments into cloth. The methods became more sophisticated with time and techniques using natural dyes from crushed fruits, berries

and other plants, which were boiled into the fabric and gave light and water fastness (resistance), were developed.

Some of the well known ancient dyes include madder, a red dye made from the roots of the *Rubia tinctorum*, blue indigo from the leaves of *Indigofera tinctoria*, yellow from the stigmas of the saffron plant, and dogwood, an extract of pulp of the dogwood tree.

In many of the world's developing countries, however, natural dyes can offer not only a rich and varied source of dyestuff, but also the possibility of an income through sustainable harvest and sale of these dye plants. Many dyes are available from tree waste or can be easily grown in market gardens. In areas where synthetic dyes, mordants (fixatives) and other additives are imported and therefore relatively expensive, natural dyes can offer an attractive alternative.

1.4 METHODS OF DYEING

There are a number of methods of applying dye to a fabric. Although the most common method used for applying natural dyes is the vat method, there are techniques which have been developed to allow patterns to be incorporated during the dyeing process. It is worth bearing in mind that using natural dyes is a complex art and the skills required for using natural dyes are learned over many years.

1.4.1 VAT DYEING

In the simplest form of dyeing a textile material is immersed in dye and gradually brought to the boil. Alternatively the fibre is allowed to sit and soak for several hours or days. During this period, agitation is necessary to allow full penetration of the textile by the dyestuff. Depending on the type of fabric and dyestuff used, certain salts or acids may be added to assist absorption of the dye. The principal difficulty in dyeing mixed yarns and fabrics is to achieve the same colour in both fibres. Cotton fibres may, for instance, absorb dyes rapidly, while the wool fibres will have to be boiled over an extended period to reach the same depth of shade. This could lead to significant damage to the material. In this case a chemical compound would need to be used to restrain the rate at which the cotton fibre takes up the dyestuff.

1.4.2 BATIK

Batik is a starch resist-dyeing process, developed on the Island of Java in modern-day Indonesia. Colour is prevented from reaching certain areas of a fabric by covering these areas with molten wax. The fabric is starched prior to the design being drawn upon it. The wax is applied with a type of cup with a fine pouring spout, usually made of copper. The technique has been developed to a high art form in Indonesia from where it is exported to many parts of the world. Batik paintings, as well as sarongs and lengths of fabric, are produced. When the fabric is dyed, all waxed areas resist the dyestuff. The wax is then removed by placing the fabric in boiling water. For patterns with many colours the same procedure is repeated until the full design is completed.

1.4.3 TIE-DYEING

Tie-dyeing is another popular artisanal dyeing technique. In this resist-dyeing process, waxed thread is tightly tied around the areas chosen to resist the coloured dyestuff, and the fabric is dipped into the dye. The waxed thread is then removed and the fabric dried. This process can be repeated for each colour to be added.

1.4.4 DIRECT DYEING

When a dye is applied directly to the fabric without the aid of an affixing agent, it is called direct dyeing. In this method the dyestuff is either fermented (for natural dye) or chemically reduced (for synthetic vat and sulphur dyes) before being applied. The direct dyes, which are largely used for dyeing cotton, are water soluble and can be applied directly to the fibre from an aqueous solution. Most other classes of synthetic dye, other than vat and sulphur dyes, are also applied in this way.

1.4.5 YARN DYEING

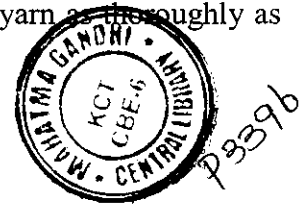
When dyeing is done after the fibre has been spun into yarn, it is called Yarn dyeing. There are many forms of yarn dyeing- Skein (Hank) Dyeing, Package Dyeing, Warp-beam Dyeing, and Space Dyeing.

1.4.5.1 SKEIN (HANK) DYEING

The yarns are loosely arranged in skeins or hanks. These are then hung over a rung and immersed in a dye bath in a large container. In this method, the colour penetration is the best and the yarns retain a softer, loftier feel.

1.4.5.2 PACKAGE DYEING

The yarns are wound on spools, cones or similar units and these packages of yarn are stacked on perforated rods in a rack and then immersed in a tank. In the tank, the dye is forced outward from the rods under pressure through the spools and then back to the packages towards the centre to penetrate the entire yarn as thoroughly as possible.



1.4.5.3 WRAP-BEAM METHOD

It is similar to package dyeing but more economical. Here, yarn is wound on to a perforated warp beam, immersed in a tank and dyed under pressure.

1.4.5.4 SPACE DYEING

In this method, the yarn is dyed at intervals along its length. For these two procedures- knit- deknit method and OPI Space-Dye Applicator- are adopted. In the first method, the yarn is knitted on either a circular or flat-bed knitting machine and the knitted cloth is then dyed and subsequently it is deknitted. Since the dye does not readily penetrate the areas of the yarn where it crosses itself, alternated dyed and undyed spaces appear. The OPI Space-Dye Applicator technique produces multi coloured space- dyed yarns. The yarns are dyed intermittently as they run at high speeds of upto 1000 yards (900 m) per minute through spaced dye baths with continuous subjection to shock waves produced by compressed air assuming supersonic velocities.

1.5 ECOLOGICAL ASPECTS

The most problematic industries in terms of dye release to the environment in the form of wastewater are the production of dyes and the dyeing industry. The uncontrolled release of these compounds in the environment causes severe problems. Since they are designed to be chemically and photolytically stable they are highly

persistent in natural environments. For instance, the half-life of hydrolysed Reactive Blue 19 is about 46 years at pH 7 and 25°C (Hao *et al.* 2000) In fact they are xenobiotic compounds because they do not exist as natural products and therefore contain structural elements that cannot be synthesized biochemically (Stolz *et al.*, 2001, Rieger *et al.* 2002). During evolution of catabolic enzymes and pathways microorganisms were not exposed to these structures and have not developed the capability to use those compounds as sole sources of carbon and energy. Dyes, by decreasing light absorption, may significantly affect photosynthetic activity of aquatic life and may be toxic due to the presence of aromatics or heavy metals (Banat *et al.*, 1996, Slokar and Le Marechal 1998, Zanoni and Carneiro 2001, Kunz *et al.*, 2002, Carneiro *et al.*, 2004).

1.6 CHARACTERISATION OF TEXTILE WASTEWATERS

Wet processing in textile industry generates large amounts of a wastewater whose pollution load arises not only from the removal of impurities from the raw materials themselves but also from the residual chemical reagents used for processing. The extreme diversity of raw materials and production schemes employed poses problems in assessing effluent characteristics and subsequently defining pollution control technologies (Correia *et al.*, 1994). Production of textile involves bleaching, mercerizing, carbonizing and dyeing, etc. Polyvinyl, alcohol, gums, PCP, cellulose materials, dyes and other substances are present in textile effluent. Azo and reactive dyes constitute the largest class of dyes used commercially in textile industries for dyeing nylon, polyacrylonitrile modified nylon, wool, silk, cotton, etc. Dyes are recalcitrant molecules difficult to be degraded biologically. Heavy metals as cadmium, chromium, copper, lead, zinc are present in the dyes. (Black *et al.* 1980) have demonstrated that aniline dyes could be mutagenic and carcinogenic to biota. These chemicals have been suggested to be responsible for the promotion of tumor growth in several bottom-feeding species of fish (Diachenko *et al.*, 1979).

The amount of dye lost is dependent upon the dye application class, varying from only 2% loss when using basic dyes to a 50% loss when certain reactive dyes are used (O'Neill *et al.*, 1999, McMullan *et al.*, 2001)

Pollutants	Chemical types	Process of origin
Organic load	Starches, enzymes, fats, greases, waxes, surfactants and acetic acid	Ds, S, W, Dy
Colour	Dyes, scoured wool impurities	Dy, S
Nutrients (N, P)	Ammonium salts, urea, phosphate-based buffers and sequestrants	Dy
pH and salts	NaOH, mineral/organic acids, sodium chloride, silicate, sulphate, carbonate	S, Ds, B, M, Dy, N
Sulphur	Sulphate, sulphite and hydrosulphite salts, sulphuric acid	Dy
Toxic compounds	Heavy metals, reducing agents (sulphide), oxidising agents (chlorite, peroxide, dichromate, persulphate), biocides, quaternary ammonium salts	Ds, B, Dy, F
Refractory organics	Surfactants, dyes, resins, synthetic sizes (PVA), chlorinated organic compounds, carrier organic solvents	S, Ds, B, Dy, W, F

Table 1.3. Major pollutant types in textile wastewater, chemical types and process of origin (adapted from Delle *et al.*, 1998).

Ds – desizing; **S** – scouring; **W** – washing; **Dy** – dyeing; **B** – bleaching; **M** – mercerising; **N** – neutralisation; **F** – finishing.

The principal air pollutants from dye manufacturing are volatile organic compounds (VOCs), nitrogen oxides (NO_x), hydrogen chloride (HCl), and sulfur oxides (SO_x). Liquid effluents resulting from equipment cleaning after batch operation can contain toxic organic residues. Cooling waters are normally recirculated. Wastewater generation rates are of the order of 1–700 liters per kg (l/kg) of product except for vat dyes. The wastewater generation rate for vat dyes can be of the order of 8,000 l/kg of product. Biochemical oxygen demand (BOD) and chemical oxygen demand (COD) levels of reactive and azo dyes can be of the order of 25 kg/kg

of product and 80 kg/ kg of product, respectively. Values for other dyes are, for example, BOD, 6 kg/kg; COD, 25 kg/kg; suspending solids, 6 kg/kg; and oil and grease, 30 kg/kg of product. Major solid wastes of concern include filtration sludges, process and effluent treatment sludges, and container residues. Examples of wastes considered toxic include wastewater treatment sludges, spent acids, and process residues from the manufacture of chrome yellow and orange pigments, molybdate orange pigments, zinc yellow pigments, chrome and chrome oxide green pigments, iron blue pigments, and azo dyes.

1.7 TREATMENT OF TEXTILE WASTEWATERS

A large number of methods are available to treat the waste-water containing dyes (Senior *et al.*, 1976). Colour is the first contaminant to be recognized in wastewater and has to be removed before discharging into water bodies. The presence of very small amounts of dyes in water (less than 1 ppm for some dyes) is highly visible and affects the aesthetic quality water transparency and gas solubility in lakes, rivers and other water bodies. The removal of colour is often more important than the removal of the soluble colourless organic substances, which usually contribute the major fraction of the biochemical oxygen demand (BOD). Methods for the removal of BOD from most effluents are fairly well established. Dyes, however, are more difficult to treat because of their synthetic origin and complex aromatic molecular structures (Banat *et al.*, 1996). The electron-withdrawal character of the azo-group generates electron deficiency. This makes the compounds less susceptible to oxidative catabolism and as a consequence many of these chemicals tend to persist under aerobic environmental conditions (Knackmuss *et al.*, 1996).

Dyes must have a high degree of chemical and photolytic stability in order to be useful, due to the harshness of the conditions to which they are submitted during and after the dyeing process (Gomes *et al.*, 2001). Therefore they will, in general, give negative results in short-term tests for aerobic biodegradability (Ollgaard *et al.*, 1998). Stability against microbial attack is also a required feature of azo dyes (Pagga and Brown 1986), because it will prolong the lifetime of the products in which azo dyes are applied.

When designing the solution for a particular wastewater there are several conditionings that should be considered in this choice. Due to the high variability of composition of this type of wastewater, not only in quantity but mainly in quality, usually there is the need of more than one type of treatment to achieve the necessary quality of treatment. There are two possible locations for any technology that could be used to remove the colour that is present in the wastewater. The first possibility is in the dye house, allowing the partial or full re-use of water. This possibility deals with a considerable smaller amount of wastewater than the next possibility which is the treatment on a municipal (or particular) wastewater treatment plant, usually with a biological step. In this case, the coloured wastewater is mixed with domestic wastewater to provide organic load for the biological treatment. And in this hypothesis it can be used before the current biological or chemical treatment or as a final polishing step (Pearce *et al.*, 2003). A wide range of methods have been developed for the removal of synthetic dyes from waters and wastewaters to decrease their impact on the environment. They are divided in three major categories: physical, chemical or biological. Biological and chemical methods involve the destruction of the dye molecule, whilst physical methods usually transfer the pollutant to another phase. In the dyeing industry the waste must be reduced and then the waste should be segregated and based on the segregation the treatment should be done. Some of the treatment techniques are as follows:

1.7.1 PHYSICAL TREATMENTS

1.7.1.1 SORPTION

Remediation based on sorption phenomena involves binding of soluble or suspended pollutants to a solid organic or inorganic matrix

1.7.1.2 MEMBRANE FILTRATION.

This technology has emerged as a feasible alternative to conventional treatment processes of dye wastewater and has proven to save operation costs and water consumptions by water recycling (Sójka-Ledakowicz *et al.*, 1998, Koyunco *et al.*, 2001, Kim *et al.*, 2004a, Koyunco *et al.*, 2004).

1.7.1.3 ION EXCHANGE

In this technique wastewater is passed over the ion exchanger resin until all available exchange sites are saturated. Both anionic and cationic dyes are efficiently removed by this method. A disadvantage of this method is the high cost of organic solvents to regenerate the ion-exchanger (Slokar and Le Marechal 1998, Robinson *et al.*, 2001).

1.7.1.4 COAGULATION/FLOCCULATION

This method is often applied in the treatment of different types of wastewaters and it is used to enhance the degree of removal of total suspended solids (TSS), biochemical oxygen demand (BOD), chemical oxygen demand (COD) and colour (Semerjian and Ayoub 2003, Allegre *et al.*, 2004, Peres *et al.*, 2004, Aguilar *et al.*, 2005). The first step, coagulation, consists in the addition of a coagulant to the wastewater and mixing. This coagulant destabilizes the colloidal particles that exist in the suspension, allowing particle agglomeration. Flocculation is the physical process of bringing the destabilized particles in contact to form larger flocs that can be more easily removed from the solution (Zee *et al.*, 2002, Semerjian and Ayoub 2003, Golob and Ojstršek 2005). This is usually achieved by a slow mixing step.

1.7.2 CHEMICAL TREATMENTS

1.7.2.1 FENTON'S REAGENT

H_2O_2 -Fe(II) salts are used when the wastewater is resistant to biological treatment or is toxic to the biomass (Slokar and Le Marechal 1998). The reaction, that occurs at acidic pH, results in the formation of a strong oxidative hydroxyl radical (HO \cdot) and ferric iron (from the oxidation of ferrous iron).

1.7.2.2 OZONE

This is a very good oxidizing agent due to its high instability (reduction potential 2.07V) when compared to chlorine (1.36V) and H_2O_2 (1.78V). It degrades a high number of pollutants like phenols, pesticides and aromatic hydrocarbons and it is used since the early 1970s in wastewater treatment (Robinson *et al.*, 2001, Özbelge *et al.*, 2002, Pera-Titus *et al.*, 2004).

1.7.2.3 PHOTOCHEMICAL

Processes like UV/H₂O₂, UV/TiO₂, UV/Fenton's reagent, UV/O₃ and others are photochemical methods based on the formation of free radicals due to UV irradiation. Degradation is caused by the production of high concentrations of hydroxyl radicals and the dye molecule is degraded to CO₂ and H₂O (Peralta-Zamora *et al.*, 1999, Robinson *et al.*, 2001, Gogate and Pandit 2004a).

1.7.2.4 SODIUM HYPOCHLORITE (NaOCl)

Coloured wastewaters can be chemically oxidized by chlorine compounds although, for environmental reasons (release of toxic organochlorinated compounds), its use is becoming less frequent (Slokar and Le Marechal 1998, Pizzolato *et al.*, 2002).

1.7.2.5 ELECTROLYSIS

Electrolysis is based on applying an electric current through to the wastewater by using electrodes. Organic compounds like dyes react through a combination of electrochemical oxidation, electrochemical reduction, electrocoagulation and electroflotation reactions.

1.7.2.6 WET AIR OXIDATION (WAO)

In this process the primary oxidant species is oxygen and it is used to produce OH-radicals which actually react with organic and inorganic species under sub-critical conditions, i.e. temperature between 175 and 320°C and pressure between 60 and 200 bar (Heimbuch and Wilhelmi 1985, Jeworski and Heinzle 2000, Arslan-Alaton and Ferry 2002, Gogate and Pandit 2004b).

1.7.2.7 ULTRASOUND

This technology can also be used in textile dye remediation but is still a very recent technology in phase of development (Gogate and Pandit 2004a, Vajnhandl and Le Marechal 2005).

1.7.3 BIOLOGICAL TREATMENTS

The fate of environment pollutants is largely determined by abiotic processes, such as photooxidation, and by the metabolic activities of microorganisms. Since catabolic enzymes are more or less specific, they can act on more than their natural substrate. This explains why the majority of xenobiotics are subject to fortuitous metabolism (cometabolism) (Knackmuss *et al.*, 1996) and several groups explore these microbial capacities for the bioremediation of dyes.

1.7.3.1 BACTERIA

Actinomycetes, particularly *Streptomyces* species, are known to produce extracellular peroxidases that have a role in the degradation of lignin and were also found effective in the degradation of dyes (Paszczynski *et al.*, 1991, Zhou and Zimmerman 1993, Cao *et al.*, 1993, McMullan *et al.*, 2001, Bhaskar *et al.*, 2003).

1.7.3.2 FUNGI

The most widely studied dye-decolourising microorganisms are the white-rot fungi like *Phanerochaete chrysosporium* (Mielgo *et al.*, 2002), *Trametes versicolor* (Tekere *et al.*, 2001), *Coriolus versicolor* (Kapdan and Kargi 2002) and *Bjerkandera adusta* (Weidtmann *et al.*, 2001).

1.7.3.3 ALGAE

The use of algae for the degradation of dyes is mentioned in only few reports and is achieved by *Chlorella* (Acuner and Dilek 2004), *Oscillatoria* (Jinqi and Houtian 1992) and *Spirogyra* (Mohan *et al.*, 2002) species.

1.7.3.4 ENZYMES

In the studies of biological degradation of dyes an effort has been made in order to identify, isolate and test the enzymes responsible for the decolourisation. In the case of extracellular fungal enzymes, like manganese and lignin peroxidases and laccases, or cytosolic azoreductases from bacteria, this has been achieved by several groups (Maier *et al.*, 2004).

1.8 MALACHITE GREEN

Malachite green is a basic dye. Basic dyes are salts of the colored organic bases containing amino and imino groups and also combined with a colorless acid,

such as hydrochloric or sulfuric. They are brilliant and most fluorescent among all synthetic dyes. Basic dyes are cationic which has positive electrical charge and are used for anionic fabrics which are negative-charge-bearing, such as wool, silk, nylon, and acrylics where bright dyeing is the prime consideration. This chemical dye is primarily designed to be used as a dye for silk, leather, and paper. Malachite green in dilute solution is widely used medicinally as a local antiseptic. It is effective against parasites, fungal infections and gram-positive bacteria. In combination with formalin as a synergist, malachite green is a common antiseptic agent against the fungus.

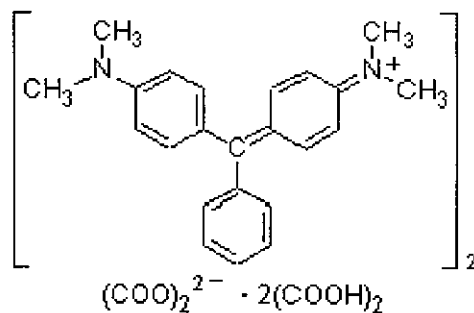


Fig 1.1 Structure of Malachite green.

1.9 LIQUID -LIQUID EXTRACTION

Liquid-Liquid extraction is a mass transfer operation in which a liquid solution (the feed) is contacted with an immiscible or nearly immiscible liquid (solvent) that exhibits preferential affinity or selectivity towards one or more of the components in the feed. Two streams result from this contact: the extract, which is the solvent rich solution containing the desired extracted solute, and the raffinate, the residual feed solution containing little solute. The following need to be carefully evaluated when optimizing the design and operation of the extraction processes.

- Solvent selection
- Operating Conditions
- Mode of Operation
- Extractor Type
- Design Criteria

1.8.1 SOLVENT SELECTION

Solvents differ in their extraction capabilities depending on their own and the solute's chemical structure. Based on the Organic-Group interactions one can identify the desired functional group(s) in the solvent for any given solute.

1.8.2 SELECTION OF EXTRACTION CONDITIONS

Depending on the nature of the extraction process, the temperature, pH and residence time could have an effect on the yield and selectivity. Operating pressure has a negligible effect on extraction performance and therefore most extractions take place at atmospheric pressure unless governed by vapour pressure considerations.

1.8.3 SELECTION OF MODE OF OPERATION

Extractors can be operated in crosscurrent or counter-current mode.

1.8.4 DESIGN CRITERIA

The basic function of extraction equipment is to mix two phases, form and maintain droplets of dispersed phase and subsequently separate the phases. The following outlines some of the factors that need to be considered while designing and optimizing extraction equipment.

1.8.4.1 MIXING

The amount of mixing required is determined by physical properties such as viscosity, interfacial tension and density differences between the two phases. It is important to provide just the right amount of mixing. Less mixing causes the formation of large droplets and decreases interfacial area (interfacial area varies with the square of the droplet diameter). This reduces mass transfer and decreases stage efficiency. Higher agitation (more mixing) minimizes mass transfer resistance during reactions and extraction but contributes to the formation of small and difficult-to-settle droplets or emulsions.

1.8.4.2 SETTLING

The settling characteristics depend on the fluid properties (density difference, interfacial tension, and continuous phase viscosity) and the amount of mixing. Settling in agitated batch vessels is carried out by stopping the agitator. In

continuous columns, a settling section is provided either as a part of the extractor or as a separate piece of equipment downstream of the extractor.

1.8.4.3 SELECTION OF CONTINUOUS AND DISPERSED PHASE

In column extractors, the phase with the lower viscosity (lower flow resistance) is generally chosen as the continuous phase. Also note that the phase with the higher flow rate can be dispersed to create more interfacial area and turbulence. This is accomplished by selecting an appropriate material of construction with the desired wetting characteristics. In general, aqueous phases wet metal surfaces and organic phases wet non-metallic surfaces. Change in flows and physical properties along the length of extractor should also be considered.

1.9 LACCASE

Laccase (*p*-diphenol:oxygen oxidoreductase; EC 1.10.3.2) is a copper-containing enzyme that catalyzes the oxidation of a phenolic substrate by coupling it to the reduction of oxygen to water. Fungal laccases display a wide substrate range, are known to catalyze the polymerization, depolymerisation, and methylation and/or demethylation of phenolic compounds (Leonowicz *et al.*, 1985, Leonowicz *et al.*, 1979), and may play a role in plant pathogenicity (VanEtten *et al.*, 1989, VanEtten *et al.*, 1995) or lignin degradation (Hatakka *et al.*, 1998). Laccases are the most numerous members of the multicopper protein family, which also includes tyrosinases, monooxygenases, and dioxygenases. Phylogenetically, these enzymes have developed from small sized prokaryotic azurins to eukaryotic plasma proteins ceruloplasmin (Claus *et al.*, 2003). They contain four histidine-rich copper-binding domains, which coordinate the types 1– 3 copper atoms that differ in their environment and spectroscopic properties (Messerschmidt and Huber 1990). The bacterial azurins, e.g., crystallized rusticyanin from *Thiobacillus ferrooxidans*, which contain only type 1 copper, can be regarded as the precursor protein of laccases (Hough *et al.*, 2001).

The description of the first laccase mediator, 2,2-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) to more recent use of the—NOH-type synthetic mediator, including 1-hydroxybenzotriazole (HBT), violuric acid (VLA) and N-hydroxyacetanilide (NHA), a large number of studies have been produced on the

mechanisms of oxidation of non-phenolic substrates (Baiocco *et al.*, 2003). The use of naturally occurring mediators would present environmental and economic advantage (Camarero *et al.*, 2005).

The enzyme possesses great biotechnological potential because of its wide reaction capabilities as well as broad substrate specificity. Promising applications include biosensors for drug analysis and phenols in tea (Ghindilis *et al.*, 1992, Peter and Wollenberger 1997), polymer synthesis (Huttermann *et al.*, 2001), textile-dye bleaching (Claus *et al.*, 2002), bioremediation (Murugesan *et al.*, 2003, Wesenberg *et al.*, 2003), fungicides (Spillman *et al.*, 2003), pulp bleaching (Palonen and Viikari 2004), clarification of juices and wines (Ygshinwa *et al.*, 2004).

1.9.1 DISTRIBUTION OF LACCASES

Laccases are common enzymes in nature. The first laccase was reported in 1883 from *Rhus vernicifera*, the Japanese lacquer tree (Reinhammar *et al.*, 1984), from which the designation laccase was derived. Subsequently laccases have been discovered in numerous other plants, e.g., peach (Lehman *et al.*, 1974), sycamore (Bligny and Douce 1983), tobacco (De Marco and Roubelakis-Angelakis 1997) and poplar (Ranocha *et al.*, 1999). The plant laccases have not been characterized or used extensively despite their wide occurrence, because their detection and purification is often difficult, as the crude plant extracts contain a large number of oxidative enzymes with broad substrate specificities (Ranocha *et al.*, 1999). The majority of laccases characterized so far have been derived from fungi especially from white-rot basidiomycetes that are efficient lignin degraders. Well-known laccase producers also include fungi belonging to the ascomycetes, deuteromycetes, basidiomycetes, and cellulolytic fungi such as *Neurospora crassa* (Froehner and Eriksson 1974), *Agaricus bisporus* (Wood *et al.*, 1980), *Botrytis cinerea* (Marbach *et al.*, 1984), *Pleurotus ostreatus* (Sannia *et al.*, 1986), *Phlebia radiata* (Niku-Paavola *et al.*, 1988), *Trametes versicolor* and *Coriolus polyporus* (Rogalski *et al.*, 1991), *Pycnoporus cinnabarinus* (Eggert *et al.*, 1996), *Chaetomium thermophilum* (Chefetz *et al.*, 1998), and *Coprinus cinereus* (Schneider *et al.*, 1999).

Literature Review

2. LITERATURE REVIEW

The release of dyes into the environment constitutes only a small proportion of water pollution, but dyes are visible in small quantities due to their brilliance. Removal of dyes from effluents is by physio-chemical means. Such methods are often very costly and although the dyes are removed, accumulation of concentrated sludge creates a disposal problem. There is a need to find alternative treatments that are effective in removing dyes from large volumes of effluents and are low in cost, such as biological or combination systems (Nigam *et al.*, 2001).

2.1 LIQUID-LIQUID EXTRACTION

Liquid-liquid extraction of Black B dye from batik industry waste water using tridodecylamine (TDA) in kerosene (Othman *et al.*, 2011).

The solvent extraction of methyl violet from aqueous acidic solutions by salicylic acid in xylene was done (Muthuraman and Teng 2010).

Liquid–liquid extraction (LLE) of methylene blue (MB) from industrial wastewater using benzoic acid in xylene has been studied at 27°C. The extraction of the dye increased with increasing extractant concentration. The extracted dye in the organic phase can be back extracted into sulphuric acid solution. The resultant recovered organic phase can be reused in succeeding extraction of dye with the yield ranging from 99 to 87% after 15 times reused, depending on the concentration of the initial feed solution (Muthuraman *et al.*, 2009).

Tetrabutyl ammonium bromide (TBAB) has been demonstrated to be a very effective reagent for selective extraction of anionic dyes from aqueous solution. The solution of TBAB in methylene chloride is able to extract 98% of 50 mg L⁻¹ concentration of dye solution. The distribution ratio is reasonably high (D=49.0) even in the presence of excess chloride and sulphate ions. Extracted dye in the organic phase can be back extracted with excess of salicylic acid and sodium carbonate solution (Muthuraman and Palanivelu 2004).

2.2 LACCASE

Laccases (benzenediol oxygen oxidoreductases, EC 1.10.3.2) are polyphenol oxidases (PPO) that catalyze the oxidation of various substituted phenolic compounds by using molecular oxygen as the electron acceptor. The ability of laccases to act on a wide range of substrates makes them highly useful biocatalysts for various biotechnological applications. To date, laccases have mostly been isolated and characterized from plants and fungi, and only fungal laccases are used currently in biotechnological applications. In contrast, little is known about bacterial laccases (Capalash *et al.*, 2006).

The bacterial azurins, e.g., crystallized rusticyanin from *Thiobacillus ferrooxidans*, which contain only type 1 copper, can be regarded as the precursor protein of laccases (Hough *et al.*, 2001). Laccases are the model enzymes for multi-copper oxidases and participate in (1) cross-linking of monomers, (2) degradation of polymers, and (3) ring cleavage of aromatic compounds (Kawai *et al.*, 1988).

For catalyzing the oxidation of non-phenolic substrates, laccase requires the presence of a mediator in the medium. A mediator is a small molecule that behaves like an 'electron shuttle' between laccase and substrate and these small molecular-mass compounds are converted into stable radicals by means of enzymatic oxidation. They act as redox mediators and oxidize other compounds that, in principle, are not substrates of laccase.

The use of naturally occurring mediators would present environmental and economic advantage (Camarero *et al.*, 2005). The enzyme possesses great biotechnological potential because of its wide reaction capabilities as well as broad substrate specificity. Promising applications include biosensors for drug analysis and phenols in tea (Ghindilis *et al.*, 1992; Peter and Wollenberger 1997), polymer synthesis (Huttermann *et al.*, 2001), textile-dye bleaching (Claus *et al.* 2002), bioremediation, fungicides (Spillman, 2003) pulp bleaching (Palonen and Viikari 2004), clarification of juices and wines.

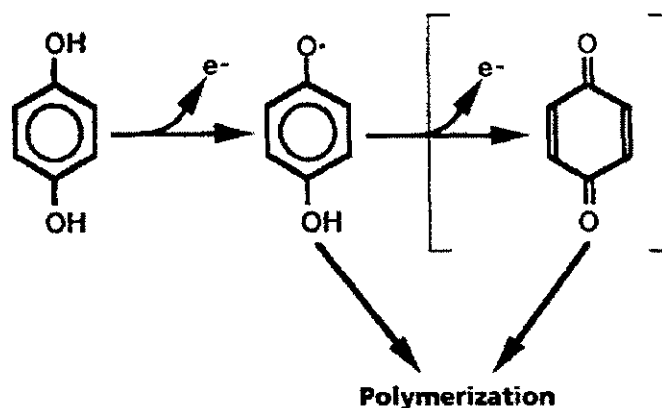


Fig.2.1. A typical laccase reaction where a diphenol (hydroquinone) undergoes one-electron oxidation to form an oxygen-centred free radical.

2.2.1 STRUCTURE OF LACCASE

The overall fold of laccases comprises three cupredoxin-like domains A, B and C, that are about equal in size. The cupredoxin fold is common among copper-containing proteins, and it has also been found in the simple copper proteins plant plastocyanin and bacterial azurin, as well as in the more complex multicopper oxidases ascorbate oxidase (Messerschmidt *et al.*, 1990) and ceruloplasmin. All three domains are important for the catalytic activity of laccases: the substrate-binding site is located in a cleft between domains B and C, a mononuclear copper center is located in domain C, and a trinuclear copper center is located at the interface between domains A and C.

The mononuclear copper center contains one type-1 (T1) copper atom that is trigonally coordinated to two histidines and a cysteine. The coordination bond between T1 and SCys is highly covalent, which causes a strong absorption around 600 nm and gives laccases their typical blue color (Solomon *et al.*, 1996). T1 also has an distant axial ligand which is a leucine or phenylalanine residue in fungal laccases (Piontek *et al.*, 2002) and a methionine residue in the bacterial *Bacillus subtilis* CotA laccase and in other multicopper oxidases (Enguita *et al.*, 2003).

The trinuclear cluster contains one type-2 (T2) copper atom and a pair of type-3 (T3) coppers. The T2 copper is coordinated by two and the T3 copper atoms by six conserved histidines (Bertrand *et al.*, 2002). The T1 and T2 coppers are paramagnetic

and can be identified in electron paramagnetic resonance (EPR) spectrum. The T3 copper pair is antiferromagnetically coupled by a bridging hydroxide, which makes the T3 coppers EPR-silent (Solomon *et al.*, 1996). However, they can be detected by their characteristic absorbance at 330 nm (Solomon *et al.*, 1996).



Fig 2.2. Three-dimensional structure of *M. albomyces* laccase
(The four copper atoms are shown as yellow balls and carbohydrates as grey sticks)

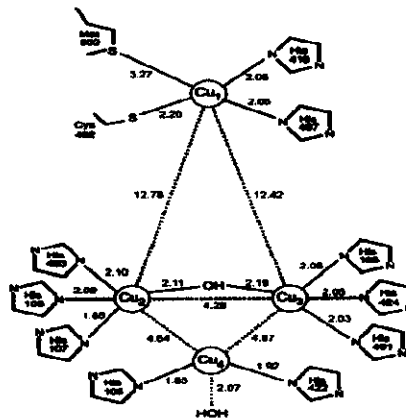


Fig 2.3. Copper centers of the laccase (CotA) from *B.subtilis* (Euguita *et al.*, 2003)

2.2.2 SUBSTRATES FOR LACCASE

Some mediators are 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonate) (ABTS), 1-hydroxyl benzotriazole (HBT), benzotriazole (BT), remazol brilliant blue (RBB), chlorpromazine (CPZ), promazine (PZ), 1-nitroso-2-naphthol-3,6-disulfonic acid (NNDS) and 4-hydroxy-3-nitroso-1-naphthalenesulfonic acid (HNNS). Mediators ABTS and HBT are those that have been used more in processes such as pulp delignification (Bourbonnais *et al.*, 1997), decolourization and detoxification of textile dyes (Reyes *et al.*, 1999), degradation of recalcitrant organo pollutants such as polycyclic aromatic hydrocarbons (PAHs) (Majcherczyk *et al.*, 1998; Bourbonnais and Paice, 1990) found that the substrate range of laccase can be extended to nonphenolic subunits of lignin by inclusion of a mediator, such as ABTS.

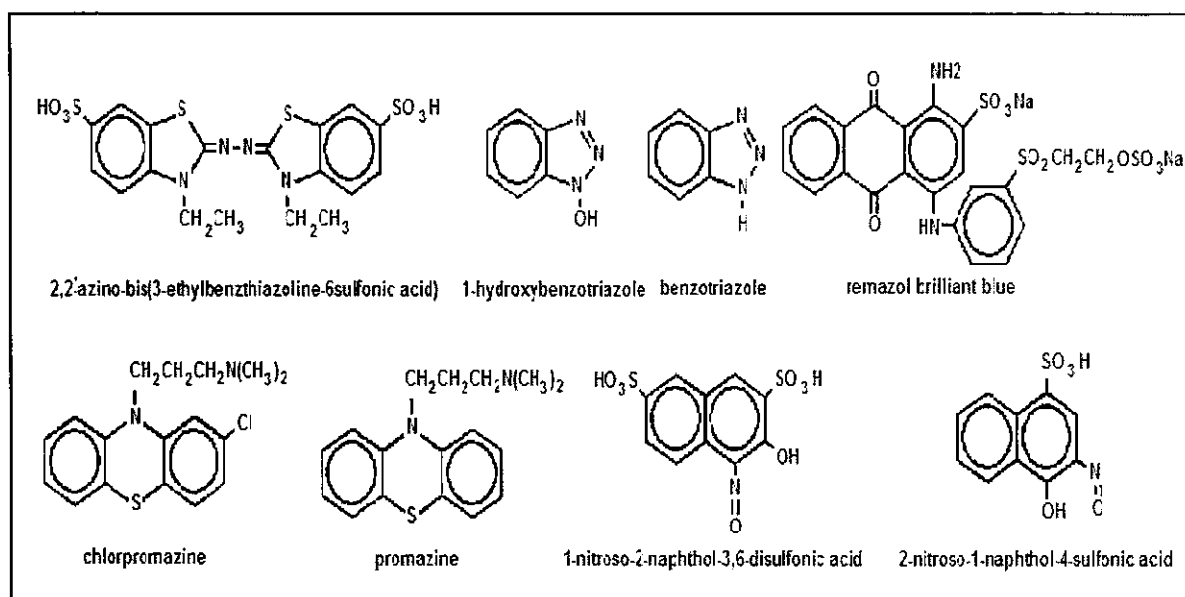


Fig. 2.4. Structures of some laccase mediators (Bourbonnais *et al.*, 1997).

2.2.2 USES OF LACCASE

The first report of prokaryotic laccase is from the rhizospheric bacterium *Azospirillum lipoferum* (Givaudan *et al.*, 1993), where laccase occurs as a multimeric enzyme composed of a catalytic subunit and one or two large chains. The enzyme plays a role in cell pigmentation and utilization of plant phenolic compounds (Faure *et al.*, 1994, 1995) and/or electron transport (Alexandre *et al.*, 1999). Another laccase has been reported from a melanogenic marine bacterium *Marinomonas mediterranea* producing two different polyphenol oxidases (PPO), an unusual multi-potent PPOable

to oxidize substrates characteristic of both tyrosinase and laccase (Solano *et al.*, 1997).

Laccase-like activity has also been found in other bacteria, e.g., CopA protein from *Pseudomonas syringae* (Mellano and Cooksey, 1988) and PcoA protein from *Escherichia coli* (Brown *et al.*, 1995). These are structurally homologous to multi-copper oxidases with respect to canonical copper binding sites and have been shown to be important for bacterial copper resistance.

The best-studied bacterial laccase is the CotA, the endospore coat component of *Bacillus subtilis*. The *cotA* gene codes for a 65-kDa protein belonging to the outer spore coat. CotA participates in the biosynthesis of the brown spore pigment, which is also thought to be a melanin-like product and seems to be responsible for most of the protection afforded by the spore coat against UV light and hydrogen peroxide. CotA protein displays similarities with multi-copper oxidases. The protein exhibits a higher thermal stability with a half-life of about 2 h at 80°C and optimum temperature 75°C (Martins *et al.*, 2002). Ligands involved in T1 copper binding are two histidines, a cysteine, and a fourth ligand that varies between the members of the multi-copper oxidase family.

In the equivalent sequence position CotA has a methionine residue (Met-502) and thus, presents a T1 copper site similar to that of ZAO but also to other fungal laccases. The single amino acid substitution of histidine 497 by an alanine (H497A) or replacement of methionine 592 with a leucine impair the copper coordination by the surface-exposed T1 center, thus altering drastically the enzymological properties of the protein.

A laccase-like enzyme activity was also found in spores of *Bacillus sphaericus* (Claus and Filip 1997). A laccase-like spore protein of a marine bacillus strain SG1 has been shown to oxidize Mn (II). Mutants in genes coding for multi-copper oxidases have lost their metal-oxidizing activities. On polyacrylamide gels, laccase activity was present as molecular mass complexes or multimers in enzymes from this bacterium (Van Waasbergen *et al.*, 1996).

2.3 DEGRADATION OF DYES

2.3.1 DEGRADATION OF DYES BY LACCASE

The decomposition of azo dyes by oxidative methods, such as enzymatic and ultrasound treatments. Each of these methods has strong and feeble sides. The laccase treatment showed high decolorization rates but cannot degrade all investigated dyes (reactive dyes), and high anionic strength led to enzyme deactivation. Ultrasound treatment can decolorize all tested dyes after 3 h at a high energy input, and prolonged sonication leads to nontoxic ionic species, which was demonstrated by ion chromatography and toxicity assays. For the first time, it was shown that a combination of laccase and ultrasound treatments can have synergistic effects, which was shown by higher degradation rates. Bulk light absorption and ion-pairing high-performance liquid chromatography (IP-HPLC) were used for process monitoring, while with reversed phase HPLC, a lower number of intermediates than expected by IP-HPLC was found.

Liquid chromatography mass spectrometry indicated that both acid orange dyes lead to a common end product due to laccase treatment. Acid Orange 52 is demethylated by laccase and ultrasound treatment. The results confirmed that the main effect of ultrasound is based on $\cdot\text{OH}$ attack on the dye molecules (Rehorek *et al.*, 2005).

Reactive Black 5 industrial dyeing effluent was decolourized by free and immobilized laccase. The stability of the enzyme (194 h free and 79 h immobilized) depended on the dyeing liquor composition and the chemical structure of the dye. In the decolourization experiments with immobilized laccase, two phenomenon were observed decolourization due to adsorption on the support (79%) and dye degradation due to the enzyme action (4%).Dyeing in the enzymatically recycled effluent provided consistency of the colour with both bright and dark dyes (Paulo *et al.*, 2003).

2.3.2 DEGRDATION OF DYES BY BACTERIA

A bacterial strain, CK3, with remarkable ability to decolorize the reactive textile dye Reactive Red 180, was isolated from the activated sludge collected from a textile mill. Phenotypic characterization and phylogenetic analysis of the 16S rDNA sequence indicated that the bacterial strain belonged to the genus *Citrobacter*.

Bacterial isolate CK3 showed a strong ability to decolorize various reactive textile dyes, including both azo and anthraquinone dyes (Zeng *et al.*, 2009).

Proteobacterium JB, an alkali-tolerant soil isolate, produced laccase constitutively in unbuffered medium. The enzyme was purified to homogeneity by ammonium sulphate precipitation, DEAE-sepharose anion exchange chromatography and preparatory polyacrylamide gel electrophoresis (Sharma *et al.*, 2007).

Four bacterial isolates identified as *Bacillus cereus* (BN-7), *Pseudomonas putida* (BN-4), *Pseudomonas fluorescens* (BN-5) and *Stenotrophomonas acidaminiphila* (BN-3) capable of completely decolorizing C.I. Acid Red 88 (AR-88), were used to develop consortium designated HM-4. The concerted metabolic activity of these isolates led to complete decolorization of AR-88 (20 mg L⁻¹) in 24 h, whereas individual cultures took more than 60 h to achieve complete decolorization of the added dye (Saini *et al.*, 2005).

A mixed anaerobic bacterial culture decolorized Drimaren Orange K-GL, Everzol Red RBN and Everdirect Supra Yellow PG dyestuffs at 200 mg dyestuff over 24 h. Improved performance with complete decolorization within 24 h was achieved by incubation with 5 g yeast extract compared to glucose, lactose and sucrose though 50 mg yeast extract supplemented with 5 g lactose or 5 g sucrose also resulted in complete decolorization within 24 h (Kapdan *et al.* 2000).

Azospirillum lipoferum is a soil bacterium known for its ability to colonize roots and to promote plant growth. Recently, a laccase-like polyphenol oxidase activity has been reported in a strain isolated from the rhizosphere of rice. The purified enzyme was found to be thermostable up to 70°C for 10 min, had an optimal pH of 6.0, and was inhibited by tropolone, a known inhibitor of metal-containing enzymes. Although the natural substrate of this enzyme is unknown, its biochemical characterization may facilitate further investigations on the ecological role of this laccase in the process of root colonization by *Azospirillum lipoferum* (Diamantidis *et al.*, 2000).

2.3.3 DEGRADATION OF DYES BY FUNGI

The decolorization and degradation of a triphenylmethane dye, malachite green by two fungal microorganisms, *Aspergillus flavus* and *Alternaria solani*. Both the species were able to decolorize different concentrations of malachite green (10 to 50M) almost completely (> 96 %) within 6 days (Ali *et al.*, 2009).

The *Pseudomonas* sp. LBC1 produced extracellular laccase when grown in the nutrient broth. The enzyme was purified using acetone precipitation and an anion-exchange chromatography. The molecular weight of the purified laccase was estimated as 70 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis. An enzyme showed maximum substrate specificity towards o-tolidine than other substrates of laccase including 2,2_-azinobis, 3-ethylbenzothiazoline-6-sulfonic acid, hydroquinone, N,N_-dimethyl phenylene diamine, syringic acid and veratryl alcohol (Govindwar *et al.*, 2009).

Spent substrate of *Agaricus biosporus*, *Pleurotus sajor –caju* and *Lentinula edodes* was evaluated for decolorisation of four different dyes. Potato dextrose broth supported highest decolorisation, 90% of Rhodamine B and Methyl Violet 2B, 93% of Chicago Sky Blue 6B and 88% of Quinaldine Red (Ahlawat and Singh 2009).

This study presents decolorisation of textile azo dye, orange II, by white rot fungus, *Phanerochaete chrysosoprium*. Decolourisation ability of fungus was correlated to lignolytic activity (Singh *et al.*, 2008).

The extracellular enzymes from *Pleurotus saro-caju* for the lignin degrading enzyme patterns and dye decolourisation potential. The results from the native page revealed that there were at least two isoenzymes. The crude enzyme had a pH and temperature optimum at 6 and 40°C respectively when 2,2 azino –bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was used as the substrate (Saranthim and Khammuang 2008).

A number of direct, vat, basic and other (Ranomill yellow and Texacid fast red A) dyes have been decolourised using concentrated culture filtrate and purified laccase of white-rot fungus *Cyathus bulleri*. Decolourisation (>50%) was achieved

and there was significant time reduction when purified laccase of high specificity was used (Mishra *et al.*, 2007).

The two species of white rot fungi were evaluated for their ability to decolorize Blue CA, Black B133, Corazol Violet SR. *Trametes hirsute* and *Pleurotus florida* displayed the greatest extent of decolorization. Laccase is the lignolytic enzyme from these fungi (Moorthi *et al.*, 2006).

Sinorhizobium meliloti CE52G strain produces a periplasmic laccase that has been purified by a two-step procedure involving heat treatment and immobilized metal affinity chromatography. It is a homodimeric protein with an apparent molecular mass of 45 kDa each subunit and an isoelectric point of 6.2. CE52G laccase was inhibited by halides (sodium chloride and sodium fluoride), ions (ferrous, manganese and copper), sulfhydryl organic compounds (mercaptoethanol and reduced glutathione), and electron flow inhibitors (sodium cyanide and sodium fluoride). Laccase activity was strongly enhanced by potassium sulphate sodium sulphate and ammonium sulphate. CE52G laccase is a pH and thermo-stable protein (Sowinski *et al.*, 2005)

White-rot fungi produce various isoforms of extracellular oxidases including laccase, Manganese peroxidase and lignin peroxidase which are involved in the degradation of lignin in their natural lignocellulosic substrates. This ligninolytic system of white-rot fungi is directly involved in the degradation of various xenobiotic compounds and dyes (Wesenberg *et al.*, 2003).

Trametes hirsuta and a purified laccase from this organism were able to degrade triarylmethane, indigoid, azo, and anthraquinonic dyes. Initial decolorization velocities depended on the substituents on the phenolic rings of the dyes. Immobilization of the *Trametes hirsuta* laccase on alumina enhanced the thermal stabilities of the enzyme and its tolerance against some enzyme inhibitors, such as halides, copper chelators, and dyeing additives. Textile effluents decolorized with *Trametes hirsuta* or the laccase were used for dyeing. However, when the effluents were decolorized with immobilized laccase, they could be used for dyeing and acceptable colour differences below 1.1 were measured for most dyes (Gublitz *et al.*, 2000).

White-rot fungi were studied for the decolorization of 23 industrial dyes. Laccase, manganese peroxidase, lignin peroxidase, and aryl alcohol oxidase activities were determined in crude extracts from solid-state cultures of 16 different fungal strains grown on whole oats. All *Pleurotus ostreatus* strains exhibited high laccase and manganese peroxidase activity, but highest laccase volumetric activity was found in *Trametes hispida*. Solid-state culture on whole oats showed higher laccase and manganese peroxidase activities compared with growth in a complex liquid medium. Only laccase activity correlated with the decolorization activity of the crude extracts. Two laccase isoenzymes from *Trametes hispida* were purified, and their decolorization activity was characterized (Rodriguez *et al.*, 1999).

In addition to excreting lignin-degrading peroxidases, the white rot fungus *Phanerochaete flavido-alba* also excretes a laccase. This protein was purified to homogeneity and found to have a molecular weight of 94,000 and an isoelectric point lower than 3.55. Its UV-visible spectrum is typical of copper-containing proteins (Martinez *et al.*, 1996).

Objectives

3. OBJECTIVES

- To extract malachite green in a simulated effluent by liquid-liquid extraction method.
- To extract the synthetic dye from real textile effluent by liquid-liquid extraction method.
- To distill to recover the solvent that can be recycled.
- To degrade the dye using bacteria and fungi.

Materials and Methods

4. MATERIALS AND METHODS

4.1 MATERIALS

All chemicals used were of analytical grade, unless specified otherwise. Distilled water was used for experiments.

4.2 PREPARATION OF SIMULATED DYE

Different concentrations of the dye malachite green were prepared ranging from 0.004ppm to 0.02ppm (1-5mg of dye in 250mL of distilled water) and 0.04ppm to 0.12ppm (10-30mg of dye in 250mL of distilled water).

4.3 SELECTION OF SOLVENT FOR LIQUID-LIQUID EXTRACTION

Different organic solvents were used to extract the simulated dye malachite green. 5mL of the dye (0.02ppm) was taken and to this equal volume of different organic solvents were added. The mixture was vortexed for a definite time period of 1 minute. The phases were left to separate by allowing an equilibration time of 10 minutes. Table 4.1 shows the results obtained.

Solvents	Extraction of malachite green
Toluene	Nil
Chloroform	Positive
Cyclohexane	Nil
Diethyl ether	Nil
Ethyl acetate	Moderate
Petroleum ether	Nil
Methoxy propanol	Nil
Propanol	Nil
Isoamyl alcohol	Positive
Benzene	Nil

Table 4.1. Selection of solvent for extraction of malachite green

It was found that simulated dye malachite green was extracted using chloroform.

4.4 LIQUID-LIQUID EXTRACTION

For the extraction process, initially 2ml of stimulated malachite green solution (0.02ppm) and 2ml of chloroform AR were taken in a test tube and mixed in the cyclo mixer for one minute and allowed for phase separation for 10 minutes. Then the organic layer and aqueous layer were separated and absorbance was measured using UV Vis spectrometer SL159. The wavelength of maximum absorption for malachite green is 620nm (Ramalingan *et al.*, 2010). Then the concentration of extracted dye was obtained from a standard calibration graph and the percentage of extraction was determined using the formula

$$E = \frac{[\text{Dye}]_{\text{aqo}} - [\text{Dye}]_{\text{aq}}}{[\text{Dye}]_{\text{aqo}}} \times 100$$

Where

- [Dye]_{org} = Concentration of dye in organic phase.
- [Dye]_{aq} = Concentration of dye in aqueous phase.
- [Dye]_{aqo} = Initial concentration of dye.

On a large scale about 100 ml of dye (0.02 ppm) and 100 ml of chloroform were added in a 250 ml Erlenmeyer flask and was tightly closed to prevent the evaporation of chloroform. The flask was kept on an orbital shaker (Scigenics, Chennai) maintained at 37°C and 100 rpm for 1 h. The contents were transferred to a 250 ml capacity separating funnel for phase separation and the absorbance of the dye in each phase was measured individually.

The efficiency of the extraction process was studied under different conditions of pH, temperature, equilibrium time, dye concentrations and volumes of extractant.

4.4.1 EFFECT OF pH

The experiment was carried out in a fixed dye concentration (0.002ppm). The pH was varied from 2-12 using NaOH (to adjust to alkaline pH) and HCl (to adjust to acidic pH). The aqueous and organic layers were separated and their optical densities were recorded at 620nm.

4.4.2 EFFECT OF TEMPERATURE

The efficiency of extraction was studied at different temperatures from 20°C to 55°C. The simulated dye malachite green (0.002ppm) was maintained at a particular temperature for 10 minutes after which it was extracted using chloroform. The optical density readings of the organic and aqueous layers were measured at 620nm.

4.4.3 EFFECT OF EQUILIBRIUM TIME

The extraction of dye at different times (upto 14 minutes) was carried out. The time intervals for vortexing were varied as 2, 4,6,8,10,12 and 14 minutes. The phases were let to separate by allowing an equilibration time of 10 minutes and the optical densities were recorded at 620nm for both the aqueous and organic layers.

4.4.4 EFFECT OF CONCENTRATION OF THE DYE

Extraction was carried out for the simulated dye malachite green at different concentrations (0.004ppm to 0.02ppm and 0.04ppm to 0.12ppm) using equal volumes of chloroform. The optical densities were recorded at 620nm for the organic and aqueous layers.

4.4.5 EFFECT OF VOLUME OF THE EXTRACTANT

The aqueous phase to organic phase volumes were varied in the ratios of 1:0.5, 1:1, 1:1.5, 1:2 and 1:3 to study the effect of phase ratio on the extraction efficiency of the initial dye concentrations ranging from 0.004 ppm to 0.12 ppm. The optical density readings of the aqueous and organic layers were recorded at 620nm.

4.4.6 MULTISTAGE EXTRACTION

It was observed that at higher concentrations (0.04ppm to 0.12ppm) the efficiency of extraction was found to decrease with increasing concentrations. Hence, in order to achieve 100% efficiency of the extraction process, the aqueous layer was further re-extracted using the solvent, chloroform until 100% extraction of the simulated dye was achieved.

4.5 DISTILLATION

The chloroform containing the extracted dye was subject to distillation using an AlMech's Simple Distillation apparatus. About 340mL of the used chloroform was

added into the distillation unit. The process was carried out for 30 minutes. After distillation, the volume of the distillate was found to be 270mL

4.5.1 EXTRACTION USING RECOVERED CHLOROFORM

The distillate (chloroform) was then used as an extractant to check the efficiency of the chloroform that was recovered. Equal volumes of the simulated dye, malachite green and recovered chloroform were added in test tubes, vortexed for 1 minute. The phases were allowed to settle down for 10 minutes. The aqueous and organic layers were separated and their optical densities were recorded at 620nm.

4.6 BIOLOGICAL DEGRADATION OF SYNTHETIC DYES

The degradation of synthetic dyes was carried out by biological means using bacteria and fungi.

4.6.1 FUNGAL DEGRADATION

4.6.1.1 PREPARATION OF CZEPAK-DOX BROTH

Components	Grams per 100mL
Glucose	2
Potassium dihydrogen phosphate (KH ₂ PO ₄)	0.1
Potassium chloride (KCl)	0.05
Magnesium sulphate (MgSO ₄ ·7H ₂ O)	0.05
Sodium nitrate (NaNO ₃)	0.1
Zinc sulphate (ZnSO ₄)	0.005
Ferrous sulphate (FeSO ₄)	0.001
Sodium molybdate (NaMoO ₄)	0.005
Copper sulphate (CuSO ₄)	0.0001
Manganese sulphate (MnSO ₄)	0.002

Table 4.1. Components of Czepak-Dox broth.

4.6.1.2 PREPARATION OF MASTER PLATES

The spawn of the fungal species *Pleurotus eous* (Wesenberg *et al.*, 2003) was obtained from Tamil Nadu Agricultural University, Coimbatore and inoculated in 250mL conical flasks containing wheat bran ensuring sterile conditions. The culture was allowed to grow for 5 days under static conditions. The grown culture was then

inoculated into sterilized plates containing Czepak-Dox agar and allowed to grow for 5 days. This served as the master plate.

4.6.1.3 EXTRACTION OF THE ENZYME

Culture from the master plate was then inoculated into 250mL conical flasks containing sterilized Czepak-Dox broth and allowed to grow under static conditions for about 5 days with frequent mixing of the broth each day. The broth was then centrifuged at 10,000rpm at 10 minutes to obtain the extracellular laccase enzyme.

4.6.1.4 LACCASE ASSAY

The method used for laccase assay was based on the oxidation of 2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) at 420 nm ($\epsilon = 36\ 000\ \text{M}^{-1}\ \text{cm}^{-1}$). The reaction mixture consist of 940 μl of sodium acetate buffer (0.1M, pH 4.5), 10 μl of ABTS (10mM), 50 μl of enzyme solution and the reaction was stopped by adding 50 μl of trichloro acetic acid. (Bourbonnais and Paice, 1990)

Sl. no.	Sodium Acetate buffer (pH 4.5) (μl)	Volume of Enzyme (μl)	Volume of Substrate (μl)	Incubate the tubes at 37°C for 10 min.	Volume of 50% TCA (μl)	Optical Density at 420 nm
Blank	990	0	10		50	
1	940	50	10		50	
2	940	50	10		50	

Table 4.3. Assay of laccase by using ABTS as substrate.

4.6.1.5 TREATMENT OF DYE

- 500 mL of Czepak-Dox broth was prepared and sterilized.
- Three dye solutions, malachite green, bromophenol blue and amaranth were subject to degradation by fungi.
- The dye solutions were prepared in the concentration of 5 mg in 250 mL of distilled water and sterilized.
- The broth was then equally distributed among three conical flasks and 10mL of the sterilized dyes malachite green, bromophenol blue and amaranth were

added, one in each flask. The broth containing the dye was inoculated with *Pleurotus eous* culture and placed under static conditions to observe for degradation of the dyes.

- The samples were filtered and collected on the 0th day, 3rd day and the 5th day of inoculation and an optical density scan was done from 260nm to 660nm. The percentage of dye degradation was calculated using the following formula.

$$\text{Dye decolourisation (\%)} = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100$$

4.6.2 BACTERIAL DEGRADATION

4.6.2.1 PREPARATION OF BUSHNELL AND HAAS MEDIUM

Components	Grams per 1000mL
Ammonium nitrate (NH ₄ NO ₃)	1
Calcium chloride (CaCl ₂)	0.02
Ferric chloride (FeCl ₃)	0.05
Magnesium sulphate (MgSO ₄ 7H ₂ O)	0.2
Dipotassium phosphate (K ₂ HPO ₄)	1
Glucose	30
Yeast extract	0.5

Table 4.4. Components of Bushnell and Haas medium.

B. licheniformis which produce laccase was obtained from the Department microbial culture collections.

4.6.2.2 LACCASE ASSAY

The method used for laccase assay was based on oxidation of o-dianisidine at 460 nm ($\epsilon = 11300 \text{ M}^{-1} \text{ cm}^{-1}$). The reaction mixture per ml: 200 μl acetate buffer (0.1M, pH 4.5), 100 μl o-dianisidine (1 mM), 600 μl culture medium and 100 μl hydrogen peroxide (2 mM) (Robles *et al.*, 2000).

Sl. no.	Acetate buffer (pH 4.5) (µl)	Volume of Enzyme (µl)	Volume of Substrate (µl)	Incubate the tubes at 37°C for 15 min.	Volume of H ₂ O ₂ (µl)	Optical Density at 420 nm
Blank	800	0	100		100	
1	600	200	100		100	
2	600	200	100		100	

Table 4.5. Assay of laccase by using o-dianisidine as substrate.

4.6.2.3 TREATMENT OF DYE

- 250mL of Bushnell and Haas broth was prepared and sterilized.
- Amaranth dye solution was prepared at a concentration of 5mg in 250mL of distilled water and was also sterilized.
- The broth was then divided among two conical flasks and this 10mL of the sterilized dye, amaranth was added.
- The broth containing the dye was then inoculated with the laccase producing *B. licheniformis* and was placed under static conditions to observe for decolourization of the dye.
- The samples were filtered and collected on the 0th day, 3rd day and the 5th day of inoculation and an optical density scan was done from 260nm to 660nm. The percent degradation of the dye was found to be

$$\text{Dye decolourisation (\%)} = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100$$

4.7 LIQUID-LIQUID EXTRACTION OF REAL-TIME EFFLUENT

Extraction of a real-time textile effluent was performed. 5mL of the effluent was taken in a test tube and to this equal volume of chloroform was added. The mixture was vortexed for 5 minutes and the test tube was kept aside for phase separation.

Results and Discussions

5. RESULTS AND DISCUSSIONS

5.1 EFFECT OF pH ON DYE EXTRACTION

The effect of pH on the extraction of dye was studied by varying the pH of the dye solution from 2 to 12 at 0.02 ppm dye concentration. The results showed that extraction of dye increases with an increase in pH (from 2 to 6). It is evident from Fig.4.1 that the maximum dye extraction (99%) has occurred for pH 6. There was about 99% extraction for pH 6-10. There was no extraction for pH 11 and 12. There are very few reports available in the literature for the solvent extraction of dyes and are presented in Table 5.1

Dye	Solvent	Effect of pH	Reference
Black B	Tridodecylamine	High extraction in the acidic ph (4).	Othman <i>et al.</i> ,2011.
Methyl Violet	Salicylic acid	As the concentration of salicylic acid increases the extraction increase from Ph 3-6.	Muthuraman and Teng 2010.
Methylene Blue	Benzoic acid	High extraction at alkaline PH (12).	Muthuraman <i>et al.</i> ,2009.

Table 5.1. Effect of pH- References.

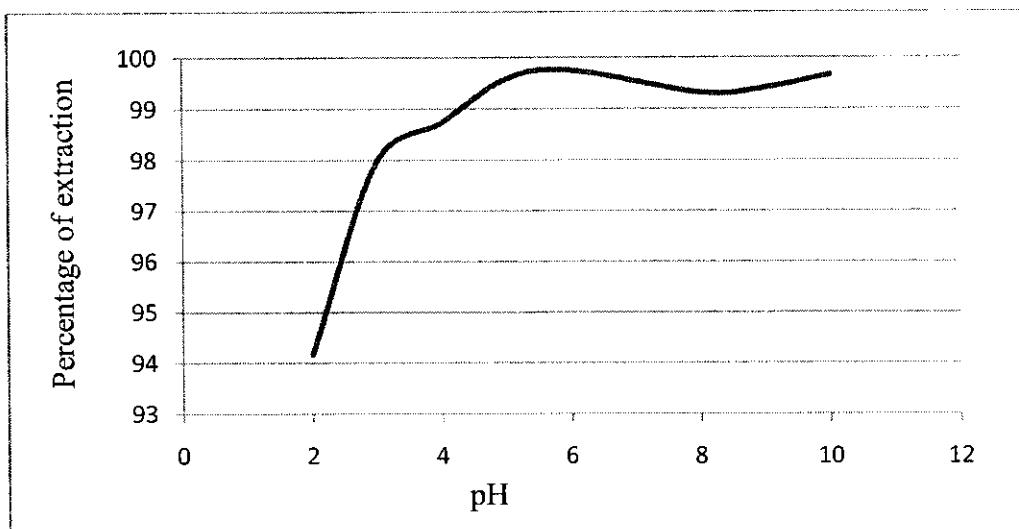


Fig. 5.1. Effect of pH on simulated dye malachite green (experimental conditions: volume of dye -5 mL, volume of solvent - 5 mL, dye concentration-0.02 ppm, equilibration time -10 min).

5.2 EFFECT OF TEMPERATURE

The effect of temperature (20-55°C) was studied. Fig 5.2 shows the effect of temperature on the extraction of dye. The efficiency of extraction remained unchanged for 20-30°C and then as the temperature was increased to 35-50°C a decrease in extraction was observed. At 30°C, the dye was completely transferred to the organic phase (99.03%). So the experiments for further study were carried out at 30°C. In the extraction of methylene blue using benzoic acid the extraction efficiency increased from 20-27°C and as the temperature was further increased there was a decrease in extraction efficiency(Muthuraman *et al.*, 2009) and for methyl violet the extraction efficiency remained unchanged from 20-30°C and decreased as the temperature increased.(Muthuraman and Teng 2010).

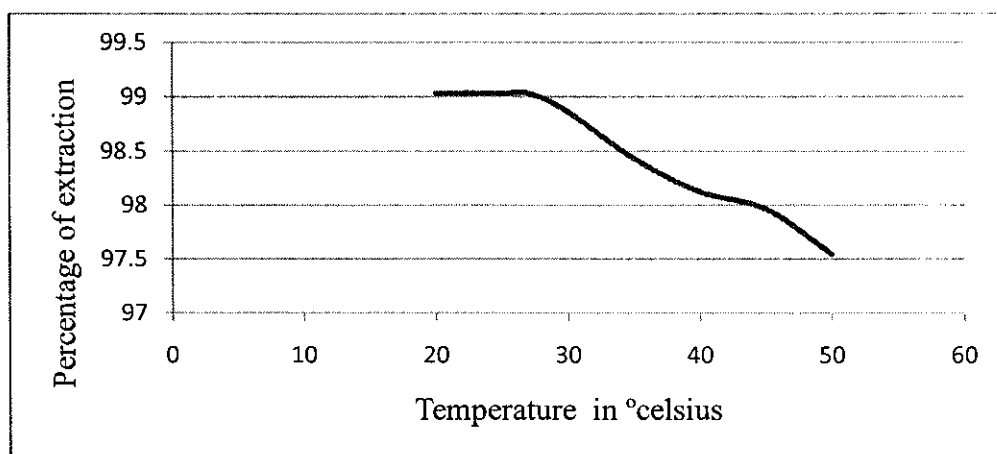


Fig. 5.2. Effect of temperature on simulated dye malachite green (experimental conditions: volume of dye – 5ml, volume of solvent – 5ml, dye concentration – 0.02 ppm, equilibrium time – 10min).

5.3 EFFECT OF EQUILIBRIUM TIME

The extraction of dye at different times (upto 15 min) were studied. Fig. 5.3 shows that the maximum extraction of dye (0.02 ppm). As time increases, the efficiency of extraction was increased. The maximum extraction was achieved at 10 min. In 10 minutes, all the dye was transferred. So, further studies were carried out at a time interval of 10 min. in the extraction of methyl violet using salicylic acid it was estimated that as time increases the extraction efficiency increases and the maximum extraction was obtained at 4th min(Muthuraman and Teng 2010) and for extraction of methylene blue using benzoic acid the equilibrium time was found to be 10 min(Muthuraman *et al.*, 2009).

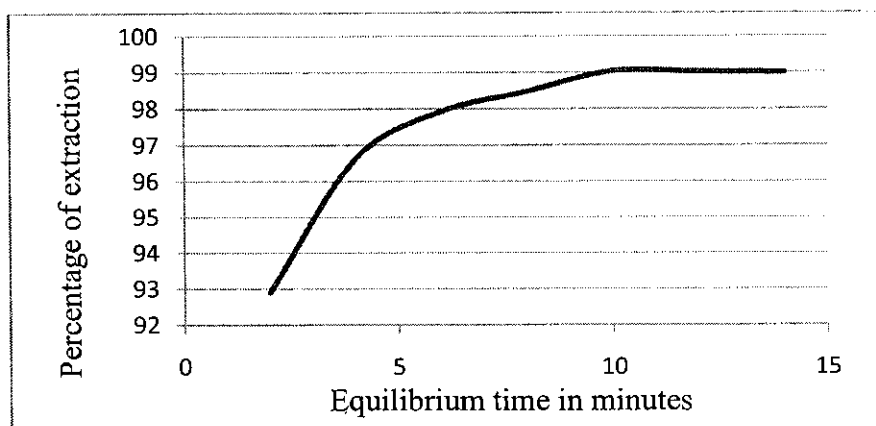


Fig. 5.3. Effect of equilibrium time on simulated dye malachite green (experimental conditions: volume of dye -5 ml, volume of solvent -5 ml , dye concentration – 0.02 ppm).

5.4 EFFECT OF DYE CONCENTRATION

Different dye concentrations ranging from 0.004 ppm (1 mg in 250 ml of distilled water) to 0.12 ppm (30 mg in 250 ml of distilled water) were used to study the effect of dye concentration in liquid-liquid extraction. It is clear from fig 5.4 that the percentage extraction decreased with an increase in dye concentration and it can also be concluded that at low dye concentration the extraction was about 99% for 0.004ppm to 0.02ppm and about 90% for 0.04ppm to 0.12ppm for equal volume of dye and extractant. The mixture of tetra butyl ammonium bromide in methylene chloride extracted 98% of anionic textile dyes (Muthuraman *et al.*, 2010). In the extraction of black B dye using tridodecylamine at lower concentration there was 100% efficiency and as the concentration increased the extraction efficiency decreased to 85%. (Othman *et al.*, 2011).

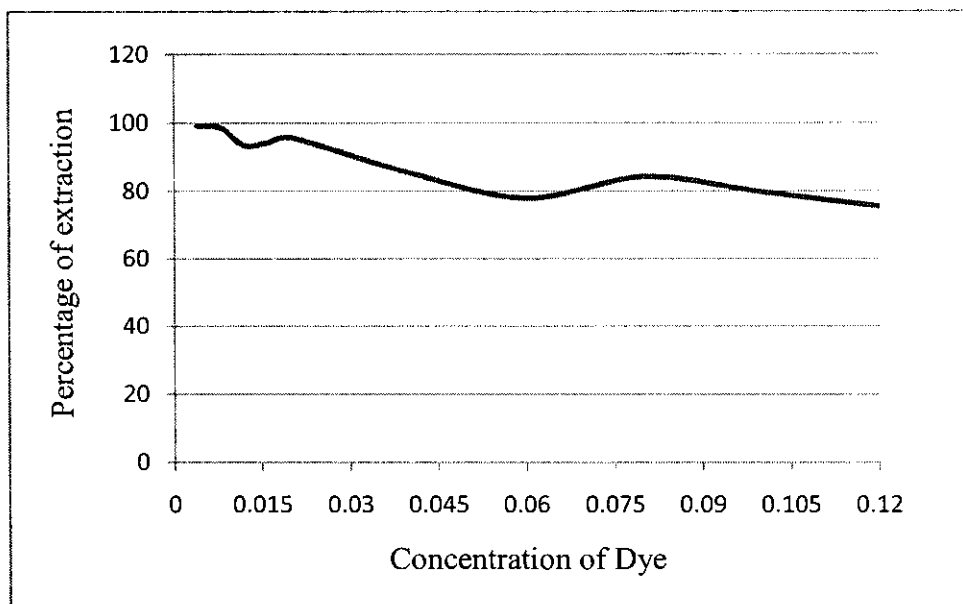


Fig. 5.4. Effect of concentration of dye on extraction (experimental conditions: volume of dye-4mL, volume of solvent - 4 mL, dye concentration - 0.004 ppm, equilibration time -10 min).

5.5 EFFECT OF VOLUME OF EXTRACTANT

The effect of solvent level on dye extraction was also studied. Different volumes of extractant were added to varying dye concentrations. Fig. 5.5 clearly shows that as the volume of extractant increases the extraction efficiency increases. There is almost complete extraction for lower concentration (0.004ppm to 0.02ppm).

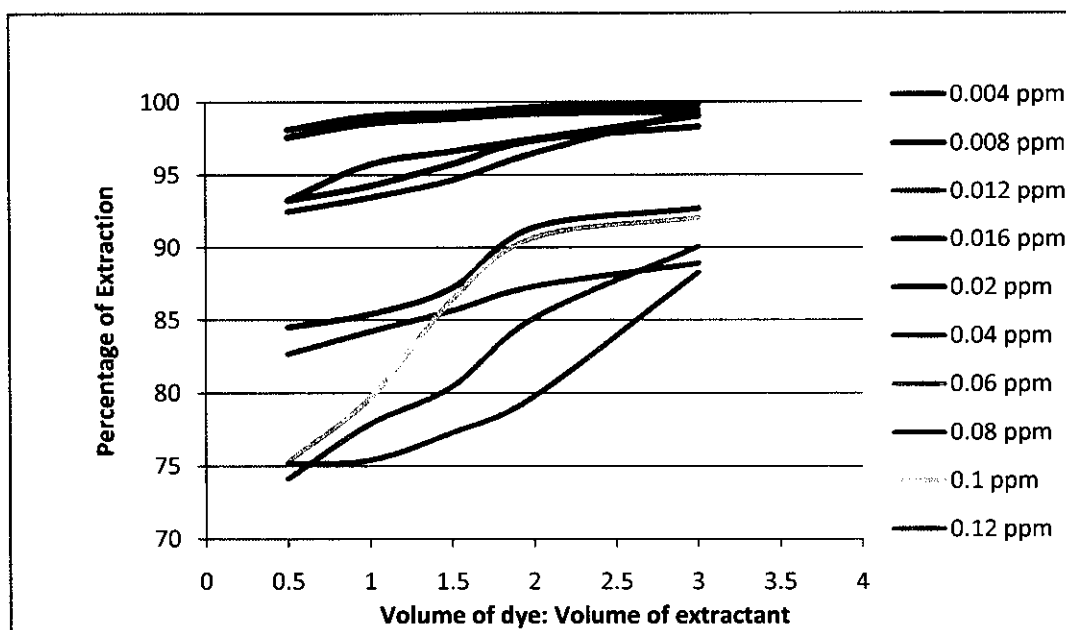


Fig. 5.5. Effect of volume of extractant on different dye concentrations (experimental conditions volume of dye-2mL, volumes of extractant-0.5,1,1.5,2,3 mL, concentrations of dye- 0.004ppm to 0.12 ppm, equilibrium time- 10min).

5.6 EFFECT OF EXTRACTION RATIO

The aqueous phase to organic phase volumes were varied in the ratios of 1:0.5, 1:1, 1:1.5, 1:2 and 1:3 to study the effect of phase ratio on the extraction efficiency of the initial dye concentrations ranging from 0.004 ppm to 0.12 ppm. It is evident from Fig. 4.6 that at 1:3 ratio of dye:solvent, there was maximum transfer for all the concentration. Maximum extraction (99%) was observed for a ratio 1:1 of dye:solvent and for 0.004 ppm and 1:2 for 0.008 ppm. The efficiency of transfer decreased from 98.1% to 75.14% for 0.004 ppm to 0.12 ppm. This clearly indicates that as the concentration increases the extraction decreases. For methylene blue when benzoic acid was used as the solvent the best extraction was obtained at 1:1 (Muthuraman *et al.*, 2009).

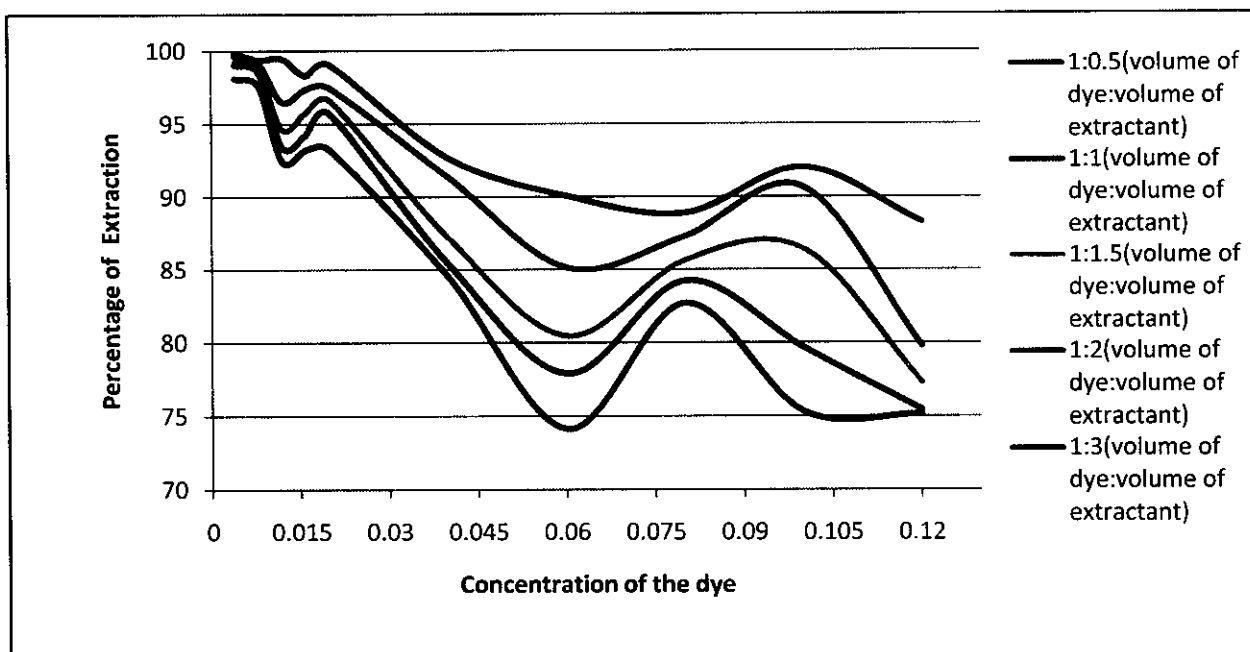


Fig. 5.6. Effect of ratio of aqueous layer:organic layer on dye extraction (experimental conditions: ratio of aqueous layer:organic layer - 1:0.5, 1:1, 1:1.5, 1:2, and 1:3 (concentrations of dye: 0.004 to 0.12 ppm; equilibrium time: 10 min).

5.7 EFFECT OF MULTISTAGE EXTRACTION

For 100% efficiency of extraction, multistage extraction was done for higher concentration (0.04ppm to 0.12ppm). Dyes in the aqueous layer was extracted with chloroform and subsequent extractions were also done chloroform at 1:1 (v/v) ratio. For the highest concentration (0.12ppm), the efficiency of extraction in the 1st, 2nd, 3rd and 4th stages were found to be 75.14%, 83.08%, 96.13% and 100%, respectively.

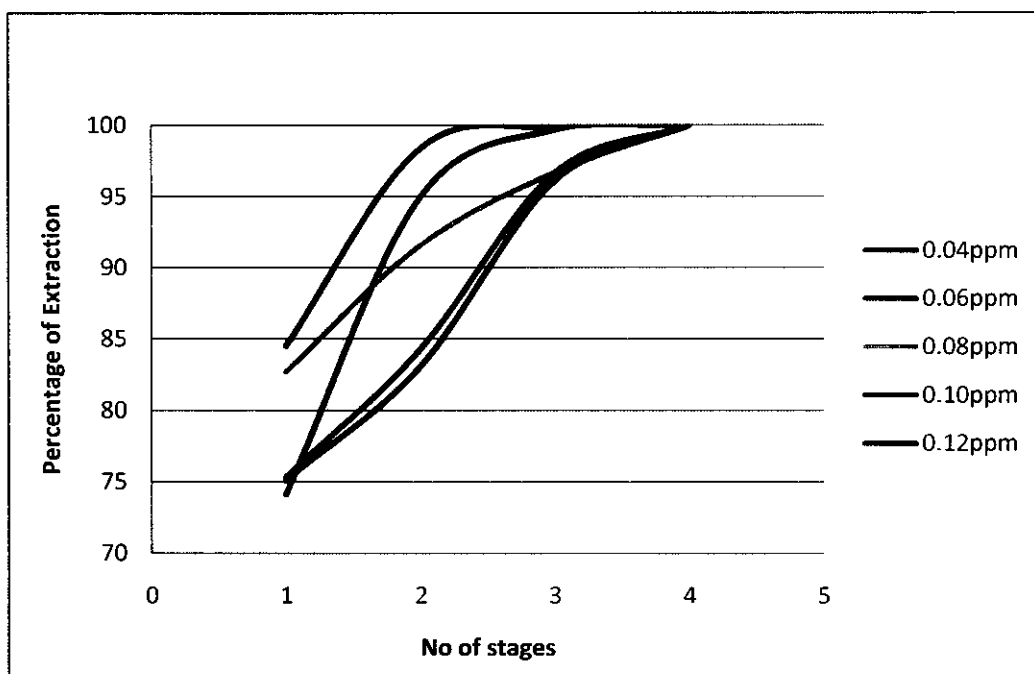


Fig. 5.7. Effect of multistage extraction on dye extraction (experimental conditions: volume of dye- 4 ml; volume of solvent: 2 to 12 ml; concentration of dye: 0.04 to 0.12 ppm; equilibrium time: 10min).

5.8 DISTILLATION

340 ml of extracted chloroform was subject to distillation. From this 270ml of the chloroform was recovered. The recovered chloroform was further reused for the process of extraction.

5.9 EXTRACTION USING RECOVERED CHLOROFORM

The recovered chloroform from the distillation process was reused as an extractant to check the efficiency of recovered chloroform. It was found that it gave the same efficiency as the commercially available chloroform.

5.10 EXTRACTION OF REAL TIME EFFLUENT

The real effluent was also subjected to liquid-liquid extraction by using chloroform as extractant. There was no considerable extraction observed. The reason is however not clear.

5.11 FUNGAL DEGRADATION

The dyes malachite green, bromophenol blue and amaranth were degraded using white rot fungus *Pleurotus eous*. A decrease in colour intensity was observed. A wavelength scan from 260 to 660nm was done to calculate the percentage of degradation of dyes. The results for percentage of degradation of malachite green, bromophenol blue and amaranth dyes on 5th day was calculated to be 95%, 87% and 82% respectively.

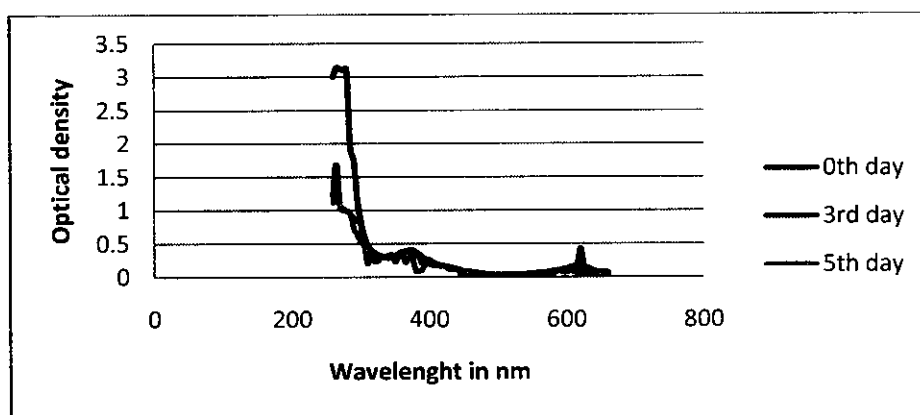


Fig. 5.8. Degradation of malachite green using *Pleurotus eous*.

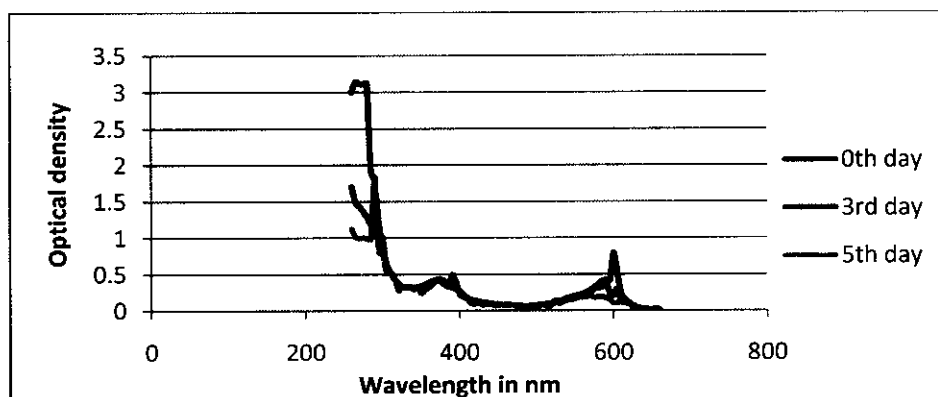


Fig. 5.9. Degradation of bromophenol blue using *Pleurotus eous*.

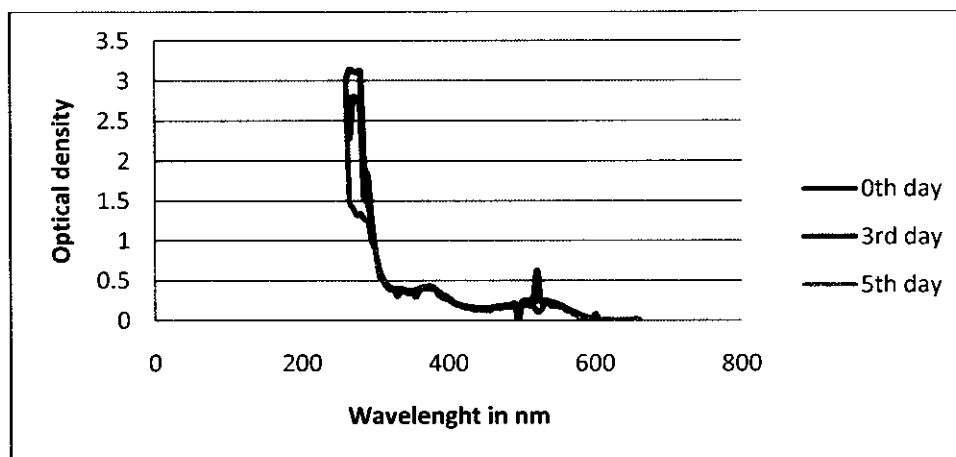


Fig. 5.10. Degrdaton of amaranth using *Pleurotous eous*.

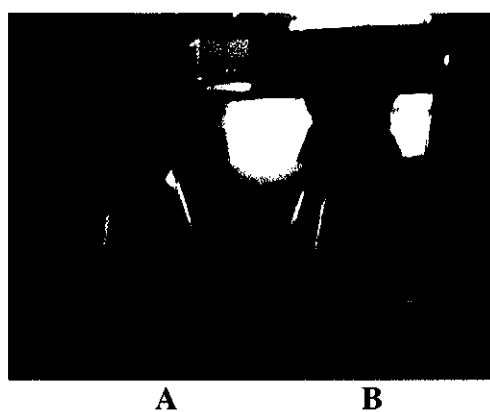


Fig 5.11. Decolourisation of dye malachite green in Czapek-Dox media (A:malachite green before decolourisation ; B: malachite green after decolourisation).

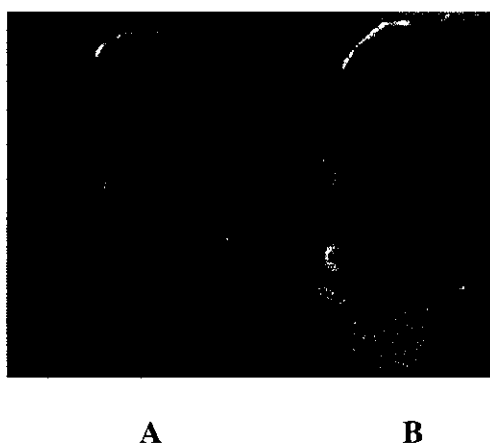


Fig 5.12. Decolourisation of dye bromophenol blue in Czapek-Dox media (A:bromophenol blue before decolourisation ; B:bromophenol blue after decolourisation).

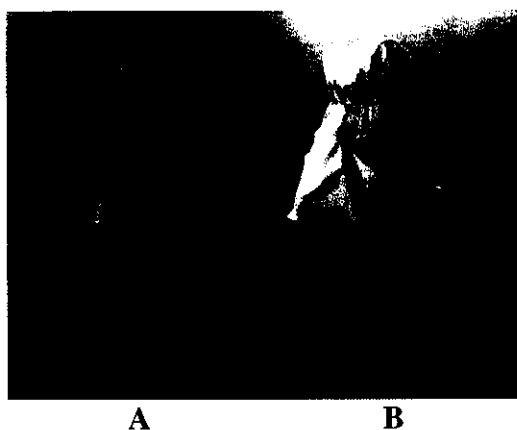


Fig 5.13. Decolourisation of dye amaranth in Czapek-Dox media (A:amaranth before decolourisation ; B:amaranth after decolourisation).

5.12 BACTERIAL DEGRADATION

The dye amaranth was degraded by *Bacillus licheniformis*. A decrease in colour intensity was observed. A wavelength scan (from 260 to 660 nm) was done for the degraded products of amaranth dye and the percentage degradation of was found to be 91%.

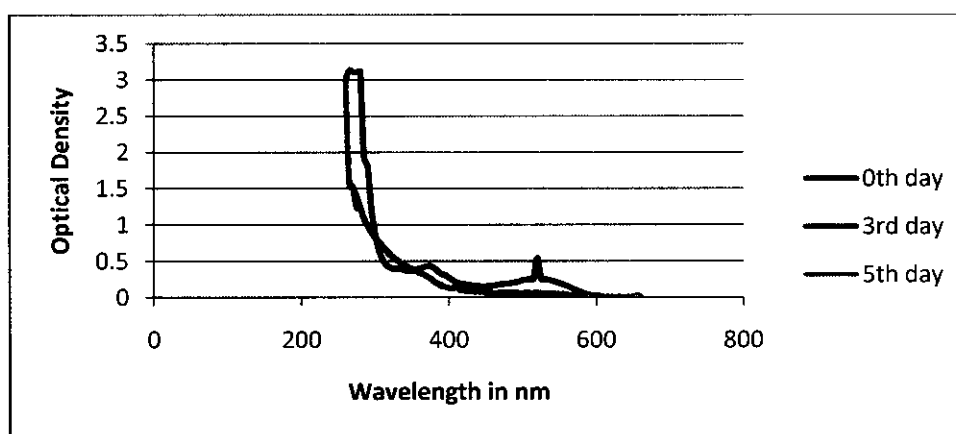


Fig. 5.14. Degradation of amaranth using *Bacillus licheniformis*.



A

B

Fig 5.15. Decolourisation of dye amaranth in Bushnell and Haas media (A:amaranth before decolourisation ; B:amaranth after decolourisation).

Conclusions

6. CONCLUSIONS

- Based on the results, liquid-liquid extraction using chloroform is effective for the extraction of malachite green.
- Results indicated that 100% extraction of malachite green is possible using chloroform as an extractant (solvent).
- The efficiency of extraction is not affected by pH while maximum extraction was observed at 30°C.
- The percentage of extraction increases as the volume of the solvent for a particular concentration of malachite green is increased while the efficiency of extraction decreases as the dye concentration increases.
- At higher dye concentrations, multiple stages of extraction was efficient than single stage extraction for the complete removal of dyes.
- The solvent used for extraction was recovered by distillation. The recovered solvent can be reused for extraction and was found to be as effective as the actual chloroform.
- Utmost care should be taken to prevent the loss of chloroform during extraction process and distillation.
- The white rot fungus *Pleurotus eous* was efficient in degrading the dyes malachite green, bromophenol blue and amaranth and the percentage of degradation was found to be 95%, 87% and 82%, respectively.
- *Bacillus licheniformis* also decolorized the amaranth dye by degradation and the percent decolorization was found to be 91%.

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