Chapter I

Introduction
1. Introduction

1.1. History of dyes

The term dye, is derived from old English word daeg or daeh meaning “color”. The exact period of the commencement of the art of dyeing in the world could not be ascertained correctly. It is presumed that the appearance of dye-vat occurred in the history along with the brick-kiln. Archaeological evidence however shows that dyeing was an wide-spread industrial enterprise in Egypt, India and Mesopotamia round about third millennium B.C. (Chippindale et al., 1953). Ever since the beginning of humankind, people have been using colorants for painting and dyeing of their surroundings, skins and clothes. Until the middle of the 19th century, all colorants applied were from natural origin. Inorganic pigments such as soot, manganese oxide, hematite and ochre have been utilized within living memory. William Henry Perkin discovered the first synthetic dye stuff "Mauve" (aniline, a basic dye) while searching for a cure for malaria and a new industry was begun. It was a brilliant fuchsia type color, but faded easily. Synthetic dyes can be named according to the chemical structure of the chromophoric group (azo dyes, anthraquinonic dyes, xanthene dyes, triphenylmethane dyes, etc) (Zollinger, 2003).

Coloristic properties of dyes were divided according to the dyeing method (in function of their mode of binding to the fibre) as reactive dyes, direct dyes, cationic dyes, etc. Since then thousands of dyes have been synthesized, and dye manufacture has become a significant part of the chemical industry. Nowadays, when care of the environment is a major issue, it is tempting to assume that the use of natural colors is an environmental friendly alternative to present-day practice. There are several groups studying the use of natural dyes in modern dyeing industry. Some of the advantages of the use of this type of compounds are the absence of toxicity upon humans, the use of sustainable sources and the fit into the natural pathways of biodegradation of the released dye baths (Tsatsaroni and Liakopoulou, 1995; Angelini et al., 1997, 2003; Kamel et al., 2005).

1.2. Dye classification

Dyes are compounds that absorb light with wavelengths in the visible range, i.e., 400 to 700 nm. The major structure element responsible for light absorption in dye molecules is the chromophore group (Marías, 1976; Van der Zee, 2002). Both dyes and pigments appear to be colored because they absorb some wavelengths of light.
Absorption of light is depending on the color of the compound. Dyes and pigments both indicate coloring matter but they differ mainly in their respective properties and technique of use. 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:

\[ \Delta E = E_1 - E_0 = h \nu = \frac{hc}{\lambda} \]

Dyes contain chromophores, delocalized electron systems with conjugated double bonds, and auxochromes, electron-withdrawing or electron donating substituent that cause or intensify the color of the chromophore by altering the overall energy of the electron system. Usual chromophores are -C=C-, -C=N-, -C=O, -N=N-, -NO₂ and quinoid rings, usual auxochromes are -NH₃, -COOH, -SO₃H and -OH. Based on chemical structure or chromophore, 20-30 different groups of dyes can be discerned (Table 1). Azo (monoazo, disazo, triazo, polyazo), anthraquinone, phthalocyanine and triarylmethane dyes are quantitatively the most important groups. Other groups are diarylmethane, indigoid, azine, oxazine, thiazine, xanthene, nitro, nitroso, methine, thiazole, indamine, indophenol, lactone, aminoketone and hydroxyketone dyes and dyes of undetermined structure (stilbene and sulfur dyes).

**Table 1.** Structural formulas of different groups of dyes.

<table>
<thead>
<tr>
<th>CATEGORY</th>
<th>CHROMOPHORE GROUP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acridine dyes</td>
<td><img src="" alt="Acridine" /></td>
</tr>
<tr>
<td></td>
<td>(derivatives of acridine)</td>
</tr>
<tr>
<td>Anthraquinone dyes</td>
<td><img src="" alt="Anthraquinone" /></td>
</tr>
<tr>
<td></td>
<td>(derivatives of anthracene)</td>
</tr>
<tr>
<td>Diarylmethane dyes</td>
<td><img src="" alt="Diarylmethane" /></td>
</tr>
<tr>
<td></td>
<td>(derivatives of diphenyl methane)</td>
</tr>
</tbody>
</table>
Triarylmethane dyes

Azo dyes

Cyanine dyes

Diazonium dyes

Nitro dyes

Nitroso dye

Phthalocyanine dyes

Quinone-imine dyes
Safranin dyes

Indophenol dyes

Oxazin dyes

Oxyzone dyes

Thiazine dyes

Thiazole dyes

Xanthene dyes

Fluorene dyes

Pyronin dyes
Rhodamine dyes

(derivative of rhodamine)

Fluorone dyes

(derivative of fluorone)

The main sources of wastewater generated by the textile wet-processing industry originate from the washing (or scouring) and bleaching of natural fibers and from the dyeing and finishing steps. Given the great variety of fibers, dyes, process aids and finishing products in use, these processes generate wastewaters of great chemical complexity and diversity which are not adequately been treated. The chemical composition of textile mill effluents is also changing rapidly as a result of shifting consumers preferences. Most significant is the current popularity of cotton fabrics and bright colors leading to greater usage of reactive and azo dyes (Vandevivere et al., 1998).

1.2.1. Reactive dyes

First appeared commercially in 1956 and were used to dye cellulose fibers. The dyes contain a reactive group that, when applied to a fiber in a weakly alkaline dye bath, form a chemical bond with the fiber. These dyes are with reactive groups that are capable of forming a covalent bond between carbon atoms of dye molecule and -OH, -NH, -SH groups in fibers. The reactive group is often a heterocyclic aromatic ring substituted with a chloride or fluoride atom, e.g. dichlorotriazine. Another common reactive group is vinyl sulfone. Reactive dyes can also be used to dye wool and nylon, in the later case they are applied under weakly acidic. In the Color Index, the reactive dyes form the second largest dye class. Mostly reactive dyes (80%) are azo or metal complex azo compounds but also anthraquinone and phthalocyanine reactive dyes are applied, especially for green and blue color (O’Neill et al., 1999).
1.2.2. Acid dyes

It is named acid dyes because they work best when applied in an acid bath. These dyes come in a wide variety of color, it is fairly fast to light and washing. When in solution are negatively charged. Water soluble anionic dyes are applied to fibres such as silk, wool, nylon and modified acrylic fibers. Attachment to the fibre is attributed, at least partly, to salt formation between anionic groups in the dyes and cationic groups in the fibre. This group contains azo, anthraquinone and triarylmethane compounds. The pH of the dye baths is acidic in nature (Knackmuss, 1996).

1.2.3. Basic dyes

This type of dye is just fair when it comes to fastness to light and washing. Cationic compounds that bind to the acid groups of the fibre. It is water soluble cationic dyes that are applied to wool, silk, cotton and modified acrylic fibres. Usually acetic acid is added to the dye bath to help to take up the dye onto the fibre. Most of the basic dyes are diarylmethane, triarylmethane, anthraquinone or azo compounds (Rocha, 2001).

1.2.4. Direct dyes

It is one of the easiest to use and has a wide range of colors, it is not fast to washing, but its fastness is often improved by more treatment. These dyes bound by Vander Waals forces to the cotton fibre, dyeing is normally carried out in a neutral or slightly alkaline dye bath, under boiling conditions, with the addition of either sodium chloride (NaCl) or sodium sulfate (Na₂SO₄). Direct dyes are used on cotton, paper, leather, wool, silk and nylon. They are also used as pH indicators and as biological stains. Direct dyes contain multi azo, phthalocyanine, stilbene and oxazine (De las Marias, 1976).

1.2.5. Mordant dyes

The mordant dye is fairly fast to light and washing, it combines with chemicals (tannic acid, alum, chrome alum, and other salts of aluminium, chromium, copper, iron, potassium, and tin) for dyeing; it is especially useful for black and navy shades. As the name suggests these dyes require a mordant that improves the fastness of the dye on the fibre. The choice of mordant is very important as different mordents can change the final color significantly (Ingamells, 1993).
1.2.6. Vat dyes

It is superior compared to the other dyes when it comes to its fastness to light and washing. These dyes are essentially insoluble in water and incapable of dyeing fibres directly. However, reduction in alkaline liquor produces the water soluble alkali metal salt of the dye. In this leuco form these dyes have an affinity for the textile fibre. Subsequent oxidation reforms the original insoluble dye. This group contains anthraquinones and indigoid compounds (Slokar and Le Marechal, 1998).

1.2.7. Metal complex dyes

The earliest metal-complex dyes were produced directly within the fibre material by reaction of a metal (usually chromium, copper, cobalt or nickel) and one or two dye molecules (acid or reactive). Among the popular metal-complex dyes, a variety known as 1:2 metal-complex dyes finds application for dyeing polyamide fibres. For dyeing wool, metal complex dyes are the most favored (Slokar and Le Marechal, 1998).

1.2.8. Disperse dyes

Disperse dyes originally developed for the dyeing of cellulose acetate, and are water insoluble. These dyes are finely ground in the presence of a dispersing agent and sold as a paste, or spray-dried and sold as a powder. Their main use is to dye polyester but they can also be used to dye nylon, cellulose triacetate, and acrylic fibres. In some cases, a dyeing temperature of 130 °C is required, and a pressurized dye bath is used. The very fine particle size gives a large surface area that aids dissolution to allow uptake by the fibre. The dyeing rate can be significantly influenced by the choice of dispersing agent used during the grinding (Slokar and Le Marechal, 1998).

1.2.9. Pigment dyes

Pigment dyes (i.e. organic pigments) represent a small but increasing fraction of the pigments, the most widely applied group of colorants. These insoluble, non-ionic, insoluble salts retain their crystalline or particulate structure throughout their application. Pigment dyeing is achieved from a dispersed aqueous solution and therefore requires the use of dispersing agents. Most pigment dyes are azo compounds (yellow, orange, and red) or metal complex phthalocyanines (blue and green). Also anthraquinone and quinacridone pigment dyes are applied (Robinson et al., 2001).
1.2.10. Azoic and ingrain

These dyes are the insoluble products of a reaction between a coupling component (usually naphthols, phenols or acetoacetylamides) and a diazotised aromatic amine. This reaction is carried out on the fibre. With suitable adjustment of dyebath conditions the two components react to produce the required insoluble azo dye. This technique of dyeing is unique, in that the final color is controlled by the choice of the diazoic and coupling components. These dyes are used in for cotton, viscose, cellulose acetate and polyester (O’Neill et al., 1999).

1.2.11. Sulphur dyes

Sulfur dyes are the most commonly used dyes manufactured for cotton in terms of volume. They are cheap, generally have good wash-fastness and are easy to apply. Sulfur dyes are water insoluble. They have to be treated with a reducing agent and an alkali at temperature of around 80 °C where the dye breaks into small particles which then becomes water soluble and hence can be absorbed by the fabric. The dyes are absorbed by cotton from a bath containing sodium sulfide or sodium hydrosulfite and are made insoluble within the fiber by oxidation. During this process these dyes form large complex molecules which are the basis of their good wash-fastness. Their main use is to dye cellulose fibres, cotton and viscose (Rocha, 2001).

1.2.12. Solvent dyes

A solvent dye is a dye soluble in organic solvents. It is usually used as a solution in an organic solvent. They are used to color organic solvents, hydrocarbon fuels, waxes, lubricants, plastics, and other hydrocarbon-based non polar materials. Solvent dyes are used for gold imitation (and other transparent metallic effects) of metalized polyester films. Also used in marking inks, inkjet inks, glass coloration (O’Neill et al., 1999).

1.3. Environmental toxicity aspects

Dyestuff toxicity has been investigated in numerous researches. The color in wastewater is highly visible and affects esthetics, water transparency and gas solubility in water bodies, This toxicity (i.e. mortality, genotoxicity, mutagenicity and carcinogenicity) studies diverge from tests with aquatic organisms (fish, algae, bacteria, etc.) to tests with mammals. Furthermore, research has been carried out to study the effects of dyestuffs and
dye containing effluents on the activity of both aerobic and anaerobic bacteria in wastewater treatment systems.

The acute toxicity of dyestuffs is generally low. Algal growth (photosynthesis), tested with respectively 56 and 46 commercial dyestuffs, was generally not inhibited at dye concentrations below 1 mg l\(^{-1}\). The most acutely toxic dyes for algae are cationic basic dyes (Greene and Baughman, 1996; Little and Chillingworth, 1974) Fish mortality tests showed that 2\% out of 3000 commercial dyestuffs tested had LC50 values below 1 mg l\(^{-1}\). The most acutely toxic dyes for fish are basic dyes, especially those with a triphenylmethane structure. Fish also seem to be relatively sensitive to many acid dyes. Mortality tests with rats showed that only 1\% out of 4461 commercial dyestuffs tested had LD50 values below 250 mg kg\(^{-1}\) body weight (Clarke and Anliker, 1980). Therefore, the chance of human mortality due to acute dyestuff toxicity is probably very low. However, acute sensitization reactions by humans to dyestuffs often occur. Especially some disperse dyestuffs have been found to cause allergic reactions, i.e. eczema or contact dermatitis (Specht and Platzek, 1995).

Chronic effects of dyestuffs, especially of azo dyes, have been studied for several decades. Researchers were traditionally mostly focused on the effects of food colorants, usually azo compounds. Furthermore, also the effects of occupational exposure of human workers to dyestuffs in dye manufacturing and dye utilizing industries have received attention. Azo dyes in purified form are seldom directly mutagenic or carcinogenic, except for some azo dyes with free amino groups (Brown and Devito, 1993). However, reduction of azo dyes, i.e. cleavage of the dye’s azo linkage(s), leads to formation of aromatic amines and several aromatic amines are known mutagens and carcinogens. In mammals, metabolic activation (or reduction) of azo dyes is mainly due to bacterial activity in the anaerobic parts of the lower gastrointestinal tract. Various other organs, especially the liver and the kidneys, can, however, also reduce azo dyes. After azo dye reduction in the intestinal tract, the released aromatic amines are absorbed by the intestine and excreted in the urine. The acute toxic hazard of aromatic amines is carcinogenesis, especially bladder cancer. The carcinogenicity mechanism probably includes the formation of acyloxy amines through N-hydroxylation and N-acetylation of the aromatic amines followed by O-acylation. These acyloxy amines can be converted to nitremium and carbonium ions that bind to DNA and RNA, which induces mutations and tumor formation (Brown and Devito, 1993). The mutagenic activity of aromatic amines is strongly related to molecular structure. In 1975 and 1982, the International Agency for
Research on Cancer (IARC) summarized the literature on suspected azo dyes, mainly amino-substituted azo dyes, fat-soluble azo dyes, benzidine azo dyes, and also a few sulfonated azo dyes (IARC, 1975, 1982).

1.4. Production and discharge statistics of dyes

Dyestuff industry plays an important role in the economic development of India. The Indian dyestuff industry, which was primarily started to cater the needs of domestic textile industry, now not only meets more than 95% requirement of the domestic market, but has gradually made a dent in the global market. All ranges of dyes such as disperse, reactive, vat, pigment and leather dyes are now being manufactured in India. This industry forms an important link in the chain of other chemical industry such as textiles, leather, plastic, paper, packaging, printing inks, paints, polymers, etc. Today nearly one million metric tonnes of dye is annually produced in the world of which azo dyes (R₁-N=N-R₂), represent about 70% on weight basis (Dos Santos et al., 2003). In India dyestuff industry produces around 60,000 metric tonnes of dyes, which is approximately 6.6% of total world output (Shenai, 1995). The largest consumer of these dyes is the textile industry accounting for two third of its market. Recent estimates indicate that 12% of the synthetic textile dyes used yearly are lost to wastewater streams. Approximately 20% of these enter the environment through effluents from wastewater treatment plants (Kirk and Farrell, 1994).

1.5. Treatment of textile dye wastewater

Presence of color and its causative compounds has always been undesirable in water used for either industrial or domestic needs. Color is a visible pollutant. Common man may not object to the discharge of colorless effluents loaded with toxic and hazardous pollutants. On the other hand the discharge of colored effluents, though less toxic, is often objected by the public on the assumption that color is an indicator of pollution. It is therefore, not surprising to note that color in wastewater has now been considered a pollutant that needs to be treated before discharge. Different coloring agents like dyes, inorganic pigments, tannins, lignins, etc usually impart color. Amongst complex industrial wastewater with various types of coloring agents, dye wastes are predominant (Anjaneyulu et al., 2005).

Color is contributed by phenolic compounds such as tannins, lignins (2-3%) and organic colorants (3-4%) (Clarke and Steinle, 1995) and with a maximum contribution
from dye and dye intermediates, which could be sulfur/mordant/reactive/cationic/disperse/azo/acid/vat dye (Raghavacharya, 1997). Dyes are difficult to be decolorized due to their complex structure, synthetic origin and recalcitrant nature, which make it obligatory to remove them from industrial effluents before being disposed into water bodies (Brown et al., 1987).

The color of water, polluted with organic colorants, reduces when the cleavage of the $-\text{C}=\text{C}-$ bonds, the $-\text{N}=\text{N}-$ bonds and heterocyclic and aromatic rings occurs. The absorption of light by the associated molecules shifts from the visible to the ultraviolet or infrared region of the electromagnetic spectrum (Strickland and Perkins, 1995). There are about 12 classes of chromogenic groups, the most common being the azo type, which makes up to 60–70% of all textile and tannery dyestuff produced (Carliell et al., 1996), followed by the anthraquinone type (Buckley, 1992). A dye house effluent typically contains 0.6-0.8 g dye l$^{-1}$ (Gahr et al., 1994). Wastewater treatment using physical, chemical and biological or combinations of these methods are well established for color removal. The advantages and limitations of various treatment methodologies besides emerging technologies are discussed below.

**1.6. Physico-chemical methods**

Dyes can be removed from wastewater by chemical and physical methods (Lin and Liu, 1994; Lin and Chem, 1996). Physicochemical techniques include adsorption, membrane filtration, coagulation/ flocculation, precipitation, flotation, ion exchange, ion pair extraction, ultrasonic mineralization, electrolysis, advanced oxidation (chlorination, bleaching, ozonation, Fenton oxidation and photo catalytic oxidation) and chemical reduction. Each process alone may not be able to meet the requirements. In several studies, many techniques have not been able to achieve decolorization because of many factors like, UV/$\text{H}_2\text{O}_2$ process are the relatively high costs and the occasional lack of effectiveness (Fu and Viraraghavan, 2002). Ozone rapidly decolorizes water-soluble dyes but not the non-soluble dyes (Marmagne and Coste, 1996). These techniques are expensive, large energy requirements, limited life time, formation of unwanted byproducts, foaming, etc. These byproducts may be more toxic than the parent compounds. Thus, the extent of the mineralization in waste decolorization should be evaluated (Table 2.).
Table 2. Application categories of treatment system and its advantages and disadvantages

<table>
<thead>
<tr>
<th>Physical/chemical method</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane filtration</td>
<td>Its ability is to clarify, concentrate and most importantly, to separate dye continuously from effluent, remove all dye types</td>
<td>Flux decline, fouling, necessitating frequent cleaning and regular replacement of the modules, ozonation is required, expensive.</td>
</tr>
<tr>
<td>Fenton’s reagent</td>
<td>Effective decolorization of both soluble and insoluble dyes</td>
<td>Sludge generation</td>
</tr>
<tr>
<td>Ozonation</td>
<td>Applied in gaseous state: no alteration of volume</td>
<td>Short half-life (20 min), effective only in certain pH range</td>
</tr>
<tr>
<td>Photochemical NaOCl</td>
<td>No sludge production, initiates and accelerates azo-bond cleavage</td>
<td>Formation of by-product, release of aromatic amines</td>
</tr>
<tr>
<td>Cucurbituril</td>
<td>Good sorption capacity for various dyes</td>
<td>High cost</td>
</tr>
<tr>
<td>Electrochemical destruction</td>
<td>Breakdown compounds are non hazardous</td>
<td>High cost of electricity</td>
</tr>
<tr>
<td>Activated carbon</td>
<td>Good removal of wide variety of dyes</td>
<td>Very expensive</td>
</tr>
<tr>
<td>Peat</td>
<td>Good adsorbent due to cellular structure</td>
<td>Specific surface area for adsorption are lower than activated carbon</td>
</tr>
<tr>
<td>Wood chips</td>
<td>Good sorption capacity for acid dyes</td>
<td>Requires long retention times</td>
</tr>
<tr>
<td>Silica gel</td>
<td>Effective for removal of basic dyes</td>
<td>Side reactions prevent commercial application</td>
</tr>
<tr>
<td>Ion exchange</td>
<td>Regeneration: no adsorbent loss</td>
<td>Not effective for all dyes, High operation cost</td>
</tr>
<tr>
<td>Irradiation</td>
<td>Effective oxidation at lab scale</td>
<td>Requires a lot of dissolved O₂</td>
</tr>
<tr>
<td>Electro kinetic coagulation</td>
<td>Economically feasible</td>
<td>High sludge production</td>
</tr>
</tbody>
</table>
1.7. Biological methods

The ability of biological treatment process for decolorization of industrial effluents is ambiguous, different and divergent. Observations indicate that dyes themselves are not biologically degradable since microorganisms do not utilize the color constituents as a source of food. Most currently used laboratory methods for biodegradation involve aerobic microorganisms, which utilize molecular oxygen as reducing equivalent acceptor during the respiration process. Yet, environmental conditions with lack of molecular oxygen are not uncommon. In these anoxic and hypoxic environments, microorganisms survive by using sulfates, nitrates, carbon dioxide, etc as electron acceptors (Birch et al., 1989). An anaerobic step followed by an aerobic step may represent a significant advancement in biological treatment and decolorization in the future (Bahorsky, 1998; Ong et al., 2005). An advantage of biological treatment over certain physico chemical treatment methods is that over 70% of the organic material present that is measured by the COD test may be converted to biosolids.

1.8. Fungal biodegradation

Lignin-degrading fungi, white-rot fungi, can degrade a wide range of aromatics. This property is mainly due to the relatively non-specific activity of their lignolytic enzymes, such as lignin peroxidase, manganese peroxidase and laccase. The reactions catalysed by these extracellular enzymes are oxidation reactions, e.g. lignin peroxidase catalyses the oxidation of non-phenolic aromatics, whereas manganese peroxidase and laccase catalyse the oxidation of phenolic compounds (McMullan et al., 2001). The degradation of dyes by white-rot fungi was first reported in 1983 (Glenn and Gold, 1983) and has since then been the subject of many research papers. Virtually all dyes from all chemically distinct groups are prone to fungal oxidation but there are large differences between fungal species with respect to their catalyzing power and dye selectivity. Fungal degradation of aromatic structures is a secondary metabolic event that starts when nutrients (C, N and S) become limiting (Kirk and Farrell, 1987). Therefore, while the enzymes are optimally expressed under starving conditions, supplementation of energy substrates and nutrients are necessary for propagation of the cultures. Other important factors for cultivation of white-rot fungi and expression of lignolytic activity are the availability of enzyme cofactors and the pH of the environment. Although stable operation of continuous fungal bioreactors for the treatment of synthetic dye solutions has been achieved (Mielgo et al., 2001; Palma et al., 1999; Zhang and Yu, 2000), application
of white-rot fungi for the removal of dyes from textile wastewater faces many problems. As wastewater is not the natural environment of white-rot fungi, the enzyme production may be unreliable (Robinson et al., 2001) and the biomass growth and retention in bioreactors will be a matter of concern (Stolz, 2001). As treatment of large water volumes may be difficult, extraction and concentration of dyes prior to fungal treatment, may be necessary (Nigam et al., 2000). Furthermore, the low optimum pH for lignin peroxidase (4.5-5.0) requires extensive acidification of the usually highly alkaline textile wastewater and causes inhibition of other useful microorganisms like bacteria (Swamy and Ramsay, 1999). Moreover, other wastewater constituents, especially aromatics, may interfere with fungal dye degradation (Stolz, 2001).

1.9. Algal biodegradation

Degradation of a number of azo dyes by algae has been reported in a few studies (Jinqi and Houtian, 1992; Semple et al., 1999). The degradation pathway is thought to involve reductive cleavage of the azo linkage followed by further degradation (mineralization) of the formed aromatic amines. Hence, algae have been demonstrated to degrade several aromatic amines, even sulfonated ones (Luther and Soeder, 1987; Luther and Soeder, 1991; Soeder et al., 1987). In open wastewater treatments systems, especially in (shallow) stabilization ponds, algae may therefore contribute to the removal of azo dyes and aromatic amines from the water phase.

1.10. Bacterial biodegradation

1.10.1. Azo dye decolorization under aerobic conditions

Several bacterial strains that can aerobically decolorize azo dyes have been isolated during the past few years. Many of these strains require organic carbon sources, as they cannot utilize dye as the growth substrate (Stolz, 2001). *Pseudomonas aeruginosa* decolorized a commercial tannery and textile dye, Navitan Fast blue S5R, in the presence of glucose under aerobic conditions. This organism was also able to decolorize various other azo dyes (Nachiyar et al., 2003). There are only very few bacteria that are able to grow on azo compounds as the sole carbon source. These bacteria cleave –N=N– bonds reductively and utilize amines as the source of carbon and energy for their growth. Such organisms are specific towards their substrate. Examples of bacterial strains with this trait are *Xenophilus azovorans* KF 46 (previously *Pseudomonas* sp. KF46) and *Pigmentiphaga*
kullae K24 (previously Pseudomonas sp. K24), which can grow aerobically on carboxy-orange I and carboxy-orange II, respectively (Zimmermann et al., 1982). These organisms, however, could not grow on structurally analogous sulfonated dyes, acid orange 20 (Orange I) and acid orange 7. Long adaptation of 4-aminobenzenesulfonate (4-ABS) degrading Hydrogenophaga intermedia strain for growth on 4-carboxy-4'-sulfoazobenzene (CSB) as the sole organic carbon source led to the isolation of other strain, which reduced CSB and utilized the two amine metabolites (Blumel et al., 1998). Sphingomonas sp, strain 1CX, an obligate aerobe, can grow on an azo dye, acid orange 7, as sole carbon, energy and nitrogen source (Coughlin et al. 1999). This strain degraded only one of the component amines (1-amino 2-naphthol) formed during acid orange 7 decolorization and 4-aminobenzene sulfonate (4-ABS) degradation.

1.10.2. Azo dye decolorization under anaerobic conditions

Methanogenesis from complex organic compounds requires the coordinated participation of many different trophic groups of bacteria, including acidogenic, acetogenic and methanogenic bacteria (Wuhrmann et al., 1980). Dye decolorization under these conditions requires an organic carbon/energy source. Simple substrates like glucose, starch, acetate, ethanol and more complex ones, such as whey and tapioca, have been used for dye decolorization under methanogenic conditions (Chinwetkitvanich et al., 2000; Willetts et al., 2000; Talarposhti et al., 2001; Yoo et al., 2001; Isik and Sponza, 2004; Van der Zee and Villaverde, 2005). Extensive studies have been carried out to determine the role of the diverse groups of bacteria in the decolorization of azo dyes. (Carliell et al., 1996; Razo-Flores et al. 1997) have associated the decolorization with methanogens, whereas studies by other investigators showed that acidogenic as well as methanogenic bacteria contribute to dye decolorization (Chinwetkitvanich et al., 2000; Bras et al., 2001).

Reduction under anaerobic conditions appears to be nonspecific, as varied group of azo compounds are decolorized, although the rate of decolorization is dependent on the added organic carbon source, as well as the dye structure (Bromley-Challenor et al., 2000; Stolz, 2001). Furthermore, there is no correlation between decolorization rate and molecular weight, indicating that decolorization is not a specific process and cell permeability is not important for decolorization. Thus, anaerobic azo dye decolorization is a fortuitous process, where dye might act as an acceptor of electrons supplied by carriers of the electron transport chain. Alternatively, decolorization might be attributed
to non-specific extracellular reactions occurring between reduced compounds generated by the anaerobic biomass (Van der Zee et al., 2001).

1.10.3. *Azo dye decolorization under anoxic conditions*

Anoxic decolorization of various azo dyes by mixed aerobic and facultative anaerobic microbial consortia has been reported (Nigam et al., 1996; Kapdan et al., 2000; Padmavathy et al., 2003; Khehra et al., 2005; Moosvi et al., 2005). Although many of these cultures were able to grow aerobically, decolorization was achieved only under anaerobic conditions. Pure bacterial strains, such as *Pseudomonas luteola*, *Aeromonas hydrophila*, *Bacillus subtilis*, *Pseudomonas* sp. and *Proteus mirabilis*, decolorized azo dyes under anoxic conditions (Chang et al., 2001; Chen et al., 1999; 2003; Yu et al., 2001). Azo dye decolorization by mixed, as well as pure, cultures generally required complex organic sources, such as yeast extract, peptone, or a combination of complex organic source and carbohydrate (Chen et al., 2003; Khehra et al., 2005). Glucose is the preferred substrate in anaerobic dye decolorization under methanogenic conditions, but its suitability for anoxic dye decolorization by facultative anaerobes and fermenting bacteria seems to vary, depending on the bacterial culture. Decolorization of Mordant Yellow 3 by *Sphingomonas xenophaga* strain BN6 was greatly enhanced by glucose, whereas a significant decrease in azo dye decolorization in its presence was reported for *Pseudomonas luteola*, *Aeromonas* sp. and few other mixed cultures (Haug et al., 1991; Kapdan et al., 2000; Chang et al., 2001; Chen et al., 2003). The negative effect of glucose on anoxic decolorization has been attributed either to a decrease in pH due to acid formation, or to catabolic repression (Chen et al., 2003).

1.11. Combined anaerobic-aerobic bacterial biodegradation of azo dyes

1.11.1. *First stage: anaerobic azo dye reduction*

Anaerobic azo dye reduction is the reductive cleavage of azo linkages, i.e. the transfer of reducing equivalents resulting in the formation of aromatic amines. As aromatic amines are generally colorless, azo dye reduction is also referred to as azo dye decolorization. The first study on azo dye reduction was published as early as 1937, when the decolorization of food azo dyes by lactic acid bacteria isolated from the human gut was reported (Brohm and Frohwein, 1937). Hence, as the formation of toxic aromatic amines in humans is a matter of concern, research on bacterial azo dye reduction has
traditionally mostly been focused on the activity of (facultative) anaerobic bacteria from mammalian intestines (Brown, 1981; Chung et al., 1978; Chung et al., 1992; Dieckhues, 1960; Rafii et al., 1990; Walker, 1970). Later, when the removal of dyes from wastewater became a topic, also bacteria from other origins were used to investigate anaerobic azo dye reduction, e.g. pure cultures (Wuhrmann et al., 1980), mixed cultures (Haug et al., 1991), anaerobic sediments (Weber and Wolfe, 1987), digester sludge (Beydilli et al., 1998; Brown and Laboureur, 1983; Carliell et al., 1994), anaerobic granular sludge (Flores et al., 1997) and activated sludge under anaerobic conditions (Bromly-Challenor et al., 2000). The large number of azo dyes that can be reduced by so many different bacteria indicates that azo dye reduction is a non-specific reaction and that the capability of reducing azo dye can be considered as a universal property of anaerobically incubated bacteria.

1.11.2. Second stage: aerobic oxidation of aromatic amines

Various (substituted) amino-benzene, amino-naphthalene and amino-benzidine compounds have been found aerobically biodegradable (Baird et al., 1977; Brown and Laboureur, 1983; Ekici et al., 2001). The conversion of these compounds generally requires enrichment of specialized aerobes. In some cases, biodegradation was only achieved in nitrogen-free medium (Konopka et al., 1993). Especially sulfonated aromatic amines are difficult to degrade. This low biodegradability is due to the hydrophilic nature of the sulfonate group, which obstructs membrane transport. Generally, biodegradation of sulfonated aromatic amines has only been demonstrated for relatively simple sulfonated aminobenzene and aminonaphthalene compounds (Sumathi et al., 2001). Another transformation that aromatic amines may undergo when being exposed to oxygen is auto oxidation. Especially aromatic amines with ortho substituted hydroxyl groups are susceptible to auto oxidation (Kudlich et al., 1999). Many aromatic amines, e.g. substituted anilines, aminobenzidines and naphthylamines, have been found to oxidize, initially to oligomers and eventually to dark-colored polymers with low solubility that are easily removed from the water phase (Field et al., 1995; Klibanov et al., 1981).

1.11.3. Combined anaerobic-aerobic treatment

The prerequisite of (reductive) fission of the azo linkage in azo dye molecules prior to (oxidative) further degradation, makes a process in which anaerobic and aerobic conditions are combined the most logical concept for the biological removal of azo dyes
(Field et al., 1995; Knackmuss, 1996; Zitomer et al., 1993). Two different approaches can be discerned: sequential treatment in separate reactors and integrated treatment in a single reactor. The integrated approach is based on temporal separation of the anaerobic and the aerobic phase, like in sequencing batch reactors (SBR) or on the principle that diffusion of oxygen in microbial biofilms is usually limited to 10-100 µm (Lens et al., 1995) so that anaerobic and aerobic conditions coexist in a single environment. The removal of color achieved in the anaerobic stages was generally high, mostly higher than 70% and in several cases almost 100%. Color removal efficiencies differed between dyes, when the removal of different azo dyes was tested under similar conditions.

The reaction time is an important factor in the anaerobic removal of azo dyes, decreasing the hydraulic retention time of the anaerobic stage was found to result in lower color removal efficiencies and anaerobic azo dye reduction is a rather slow reaction (An et al., 1996; Seshadri et al., 1994). The biomass concentration also plays a role in the anaerobic removal of azo dyes lowering the biomass and the solid retention time of a sequencing batch reactor resulted in a lower color removal efficiency (Lourenço et al., 2000). The studies that paid attention to azo dye reduction leading to the formation of aromatic amines. The recovery percentages ranged between <1% to almost 100%. This wide range may partly be explained by the difficulties encountered in analyzing these often chemically unstable compounds. The fate of aromatic amines in the aerobic stage cannot be conclusively determined. Partial or complete removal of many aromatic amines can be suspected from the decrease or disappearance of the sometimes unidentified peaks in HPLC chromatograms (FitzGerald and Bishop, 1995; Harmer and Bishop, 1992; Jiang and Bishop, 1994; Kalyuzhnyi and Sklyar, 2000; Lourenço et al., 2000; O’Neill et al., 2000; Sarsour et al., 2001; Sosath and Libra, 1997; Tan et al., 2000) as well as from the decrease of UV absorbance (Cruz and Buitrón, 2001). Moreover, a large decrease of toxicity to aerobic bacterial activity was measured between the effluent of the anaerobic stage and the effluent of the aerobic stage (O’Neill et al., 2000; Sosath et al., 1997). To summarize, combined anaerobic-aerobic biological treatment holds promise as a method to remove azo dyes from wastewater. However, it can be concluded that there are two possible bottlenecks: (i) anaerobic azo dye reduction is a time-consuming process, reflected by the requirement of long reaction times and that (ii) the fate of aromatic amines during aerobic treatment is not conclusively elucidated.
1.12. Enzymes in biodegradation

1.12.1. Cytochrome P450

Cytochrome P450 (P450) represent one of the largest and oldest gene superfamilies (Degtyarenko and Kulikova, 1993) coding for enzymes present in the genomes of all biological kingdoms. The terminology P450 is uncommon for enzymes because it is not based on function, but describes originally the spectral properties of this b-type heme containing red pigments, which display a typical absorption band at 450 nm of their reduced carbon-monoxide bound form (Omura and Sato, 1962). This for cytochromes unusual spectral feature is induced by a cysteine thiolate group (Ichikawa and Yamano, 1967; Murakami and Mason, 1967) forming the fifth ligand of the heme iron and classifies cytochrome P450 enzymes as hemethiolate proteins (NC-IUB et al., 1991). Usually, they act as terminal monooxygenases (Fig. 1) (Ruckpaul and Blanck, 1989) in a range of reactions all including the transfer of molecular oxygen to X-H bonds (X: -C, -N, S) of a substrate with the concomitant reduction of the other oxygen atom to

![Fig. 1. Electron transfer mechanism of cytochrome P450](image-url)
water (Mansuy, 1998). The reactions catalyzed can be extremely diverse as hydroxylations, N-, O- and S-dealkylations, sulfoxidations, epoxidations, deaminations, desulfurations, dehalogenations, peroxidations, and N-oxide reductions (Sono et al., 1996; Bernhardt, 2006). Since many of the individual P450s catalyze multiple reactions, the usual method of naming enzymes is inadequate for this group of proteins, and a systematic nomenclature has been derived based on structural homology (Nelson et al., 1993; Nelson et al., 1996). The P450 genes are identified by the abbreviation CYP followed by a number denoting the family (proteins with more than 40% sequence identity), a letter designating a subfamily (more than 55% identity) and a number representing the individual gene within the subfamily, for example, CYP106A2. At present, 267 families with more than 5000 genes are notified. Even though the sequence conservation among P450 proteins of different families may be less than 20%, their general topography and structural fold are highly conserved and point to a common mechanism of oxygen activation. This explains the remarkable variety of chemical reactions catalyzed and the enormous number of substrates attacked. Some P450s do not require any other protein component to achieve the reductive activation of molecular oxygen (Degtyarenko and Kulikova, 2001) while the vast majority of P450s performs the diverse range of chemical reactions after interaction with one or more redox partners to source their redox equivalents from electron transfer chains. Most of the electron transfer reactions begin with the transfer of electrons from NAD(P)H and end with the reductive cleavage of oxygen and the incorporation of one oxygen atom into the substrate by the terminal P450s (Porter and Coon, 1991). These so called monooxygenase systems have revealed an unexpected diversity (McLean et al., 2005) after a dramatically growing number of genes has been discovered in recent genome projects (Hannemann et al., 2007).

1.12.2. Azoreductase

Azoreductase is one of the biotransforming enzyme involved in decolorization of azo dyes by breaking azo bond. Azo dyes can be used by microorganisms as sole source of carbon, energy and nitrogen with the action of azoreductase enzyme. Azoreductase requires NADH/NADPH as electron donor to catalyze the reductive cleavage of azo bond (Fig. 2). It has been known for a long time that metabolic processes such as azo reduction can activate, and sometimes also detoxify, azo compounds (Combes and Haveland-Smith, 1982). The reduction of the azo bonds in azo dyes is important in their toxicity,
mutagenicity and carcinogenicity (Garner et al., 1984). It has been shown that intestinal bacteria carried out reduction of azo compounds, to produce aromatic amines. Some 45 different intestinal bacteria, have been found that expresses azoreductases (Moller et al., 2000), with a diverse collection of bacteria, including both strictly anaerobic Clostridium species and facultative anaerobic Enterobacteriaceae. It is not clear how many different types of azoreductase enzymes are in the human gut, or which of these may be relevant for metabolism.

During the aerobic, “semi-aerobic” (in static anoxic) or anaerobic incubation of bacteria with azo compounds, amines were often detected that originated from a reductive cleavage of the azo bond. The aerobic reductive metabolism of azo dyes requires specific enzymes (aerobic azoreductases) that catalyze these reactions in the presence of molecular oxygen. In contrast to few reports of aerobic decolorization of azo dyes, a wide range of organisms are able to reduce azo compounds under anaerobic/anoxic conditions. This has been shown for purely anaerobic (e.g. Bacteroides sp., Eubacterium sp., Clostridium sp.), facultatively anaerobic (e.g. Proteus vulgaris, Streptococcus faecalis), and aerobic (e.g. Bacillus sp., Sphingomonas sp.) bacteria, yeasts, and even tissues from higher organisms (Adamson et al., 1965; Bragger et al., 1997; Dieckhues, 1960; Dubin and Wright, 1975; Mecke and Schmähl, 1957; Rafii et al., 1990; Scheline et al., 1970; Walker, 1970; Wuhrmann et al., 1980).

Cell extracts generally showed much higher rates of anaerobic reduction of azo dyes than of resting cells (Wuhrmann et al., 1980). Reductases like NADH-DCIP, azo and riboflavin were reported for the degradation of different dyes, but the actual role of
different reductases is still unknown, FMN-dependent NADH-azoreductase found to
degradate azo compounds that catalyses the reductive cleavage of an azo group by a ping-
pong mechanism (Fig. 2) (Ooi et al., 2009).

1.13. Objectives of the study

1. Screening, isolation and identification of microorganisms involved in the degradation
   of different dyes.

2. Standardization of the media conditions and other parameters for maximum
   degradation of dyes.

3. Study of enzyme system responsible for biotransformation reactions.

4. Structure determination of metabolites formed during biodegradation of dyes by UV,
   FT-IR, GCMS, HPLC, etc

5. Evaluation of the cytotoxicity and genotoxicity of dye and degraded metabolites.

6. Designing of the consortia for effective decolorization of dyes.

7. Designing of bioreactor for the decolorization of dyes.

Fig. 3 DCPIP reductases activity by using DCPIP as substrate