1. INTRODUCTION:

1.1 Introduction to Carpet Industry:

Indian Carpet Industry comprises of hand knotted carpet, hand tufted and machine made carpet. However, Indian hand-knotted carpets receive worldwide acclaim. Perhaps the most famous carpet-producing region in India is the "carpet belt" of Uttar Pradesh. This belt is located near Varanasi in the south eastern corner of Utter Pradesh, one of the State's economically poorest regions, and is capable of producing virtually every variety of carpet. Production and trade activities are primarily concentrated in the Mirzapur-Bhadohi area and extend outwards to surrounding districts of Utter Pradesh. This industry is endowed with a fully large and diversified production base with an estimated 3 lakhs looms which is providing employment to 1.6 million as carpet weavers and others engaged in allied activities. Bhadohi-Mirzapur belt in U.P. accounts for around 80% of activities pertaining to production and export**.** The Industry is export oriented and positive Net Foreign Exchange (NFE) earner contributing to the growth of Indian Economy in general and textile industry in particular.

Carpets were probably first made by Nomadic people to cover the earthen floor in their tents. Carpets were known in India as early as 500 B.C. References to woven mats and floor coverings can be found in ancient and medieval Indian literature. Precisely, the birth of the Indian style carpet which is overwhelmingly rich with a sheer range of patterns, styles and designs on their bristled surface is definitely from the Persian style carpets. From the beginning, wool or silks have been the basic raw material of the

knotted carpets. The wool may have a variety of origins as per the type of carpet being made. Silk knots are also used in Kashmir region. The patterns varied from vines and floral patterns, animal and bird figures and geometric and calligraphic patterns. Though the floor coverings in India are one of the most ancient crafts followed till date yet the typical pilled carpets, more commonly known as the Kashmiri carpet are being used in India since ages. Since then the carpet industry has developed leaps and bound in terms of production, styles and patterns and so in the markets. There are more than 3000 carpets manufacturing units recorded in India and out of that more than 1000 are existing in this region. The carpet-manufacturing units are categorized into three categories, small scale, large scale and medium scale. In Bhadohi region, the large scale units are about 20 in number, medium scale are 60 whereas, number of small-scale units are more than 900. Some of the small-scale manufacturers are housed in their own homes and most of the family members are involved in Carpet manufacturing. About 200,000 personnel including women are involved in carpet manufacturing and its allied areas. The work force includes skilled, unskilled and allied. The skilled manpower includes designers, weavers, dyers and washers. The unskilled manpower includes finishers, clippers and yarn openers. The allied industries work forces include loom makers, yarn spinners and toolmakers. Recognizing, the unique position of Carpet industry as a self-reliant industry from the production of raw materials to the delivery of finished products, with substantial value- addition at each stage of processing and its major contribution to the country's economy this industry should be considered as a representative cluster at Bhadohi.

The SME Carpet Cluster projected at Bhadohi, is unique in the sense being unorganized, yet having a characteristic export potential and market worth 25 million $ which is about 90% of the total sales from India. The specific characteristic of this cluster is that very few industrial houses are having all the facilities for the manufacturing of carpets and even these houses are getting weaving done on contract through the weaving centers spread in nearly every village of the Bhadohi carpet cluster. These centers are registered unit by the Textile Ministry of the Government of India. Typically, every weaving center employ around 10-15 registered weavers. More than two lakhs registered weavers are working in these weaving centers. In addition to these about one lakh unregistered weavers from neighboring states are also engaged in the weaving.

The carpet industry is using large quantities of dyes**.** Only big carpet houses of the cluster are having their own dye house facility. Majority of medium player and small manufacturers are taking the services of independent dye house present in the cluster. Most of the smaller manufacturers are using old technique of manual dyeing, which have all kind of deficiencies in terms of product quality and environmental impact. Majority of the dye houses of the cluster are also not following state of art technology, which are being practiced in other part of the globe. The dyeing chambers are also based on the older technology resulting into non-uniform product quality. The improper control of the operating parameters is the main reason for this. Lots of steam is being wasted which results into loss of energy. The boilers being used for steam generation are also not properly maintained, and in some cases we have found boilers of pre-independence era, resulting into great loss of energy. No industry in the cluster is even aware of importance

of demineralization plant prior to boiler feeding. Most of the boiler utilized in carpet industries are heavily contaminated by the scales as the ground water of the region is very hard, which is also reflected in the water sampling and testing done during the study.Due to improper control of dyeing parameters there is variation in color. None of the dye houses are practicing proper effluent treatment processes, so environment is certainly being adversely affected. Use of natural dye is entirely missing from the cluster. Deeper study of European market suggests that there is more market of naturally dyed and washed carpets. Extra effort is needed to introduce these novel concepts in this cluster.

1.2 Synthetic Azo Dye

The use of natural dye has been found in ancient time even back to 3500 B.C. which were extracted from fruits, vegetables, flowers certain insect's fish dating. In old day's fabric were dyed with natural dyes. Natural dyes gave a limited and a dull range of colors. The synthetic dyes were first synthesized by W.H. Perkins in 1856 has provided a wide range of dyes that are color fast and come in a wider color range and different shades. As a result, dye application has become a massive industry today. The azo dyes are used for permanent coloring of fibers and other consumer products including food, cosmetics, pharmaceutics, papers etc. The annual production of these dyes is more than 7×10^7 ton out of which about 30,000–150,000 tons are discharged into running water stream [Anjaneya *et al.* (2011)]. The azo dyes derive its name due to the presence of azo group (-N=N-) [Chang *et al.* (2000)]. Most of the azo dyes, except naturally occurring 4-4'-dihydroxyazobenzene, are synthetic [Gill and Straunch (1984)]. The wide application of azo dye is due to its availability in various color combinations, fast coloring and

binding with natural and synthetic fibers**.** Besides these; they have showed low color fastness when exposed to washing and sunlight. Synthetic dyes are classified on the basis of chemical structure, applications and Color Index number developed by the Society of Dyers and Colourist [Society of Colourlist (2010); Saratale *et al*. (2013)]. On the basis of chemical structure, they are categorized as acid, basic, direct, disperse, mordant, reactive, sulfur azoic, vat dye, sulfur reactive, metal complexes etc., including antraquinone, indigo, triphenylmethyl, xanthenes and phthalocyanine derivatives. For example, acid means the dye is negatively charged, basic when it is positively charged, reactive if it is an anionic dye used in the textile industry, mordant if it contains a metallic ion, Vat when it derives from natural indigo, and disperse when it is a non-ionic dye used in aqueous dispersion and so on. The nomenclature of various dyes, followed by the name of its color and an order number, gives the color index (C.I.) of the dye [Asad *et al*. 2007]. The wastewaters are generated at various steps such as sizing, scouring, bleaching, mercerizing, dyeing, printing and finishing (EPA, (1997)). The effluents discharges from these industries are complex in nature. The contaminants present into these wastewaters are dyes, their degraded products and other left over chemicals being used in dying and washing process such as surfactant, salt and other toxic products **[**Sen *et al*. (2003)]. Presently, more than 3000 different kind of azo dyes are being used in textile and many other industries [Spadaro *et al*. (1994); Spadaro *et al*. (1992)]. The azo dyes are recalcitrant to biodegradation due to their origin and chemical structure [Qu *et al*. (2010); Sharma and Singh, (2011)]. The azo dyes and their intermediate aromatic amines are toxic, carcinogenic, mutagenic and teratogenic to various living organism [Marini *et al*.

(1996); Tokiwa *et al*. (1986); Padda *et al*. (2003); Parshetti *et al*. (2010)]. Thus, presence of dye and their degradation products in environment would pose threat to living organisms in short-term or long-term threats. Azo dye and its degradation products exhibited inhibitory effect on the microbial population [Carliell *et al*. (1994); Masour *et al*. (2011); Pandey *et al*. (2007); Neumann *et al*. (2010)]. The colored effluents of textile industries discharged into large water bodies without treatment disturb the ecological system of receiving aquatic system due their high color content and high dissolved solids [Doble *et al*. (2005); Vandevivere *et al*. (1998)]. Highly colored wastewater with low light penetration reduces photosynthesis and oxygen level in aquatic system **[**Champagne *et al.* (2010)]. In addition to the surface water, ground water quality is also adversely affected by colored dye wastewater [Rajaguru *et al*. (2002); Umbuzeiro *et al*. (2005)]. In terrestrial ecosystem, colored effluents decreases seed germination and plant growth of crops and yield [Kapustka *et al*. (1993); Ghodake *et al.* (2009b)] by altering chemical and biological status of the soil. Azo dyes metabolization at the intestinal wall and liver produces free aromatic amines that are potentially carcinogenic and mutagenic [Baughman *et al.* (1988); Weber *et al.* (1987)]. The aromatic amines were responsible for increased cancer of the urinary bladders [Saratale *et al*., (2011)]. The benzidine induces urinary bladder cancer in humans and tumors in some experimental animals [Combes *et al.* (1982)]. German painters developed bladder cancer due prolonged exposure to azo dyes [Myslak*et al*. (1991)]. The discharge of textile dye effluent from textile industries in River Cristais, the major drinking water source in Brazil, has been identified to be one of the reasons for mutagenicity in inhabitants [Lima *et al*., (2007)].

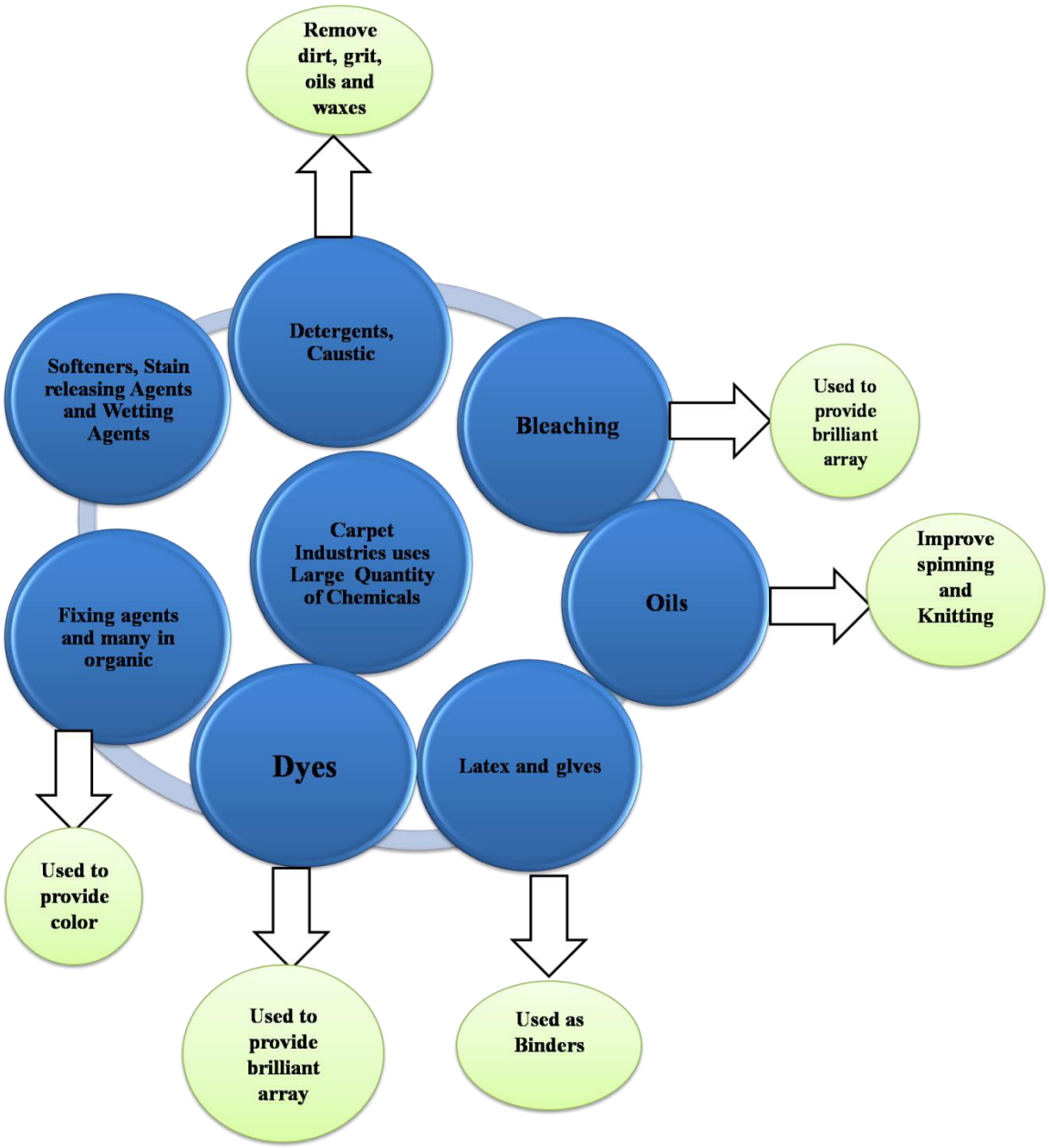

Figure 1.1: Showing the various steps of Carpet Manufacturing Unit.

1.3 Carpet Manufacturing At a Glance

There are various steps in dying process in carpet manufacturing unit as shown in *Figure 1.1*:

- ***Scouring***: This is first step of dying process which is carried out with or without chemicals at room temperature or at suitable elevated temperature with the addition of suitable wetting agents like alkali. This process involves washing the wool in hot soapy water to remove dirt, grease and dry plant matter from fleece. With the removal of all these impurities scouring process finally makes the raw textile material hydrophilic or water absorbent. The wastewater generated at this stage is consisted of disinfectants and insecticide residues, sodium hyroxide, detergents, fats, oil, pectin, wax, lubricants, spin finishes and spent solvent with the characteristics of high BOD, COD, TOC and neutral pH.
- ***Desizing:*** This process involves impregnation of fabric with desizing agent to degrade or solubilize the size material and finally to wash out the degradable products formed during the process prior to dying and weaving. The nature of this process depends upon the type of size material applied. Water soluble size may simply be washed out, whereas water insoluble size must first be subjected to chemical or enzymatic degradation which prevents size recovery and so a large burden is placed on wastewater stream. The wastewater produced at this step is having high BOD, high TSS and neutral pH.

- ***Bleaching***: Bleaching is the process used to remove unwanted color from fibers by using chemicals such as sodium hypochlorite and hydrogen peroxide. The wastewater has following characteristics such as high BOD, high TS and alkaline wastewater.
- ***Dyeing:*** is the process of adding color to the fibers, which normally requires large volumes of water not only in the dye bath, but also during the rinsing step. This process is normally carried out in a special solution containing appropriate dye and some particular chemicals like metal salts, surfactants, organic processing aids, sulphide and formaldehyde etc. The added chemicals improve the dye adsorption on the fibers. The large amount of wastewater is produced at this stage has following characteristics consisting of synthetic dyes, high BOD, high COD, TS and alkali.

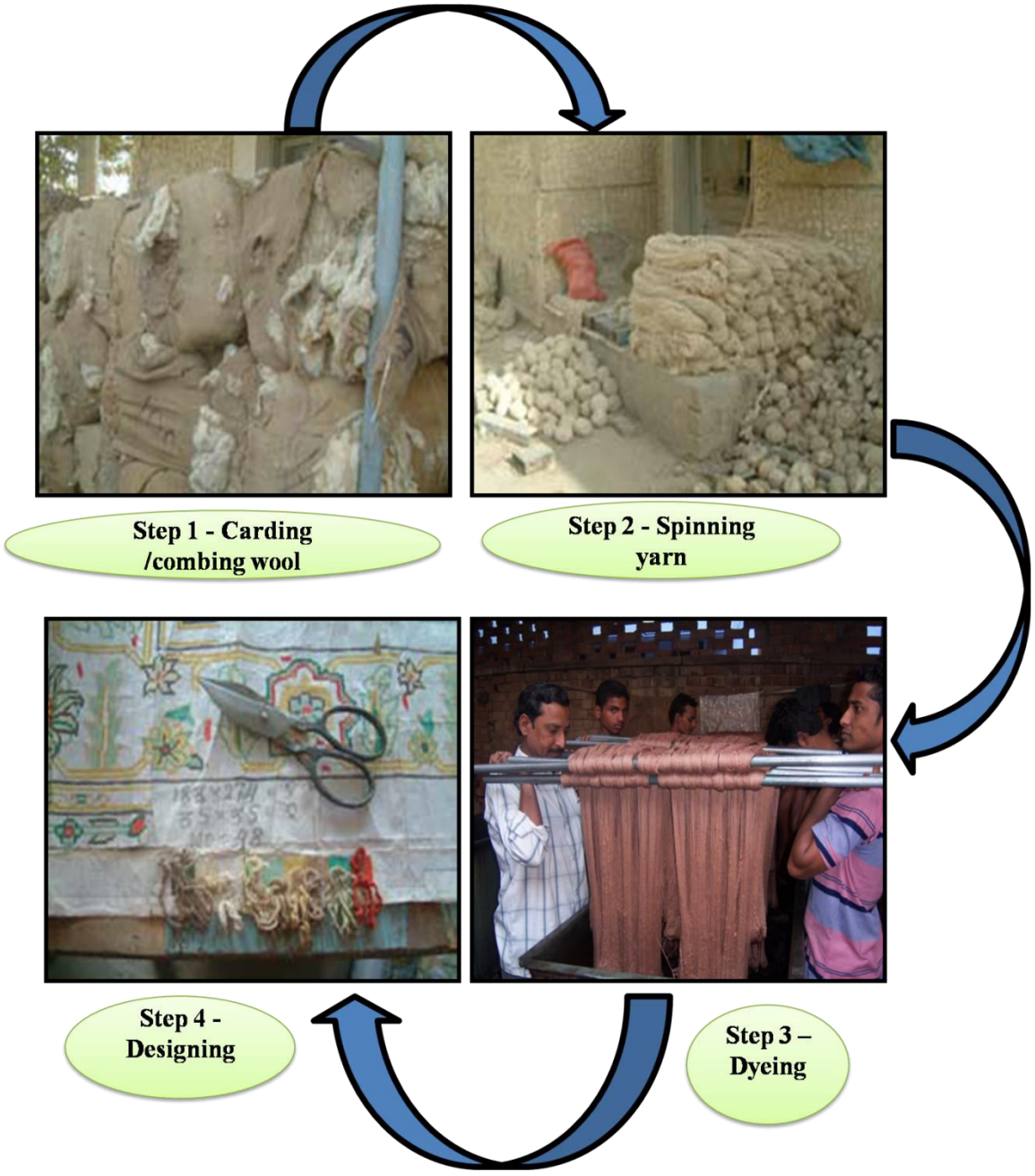
Step 1 - Carding /combing wool
Step 2 - Spinning yarn
Step 3 – Dyeing
Step 4 - Designing

Figure 1.2: Carpet Manufacturing Unit at a Glance

1.4 Environmental and Health Hazard Due to Dye bearing wastewater

The wastewater coming from dye based industries contaminates nearby water bodies like river, ground water and other water ways. The carpet industry discharged their effluent which contain azo dyes and others chemical compounds like bleaching agents, salt, acids and alkali into nearby ponds and drains without any treatment. Heavy metals like cadmium, copper, zinc, chromium and iron are also found in the dye effluents. The discharge of untreated effluents into the aquatic environments responsible to severe contamination of surface and underground water and have adverse effects on aquatic flora and faun [Champagne *et al.* (2010)]. The releases of large amount of industrial effluent not only alter the physico-chemical properties of water bodies but it is also proved to be hazardous to aquatic organism. The presence of very low concentration of dye in water affects dissolve oxygen content and photosynthesis. The discharge of industrial effluent into nearby water bodies severally affects soil fertility and plant growth. The dyes and other compounds in the effluent alter the chemical and biological status of soil and water, which may affect the growth and productivity of plants. Effluents also affect the susceptibility of plants to various pathogens. The untreated effluents inhibit elongation of shoot and root of seedlings. Due to presence of color in the wastewater reduces the light penetration and dissolve oxygen, which resulted into restriction of growth and development of seedlings. The inhibition of seed germination may be due to the high dissolved solids in the effluent. The wide use of azo dye in food industries as coloring agent and their degraded products on human health concern over a number of year, in spite of legislation controlling their use in several countries. The US

food and Drugs Administration allow various azo dyes for use in food, drugs and cosmetics; they constituted the largest group of FDA certified colorants. The dyes and other products present in the effluents causes various diseases in human beings like bladder cancer, splenic sarcomas, hepato carcinomas, nuclear anomalies in experimental animals and chromosomal aberrations in mammalian cells. The toxic dyes and its intermediates can act as mutagenic, carcinogenic and terogenic in human beings. The workers exposed to large quantities of azo dyes is more susceptible for bladder cancer [de Aragao Umbuzeiro G *et al*. (2005)]. Azoreductase is enzyme responsible for cleavage of azo group into amines which is mutagenic and carcinogenic in nature. The effluents bring change in the water quality causes several physiological and biochemical disturbances in fish. The chemicals present in the textile industry effluent affect the normal life of aquatic animals. Toxic compounds from dye effluent get into aquatic organisms, pass through food chain and ultimately reach to man and cause various physiological disorders like hypertension, sporadic fever, renal damage, cramps etc. Bioaccumulation of toxicants depends on availability and persistence of the contaminants in water, food and physiochemical properties of the toxicants.

1.5 Characteristics of the carpet industries wastewater

The majority of carpet industry used large amount of chemical additives which are impregnated during the manufacture of the carpet fiber or are introduced externally as topical treatments on the final product. The proposed purpose of some of these chemicals is to protect against dust mites, bacteria, moulds and fungi. During the production of carpet large amount of chemicals and dyes that are applied are not going too fixed on the

carpet. Some portion of these always remains unfixed to the carpet and gets washed out. These unfixed dyes and chemicals are found to be in high concentrations in their effluents. The amount of water consumed and released also varies depending on the type of carpet produced. Carpet Industry alone use approximately 600 M. T. chemicals and approximately 6 M.T. of sulphuric acid out of which about 1 crore liter of industrial waste water are generated annually in Bhadohi. Some of the important characteristics of the carpet industries wastewater are described herewith:

1.5.1 pH Value

pH is a measure of acidity or basicity of a solution. In the carpet industry pH plays an important role. The solubility of dyes depends on pH. The pH range of wastewater coming from industries is 7 to 10. The harmful effect of high pH on microorganisms, aquatic plants, animals and enzymes are well documented. High pH of the effluents is also responsible for the change in soil permeability which results in polluting underground resources of water. The pH value of aqueous extracts of the textiles is also an important parameter with respect to human health.

1.5.2 Temperature

The temperature fluctuation is observed in effluent coming from these industries. Temperature is an important parameter as it is characteristics of wastewater. It greatly influences vital activities like metabolism, behavior, reproduction and development of microorganisms (Saxena *et al.* 1990).

1.5.3 Acidity

Acidity is defined as quantitative capacity of water to react with a strong base. Acidity is a measure of an aggregate property of water and can be interpreted in terms of specific substances only when the chemical composition of sample is known. If highly acidic effluents are released into the natural water ways or soil, it essentially decreases the pH, which can subsequently cause problems for the whole biota.

1.5.4 Biological Oxygen Demand

Biological Oxygen Demand is defined as the amount dissolved oxygen required for the complete degradation of biologically degradable organic matter present in any water. The greater the biologically degradable organic content, greater will be BOD values. The maximum permissible limits for BOD value was set by WHO for all the samples of water and waste water. The BOD value for dye bearing waste water was found to be in the range of 221 mg/L to 699 mg/L. This indicates that there could be low oxygen available for living organisms in the wastewater. Therefore, consistent analysis of this parameter needs to be encouraged for the textile industry effluent. Oxygen demand is important because organic compounds are generally unstable and may be oxidized biologically to a stable inert end product. An indication of organic oxygen demand content of wastewater can be obtained by measuring the amount of oxygen required for its stabilization either as BOD or COD. BOD/COD ratio is an important factor in evaluating the extent of organic pollution ratio which reveals the treatability of waste water. If the ratio of BOD/COD for untreated effluent is above 0.5, the waste water is considered to be easily treatable by biological means. If the ratio is less than 0.3, the

waste water is deemed to undergo either a chemical treatment prior to routine biological treatment or acclimated microorganisms may be required in its complete stabilization. The textile wastewaters exhibit low BOD/COD ratios (< 0.1) indicating presence of some toxic components [Azbar *et al*. (2004)].

1.5.5 Chemical Oxygen Demand (COD)

High COD levels imply toxic condition and the presence of biologically resistant organic substances. The maximum permissible limits limit of COD for waste water is specified 150 mg/L by WHO. The COD value for dye bearing wastewater was found in the range of 1170 mg/L to 3998 mg/L. COD measures overall oxygen requirements of the wastewater sample including all oxdisable components that are non-biodegradable and so not detected in BOD analysis. COD is used to measure the oxygen equivalent to organic material in the wastewater that can be oxidized chemically using dichromate as oxidizing agent in an acid solution. In textile dye effluent, the high molecular weight chromophores contribute little to BOD. However, these compounds are major contributors to COD and color toxicity due to their inability to pass through the cell membranes. [Eriksson *et al.* (1985)].

1.5.6 Color

The effluents coming from the carpet industries are colored due to presence of dyes, pigments and other colored compounds. The nature of wastewater is complex due to presence of different types of dyes and others chemicals [Correia *et al*. (1994)]. Moreover, the textile dyes have complex structures, synthetic origin and recalcitrant

nature, which makes them obligatory to remove from industrial effluents before being disposed into natural hydrological systems [Anjaneyulu *et al*. (2005)].

1.5.7 Total Suspended Solids and Total Dissolved Solids

The effluents contain low TSS levels and moderate to high TDS levels. In addition to particular types of dye, some chemicals like electrolytes, acids and alkali are also being used in different stages of dying contribute to the total solids in wastewaters. Besides effecting the growth of the plants directly, the solids present in the waste water, also affects the soil structure, permeability and aeration thus affecting the plant growth.

1.5.8 Formaldehyde

The Formaldehyde released from fabrics has been a matter of great concern as it causes dermatitis and respiratory problems. The Formaldehyde thresholds lie between 20 and 300 mg/kg for different groups of textile articles. Formaldehyde has irritant effect on the respiratory tract and mucoss in concentrations above 0.5 mg/m^3 air. The limit value is 0.1 mg/m^3 air for the formaldehyde emissions into the air from Carpets. However, around 300 mg formaldehyde per kg of garment can trigger allergic effects in sensitized persons.

1.5.9 Heavy Metals

Heavy metals thresholds relate to the extractable part which can migrate to the skin under wearing conditions, not to the total heavy metal content of a textile substrate. The very low limits are set by the statutory regulations for heavy metal which is equivalent to drinking water. Clear differentiation need to be made between the highly toxic heavy metals like mercury, arsenic, cadmium and lead on the one hand and those metals which are classed as heavy metals because their specific gravity exceeds 2.5 g/cc

such as copper, chromium or zinc. Poisonous heavy metals (Hg, As, Cd, Pb) are not contained in high quality dyes and auxiliaries. However, other heavy metals frequently used in textile finishing are in some cases, irreplaceable. Some copper compounds improve the light fastness of polyamides based carpets, while chromium compounds can be used as oxidants in sulphur and vat dyeing processes or as mordents in certain wool dyes. The heavy metals copper, chromium and nickel have particular significant for the metal complex dyes. These dyes form many ranges in the blue, marine, turquoise, green and grey shades. With all these dyes the metal is a complex bonded, integral parts of the dye molecule. It is released together with the dye values obtained for extractable heavy metals, hence correlate fairly to wet fastness value, or more precisely, perspiration fastness values for each particular dye.

1.5.10 Pesticides

The pesticide content in textiles come from the grey cotton and other natural fibers which may cause long term damage to the health of the wearer by accumulating in the body. They irritate the skin and affect the central nervous, gastrointestinal respiratory as well as reproductive system.

Tests are performed for 22 halogenated pesticides, most of which are no longer used today in sheep rearing and cotton growing due to the ban. However, even traces are chemically destroyed or washed out during the various pretreatment phases such as scouring, desizning, kier boiling and bleaching.

1.5.11 Pentachlorophenol

Highly toxic chlorophenols such as pcp (pentachlorophenol) are still used to prevent rot and mould in raw fibers and textiles during prolonged storage and transport. PCP is stable to natural degradation process. In humans, its bioaccumulation takes place and hence poses a severe health hazard.

1.5.12 Prohibited Dye Stuffs

Azo dyes stuffs are pigments which release any one of 22 listed aryl amines following azo cleavage may not be used in the production of textile goods. Eco laboratories test are always performed for compliance during the certification procedure. A prohibited dye is deemed to have been used only when greater than 20 ppm amines per kg textile is detected.

1.6 Status of Ground Water in Cluster

Bhadohi is a hub of carpet industries of Eastern U.P. The Carpet Industry of Bhadohi is not localized but is spread door to door. Different steps, involved in carpet manufacturing, are accomplished by the labours at their home. Consequently, it provides employment to almost every door of Bhadohi residents.

There are seven main steps in carpet making, viz. scouring of yarn, bleaching, dyeing, designing, weaving, washing and finishing. Finally, the carpet is ready for marketing. Each of the seven steps, except weaving, is carried out by using some chemicals, which include sulphuric acid, acetic acid, caustic soda, soda ash, bleaching powder, dyes, etc. These chemicals are drained in open area and along with dyes where they percolate underground. The extent of percolation is alarming in some places. The

negative impact of these chemicals has put the underground water under pressure of contamination. As the demand of ground water is increasing day by day, an urgent attention should be paid to check the polluting chemicals.

The discharge of effluent from these industries contaminate nearby ground water. The results of testing ground water samples showed total hardness a minimum of 228 ppm at Kansaraipur, Bhadohi and 768 ppm at Rajpura Chauraha. This increase in hardness of underground water of this region has created a lot of scaling problem for the boiler and compels frequent de-scaling, which makes the boilers less energy efficient. Furthermore, substances and mixtures of chemicals used for de-scaling render surface water quality lower to the prescribed mark. Another noteworthy negative impact of hardness of the ground water is on dyeing process. It has been observed that when carpet dyeing is carried out at different places, there is a significant variation of shades even under identical dyeing conditions. This variation in shade is attributed to the varying degree of hardness of water used for dyeing. This increase in hardness of the ground water is ascribed to the use of bleaching powder, soda ash, lime etc. These chemicals present in water are drained and they undergo a slow process of percolation dissolving $Ca^{2+}$and Mg^{2+} down the earth.

Extensive use of sulphuric acid, acetic acid, caustic soda, soda ash, bleaching powder etc., cause variation in alkalinity of the ground water. The lowest alkalinity, 240 ppm, was found at Kushiyara while that of highest, 529 ppm was at Chauri Bazar. A critical examination of testing report reveals that alkalinity due to CO_3^{2-} is much more less that due to HCO_3^-. For example, at Chauri Bazar alkalinity due to CO_3^{2-} was merely

39.2 ppm while that due to HCO_3^- was strikingly 490 ppm. This may be due to greater solubility of HCO_3^- in aqueous medium than that of CO_3^{2-} . The alkalinity has a negative impact on dyeing process. A varying amount of acid/base has to be used in dye bath for achieving suitable pH. Even a slight variation in pH results in varying degree of shades of the carpet. In addition, the total dissolved solids (TDS) also have negative impact on various stages of carpet manufacturing. Though the pH of ground water is at permissible limit yet variation in its range gives a warning signal. The lowest pH 6.2 was found at Churi Road, Bhadohi while the highest 8.1 was at Indira Mill Chauraha, Handpump. This pH variation of about 2 is insignificantly significant, as it gives a glimpse of future when alkalinity of water touches a mark where water will be too alkaline to drink.

In some places of Bhadohi Cr (VI) has been detected, though lower than the prescribed limit of 0.05 ppm. Nevertheless, it is a cause concern because Cr (VI) is definitely carcinogenic. The water sample from Jamuripur, Bhadohi and Indira Mill Chauraha, Handpump contains 0.004 ppm and 0.005 ppm Cr (VI) in the ground water samples of near dye plant. Evidently it has percolated from dye-drain, which contains various metal-dyes and chrome-dyes. In some places test results show that there is presence of As (III), which is under permissible limit. The presence of Arsenic (III) has not correlated with carpet industry. Fortunately, enough, all the ground water samples contain As(II I) below the detectable limits.

1.7 Literature Review

A. Methods for Dye Degradation

1.7.1. Non-Biological Methods:

Various physical and chemical processes have been employed to remove such contaminants from wastewater. The physio-chemical processes like coagulation and flocculation [Kartikeyan *et al.* (1990); Liakou *et al.* (1997); Babu *et al.* (2007)], sedimentation, adsorption, reverse osmosis **[**Bousher *et al.* (1997); Mall *et al.* (1996)]; filtration [Zaidi *et al.* (1992); Cartier *et al.* (1997)] and chemical methods (reduction, oxidation, ion exchange, neutralization, electrolysis, ozonation) have been employed for azo dye removal [Churchley *et al.* (1994); Strickland *et al.* (1995)]. The photocatalyic processes [Bhatkhande *et al.* (2002); Blake (1997); Herrmann *et al.* (1999); Yawalkar *et al.* (2001)], as well as photochemical and complexometric methods are also used for complete mineralization of dye bearing effluent [Forgas *et al.* (2004)]. Adsorption technique have also been used for treatment of textile wastewater [Vecino *et al.* (2013); Devesa-Rey *et al.* (2011); Zahid *et al.* (2011)], however, it is time consuming processes. Few of them are effective but they have their own limitations like high cost, excessive chemical utilization, sludge accumulation and lack of effective color reduction [Verma *et al.* (2003); Eichlerova *et al.* (2006**).** To overcome these limitations of the conventional methods for treatment of dye wastewater, advanced oxidation processes (AOP) have been suggested [Oller *et al.* (2011)] which can provide effective technological solutions for waste water treatment. In this method contaminants are oxidized by four different reagents like ozone, hydrogen peroxide, oxygen, and air, in precise, preprogrammed

dosages, sequences, and combinations. These procedures may also be combined with UV irradiation which results in the generation of highly reactive free radicals viz hydroxyl radicals (OH), an effective species responsible to degrade certain toxic contaminants. A well-known example of AOP is the use of Fenton's reagent which has proven a promising and attractive treatment method for the effective decolorization and degradation of dyes without production of any solid waste **[**Vandevivere *et al.* (1998)**;** Alaton *et al.* (2002)**;** Al-Kdasi *et al*. (2004)**]**.

Recently, great attentions have been paid to microbial fuel cells (MFCs) due to their mild operating conditions and using variety of biodegradable substrates as fuel. Murali and coworkers (2013) reviewed the content related to microbial fuel cells that focuses on simultaneous power generation and effluent treatment using azo dye as the substrate. They addressed major challenges in exploration and operational modification for complete azo dye removal and maximum power generation and suggested a sequential treatment system for complete mineralization of azo dye.

1.7.2 Biological Methods

Microorganisms have capabilities to degrade a wide range of xenobiotic compounds. The azo dye degradation has also been reported from different class of microorganisms.

1.7.2.1 Cyanobacteria and algae

Photosynthetic cyanobacteria have been used for bioaccumulation of dye under thermophilic conditions [Sadettin and Donmez, (2006); Dilek *et al*. (1999)] as well as bio-adsorbent (dead biomass) to remove dyes from aqueous solutions [Mona *et al.* (2011); Priya *et al*. (2011)]. Free floating algal cells has also been employed for the dye degradation process [Khataee *et al*. (2009); Shukla *et al*. (1994)] reported inhibition of *Nostoc muscorum* growth in the presence of azo dye Metomega Chrome Orange GL at the concentration of 20 ppm. Similarly, growth of a nitrogen fixing cyanobacterium, *Anabaena* sp. was reduced to 50% in 5ppm of azo dye CI Reactive 22 [Hu *et al*. (2001)]. In contrast, *Phormidium* sp. can tolerate highest concentration of azo dye without any significant reduction of their growth and hence able for bioaccumulation of azo dye.

1.7.2.2 Fungi

The heterotrophically energy deriving fungi [Zheng *et al*. (1999)] has been used by many investigator to degrade azo dye due to the activity of extracellular enzymes such as laccases, peroxidases and ligninolytic enzymes [Stolz, (2001); Robinson *et al.* (2001a)**;** Hao *et*. *al*. (2007); McMullan *et al*. (2001); Wesenberg *et al*. (2003); Forgacs *et al*. (2004); Harazono and Nakamura, (2005); Pazarlioglu *et al*. (2005); Toh *et al*. (2003); Mohorcic *et al*. (2006); Madhavi *et al*. (2007)]. A wide spectrum of microorganisms including bacteria, filamentous white rot fungi, yeasts and algae are capable of decolorizing a wide range of dyes via aerobic, anaerobic and sequential anaerobic-aerobic treatement processes [Martins *et al*. (1999); Asgher *et al*. (2012)]. In the recent years

many efforts have been made for the development of bioremediation processes using fungi [Azmi *et al*. (1998); Coulibaly *et al*. (2003); Brar *et al* .(2006)]. Fungus has proved to be a suitable organism for the treatment of textile effluent and dye removal. White rot fungi have been utilized for the decolorization of various different dyes. The most widely used for dye decolourising microorganisms are the white rot fungi. This group of fungi plays an important role in global carbon cycle and their ability to minileralise the woody plant material lignin which has a complex polymeric structure. Due to property of this white rot fungi have been found to be capable of mineralizing a diverse range of persistent organic pollutants which distinguishes them relatively. The fungi are extensively studied mainly because of their ability to degrade wood cell walls, which lead to extensive damage to and failure of wooden products in service. The white rot fungi their enzymes for useful purposes and rapid are being made in a wide range of biotechnological applications for detoxification of environmental pollutants. Basidiomycete fungi in biotechnology white and a brown rot fungus is introduced, with a brief mention also made of less widely used soft rot fungi. White rot fungi are common in nature. They are particularly abundant in forest ecosystems; rotting stumps as forest residues with a 'bleached' appearance are a common site in hardwood forests, as hardwoods are more susceptible to white rot attack than softwoods [Blanchette *et al*. (1992)].

The advantage of fugus used for dye degradation over bacteria is due to increased cell to surface ratio, fungi have a greater physical and enzymatic contact with the environment. The fungus extracted extracellular enzyme is advantageous in tolerating

high concentration of the toxicants. The fungi used for dye degradation is living, dead and pure or mixed consortia. The dye degradation by fugus is based on following mechanism such as biosorption, biodegradation and bioaccumulation. Biosorption is defined as binding of solutes to the biomass by processes which do not involve metabolic energy or transport, although such processes may occur simultanepusly where live biomass is used. Therefore, it can occur in either living or dead bimass [Tobin *et al.* (1994)]. Biodegradation is defined as energy dependent process and involves the breakdown of dye into various by products through the action of various enzymes. Bioaccumulation is the accumulation of pollutants by actively growing cells by metabolism [Aksu *et al.* (2005)]. The most of work related to biodegradation of textile dyes by fungi concentrates on the use of wood-rot fungi which produce lignin degradaing enzymes like Laccase, Manganese Peroxidase, Lignin Peroxidase etc. whereas studies related to biosorption have been mostly conducted on fungi other than wood-rot fungi.

Biodegradation is an energy dependent process and involves the breakdown of dye into various by products through the action of various enzymes. Bioaccumulation is the accumulation of pollutants by actively growing cells by metabolism [Aksu *et al.* (2005)]. From *Table 1.2*, it is observed that the most of the work related to biodegradation of textile dyes by fungi concentrates on the use of wood rot fungi which produce lignin degrading enzymes like (Azmi *et al.* 1998).

- ***Laccase***

These fungal extracellular blue multicopper oxidases which are secreted during natural lignin degradation are capable of degrading wide variety of xenobiotic compounds including azo dye. There are some reports regarding over expression of laccases by genetic manipulation in fungal genera like *Coprinus, Aspergillus* and *Ceriporiopsis*. These recombinant laccases of *Coprinus cinereus* and *Aspergillus oryzae* were are produced on industrial scale in large fermenters [Schneider et al. (1999)]. The thermostable laccases have been produced in batch/fed batch as well as under continuous culture by fungus *Melanocarpusalbomycesin* and *Trichodermareese*I [Bailey *et al.* (2007)]. The glycosylated residue provides proteolysis resistance to this enzyme laccase at high temperature [Yoshitake *et al.* (1993)]. This enzyme could be produced in yeast cells due to fast growth and easy culture manipulation and most importantly in absence of post translational modifications [Couto *et al.* (2007)].Analysis of degradation efficiencies for two dyes [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfnate) 2,29-azinobis (3-ethylbenzthiazoline-6-sulfonate, and syringaldazine] shows recombinant laccases exhibits similar activity to that of wild type at various pH [Jonsson *et al*. (1997)]. In recent studies by Hong and coworkers (2007) identified and isolated a novel laccase producing fungus *Trametes* sp. 420 from mouldered wood which was efficiently expressed in another fungus *P. pastoris*.

- ***Ligninolytic enzymes***

Recently, the enzymatic approach has widely been used by many researchers for degradation of dyes bearing wastewater as an alternative strategy to conventional chemical, physical and biological treatments, which pose some serious limitations.

Among the various types of peroxidases, manganese peroxidases, lignin peroxidases, laccases, microperoxidase-11 and polyphenol oxidases have been particularly exploited for azo dye degradation. White-rot fungi able to produce various isoforms of extracellular oxidases, which are involved in the degradation of lignin in their natural lignocellulosic substrates. These extracellular enzymes act in nonspecific and non-stereo-selective manner [Barr *et al.* (1994); Pointing *et al.* (2001)] and is directly involved in the degradation of various xenobiotic compounds including dyes (Becker *et al.* (1993); Hatakka *et al.* (1994); Barr *et al.* (1994); Pointing *et al.* (2001); Scheibner *et al.* (1997), Glenn *et al.* (1983); Pasti-Grigsby *et al.* (1992); Paszczynski *et al.* (1992); Spadaro *et al.* (1992)]. Basidiomycete strain PV 002 isolated from decaying and decomposed plant waste near textile factories efficiently degraded Ranocid Fast Blue (96 % in 5days) and Acid Black 210 (70% in 9 days) under static conditions [Verma *et al.* (2005)].

1.7.2.3 Bacteria

A variety of culturable bacteria have efficient enzymatic systems that can carry out complete mineralization of azo dye and their metabolites (aromatic compounds) over a wide range of environmental conditions. Bacteria are suitable for dye degradation because of strongly adaptability, high activity and extensive distributions [Pearce *et al.* (2003); Dos Santos *et al.* (2007)]. In a contaminated habitat, different bacteria adapt different strategy to cope with the contaminants, so, in such toxic environmenthabitat biological dye degradation is slow due to enzyme deactivation and cell death. To cope with this limitation highly coloured industrial waste are first subjected to physicochemical treatment prior toapply anaerobic/aerobic microbial processes [Chen *et*

al. (2006); Chengalroyen *et al*. (2013); Solis *et al*. (2012)]. Most of bacterial strains degrade the azo dye under anaerobic and aerobic condition (*Xenphylus azovorans* KF46F, *Bacillus* strain*, Kerstersia*sp. Strain VKY1 and *Staphylococcus* sp.).

- ***Bacterial amendment:***

Pure and mixed bacterial strains has more widely has been isolated and utilized for the dye degradation in laboratory conditions. [Chen *et al*. (2003); Khehra *et al*. (2005)].

- ***Single bacterial culture:***

Each microbe has its own capability and efficiency to degrade azo dye. The isolated bacteria and their consortium the nonliving fraction used for azo dye degradation. The single isolated bacterial strain degrades single dye and even incompletely and leaves partially degraded toxic intermediate.

- ***Mixed bacterial culture:***

The use of mixed bacterial culture is supposed to be beneficial for complete dye degradation.As it is the case of treatment systems which composed of mixed microbial populations which achieve a higher degree of biodegradation and mineralization due to the synergistic metabolic activities of the microbial community. In a mixed bacterial culture, the toxic intermediates which is carcinogenic and mutagenic in nature, generated due to activity of one bacteria (aromatic amines produced due to cleavage of the azo bonds) are degraded by complementary organismspresent in themicrobial consortium [Forgacs *et al*. (2004); Senan *et al.* (2004); Jain *et al*. (2012); Joshi *et al.* (2008)]

Obviously, the degradation of dye will depend on origin and chemical structure of dye and microorganisms used in consortium.

Different groups of microorganisms in present in consortium have ability to degrade azo dyesunder anaerobic, anoxic or aerobic conditions (Chen *et al.* (2006): Chengalroyen *et al*. 2013, Solis *et al*. 2012).Three are several studies reported in the literature, concerning the biodegradation of colored wastewater using mixed bacterial cultures which are given in *Table 2*. Moreover, the treatment of pollutants based on consortium of microorganisms naturally present at the contaminated sites, could be more effective since the microorganisms become adapted to those particular conditions (Singh *et al*. 2004).

The azo dye degradation in reactors requires both anaerobic and aerobic steps for complete dye degradation. Under anaerobic treatment azo bond is cleaved and aromatic amines are generated that are resistant to further degradation under prevailing conditions and they further get degraded to simple nontoxic products by changing the treatment conditions to aerobic. Mixed cultures have an advantage over pure cultures, since the mixed population attains additional co-metabolic potentials and possesses higher degree of biodegradation and mineralization due to synergistic metabolic activities of microbial community [Senan *et al.* (2004); Jain *et al*. (2012)]. The different bacterial genera used for different types of azo dye degradation either in pure culture, mixed culture or consortium, isolated from various contaminated sited and their % dye degradation has been tabulated (*Table 1.1*)

- ***Mutant bacterial strains***

Genetic expression pattern of microorganism has mainly been altered by random mutagenesis, and site directed mutagenesis. Several classes of mutagenic agents has been used to generate the mutant microbes, however, the ultraviolet light has mainly been employed for efficient azo dye degrading microbes. The mutant bacteria are developed by UV light induced random mutagenesis for azo dye degradation are *Bacillus* sp. [Gopinath et al., (2009b)], *Pseudomonas* sp 1. [Joshi *et al*. (2012)] and *E. coli* NO_3 [Yeh *et al*. (2004)]. Mutant *Pseudomonas* was 23 % time efficient in complete degraded of sulfonated azo dye (Green HE4B) compared to wild type. Such time efficiency in the mutant of the bacteria strains [Joshi *et al*. (2012)] was due to boosted activity of enzymes (laccase, lignin peroxidase, veratryl alcohol oxidase, and NADH dichlorophenol indophenol reductase) participating in azo dye reduction.

The other chemical mutagens commonly used for inducing and developing mutant are ethidium bromide (EtBr) [Chandra *et al*. (2008)]. The EtBr induced mutant of *Bacillus* sp. decolorized three folds higher activity in the dye concentration of up to 3000ppm compared to wild species [Gopinath *et al*. (2009a)]. The kinetics study of azo dye decolorization mutant (*Escherichia coli* NO_3) depended on physiochemical parameters like initial dye concentration, pH, dissolved oxygen and temperature. The enzyme activity was recorded in bottom 20-45^{0}C and pH 7-9 [Chang *et al.* (2000)]. Further, in addition to the above mentioned methods, site specific mutagenesis has also been employed for generating mutants [Ito *et al*. (2008)]. The wild-type isolate of

Pseudomonas luteola removed Reactive Black B (200 ppm) only 65% whereas under similar conditions [Yeh *et al*. (2004)] mutants of E. coli NO3 decolourized ≥ 90%.

Kirk and coworker (1986) developed strains of fungi *Phenerochete chrysosporium* by use of UV lightand reported efficient dye degradation. They also reported increased production of ligninases in the mutant.

Table1.1: Biodegradation of dye by single strain and mixed culture of bacteria:

Microorganism	**Dye**	**Source of isolate**	**Removal / degradation (%)**	**Reference**
Pure culture				
*Alishewanella s*p. strain KMK6	Reactive Blue 59	Textile dye-contaminated soil	100% of 2.5 gl^{-1}, within 6 h.	[Kolekar *et al***.** (2013)]
Aeromonashydrophila	RED RBN	Sludge and mud lakes	< 90% in 8 days at 3000 ppm.	[Chen *et al*. (2009b)]
Bacillus sp.	Congo red	Tannery effluent	degradation time reduced by 6-8 h.	Gopinath *et al*. (2009a)
Bacillus sp. mutant	Congo red	Tannery effluent	Better decolorization up to 3000 ppm. 12-30% time reduction	[Gopinath *et al*. (2009b)]
Bacillus subtilis HM	Fast Red	Dye contaminated soil.	99% in 6 h under optimal condition and in 14h without process	[Mona *et al*. (2008)]

			optimization	
Bacillus cereus	ReactiveRed195 Reactive Black5, Reactive Yellow145 and Reactive Black	Bacterial strain isolated form dye house effluent	97% in 72 h.	[Modi *et al*. (2010)]
*Bacillus fusiformis*KMK 5	Disperse Blue 79 and Acid Orange 10	Textile dye contaminated soil	100% of 1.5 ppm in 48 h	[Kolekar *et al*. (2008)]
Bacillus firmus		Textile wastewater	100% in 18 h	[Ogugbue *et al.* (2011)]
Comamonas sp. UVS	Direct Red 5B	Textile dye contaminated site	100% at 40 °C and pH 6.5.	[Jadhav *et al*. (2009)]
Dyellaginsengis oli LA-4	Acid Red GR	Strain Received from Dalian University of Technology	92% in anaerobic conditions ~ 22% in aerobic conditions.	[Zhau *et al*. (2010)]
Enterococcus faecalis strain YZ66	C.I. Reactive Red 195	Contaminated soils dye industry effluent	99.5% in 90 min in static anoxic condition	[Mate *e*t. *al*. (2012)]
*Enterobacter*sp. EC3	Reactive Black 5, Reactive Red 180, Reactive Yellow 81, Reactive Blue	Activated sludge of textile mill.	Upto 92.56%	[Wang *et al*. (2009)]

	19, Reactive Blue 49			
*Exiguobacterium*sp. RD3	Navy Blue HE2R	Contaminated soil dyestuff	91.2 % of 50 ppm in 48 hr.	[Dhanve *et al*. (2008)]
*Halomonas*sp	Remazol Black B, Maxilon Blue, Remazol Black N, Entrazol Blue IBC Sulphonyl Scarlet BNLE Blue TLE Green BLE	Textile effluents	decolorize 4 days of incubation in static culture	[Asad *et al*. (2007)]
*Kerstersia*sp. strain VKY1	Amaranth, Fast Red E, Ponceau S, Congo Red, Orange II, Acid Red 151 Acid Orange 12	Coconut coir	100 ppm initial level 100% 100% 100% 100% 84% 44% 12.73%	[Kumar *et al*. (2007)]
Micrococcus glutamicus NCIM-2168	C.I. Reactive Green 19A	National Chemical Laboratory	50ppm within 42 h under static condition	[Saratale *et al*. (2009)]
Pseudomonas sp. SUK1	Reactive Red 2	Textile industries	Up to 5 ppm at 30°C and pH (6.2–7.5) in static condition	[Kalyani *et al*. (2009)]
Pseudomonas	Remazol red	dye contaminated	97% within 20 min	[Jadhav *et al*. (2011)]

aeruginosa BC H		soil		
Pseudomonas sp. SUK1	Red BLI	Soil contaminated sites of textile industry	99.28% within 1h under static anoxic condition at optimum condition.	[Kalyani *et al*. (2008)]
Pseudomonas desmolyticum N CIM 2112	Direct Blue-6	NCL, India	88.95% reduction in COD in static anoxic condition after 72 h	[Kalme *et al*. (2007)]
Pseudomonas aeruginosa CLRI BL22	Metanilic acid Sulfonated aromatic amine	Soil near the tannery site	Effective degradation upto 1,500 ppm.	[Nachiyar *et al. (*2007)]
Pseudomonas aeruginosa NBAR12	Reactive Blue 172	Soil obtained from industrial area	83% in 42 h of 500 ppm.	[Bhatt *et al.* (2005)]
Pseudomonas luteola	Methyl orange and Methyl red	Indigenous activated sludge		[Hsueh *et al*. *(*2009)]
Pseudomonas luteola	RP_2B		95% in within 5 days through shaking-static process.	[Hu *et al*. (1998)]
Proteus mirabilis	Red azo dye (RED RBN)	Sludge of dyeing wastewater treatment	95% within 20 h of 1 ppm.	[Chen *et al*. (1999)]

		plant.		
Pseudomonas mendocina MCM B-402	Methyl violet	Soil of the factory manufacturing methyl violet	100% in 48 h	[Sarnaik *et al.* (1999)]
*Rhizobium radiobacter*MTCC 8161	Reactive Red 141	Effluent treatment plant of textile and dying industry.	90% of 50ppm under optimized condition	[Telke *et al.* (2008)]
Shewanella putrefaciens	Acid Red 88 Reactive Black 5 Direct Red 81 Disperse Orange 3	Soil, activated sludge and natural asphalt	under static condition decolorization of 100 ppm dye completed in 4 h for AR-88 6 h for DR-81 3h for RB-5 and DO-3	[Khalid *et al.* (2008)]
*Sphingomonas*sp strain 1CX	Orange II, Acid Orange 8, Acid Orange 10, Acid Red 4, and Acid Red 88	Wastewater treatment plant	99% in 6 h	[Coughlin *et al.* (1999)]

Lysinibacillus s p. KMK-A	Orange M2R	Soil sample	88% in 48 h.	[Chaudhari *et al.* (2013)]
Planococcus sp. MC01	anthraquinone-2, 6-disulphonate, humus analog) and decolorize Orange I	Printing and dyeing wastewater	>94.0% Alkaline pH, Anaerobic conditions	[Ma *et al.* (2013)]
Bacillus sp. YZU1	Reactive Black 5	Soil from textile factory	95% in 120 h , Static conditions (pH 7.0 , 40 °C)	Wang *et al.* (2013)
Alcaligenes faecalis PMS-1		Soil contaminated with dyes	24.75 ppm h^{-1}	Shah *et al.* *(*2012)
Mixed bacterial culture				
Alcaligenesfaecalis, Sphingomonas sp. EBD, *Bacillus subtilis, Bacillus thuringiensis*	Direct Blue-15	Activated sludge	92.14% of 50ppm 94.46% of 100 ppm 95.63% of 250 ppm in 24 h.	Kumar *et al.* (2007)

and *Enterobacterca ncerogenus.*				
Phanerochaetec hrysosporiumStr eptomyces chromofuscust		Higher termites in Kenya		[Paszczyns ki e*t al.* (1992)]
Bacterial consortium (TJ-1)	Acid Orange 7 (AO7)	Mixed inoculums of contaminated soil and sludge of WWTP	90% of 200 ppm in 16 h.	[Joshi *et a*l. (2008)]
Consortium-GR	Scarlet R (50 mg/L)		100 % in 3 h.	[Saratale *et al. (*2009)]
Consortium PMB11	Reactive Blue 59 (50 mg/L)		100 % in 6 h.	[Patil *et al. (2008*)]
Bacillus sp. *Galactomyces geotrichum*	Brilliant Blue G	MTCC, India	100% and 33% in 12 h	[Jadhav *et al*. (2008)]
Bacterial consortium NBNJ6	Direct Red 81	Soils contaminated with Dye stuff	90% within 35 h.	[Junnarkara r *et al.* (2006)]
Bacterial consortium PMB11	Reactive Blue 59	Soil contaminated with dye	Within 3 h.	[Patil *et al.* (2008)]

1.7.3 Enzyme Azoreductase

The enzyme azoreductase is quite diverse in respect to the structure, function and evolution [Chen *et al.* (2010)]. Based on structure, flavin dependency and dinucleotide preferences, it could be classified into three groups

Group I - polymeric flavin dependent NADH preferred azoreductase

Group II - polymeric flavin dependent NADPH preferred azoreductase and

Group III - monomeric flavin independent NADPH preferred azoreductase

There are some basic differences in the structural and functional features in these azoreductases. The main difference between flavin dependent and flavin independent is substrates specificities [Burger *et al.* (2010)]

The Group I and II azoreductase have two different sites for NADH, NADPH binding and substrate binding and four electrons are sequentially transferred via flavin for azo bond reduction [Chen *et al.* (2004); Liu *et al.* (2007); Suzuki *et al.* (2001)]. The Group II azoreductases significant sequence similarities to the AZR *Bacillus* sp. OY1-2 that preffered NADPH as reductant and contain FMN in many cases. These flavin (FMN) dependent aerobic azoreductases have a wide substrate range. In contrast, Group III azoreductases simultaneously accept both NADPH and substrate in its active site. This monomeric flavin free azoreductase preferentially used NADPH as a cofactor.

The Group I azoreductase AzoA and AzoR containing bacteria *E.coli* and *Enterobacterfaecalis*, respectively, belong to enterabacteraceae [Chen *et al.* (2004, 2009);

Liu *et al*. (2007)] however, on the basis of activity AzoA is considered to be the most active azoreductase of the human intestinal track [Chen *et al*. (2004**);** Liu *et al*. (2007a)]. Both the enzymes AzoA and AzoR are closely related to FMN containing enzyme in unusual preference for NADH. The environment of the intestinal lacks oxygen so the bacteria residing in the intestinal track are adapted to this specific habitat. Several species of intestinal bacteria with azoreductase activity have been isolated and studied [Chung *et al*. (1992); Rafii *et al*. (1990); Song *et al*. (2003); Gingell *et al*. (1971)]. Bacteria from the human intestinal tract can be exposed to azo dyes from environmental contamination and from consumption of dyes in foods and drugs [Marmion *et al*. (1991); Dillon *et al***.** (1994)]. Ingested azo compounds are metabolized by azoreductases in the gastrointestinal tract, skin and liver to their respective aromatic amines.

Group II azoreductases have been found in bacteria like *Salmonella* sp. and *Bacillus* sp. Azr of *Bacillus sp*. OY1-2 [Suzuki *et al*. (2001)] and Azo1 from *S. aureus* (Chen et al. 2005). Azo 1 has been cloned and overexpressed to decolourize varieties of dyes. The *S. aureus* constitutively express tetrameric azoreductase of native molecular mass of 85 kDa. The enzyme contains four non-covalently bound FMN and exclusively requires NADPH as an electron donor. In the hydrophobic interaction chromatography, this enzyme existed as a dimeric apoprotein in absence of flavin prosthetic groups and converted to fully functional native tetrameric form on reconstitution of column with free stage FMN. Similarly, the azoreductase of *P. aeruginosa* was a homotetramer in solution with two functional dimmers having one flavinco-factor and one substrate molecule was non-covalently bound substrate to each monomer **[**Wang *et al*. (2007)**].**

Xenophilus azovorans KF46F and *Pigmentiphga kullae* K24 are bacteria express Group III azoreductase that had no significant sequence similarity to the AZR and AzoR. These acyl carrier protein phosphodiesterase (acpD) cloned and heterologously overexpressed flavodoxin-like protein of ~110 kDa in *E. coli* which was similar to the AZOR1 of *Pseudomonas aeruginosa* (paAzoR1).Recently Chen and coworker (2010) isolated gene azoB from *Pigmentiphagakullae K24* heterologously expressed in *E.coli.* The recombinant monomer enzyme of 203 AA was and uses NADH and NADPH as an electron donor for its activity.

Table 1.2: Characteristics of enzyme azoreductase isolated from various microorganisms:

Microorganism	**Reductant**	**Cloned gene**	**Recombinant enzyme characterstics**	**References**
Group I - Polymeric FMN dependent Azoreductase				
Bacillus sp.B29	NADH	***AzrA***	O_2 insensitive, 23 kDa	[Ooi *et al.* (2007)]
*R. sphaeroides*AS1.1737	NADH		O_2 insensitive	[Bin *et al.* (2004)]
E. coli SS125	NADH	Azo A	O_2 tolerant	[Sandhya *et al.* (2008)]
E. coli JM 109	NADH	Azo R	MR	[Jin *et al.* (2008)]

	NADH and/or NADPH	Azo C		
Group II - Polymeric FMN dependent Azoreductase				
Bacillus Sp. OY1-2	NAD(P)H	Azr	20 kDa, O_2 tolerant, MR, RB 5, AR 88	[Suzuki *et al*. (2001)]
Pseudomonas aeruginosa PAO1	NAD(P)H	paAzoR1	23kDa	[Wang *et al*. (2007)]
Pseudomonas aeruginosa		paAzoR2		
Pseudomonas aeruginosa		paAzoR3		
Ent. faecalis Strain ATCC 19433	NAD(P)H	AzoA, dimeric	MR, Orange II, BS, Amaranth, PonceauPonceau S.	[Chen *et al*. (2004)]
Staphylococcus aureus	NAD(P)H	Azo1	85kDa, MR, Orange II	[Chen *et al*. (2005)]
Citrobacter sp. Strain KCTC 18061P	NADPH		287aa, 30kDa	[Jang *et al*. (2005)]
Group III- Monomeric FMN independent azoreductase				

Pimentiphaga kullale K24	NAD(P)H, NADH	azoB. monomeric	22 kDa, 203 AA, O_2 tolerant	[Chen *et al.* (2010)] [Blumel *et al*. (2003)]
Pimentiphaga. kullae K24	NAD(P)H	Azo A	O_2 tolerant	[Chen *et al.* (2010)]
*Xenophilusazouorans*K F46F	NAD(P)H,	azo B	30 kDa, O_2 tolerant	[Blumel *et al*. (2002a)]
Bacillus sp. ADR			66 kDa	[Telke *et al.* (2009)]
Bacillus badius	NADH		O_2 tolerant	[Santosh *et al*. (2011)]
B. velezensis	NADH		O_2 tolerant	[Bafana *et al*. (2008)]
B. cereus	NADH.			[Pricelius *et al*. (2007)]

1.7.4 Genetically Modified Bacteria

In recent years several scientific groups across the world are involve in manipulating the bacterial genetic constitution to improve their dye degradation ability to accelerate the bioremediation process. The gene for the enzyme azoreductase has been isolated from the azo dye degrading bacteria and transferred to the other bacteria.

Azoreductase genes have been characterized and isolated from a variety of bacterial genera such as *Bacillus* sp. OY1-2(AzrA), [Suzuki *et al.* (2001)], *E. coli* (AzoR) [Nakanishi *et al.* (2001)], *Enterococcus faecalis* (AzoA) [Chen *et al.* (2004)], *Rhodobactersphaeroides* [Yan *et al.* (2004)], *Pseudomonas aeruginosa* (paAZOR1) [Wang *et al.* (2007)], and *Staphylococcus aureus* Azo1 [Chen *et al.* (2005)]. These variants of azo gene have mainly been expressed in the *E. coli*. The azoreductase of *Xenophilusazovorans* and *Pimentiphaga kullae* K24 have also been described [Zimmermann *et al.* (1984); Blumel *et al.* (2002), Blumel and Stolz, (2003)].

The azo bond is cleaved into aromatic amine and many other intermediate that are toxic and carcinogenic in nature. Sudan I is a liver and urinary bladder carcinogen in mammals and considered a possible human mutagen [Stiborova *et al.* (2002, 2005, 2006)] It can produce benzenediazonium ion during cytochrome P450 catalyzed metabolism, which is considered the possible mechanism of Sudan I activation to an ultimate carcinogen [Stiborova *et al.* (2005)]. Sudan III used in cosmetics have been raised due to potential metabolic cleavage by skin bacteria to yield 4-aminoazobenzene and aniline [Gao *et al.* (2007); Xu *et al.* (2007); Pielesz *et al.* (2002)]. Sudan IV requires reduction and microsomalactivation to be mutagenic [Brown *et al.* (1978)]. Sudan dyes and Para Red could be reduced by a human intestinal microbial consortium to produce aniline, 2,4-dimethylaniline, o-toluidine, andp-nitroaniline . These aromatic amines are toxic, water-soluble, and easily absorbed by the human intestine. Aniline, a metabolite of Sudan I and III, is considered hazardous because of its toxicity and carcinogenicit2, 4-Dimethylaniline, a metabolite of Sudan II, is able to damage DNA in the liver cells of

mice in the comet assay under alkaline conditions [Osano *et al* (2002)]. O-Toluidine (2-methylaniline), a metabolite of Sudan IV, is degraded in vivo into a number of compounds, some of which are active genotoxins [Danfbrd *et al.* (1991)]. 4-Nitroaniline, a metabolite of Para Red, is highly toxic by inhalation, ingestion and if absorbed through skin [French *et al*. (1995)].

1.7.4.1Efficiency of Genetically Modified Bacteria

Azoreductase genes have been inserted in different bacterial genera and species to develop recombinant microbes for efficient degradation of azo dyes efficiently (Sandhya *et al*. (2008); Jin *et al*. (2008), Chen *et al*. (2004), Bin *et al*. (2004). The genetically modified microorganisms have also been reported for better performance in terms of substrate range, efficiency and stress tolerance over wild type and mutants. Recombinant *E. coli* expressing Azoreductase gene of *R. sphaeroides* AS1.1737 was used for degradation of several azo dyes Bin *et al*. (2004)]. Such broad substrate specificity was also observed in *azoA* gene from *Enterococcus faecalis* in 34% (ACpD) of *E. coli* heterologously overexpressed in *E. coli*. This oxygen insensitive enzyme is responsible for azo dye degradation in the gastrointestinal tract [Chen et al. (2004)]. At same bacterial cell density of 225 mg cell/L, genetically modified *E. coli* SS125 (with genes of *Bacillus latrosporus* RRK1) efficiently decolourized Red dye compared to wild type [Sandhya *et al*. (2008)]. Whereas, genetically modified E.coli (AZR gene of *Rhodobacter sphaeroides* AS 1.1737 JM109) decolourized Direct Blue 71 in sequential batch reactor at high pH (9.0) and 1-3% salinity [Jin *et al*. (2008)]. Yeh and Chang (2004) reported that genetically modified *E. Coli* (gene insert of *Rhodococcus* sp) to have better degradation

of Reactive Blue B compared to mutant strain *E. coli* NO3 and wild type *Pseudomonas luteold*. The heterologous expression of oxygen insensitive azrA of *Bacillus* sp. strain B29 exhibited activity over a wide pH (6-10) and temperature (60-80°C) range to cleave Methyl red and other sulfonated azo dyes (Orange I and Acid Red 88). The recombinant enzyme with gene isolated of *Citrobacter* sp. strain KCTC 18061P various factors like pH, dissolve oxygen, temperature in its kinetic analysis.

1.7.5 Mechanism of Azo Dye Degradation

Azo dyes are degraded by bacteria and fungus both under aerobic as well as anaerobic conditions in the natural environment**.** The process of dye degradation by microbes may take place in extracellularlly or intracellularlly. The anaerobic azo dye decolorization by *Sphingomonas xenophaga* sp does not involve transport of azo dye across the cell membrane and it is extracellular. Most probably, enzyme located in the cell membrane reduces quinine to hydroquinone causing reduction of azo dyes in the culture supernatant by purely chemical redox reaction. For the first time**,** Overney (1979) reported that *Flavobacterium* could grow and survive aerobically on the corboxylated azo compound utilizing it as the sole source of carbon and energy. The bacteria cleave azo compounds and their respective aromatic amines that further get degraded to simpler compounds. The anaerobic intestinal bacteria degrade azo dye of food ingredients in anaerobic habitat of intestine [Levin *et al.* (1991); Bragger *et al. (*1997), Cerniglia *et al.* (1986); Zbaida *et al. (*1994); Watabe *et al. (*1980)] while other aerobic bacteria do the same in the presence of oxygen.

The steps involve in the azo dye reduction has been presented in *figure 1.3*.

- Reduction of Azo bond
- Generation of aromatic amines
- Mineralization of aromatic amine

The extracellular glucose in the medium acts as repressor and strongly inhibits dye decolorization efficiency of bacteria [Chang *et al*., (2001)]. The glucose is the preferred nutrient source and inhibits the expression of enzymes responsible for the utilization of azo compounds as alternate energy substrate, so turn off the expression of azoreductase [White, (1995)]. The glucose derepression was necessary to activate transcription of azo reductase and initiation of dye decolorization. The appropriate metabolites dosage enhances decolorization performance, however, only till certain threshold level and further increase led to product inhibition due accumulated inhibitory intermediates [Chang *et al*., (2004)].

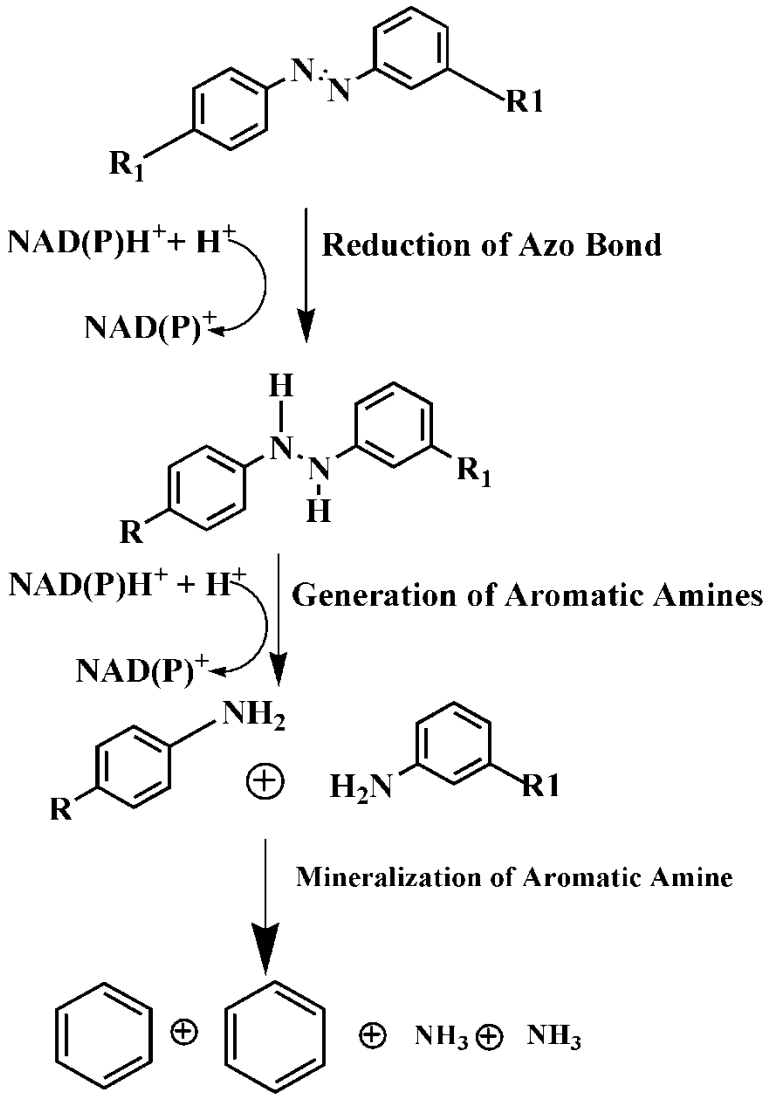

Figure 1.3: Steps innvolved in Azo Dye reduction under anaerobic condition.

1.7.5.1 Reduction of Azo bond

In FMN dependent azoreductases non-covalently bound flavin prosthetic group is must for enzymetic function. The flavin dependent azoreductases such as AzoR (*E. coli)*, AzoA (*E. faecalis)*, AZR (*R. sphaeroides*), paAZOR1 (*P. aeruginosa)*, Azo1 (*S. aureus)* (Nakanishi *et al*., (2001); Chen *et al*., (2004); Liu *et al*., (2007b)**;** Wang *et al*, (2007)] azo

bond reduction follow classical "ping-pong mechanism". FMN serves as a redox centre in the electron-transferring from NAD(P)H to the azo substrate. The cofactor flavin is first reduced by NAD(P)H, followed by transfer of reduction equivalents of flavin to the azo group to form corresponding hydrazo derivatives. The proposed mechanism for the reduction of FMN by NADPH in azoreductases is similar to that proposed for the reduction of FMN by NADH in EmoB. The hydride ion is transferred from the NADPH to the N5 of FMN. The negative charge is delocalized around the pyrimidine ring and stabilized via interactions with the backbone amide groups of Gly 146 and Gly 147. Further, aerobic azoreductase from *Bacillus* sp. strain OY1-2 and *X. azovorans* KF46F were able to decolorize Acid Red 88 and Acid Orange 7. Furthermore, it was suggested that the azoreductase from *Bacillus* sp. strain OY1-2 contains an NAD(P)H-binding motif (GXGXXG) at positions 106 to 111 of the protein and not at the amino terminus as found in the present study for the enzyme from *X. azovorans* KF46F.

1.7.5.2 Mechanism of carcinogenicity

The carcinogenicity of azo dye is due to its metabolic activation to reactive electrophilic intermediates that covalently bind to DNA. Such reactive electrophilic intermediates could be generated by

i. Metabolic oxidation of aromatic amines produced by reduction and cleavage of the azo bond
ii. Metabolic oxidation of azo dyes with free aromatic amine without azo bond reduction and
iii. Activation of azo dye via direct oxidation of the azo linkage to highly reactive electrophilic diazonium salts.

1.7.5.3 Mineralization of Intermediates

For mineralization of azo dye, strong oxidative steps are required after the reductive decolorization. So, integrated anaerobic and aerobic steps fulfill the need for complete minrelization of azo dye (Tan *et al*. (1999)]. This is due to the fact that aromatic amine generated under anaerobic condition gets completely degraded under aerobic condition [Spanzo *e*t *al*. (2002)].

1.8 Objective:

There is a lack of understanding with above mention points in the literature, my specific objectives of proposed work are listed as following:

1) Selection of microbe/ microbes: Selection of the microbe will be done through literature review or as per requirement.
2) Isolation of Bacteria from contaminated soil.
3) Growth kinetic evaluation of the isolated microbe: This will be done through sub culturing, growth kinetics studies and substrate utilization studies.
4) Several parameters consider for the efficient degradation of dye wastewater by bacteria.
5) Optimum condition find out for efficient degradation of dye wastewater.
6) Batch culture studies,
7) Experimental Design
8) Continuous culture studies.

Part 2: Batch and continuous adsorption studies, using low cost adsorbent.

Part 3: Economic feasibility of the treatment process.