# Bacterial Degradation of Azo Dye Containing Wastes

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## 1 Introduction

Till the late nineteenth century, all the dyes used were more or less natural with main sources like plants, insects and molluscs, and were mostly prepared on a small scale. It was only after 1856 that with Perkin's historic discovery of the first synthetic dye, mauveine was manufactured on a large scale. At present there are more than 100,000 commercial dyes available with a estimated production of $7 \times 10^5$–$1 \times 10^6$ tons per year (Robinson et al. 2001). These dyes are used extensively in the paper, clothing, food, cosmetic and pharmaceutical industries. Because of the diversity of the dye components available for synthesis, a large number of structurally different synthetic dyes are today utilized for coloration. Among synthetic dyes, azo dyes are the largest and versatile class of dyes which account for more than 50 % of the dyes produced annually. Azo dyes are aromatic hydrocarbons and derivatives of benzene, toluene, naphthalene, phenol and aniline (Puvaneswari et al. 2006). A wide variety of azo dyes with polycyclic, anthraquinone and triphenylmethane groups are being increasingly used in textile and printing processes. Azo dyes contain one, two, three or more azo (–N=N–) bonds and based on this, they are grouped into monoazo, diazo, trisazo and polyazo dyes. Some dyes are listed in Table 1 with their structure and molecular weight. The azo group is substituted with benzene or naphthalene groups, containing many different substituents, such as chloro (–Cl), methyl ($–CH_3$), nitro ($–NO_2$), amino ($–NH_2$), hydroxyl (–OH) and carboxyl (–COOH) and thus forms different types of azo dyes (Saratale et al. 2011). Azo dyes are highly stable and resistant to microbial attack. Due to complexity of structure and electron withdrawing capacity of azo dyes, they are considered xenobiotic compounds recalcitrant to biodegradation.

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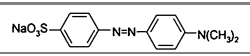
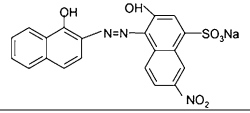
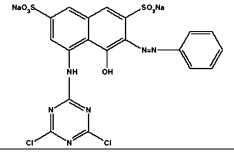
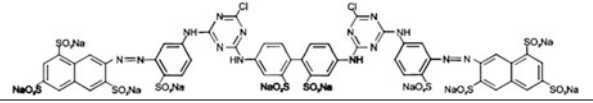
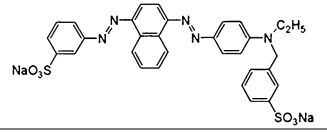
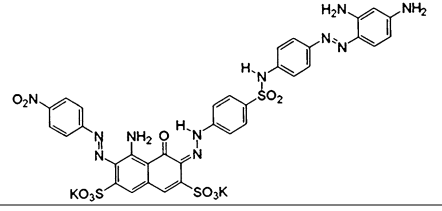
**Table 1** Chemical classes of azo dyes with their different structures and molecular weights

| Chemical class | Structure | C.I. name of dye | Molecular weight |
|---|---|---|---|
| Monoazo dyes |  | Acid Orange 52 | 327.33 |
| |  | Mordant Black 11 | 461.37 |
| |  | Reactive Red 2 | 615.34 |
| Diazo dyes |  | Reactive Yellow 84 | 1628.2 |
| |  | Acid Red 119 | 637.67 |
| Trisazo dye |  | Acid Black 210 | 938.02 |

The effluents from textile and dyeing industries are generally colored and contain high concentrations of dissolved solids, total solids, BOD/COD, salts and variation in pH and temperature. Such effluents also have the presence of heavy metals, such as Cr, Zn, Cu and Al due to presence metal-based complexed dyes. Available reports have indicated that direct and indirect toxic effects of the dyes and dyes containing waste can cause tumours, cancers and allergies in humans. Besides they inhibit the growth of bacteria, protozoan, algae, plants and different animals including human beings. Improper discharge of textile dyes effluent in aqueous ecosystems also leads to reduction in sunlight penetration, photosynthetic activity, dissolved oxygen concentration and water quality. Thus, untreated dye containing wastewater causes severe environmental and health problems worldwide (Saratale et al. 2011; Solis et al. 2012).

Now-a-days, government legislation is becoming more stringent in most developed countries, regarding the removal of dyes from industrial effluents. So, it has become mandatory to treat dye containing wastewater before discharging to sewage treatment plant or environment. Government has set limits for some parameters for quality of water to discharge in sewage treatment plant and environment. A number of biological and physico-chemical methods have been developed for the efficient removal of industrial azo dyes (Solis et al. 2012). "Bioremediation" has become a key microbial tool to deal with different pollutants, is a key research area in the field of environmental science. Biological methods are environmentally friendly, produce less sludge than physico-chemical systems, and are also relatively inexpensive, as the running cost is very low. A number of bacteria, fungi, yeasts, algae and actinomycetes have been capable of decolorizing a range of azo dyes (Saratale et al. 2011).

Particularly, bacteria are the most frequently applied microorganisms for degradation of azo dyes, as they are generally fast to multiply rapidly under aerobic, anaerobic, facultative conditions as well as in extreme environmental conditions, like high salinity and wide variations in both pH and temperature (Solis et al. 2012). In this chapter, biodegradation of azo dyes by bacteria has been discussed in details with reference to mechanism of degradation, factors affecting and detoxification.

## 2 Decolorization and Degradation of Azo Dyes by Bacteria

Biological processes provide an alternative to existing technologies for dye containing wastewater treatment, because of their cost-effectiveness and eco-friendliness. Moreover, bacteria mediated process is faster than to fungal systems with regard todecolorization and mineralization of azo dyes. Extensive studies have been carried out to determine the role of the diverse groups of bacteria in the decolorization of azo dyes under conventional anaerobic, facultative anaerobic and aerobic conditions.

## 2.1 Bacterial Degradation of Azo Dyes by Pure Culture

Attempts to isolate pure bacterial cultures capable of degrading azo dyes started way back in 1970s with isolation of *Bacillus subtilis*, *Aeromonas hydrophila* and *Bacillus cereus* (Wuhrmann et al. 1980). Application of single bacterial cultures like *Proteus mirabilis*, *Pseudomonas luteola*, and *Pseudomonas* sp., has shown very promising results for the azo dye degradation under anoxic conditions (Saratale et al. 2011). Besides, there are other several studies describing the decolorization of reactive azo dyes mediated by pure bacterial culture which have been summarized in Table 2. Apart from them, other bacterial strains of *Desulfovibrio desulfuricans, Exiguobacterium* sp., *Sphingomonas* sp., *Rhizobium radiobacter* and *Comamonas*sp. were also reported for decolorization of various azo dyes. Among these strains, *Pseudomonas* is widely used for decolorization study of azo dyes and also exploited widely to decolorize commercial textile azo dyes, such as Red BLI, Reactive Red 2, Red HE7B, Reactive Blue 172, Reactive Red 22, Orange I and II. Azo dyes are generally resistant to bacterial attack under aerobic conditions due to inhibition of azo reductase activity in presence of oxygen. However, a few selected aerobic bacterial strains possess the ability to reduce azo linkage by oxygen insensitive or aerobic azoreductases. *A. hydrophila* was studied for azo dye decolorization under aerobic conditions by Chen et al. (2009). *Micrococcus* sp. is an interesting example which decolorizes dyes faster under aerobic conditions than in anaerobic environments. In an oxygen free atmosphere ($N_2$ atmosphere), no decolorization was observed by *B. lentus*, indicating that culture required certain amount of $O_2$ for the decolorization process. Use of a pure culture system ensures reproducible data, and also becomes easier for interpretation of experimental observations. Such studies

**Table 2** Examples of degradation study of azo dyes by pure bacterial culture

| Bacterial culture | Azo dyes | References |
|---|---|---|
| *Pseudomonas* spp. | Red BLI | Kalyani et al. (2008a) |
| | Reactive Red BS | Sheth and Dave (2009) |
| | Reactive Red 2 | Kalyani et al. (2008b) |
| | Red HE7B | Kalme et al. (2007) |
| | Reactive Blue 172 | Bhatt et al. (2005) |
| | Reactive Red 22 | Hu (2001), Chang and Lin (2000) |
| | Orange I and II | Kulla et al. (1983) |
| *Proteus* spp. | Red RBN | Chen et al. (1999) |
| *Shewanella* spp. | Acid Red GR | Xu et al. (2007) |
| | Remazol Black B and Anthraquinone dye | Bragger et al. (1997) |
| *Bacillus* spp. | Acid Red 119 | Dave and Dave (2009) |
| | Disperse Blue 79 and Acid Orange 10 | Kolekar et al. (2008) |
| | Navy Blue 2GL | Dawkar et al. (2009) |

also help in exploring the mechanisms of biodegradation using the tools of biochemistry and molecular biology. This information could be also useful in modifying the strains with enhanced enzyme activities for faster degradation of dyes (Saratale et al. 2011).

## 2.2 Bacterial Degradation of Azo Dyes by Mixed Cultures and Consortia

Due to the chemical complexity of dye containing wastewater, it is necessary to develop more efficient microbial processes for decolorization. Pure strains are often specific to a type of dye and have a narrow range for different azo dyes decolorization. However, at field level, it is not possible to have mono culture process due to a large volume of wastewater containing different dyes. Hence, mixed cultures are particularly more useful in such situation, as microbial consortia can collectively carry out biodegradation which is not possible with individual pure strain. A significant advantage of consortia or mixed culture over the use of individual strains is that different strains can attack the dye molecule at different positions or may utilize toxic metabolites produced by the co-existing strains for further decomposition. No doubt treatment systems having mixed microbial populations achieve a higher degree of biodegradation and mineralization due to their synergistic metabolic activities (Nigam et al. 1996). The complete degradation of chemical substances is only possible in the presence of several enzymes produced by the mixed cultures (Khehra et al. 2005).

In general, initial cleavage of the azo bonds takes place, during azo dye degradation which results into the production of aromatic amines, which are often toxic in nature. However, in the presence of microbial consortium, these aromatic amines get degraded by complementary organisms, making the process more effective and efficient (Moosvi et al. 2005). No doubt it is very difficult to isolate a single bacterial strain from dye-containing wastewater samples and long-term adaptation procedures are needed for effective decolorization and degradation of azo dyes. On the other hand, mixed cultures only provide an average macroscopic view of what is happening in the system. In this case, the results are not easily reproduced and interpretation of the results is also quite difficult. Moosvi et al. (2007) reported three isolates *Micrococcus* sp., *M. luteus*, and *P. polymyxa*, when used in mixed culture were able to decolorize nine dyes but when individually used, they were found to be inefficient for dye removal. Similar results were also reported by Nigam et al. (1996). They also did not observe decolorization by a single strain but with a consortium, a wide range of dyes were effectively reduced. A consortium, consisting of four bacterial isolates, *P. putida*, *B. cereus, P. fluorescence* and *Stenotrophomonas acidaminiphila*, decolorized C.I. acid red 88. The dynamics of consortia capable of faster methyl red biotransformation was monitored which revealed *Klebsiella* sp., *Buttiauxella* sp. and *Bacillus* sp. as the main population

**Table 3** Degradation azo dyes by mixed cultures and consortia

| Consortium | Bacterial strains | Azo dye | References |
|---|---|---|---|
| Consortium TJ-1 | *Aermonas caviae, Proteus mirabilis* and *Rhodococcus globerulus* | Acid Orange 7 | Joshi et al. (2008) |
| Consortium JW-2 | *Paenibacillus polymyxa, Micrococcus luteus* and *Micrococcus* sp. | Reactive Violet 5R | Moosvi et al. (2007) |
| A consortium of five bacteria | *Alcaligenes faecalis, Sphingomonas* sp. EBD, *B. subtilis, B. thuringiensis* and *Enterobacter ancerogenus* | Direct Blue-15 | Kumar et al. (2007) |
| Consortium-GR | *P. vulgaris* and *Micrococcus glutamicus* | Scarlet R and mixture of 8 dyes | Saratale et al. (2009) |

under aerobic conditions and *Klebsiella* sp., *Escherichia* sp., *Bacillus* sp. and *Clostridium* sp. as dominant species under anaerobic conditions (Saratale et al. 2011). A consortium consisting of *Aeromonas caviae, P. mirabilis* and *Rhodococcus globerulus* was tested against 16 azo dyes individually and also a mixture (200 mg $l^{-1}$) and found to decolorize these dyes efficiently (Joshi et al. 2008; Saratale et al. 2011). A few examples of mixed culture and consortium reported in the literature are shown in Table 3.

The enzymatic activity of a single strain is highly influenced by the presence of other microorganisms, and the biocatalytic activity of a consortium is different than from its individual constituents. Microorganisms, in a consortium, work synergistically to enhance the decolorization activity. Jadhav et al. (2010) have also reported efficient dye effluent decolorization by a consortium than the individual strains. Consortium is found to increase rate and percentage of azo dye decolorization, decrease time for decolorization and better mineralization of azo dyes as compared to single strain. However, it is well known that enzymatic activity and enzymatic induction in consortium are not the sum of the respective enzymatic activities and induction of the individual strains (Solis et al. 2012).

In the preparation of consortia, the proportion of each microorganism is important to obtain an efficient system for the treatment of azo dyes. Khehra et al. (2005) described that an equal proportion of all four isolates of consortium increased 3-fold efficiency of dye decolorization as compared to other combinations of the same isolates. Forss and Welander (2011) reported that decolorization of Reactive Black 5 and Reactive Red 2 was more efficient with a 2:3 proportion of *Bjerkandera* sp. and native microflora than with a 2:4 proportion. Thus, a proportion of culture in consortia also varies from process to process depending on the number of consortia and nature of dye effluent.

Efficient decolorization of azo dyes by consortium also depends on a combination of aerobic and anaerobic process. Literatures are available on sequential decolorization of azo dyes by the consortium, in which during microaerophilic condition, azo dyes were partially degraded and produced some intermediates and

under aerobic conditions, these intermediates were further metabolized, resulting in complete mineralization. Not only, anaerobic-aerobic systems are able to mineralize azo dyes, but the improved degradation of Golden Yellow using a consortium was found when aerobic conditions was followed by microaerophilic conditions (Waghmode et al. 2011). Many times, aromatic amines, resulting from anaerobic azo dye decolorization, undergo partial degradation in aerobic environments, because the metabolites of azo dye are auto-oxidised upon exposure to oxygen and the products are unable to degrade further. The individual cell densities of consortium also vary significantly with the change in oxygen amount (Solis et al. 2012). Chan et al. (2011) have observed that during microaerophilic conditions, *Enterococcus casseliflavus* is predominant, while *Enterobacter cloacae* and *Citrobacter freundii* are predominant under aerobic conditions.

## 2.3 Bacterial Degradation of Azo Dyes by Immobilized Cells

Currently, immobilized bacterial cells have been used for degradation study of azo dyes. Immobilization of cells is done by two ways, i.e., attachment and entrapment. In attachment, cells adhere to surfaces of inert materials or other organisms. While in case of entrapment, bacterial cells get entrapped in the interstices of fibrous or porous materials. During last few years, different reactor designs have been proposed for an effective continuous anaerobic/aerobic treatment of azo dyes. Immobilized bacterial cells have higher level of stability to environmental perturbations, such as change in pH, or exposure to high concentrations of dyes, than their free cell counterparts. Immobilization of cells for degradation of dyes is a promising method, as it is easy to perform under aseptic condition in situ, prevents cell washouts, and allows a high cell density to be maintained in a continuous reactor and also easy to scale up. Catalytic stability and substrate uptake are often improved by immobilization because of increased availability of nutrients. Numerous carriers, such as sintered glass, nylon web, polyurethane foam, activated carbon, pine wood and porous polystyrene, are used for immobilization of bacterial cells. Barragan et al. (2007) studied degradation of several azo dyes by immobilizing cultures of *Enterobacter* sp., *Pseudomonas* sp. and *Morganella* sp., on Kaolin, bentonite and powdered activated carbon (PAC) to degrade several textile dyes. Similarly, a reactor, using polyvinyl alcohol (PVA) and co-immobilized cells of *A. hydrophila, Comamonas testosterone* and *Acinetobacter baumannii*, was used for decolorization of azo dye Red RBN and it was found that decolorization was effective even at higher concentration of dye (Chen et al. 2003). They have optimized various operating conditions, such as bed expansion, cell bead number, density and initial dye concentration, hydraulic retention time and diameters of beads for decolorization of dye (Puvaneswari et al. 2006). Moreover, bacterial entrapment within natural or synthetic materials has an application for azo dye degradation on a large scale (reactor scale), because it creates anaerobic environment favourable to dye

degradation by enzymes of bacteria. Immobilized bacterial cells, on furnace charcoal in down flow fixed film bioreactor, were reported to enhance degradation of dye manufacturing industrial effluent (Sheth and Dave 2010).

### 2.4 *Bacterial Degradation of Azo Dyes by Microbial Fuel Cells (MFCs)*

In recent years, interest in MFCs has increased tremendously, both in terms of number of researchers as well as the applications of these systems. In a MFC, microorganisms interact with electrodes using electrons, which are either removed or supplied through an electrical circuit (Rabaey et al. 2006). MFCs are the major type of bio-electrochemical systems (BESs), which convert biomass spontaneously into electricity through the metabolic activity of the microorganisms. MFC is considered to be a promising sustainable technology to meet increasing energy needs, especially using wastewaters as substrates, which can generate electricity and accomplish wastewater treatment simultaneously. Thus, it may balance the operational costs of wastewater treatment plant (Lu et al. 2009). The use of bacterial fuel cells for decolorization of azo dyes is an emerging research area. Bacterial fuel cells are capable of producing energy and reducing dye color of textile wastewater simultaneously. A new trend of bacterial fuel cells has emerged for enhancement of decolorization and at the same time generating electricity from a readily degradable organic carbon source. Sun et al. (2009) have studied decolorization of azo dye Active Brilliant Red X3 using glucose as substrate, in which improvement of decolorization activity was observed by MFCs along with electricity generation. Thus, biodegradation of dyes by MFCs has opened new horizons in bioelectricity and energy research.

## 3 Decolorization and Degradation of Textile Wastewater

Increasing industrialization and urbanization leads to environmental pollution. Textile and dye manufacturing industries are major consumers of water and hence potential sources for water pollution. The main recalcitrant component of textile and dye industry effluent is dye along with a complex mixture of many polluting substances ranging from organochlorine based waste pesticides to heavy metals. Removal of dyes from effluent has been given a top priority, because state and federal agencies in the USA and all other developed countries all over the world, have been requiring lower effluent color limits (<200 units of American Dye Manufacturers Institute, ADMI) (Banat et al. 1996). Biological treatment of industrial effluent is economically viable and cost-effective. But the main problem encountered is that dyes are highly resistant to bacterial degradation under

anaerobic conditions normally found in wastewater treatment plant. So, degradation of industrial effluent by bacteria is not that much easy as found with pure dye. Majority of the studies are conducted using synthetic/simulated dye wastewater and only a few reports are available on actual dye industrial effluent decolorization by bacteria.

## 4 Factors Effecting Bacterial Degradation of Azo Dyes

The biodegradation of azo dyes by bacteria is highly sensitive to variation in physico-chemical operational parameters, such as structure and concentration of azo dye, supplementation of different carbon and nitrogen sources, pH, temperature, aeration and agitation, salt concentrations, electron donors and redox mediators. Therefore, acclimatized bacteria, isolated from dye contaminated sites, are very efficient in decolorization process due to adaption to different extreme environmental conditions. To make the process more efficient, faster and practically applicable, prior study of the effect of each factor on the bacterial decolorization of azo dyes is essential. Optimization of such abiotic conditions will help in the development of industrial scale bioreactors for bioremediation. Following factors affecting the azo dyes decolorization by bacteria have been studied in details.

### *4.1 Structure of Azo Dye*

There are diverse structures present in the synthetic azo dyes and changes in the chemical structures, i.e. isomers or the presence of different functional groups, used to significantly affect the decolorization efficiency of bacteria. Specifically, azo compounds, with hydroxyl or amino groups, are more likely to be degraded than those with methyl, methoxy, sulpho or nitro groups. Simple structured mono azo dyes with low molecular weight are decolorized faster by bacteria compared to diazo and trisazo dyes having high molecular weight. Azo dyes with electron withdrawing groups, such as $SO_3^-$, are easy to degrade than those with electron-releasing groups. Especially, when electron withdrawing groups are present in para and ortho position, azo dyes are more susceptible to bacterial degradation than those at meta position. In fact, electron withdrawing groups in para and ortho position of the phenyl ring provide a more effective resonance effect to make azo dyes to be highly electrophilic (dos Santos et al. 2004). In addition, the steric effect of chemical structure strongly affects the color removal efficiency and less steric hindrance near the azo bond gives faster and better decolorization by bacteria. Even enzyme induction (azo reductases) in bacteria for azo dye decolorization is also influenced by the dye chemical structure (Hu 2001). The sulfonated azo dyes are easier to be degraded by bacteria than carboxylated azo dyes, because sulfonated

azo dye cannot pass through bacterial cell membrane easily. The specificity of azo reductase was found to be strongly dependent upon the electron-withdrawing ability of functional groups in the proximity of the azo linkage (Saratale et al. 2011).

## 4.2 Concentration of Azo Dye

A survey of the literature suggests that increasing the dye concentration gradually decreases the decolorization rate, probably due to the toxic effect of dyes on the bacteria. It is also due to inadequate biomass concentration and blockage of active sites of azo reductase by dye molecules with different structures. It was also observed that azo dyes with reactive groups as sulfonic acid ($SO_3H$) on their aromatic rings greatly inhibited the growth of microorganisms at higher dye concentrations (Chen et al. 2003). However, Saratale et al. (2009) found that the increasing concentration effect was reduced when bacterial co-culture was used instead of pure culture, due to the synergistic effect of both microorganisms. Moreover, Dubin and Wright (1975) did not observe any effect of dye concentration on its reduction rate. This observation is compatible with a non-enzymatic reduction mechanism that is controlled by processes that are independent of the dye concentration.

## 4.3 Aeration and Agitation

Decolorization of azo dyes occurs under strictly anaerobic, facultative anaerobic and aerobic conditions by different groups of bacteria. Biodegradation process of azo dye is highly influenced by presence or absence of oxygen. Aeration and agitation may favour or inhibit the azo dye decolorization by bacteria. Generally, aeration and agitation increase biomass and oxygen transfer between bacterial cells and nutrient medium. It also increases enzyme activity, if mechanism is aerobic, but most of the reductase enzymes are sensitive to oxygen. The effect of oxygen on azo reduction is irreversible, so in presence of oxygen, azo reductases show lower activity. Hence, for efficient reduction of color, aeration and agitation, which increase the concentration of oxygen in the solution, should be avoided. However, under anaerobic conditions, reductive enzyme activities are higher, but a small amount of oxygen is also required for the oxidative enzymes, which are involved in the degradation of azo dyes. Some studies have reported that during bacterial degradation of azo dyes, both oxidative and reductive enzymes play a significant role. The intermediates, formed during azo dye reduction reaction, like the simple aromatic compounds, are degraded via hydroxylation and ring-opening in the presence of oxygen. The aerobic condition is required for the mineralization of the azo dye molecules. Thus, for the most effective effluent treatment, an anaerobic process with subsequent aerobic treatment can be used to decolorize wastewaters containing dyes (Saratale et al. 2011; Solis et al. 2012).

## 4.4 Carbon and Nitrogen Sources

It is very difficult to decolorize azo dyes by bacteria without additional carbon and nitrogen sources. However, it can be achieved in some cases, if the acclimatization of the bacteria is done by increasing the concentration of dye with diminishing of the carbon source until they can survive only in the presence of dye. A few bacterial species are reported, such as *Pseudomonas* and consortium of *Arthrobacter* and *Rhizobium*, which utilized dye as source of carbon (Saratale et al. 2009; Ruiz-Arias et al. 2010). Generally, azo dye decolorization by mixed as well as pure cultures requires additional carbon or nitrogen sources, because some dyes are deficient in carbon and nitrogen concentrations. Carbon sources provide energy for the growth and survival of the bacteria and also transfer reducing equivalents to the dye for azo bond cleavage (Moosvi et al. 2007; Solis et al. 2012). Different bacterial strains have different metabolic profiles with respect to different carbon and nitrogen sources, which cause significant variation in extent of azo dye decolorization. Many scientists have reported enhancement of dye or textile effluent decolorization by glucose as carbon sources. Apart from glucose, starch is another common source of carbon for azo dye decolorization. Several economically cheap and raw carbon sources, such as molasses, sugarcane bagasse, tapioca and jiggery, have been used to improve decolorization and to reduce the cost of process (Solis et al. 2012). It was reported that as compared to additional carbon sources, organic nitrogen sources seemed to be highly effective in promoting dye decolorization. Using complex organic nitrogen sources, such as peptone, yeast extract, beef extract, urea and others resulted in better decolorization, because of regeneration of NADH by these sources, which is the source of electron donor for azo bond reduction (Chang et al. 2000). To make the process economically feasible and practically applicable, some investigators have used lignocellulosic agricultural waste as an additional supplement for effective decolorization. Lignocelluloses constitute a major portion of agricultural and forest wastes, and industrial effluents from the global pulp/paper, food and other industries produce up to $0.85 \times 10^{11}$ t of these substances per annum (Saratale et al. 2011). Recently, many reports are available on use of agricultural wastes, such as extract of sugarcane bagasse powder, wood straw, rice husk and rice straw, to increase the azo dye decolorization performance. Apart from the type of sources available, amount of the source also matters in decolorization process, as with insufficient amount of C sources, growth and activity of bacteria are influenced negatively. Restricted amount of carbon source in medium allows limited biomass growth but with high metabolic activity, which is necessary for significant decolorization of dyes (Kumar et al. 2009). Even in presence of high carbon source, reduction in decolorization was reported because bacteria utilise carbon source favourably to the dye (Solis et al. 2012).

## 4.5 Incubation Temperature

Temperature is also an important environmental factor for all processes associated with microbial life, including the decolorization of dye and dye effluents by bacteria. The bacterial cells adapt to temperature changes by biochemical or enzymatic mechanisms. Incubation temperature affects both bacterial growth and enzyme activity, and so, the rate of azo dyes decolorization. Narrow temperature ranges were determined as being necessary for the decolorization of azo dyes by extremely complex consortia of microorganisms inhabiting active sludge (Yu et al. 2001). It was observed that bacteria show maximum decolorization of dye at optimum temperature needed for their growth, reproduction and enzyme activities (Hazrat 2010). Beyond the optimum temperature, reduction in decolorization activity is found due to loss of cell viability, decreased rate of reproduction and denaturation of enzymes responsible for degradation (Saratale et al. 2011; Solis et al. 2012). But, for actual treatment of dye containing waste on commercial level requires stable bacterial culture to a wide change in environmental temperature. Thus, application of thermo tolerant culture for the treatment of dye effluents is advisable. Thermophilic bacteria, *Geobacillus stearothermophilus* for Orange II dye decolorization and azo reductase enzyme of *B. Badius* were reported stable even at higher temperature. Most of the reports available on azo dye degradation by bacteria indicated 25–37 °C temperature range and 35 ± 2 °C as an optimum temperature (Hazrat 2010; Solis et al. 2012).

## 4.6 Medium pH

pH is also an important factor with respect to biological decolorization of azo dyes. The medium pH has a major effect on the efficiency of dye decolorization because of enzymatic activity dependence on the pH. Color of the solution and solubility of dyes is also affected by pH. Generally, bacteria show better decolorization at neutral or basic pH and the optimal pH for color removal is often between 6.0 and 10.0 (Solis et al. 2012). The rate of color removal is the highest at the optimum pH and tends to decrease on either side of optimum pH. It is possible that pH change affects the transport of dye molecules across the cell membrane, which is considered as the rate limiting step for the decolorization (Saratale et al. 2011). An increase in pH of the medium was observed during anaerobic dye decolorization due to reduction of the azo bond to form aromatic amine metabolites, which are more basic than the parent azo dye (Willmott 1997). Generally, fluctuation in the pH slightly from neutral to alkaline side has very little effect on the dye decolorization as compared to acidic side (Jadhav et al. 2008). However, *Citrobacter* sp. CK3 is able to decolorize even in strongly acidic (at pH 4) and strongly alkaline (at pH 12) conditions (Wang et al. 2009). Thus, pH tolerance of decolorizing bacteria is quite important, as it makes them suitable for the commercial treatment of dye containing effluents.

## 4.7 Salt Concentrations

Wastewater from dyestuff manufacturing and textile processing industries shows presence of various acids, alkalis, metal ions and salts as impurities. Up to 15–20 % salt concentration has been reported in these industrial wastewaters, because high salts are needed for the dyeing process (Hazrat 2010). So, high salt-tolerant bacteria capable of dye decolorization are the organisms of choice for actual effluent treatment on a large scale. Generally, a sodium concentration above 3 % causes moderate inhibition of most bacterial activities. Thus, azo dye removal efficiency is inversely proportional to the salt concentration. However, there are a few examples of halotolerant microorganisms, such as *Exiguobacterium acetylicum, Staphylococcus gallinarum, B. firmus* and *A. hydrophila* which are able to decolorize azo dyes even in the presence of high salts (Chen et al. 2011; Ogugbue et al. 2011).

## 4.8 Electron Donors

The azo dyes and the other organic content of textile wastewater are too low to act as a sufficient substrate for the growth of anaerobic bacteria. So, it is necessary to have an external substrate (electron-donor) supply to enhance the anaerobic decolorization performance. The electron donors, sodium salts of acetate, formate, succinate, citrate and pyruvate have been shown to enhance the azo dye decolorization. The anaerobic bacterial azo reduction is a biochemical process that oxidizes the electron donors and transfers the electrons to the acceptors through a multicomponent system related to the electron transport chain. During decolorization, bacteria require NADH as an electron donor for NADH-DCIP reductase. In the presence of artificial electron donors, such as glucose, methanol, sodium acetate, sodium formate, sodium citrate, and sodium pyruvate, an induction in the reduction activity was observed. Amongst these substrates, methanol is widely used as a cost-effective electron donor for the biological treatment of wastewater. The donors not only induce the reduction mechanism, but also stimulate the enzymatic system responsible for the reduction process. In contrast, it has been observed that in the presence of some electron donors, the electron transport process is inhibited, and this might be due to the competition for electrons from the donors. Certain chemicals, such as thiomersal and p-chloromercuribenzoate, inhibit the alcohol dehydrogenase of the NADH-generating systems required to produce reducing equivalents for dye reduction. It has also been noted that the products of cell lysate residue can also function as electron donors for an anaerobic azo dye reduction (Saratale et al. 2011).

### 4.9 Redox Mediators

Redox mediators are the compounds that speed up the reaction rate by shuttling electrons from the biological oxidation of primary electron donors to the electron accepting compounds, as first described by Bourbonnais and Paice (1990). Flavin-based compounds, such as flavin adenide dinucleotide (FAD) and flavin adenide mononucleotide (FMN), and quinone-based compounds, such as anthraquinone-2,6-disulfonate (AQDS), anthraquinone-2-sulfonate (AQS), riboflavin (vitamin B2), cyanocobalamin (vitamin B12) and lawsone (2-hydroxy-1,4-naphthoquinone), have been reported as redox mediators. Redox mediators are characterized by a redox potential ranging from −200 to −350 mV. The performance of redox mediators is dependent on the redox potential. The dye removal rate is the highest, when the redox potential of the system is at its most negative, and it decreases when the redox potential of the system increases. The transfer of reducing equivalents from a primary electron donor (co-substrate) to a terminal electron acceptor (azo dye) lowers the electron density in the azo link which enhances the color removal rate. The supplementation of redox mediators accelerates the transfer of reducing equivalents to the terminal electron acceptor (i.e. azo dye), and also minimizes the steric hindrance of the dye molecule. The effect of redox mediators on the decolorization of azo dyes and textile wastewaters was investigated by Saratale et al. (2011). However, the effect of redox mediators in enhancing the decolorization of textile wastewater is still unclear due to a wide range of redox potentials among azo dyes (−180 to −430 mV) present in wastewater, high dye COD concentration (about 1.4 g $l^{-1}$) and the different properties of the dyes (dos Santos et al. 2004).

## 5 Enzyme System Involved in Azo Dye Degradation

The bacterial decolorization of azo dyes is mainly mediated by the various oxidoreductive enzymes, such as lignin peroxidase, manganese peroxidase, laccase, veratryl alcohol oxidase, tyrosinase, amino pyrine N-demethylase, DCIP reductase, azo reductase. According to reaction type, these oxidoreductase enzymes transfer electrons from a substrate to an acceptor (azo dye). As per earlier reports, it has been suggested that initial reduction of azo group takes place by reductive enzymes, such as azo reductase, NADH-DCIP reductase and resulted intermediates are further mineralized by oxidative enzymes. In general, azo dyes are electron deficient compounds due to presence of azo group (–N=N–) and other electron withdrawing groups, such as sulphonic ($SO_3^-$). Electron deficient azo dyes are not easily degraded by bacteria, as they need supplementation. Enzymatic treatment is most effective, when there is a high concentration of target contaminants and low concentration of interference.

## 5.1 Tyrosinases

Tyrosinases (E.C. 1.14.18.1) are also known as monophenol mono-oxygenase found in the variety of organisms, including bacteria, fungi, plants, insects, amphibians and mammals. They are copper-containing enzymes and catalyze two type of reactions, the *o*-hydroxylation of some monophenols (monophenolase, cresolase) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase, catecholase) using molecular oxygen (Chen and Flurkey 2002). Tyrosine and catechol are commonly used as substrates of the enzyme tyrosinase that produces dopaquinone and *o*-benzoquinone, respectively. This enzyme acts as marker of the oxidative enzymes involved in azo dye degradation. Significant induction of tyrosinase is reported during the decolorization of azo dyes by bacteria. Reports on tyrosinase induction after exposure to azo dyes are available from *Alcaligenes faecalis* PMS-1, *B. laterosporus* MTCC 2298 and consortium consisting of *Galactomyces geotrichum* MTCC 1360 and *Bacillus* sp. VUS (Saratale et al. 2011; Shah et al. 2012).

## 5.2 Laccases

Laccases (E.C.1.10.3.2) are copper-containing oxidoreductases and also known as phenol oxidases. Laccases have a high potential for biodegradation of dyes due to their wide reaction capabilities as well as broad substrate specificity along with no requirement of co-factors. The molecular structure exhibits four histidine rich copper containing binding domains, which are classified into three types: copper I, II, and III which are differentiated by their spectroscopic properties. They are mainly classified into two categories viz. the blue laccases which contain type 1 copper site and second one which lacks the type 1 copper site. The molecular weight of laccase was reported between 60 and 390 kDa. The mechanism of reaction involves the oxidation of substituted phenolic and non-phenolic compounds in the presence of oxygen as an electron acceptor to form free radicals which further undergo depolymerization, repolymerization, demethylation or quinone formation. Laccases in immobilized phase are also studied for degradation of azo dyes. This enzyme showed optimum pH 4.0 and temperature 60 °C for its activity with maximum substrate specificity for 2, 2′-azinobis, 3-ethylbenzothiazoline-6-sulfonic acid. In case of laccase mediated degradation of azo dyes, there is no formation of aromatic amines after azo bond cleavage because laccases directly cleave the azo group through non-specific free radical mechanism. The role of laccase in asymmetric cleavage of azo dye was well documented in Remazol Red degradation by *Pseudomonas aeruginosa* BCH. The laccases from *Bacillus* sp. ADR and *Pseudomonas desmolyticum* NCIM 2112 which are involved in degradation study of various azo dyes were purified and characterized (Saratale et al. 2011).

## 5.3 Lignin Peroxidases

Lignin peroxidase (EC 1.11.1.14; LiP) was first discovered from *Phanerochaete chrysosporium* and shown to attack lignin type compounds (Tien and Kirk 1984). This enzyme belongs to the family of oxidoreductases, specifically those acting on peroxide as an acceptor (peroxidases) and can be included in the broad category of ligninases. The systematic name of this enzyme class is 1, 2-bis (3, 4-dimethoxyphenyl) propane-1, 3-diol: hydrogen-peroxide oxidoreductase. LiP is glycoprotein having molecular weight between 38 and 46 kDa. LiP is haem containing peroxidase with high redox potential. The optimum pH of this enzyme is below 3.0. The mechanism of sulphonated azo dye degradation by LiP involves two successive one-electron oxidations in the phenolic ring by the $H_2O_2$-oxidised forms of LiP and produces a radical at the carbon bearing the azo linkages. Now, nucleophilic attacks by water on phenolic carbon produce phenyldiazene. This product is then oxidized by $O_2$ to a phenyl radical, and the azo linkage is eliminated as $N_2$. The most widely accepted assay for detecting lignin peroxidase is based on the oxidation of veratryl alcohol to veratraldehyde. Moreover, from the crystal structure of Lip, it can be inferred that only veratryl alcohol can enter the active site. It is not possible for the lignin polymer to interact directly with the haem-group of the enzyme. The lignolytic enzymes have been widely studied in fungi, but very few reports are available for activity of lignolytic enzymes in bacteria for azo dye degradation. However, purified LiP from *Bacillus* sp. strain UVS, *Brevibacillus laterosporous* MTCC 2298 and *Acinetobacter calcoaceticus* NCIM 2890 efficiently decolorized the various synthetic azo dyes. Invariables, the purified LiP shows better efficiency for decolorization of dyes than intact cells. Lignocellulosic substrates in medium induce the production and activity of LiP for degradation of azo dyes (Saratale et al. 2011).

## 5.4 NADH-DCIP Reductases

The NADH-DCIP reductase (EC 1.6.99.3) reduces the DCIP (2,6-dichloroindophenol) using NADH as an electron donor. The enzyme acts as a monomer with a molecular mass of 43 kDa. DCIP is blue in its oxidized form and becomes colorless after reduction by a reductase enzyme. NADH-DCIP reductase from *Bacillus stearothermophilus* was reported in 1980. Several researchers reported NADH-DCIP reductase as marker enzyme for the reduction of azo dyes. A significant induction of DCIP reductase activity during the decolorization of various dyes has been reported from *Bacillus* sp. ADR, *Pseudomonas aeruginosa* BCH, *Alcaligenes faecalis* PMS-1, *Brevibacilus laterospores*, *Acinetobacter calcoaceticus*, *Pseudomonas* sp. SUK1. Consortium GB, consisting of *Galactomycesgeotrichum* MTCC 1360 and *Bacillus* sp. VUS, also showed NADH-DCIP reductase activity for azo dye Brilliant Blue G degradation (Saratale et al. 2011; Shah et al. 2012; Solis et al. 2012).

## 5.5 Azo Reductases

Azo reductases which are reducing enzymes widely applied in field of dye degradation, catalyse reductive cleavage of electrophilic azo groups (–N=N–) and other compounds containing azo bond to produce aromatic amines. Many bacterial strains possess unspecific cytoplasmic enzymes, which act as "azo reductases". Azo reductases reduce azo bond by transferring electrons via soluble flavins to azo dyes. First report of presence of azo reductase in anaerobic bacteria was given by Rafii et al. (1990). They reported extracellular oxygen sensitive azo reductase from *Clostridium* and *Eubacterium* for azo dye degradation. Apart from bacteria, algae and yeast, azo reductases have also been detected in liver cells (Solis et al. 2012; Chengalroyen and Dabbs 2013).

Classification of azo reductases is broadly based on their oxygen requirement and structure. But due to low level of similarity in nucleotide and amino acid sequence of azo reductases, they are mainly classified based on secondary and tertiary structure. Further based on function they are classified into two groups, flavin dependent and flavin independent. Again flavin dependent azo reductases are categorized according to co-enzymes required, NADH, NADPH or both. Recently, three group classification systems have been proposed. First group is consisting of FMN dependent enzymes utilizing NADH, second NADPH utilizing enzymes and third group is flavin free reductases (Saratale et al. 2011; Solis et al. 2012; Chengalroyen and Dabbs 2013). Structurally azo reductases are mainly monomeric, but a few are also reported as dimeric and tetrameric in nature (Bafana and Chakrabarti 2008) The optimum temperature range of bacterial azo reductases is between 25 and 45 °C and pH of 7.0. Azo reductase activity is not dependent on the intracellular uptake of dye, as high molecular weight azo dyes are unlikely to pass through cell membrane of bacterial cells (Chengalroyen and Dabbs 2013).

Azo reductases are localized at intracellular or extracellular site of the bacterial cell membrane. These azo reductases required NADH or NADPH or FADH as an electron donor for the reduction of an azo bond (Russ et al. 2000). Azo reductase activities have been also observed in cell extracts. As co-factors, $FADH_2$, NADH, NADPH, $FMNH_2$ and their reducing enzymes are located in the cytoplasm, lysis of cells releases these co-factors and enzymes in extracellular environment. In case of intact cells, membrane transport system may be a prerequisite for azo dye reduction. Riboflavin can pass through cell membranes, but FAD and FMN cannot easily pass through cell walls. Similarly, many azo dyes cannot pass through cell membranes due to complex structure and high polarities, while azo reductases are found intracellular in many bacteria. Thus, cell extracts of lysed cells often show higher reductase activity of dye reduction as compared to intact cells. Many researchers have observed lack of specificity in the azo reductase system and showed the substrate specificity of azo reductases depends on the functional group present near azo bond (Saratale et al. 2011; Solis et al. 2012; Chengalroyen and Dabbs 2013).

Azo reductases of bacteria showed very less similarity with other reported reductases and hence represent novel families. Purification and biochemical

characterization of azo reductases reducing azo dyes were carried out by several investigators. Aerobic FMN dependent azo reductases, reported for azo dye decolorization, have been isolated from *E. coli, Enterococcus faecalis, Kerstercia* sp. and *Staphylococcus aureus*. NADH dependent azo reductases have been characterized in *B. cereus, B. velezensis, B. badius* and *Bacillus* sp. ADR. A FAD independent azo reductase was isolated from *Sphingomonas* sp. by Kudlich et al. (1997). For better information up to sequence level, DNA screening and probe design of NADPH dependent azo reductase (20 kDa) from *Bacillus* sp. OY1-2 was studied by Suzuki et al. (2001). They also isolated gene from *B. subtilis* ATCC6633, *B. subtilis* ISW1214 and *G. stearothermophilus*. Another 30 kDa azo reductase enzyme was identified by Blumel et al. (2002) from *Xenophilus azovorans* KF46F. Azo reductase from *Pseudomonas aeruginosa* was found to be oxygen-insensitive towards azo dye degradation (Chen et al. 2005).

## 6 Mechanism of Bacterial Azo Dye Degradation

It is very much important to know the mechanism by which azo dye decolorization is carried out by bacteria. Dye degradation studies are conducted both under aerobic as well as anaerobic conditions. But generally bacterial degradation of azo dyes comprises the reductive cleavage of azo bonds (–N=N–) with the help of an azo reductase enzyme under anaerobic conditions. During this process, four-electrons (reducing equivalents) are transferred from electron donors to the electron acceptor (azo dye) in two stages at the azo linkage, resulting in dye decolorization and generation of colorless amines. The resulting intermediate metabolites (e.g., aromatic amines) are then further degraded aerobically or anaerobically. Azo dye decolorization under anaerobic condition is simple but non-specific process. Under anaerobic conditions, a low redox potential (≤50 mV) causes the effective decolorization of the azo dyes. However, in case of aerobic degradation, respiration may dominate utilization of NADH, thus inhibiting the electron transfer from NADH to azo bonds. Alternatively, decolorization might be attributed to non-specific extracellular reactions occurring between reduced compounds generated by the anaerobic biomass. Much of the experimental work involving the anaerobic decolorization of dyes (predominantly azo dyes) was conducted using mono cultures. In anaerobic conditions, the permeation of the azo dyes through cell membrane into the microbial cells acts as the principal rate-limiting factor for the decolorization. Under aerobic conditions, mono- and di-oxygenase enzymes catalyze the incorporation of oxygen from $O_2$ into the aromatic ring of organic compounds prior to ring fission. Some aerobic bacteria are able to reduce azo compounds with the help of oxygen catalysed azo reductases and produce aromatic amines. It was also reported that the aerobic azo reductases were able to use both NAD(P)H and NADH as co-factors and reductively cleaved not only the carboxylated growth substrates of the bacteria, but also the sulfonated structural analogues. There are a 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 to their substrate. Examples of bacterial strains with this trait are *Xenophilus azovorans* KF46 (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 (McMullan et al. 2001). Only a few bacteria with specialized azo dye reducing enzymes have been found to degrade azo dyes under fully aerobic conditions (Saratale et al. 2011; Solis et al. 2012).

## 7 Analytical Methods for Evaluation of Dye Degradation Mechanism

Various analytical techniques are used to identify the intermediates and end metabolites generated during azo dye decolorization by bacteria. Amongst all, UV-Vis spectroscopy is preliminary method used by all researchers to evaluate dye decolorization. In this method, dye sample gives a sharp peak at $\lambda_{max}$ in visible region, while after bacterial treatment decolorized sample shows a decrease in intensity or complete disappearance of the peak with simultaneous increase in intensity of peaks in the UV region. For further evaluation of metabolite generation and dye degradation, Thin Layer Chromatography (TLC) and High Performance Thin Layer Chromatography (HPTLC) techniques are used which gives insights in numbers and types of metabolites generated. But for confirmation of the degradation of dye, HPLC has been used by many investigators, which show appearance of new HPLC peaks having different retention time ($R_t$) as compared to HPLC peaks of original dye chromatogram. Moreover, Fourier Transform Infrared Spectroscopy (FTIR) is widely used to determine the type and strength of interactions that occur within azo dyes containing different functional groups after treatment by bacteria. FTIR is comparatively more important to check the removal of azo group from the azo dye and generation of new type of metabolites. Another powerful techniques used for evaluation of metabolites are Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS). Both these techniques are used for qualitative determination of metabolites along with molecular weights and their structural information. This information of metabolites is used further to propose the degradation pathway of the dye. Now-a-days, apart from these techniques, advance and very complex technique like Nuclear Magnetic Resonance (NMR) is applied for further detailed quantitative study of compounds and also to get structural information concerning molecular compounds. But for dye containing wastewater degradation analysis, these techniques are not very beneficial, because composition of actual wastewater is unknown and also complex. So, analysis and interpretation by these methods are not much helpful as in case of pure dye degradation by bacteria.

So, parameters such as chemical oxygen demand (COD), biochemical oxygen demand (BOD), total organic carbon (TOC) and ADMI values are measured to analyse extent of mineralization and degradation of dye containing wastewater.

## 8 Pathway of Degradation

The pathway of azo dye degradation has been proposed on the basis of results of GC-MS and enzyme systems involved. Here a degradation pathway of is shown in Fig. 1. Acid Black 210 is a very complex azo dye containing three azo groups in its structure and has a molecular weight of 859.8. Decolorization of AB210 by a bacterial consortium under microaerophilic condition was studied by Agrawal et al. (2014).

In initial degradation, azo reductase enzyme cleaved molecule symmetrically and produced four intermediates viz. benzene 1, 2, 4-triamine, 4-amino phenyl-N-4 (aminophenyl) benzene sulfonamide, 4-nitroaniline and an unidentified compound with M.W. 364. Presence of laccase enzyme cleaved 4-amino phenyl-N-4 (aminophenyl) benzene sulfonamide asymmetrically and formed aniline and 4-amino benzene sulfonamide. Further, aniline and 4-amino benzene sulfonamide undergo deamination and desulfonation to produce benzene as end product. On the other hand nitro group was removed from 4-nitroaniline resulting in yield of aniline which was further converted to produce benzene as final product by deamination. In addition, benzene1,2,4-triamine undergoes deamination forming benzene. The presence of an unidentified product could not be detected from GCMS analysis due to its very transient existence. The unidentified compound probably undergoes deamination and desulfonation to produce 1-napthol, which on dehydroxylation yields naphthalene at the end. Similarly, enzymatic degradation pathway of azo dyes, such as Reactive Orange 13, Reactive Red BLI, Direct Brown MR, Direct Blue-6 and many others has been studied by many researchers in details (Kalme et al. 2007; Kalyani et al. 2008a; Ghodake et al. 2009; Shah et al. 2012).

## 9 Microbial Toxicity of Azo Dyes

Recently, adverse effects of hazardous chemicals present in industrial effluent on the environment and health are becoming subject of scientific study. Many azo dyes in purified form are mutagenic or carcinogenic, except for some azo dyes with free amino groups. Bacterial degradation of azo dyes under anaerobic condition produced aromatic amines, which are reported to be mutagens and carcinogens. Therefore, it is necessary to evaluate the toxicity of azo dyes and also of its metabolites resulted from bacterial degradation. Toxic effects of dyes especially of azo dyes have been studied for several decades. Brown and DeVito (1993) have postulated the mechanism of azo dye toxicity as mentioned below:

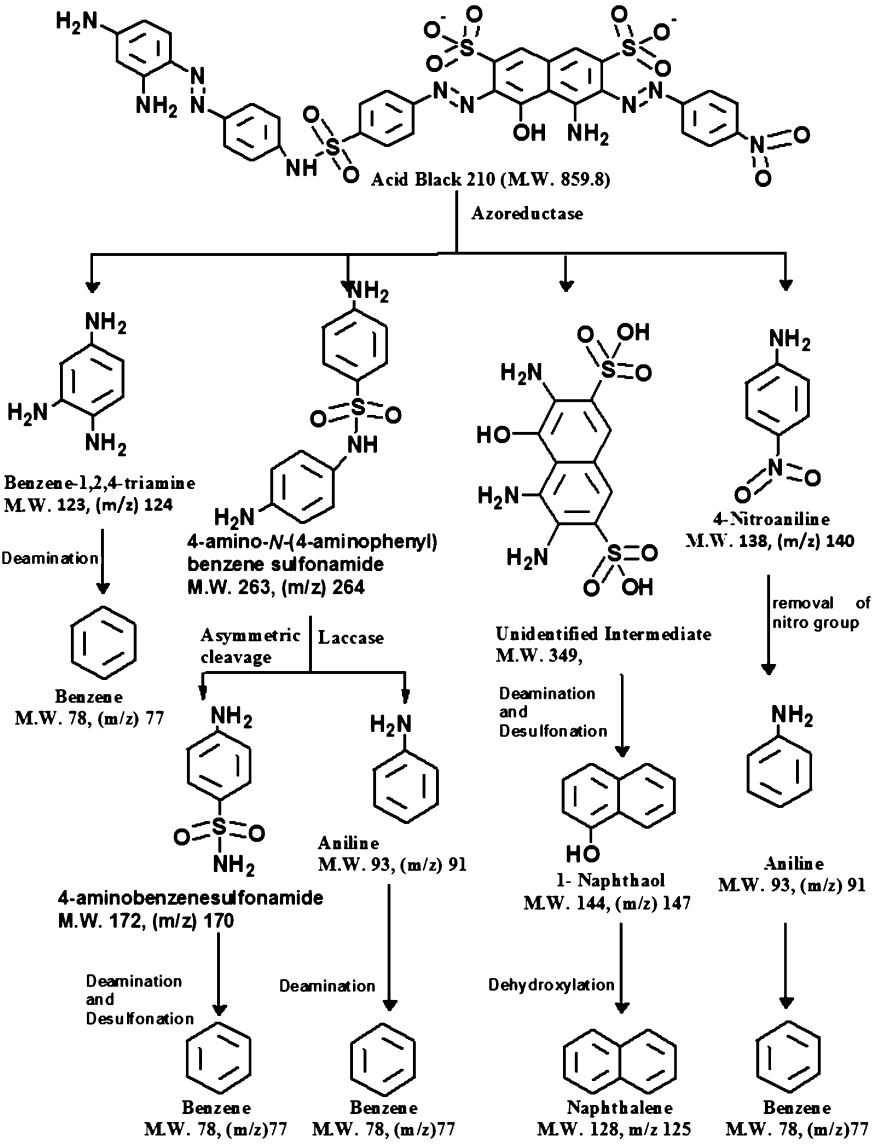



**Fig. 1** Bacterial degradation pathway of azo dye Acid Black 210

- Azo dyes may be toxic only after reduction and cleavage of the azo linkage, producing aromatic amines.
- Azo dyes, containing free aromatic amine groups that can be metabolically oxidized without azo reduction, may cause toxicity.
- Induction in azo dye toxicity may occur following direct oxidation of the azo linkage producing highly reactive electrophilic diazonium salts.

**Table 4** Methods for evaluation of toxicity of azo dyes

| Methods | Organisms |
|---|---|
| Microbial toxicity | Bacteria (*E. coli, Bacillus* spp., *Rhizobium* spp., *Vibrio fischeri*), Algae (Chlorella vulgaris, *Gloeocapsa minutus, Phormidium ceylanicum*) |
| Phytotoxicity | *Sorghum vulgare, Phaseolus mungo, Triticum aestivum, Oryza sativa, Cicer arietinum* |
| Mutagenicity (Ames test) | *Salmonella typhimurium* |
| Cytotoxicity | *A. cepa* |
| Genotoxicity (Comet assay) | *A. cepa* |
| Ecotoxicity | *Lemna minor* |
| Acute Toxicity | *Artemia nauplii* |
| Oxidative stress in plants | *A. cepa* |

Toxicity of azo dye is caused by more than one mechanism. Several methods, used to determine toxicity of azo dyes or their metabolites, are listed in Table 4.

Amongst these toxicity tests, phytotoxicity method has gained major attention because of easiness to perform, less expensiveness and simple analysis of seed germinations. However, determination of toxicity of dyes and their metabolites on standard known microorganisms is a more sensitive method with high reproducibility of results. The *Salmonella* mutagenicity assay or Ames test is widely used to detect chemical mutagenic and potential carcinogenic ability of azo dyes and their metabolites produced. Ferraz et al. (2011) reported mutagenic effect of dye Disperse Orange 1 on *Salmonella typhimurium* strains viz. TA98, YG1041, TA100 and YG1042. Similarly, toxicity of Reactive Black 5, Acid Orange 7, Food Yellow was studied using bioluminescent marine bacterium *Vibrio fischeri* by Gottlieb et al. (2003). However, Agrawal et al. (2014) studied microbial toxicity using *B. subtilis, E. coli, Azotobacter* and phosphate solubilizer and reported a decrease in toxicity of trisazo Acid Black 210 after decolorization by *Providencia* sp. SRS82. However, non toxic effect of azo dye Reactive Red BS and its metabolites on important soil bacteria *Bacillus cereus, Azotobacter* and *Rhizobium* was reported by Sheth and Dave (2009). Green unicellular alga *Pseudokirchneriella subcapitata* was also used to evaluate toxicity of dyes. Apart from phytotoxicity and microbial toxicity, cytotoxicity and genotoxicity using plant *Allium cepa* are now widely studied by researchers. Higher Plants are recognized as an excellent genetic model to evaluate mutagenicity of toxic chemicals and *A. cepa* species is having advantages of low cost and easy to handle. Chromosome aberrations in root cells of *A. cepa* are used to detect genotoxicity, while mitotic index and some nuclear abnormalities are evaluated for cytotoxicity of azo dyes. The oxidative stress response is recently studied for the assessment of toxicity of azo dyes. Analysis of antioxidant enzymes, such as ascorbate peroxidase (APX), superoxide dismutase (SOD), catalase, glutathione reductase and peroxiredoxins along with analysis of lipid peroxidation and protein oxidation, was carried out to assess oxidative stress response by toxic azo dyes

(Solis et al. 2012). Less toxicity of metabolites was reported by many researchers using above mentioned methods which indicates detoxification of azo dyes by bacterial activity.

## 10 Patents on Biological Azo Dyes Treatment

Several independent research groups have been working worldwide on biological treatment of azo dyes containing waste and they also patented their researches. US patent number 5,618,726 has been issued in 1997 on subject of biodegradable azo dyes (Paszczynski et al. 1997). In this patent, Acid Yellow 9 and other two synthesized azo dyes were mineralized by twelve *Streptomyces* species and white rot fungus *Phanerochaete chrysosporium* was reported. In 2009, biological treatment of acidic azo dye wastewater in bioreactors has been patented with Indian patent number 230896 (Sharma and Sharma 2009). In this patent, researchers have used consortium developed from activated sludge for reduction of BOD, color and COD of wastewater. They designed up flow reactors and developed biofilms on grits. They confirmed their results of degradation by UV-Vis spectroscopy, GC-MS analysis and also studied detoxification of dye containing wastewater using fishes and plants. Other US patents, such as number US 5,091,089, US 6,613,559 B2, US 4,655,926 and US 4,554,075, have been reported for the biological treatment of dye containing wastewater (Blanquez et al. 2008).

## 11 Future Perspectives

Global environmental pollution is a major threat now-a-days. Therefore, government legislations in all developed countries are becoming more stringent for environment protection. Water pollution, resulted from synthetic chemicals discharge from industries, is a big environmental issue, especially untreated dye containing effluent. Textile and dye stuff manufacturing industries have a great challenge to treat a large volume of dye effluent and recycle it which increases the cost of waste management. Till date, there is no attractive biological method available to be directly applied on a commercial scale to degrade and detoxify dye effluent. Still there is a need to work in this field using bacteria with objectives, such as use of acclimatized consortia rather than pure culture and reactor scale study in non-sterile conditions with actual dye effluent in contrast to pure dye decolorization study at flask level. There is also interesting alternative to use MFCs to treat wastewater along with generation of valuable products. Applications of molecular biology are also useful to study diversity of bacteria present in consortia and also identify genes encoding enzymes responsible for azo dye degradation. With the help of genetic engineering, genetically modified bacteria strains are created by transferring genes and recombinant strains with higher efficiency to degrade azo dye

effluent are applied. But environmentalists have apprehensions in using genetically modified organisms, as process will create new environmental problems. It is highly important to connect researchers of academic institute to industrial plants to find out a large scale treatment process. Even implementation of biological dye effluent treatment process on a commercial scale requires interdisciplinary knowledge of chemistry and biochemical engineering along with microbiology. Time will prove the significance of the application of bacterial treatment of dye containing wastewater at a commercial level.

## 12 Conclusions

Bacteria can degrade various azo dyes in anaerobic, aerobic and facultative conditions. Bacterial consortia have been proved more effective in faster mineralization of complex structured azo dyes than to single strain. Azo dye degradation process is highly affected by factors, such as pH, temperature, dye structure and concentrations, oxygen and nutrient sources. Various oxidoreductive enzymes are induced during remediation of azo dye containing waste. On the basis of different sophisticated analysis of dyes and metabolites, pathway of degradation has been also proposed. Using several biological systems, toxicity of azo dyes and generated metabolites was studied in order to prove detoxification of dyes after bacterial treatment.

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