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Microbial Degradation of Synthetic Dyes in Wastewaters

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To
parents in heavenly abode

Preface

Unlike natural dyes which are derived from plants, vegetables, and some minerals, synthetic dyes are manufactured by chemical processes and are commonly used for dying and printing in a wide range of industries. They are originally derived from coal tar derivatives, but are now synthesized from benzene and its derivatives. Synthetic dyes are usually named after the chemical structure of their particular chromophore group. The first synthetic dye, mauve, was discovered way back in 1956 by W.H. Perkin when he attempted to synthesize quinine.

Now, different synthetic dyes are produced, such as acid dyes, azoic dyes, basic dyes, chrome dyes, diazo dyes, direct dyes, disperse dyes, reactive dyes, sulfur dyes, and vat dyes. These dyes are widely used in different industries for dyeing cloth, paper, food, wood, etc. Among these chemical dyes, azo dyes are the most commonly used in dyeing units. There are more than 10,000 dyes as of now and their production has already exceeded 735 metric tons globally. Since synthetic dyes are cheaper, brighter, faster, and easier to apply to the fabric, they have changed the entire scenario worldwide.

On the other hand, dyeing industries discharge an enormous amount of synthetic dyes in wastewaters which are very toxic to both animals and plants. In order to remove synthetic dyes from wastewaters, adsorption on various sorbents and chemical decomposition by oxidation and photo-degradation are widely used. However, microbial degradation or decolorization, employing activated sludge, pure cultures and microbial consortia, degradative enzymes, etc., has been found self-driven, cost-effective, and also eco-friendly.

Therefore, I endeavored to compile the latest state-of-art on the microbial degradation of synthetic dyes in wastewaters coming from dyeing units in an edited volume which will serve as a ready reckoner to scientists, environmentalists, policy makers, teachers, students, industrialists, NGOs, and others concerned.

In this attempt, I would like to profusely thank all the contributors for their prompt response and active participation by contributing review articles on different aspects of microbial degradation of synthetic dyes. Besides, I also acknowledge the research scholars associated with me, Ms. Shweta Mishra, Ms. Nitanshi Jauhari, Mrs. Babita Kumari, and Ms. Divyata Maurya (trainee) for their academic and

technical support. Untiring service, provided by Mr. Dilip Chakraborty in preparing the manuscript meticulously, is also deeply acknowledged.

Lastly, I would also like to thank my family members: Mrs. Manorama Singh (wife), Ragini (daughter), and her kids Antra and Avantika, and Pritish (son) for their inspiration, endurance, and moral support to me in this endeavor.

S.N. Singh

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Mycoremediation of Synthetic Dyes: An Insight into the Mechanism, Process Optimization and Reactor Design

Prachi Kaushik and Anushree Malik

1 Introduction

Dye bearing industrial effluent can cause environmental problems unless it is properly treated before disposal. The complex aromatic structure of the dyes is resistant to light, biological activity, ozone and other degradative environmental conditions. Thus, conventional wastewater treatment is less effective. There are various methods for the treatment of wastewater (Forgacs et al. 2004), which broadly fall into three categories: physical (adsorption, coagulation/flocculation, membrane filtration etc.), chemical (chemical oxidation, photo-catalytic oxidation, electrolysis,Fenton reagent etc.) and biological (biosorption, enzymatic degradation etc.).Present treatment processes are largely based on the principles of flocculation with lime and ferrous sulphate, adsorption on activated carbon, nano-filtration, reverse osmosis and solar evaporation (Ranganathan et al. 2007). But owing to their high maintenance cost, prerequisite for preliminary treatment steps and land requirement, these are not economically viable for small enterprises and hence, there is a need to look for suitable decentralized technologies.Being eco-friendly, microbial decolorization (through bacteria, fungi and algae) is receiving much attention for the treatment of textile dye waste water (Sarayu and Sandhya 2012).

Considering the choice of microbes and the characteristics of textile effluent, fungi can be considered as the most suitable organism for the remediation purpose. Owing to their high cell-to-surface ratio, these organisms have a greater contact with the environment. Also, the extra-cellular nature of fungal enzymes allows them to thrive and tolerate high concentrations of toxicants. The fungi have a strong potential for mycoremediation in non-sterile open environment (Fig. 1). The mycelial growth gives a competitive advantage over single cells, such as bacteria and yeasts, especially with

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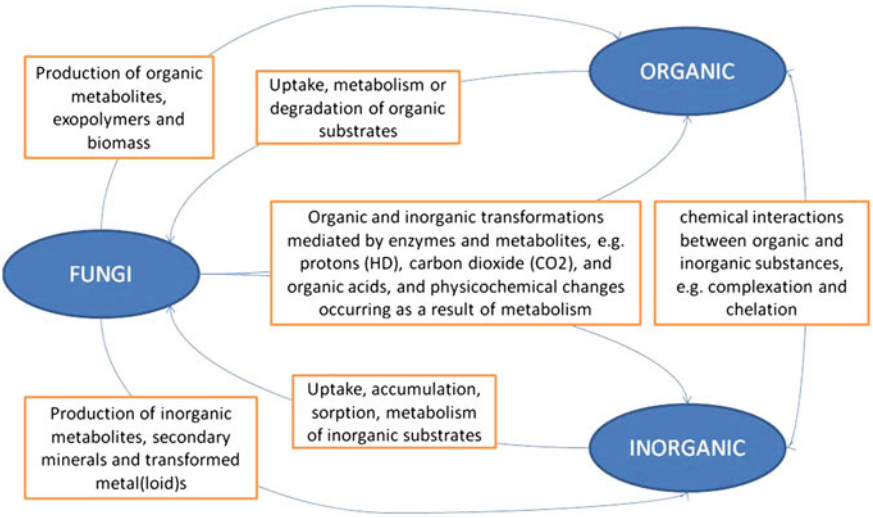

Fig. 1 Simple model of fungal action on naturally-occurring and/or anthropogenically—derived organic and inorganic substrates (modified after Gadd 2007)

respect to the colonization of insoluble substrates. They have high surface-to-cell ratio characteristics of filaments that maximize both mechanical and enzymatic contact with the substrate. The extracellular nature of thedegradative enzymes enables fungi to tolerate higher concentrations of toxic chemicals than what would be possible, if these compounds had to be brought into the cell. In this case, insoluble compounds, that cannot cross a cell membrane, are also susceptible to attack.

A lot of research has focused on mycoremediation, which could offer an attractive decentralized system. However, there has not been much success in translating these results into actual applications. A critical insight into the mechanism or pathways of dye transformation as well as process optimization is important while designing the operational strategy for mycoremediation. Several analytical and statistical tools have been described in the recent studies to achieve this. Moreover, some studies focus on the appropriate bioreactor design as per the underlying mechanism of fungal dye decolorization,while a very few investigations are available on the management of the dye laden fungal biomass. This chapter describes the recent innovations and vital advancements in mycoremediation targeting the ease of application.

2 Mechanism of Dye Removal by Fungi

Researchers have been employing various fungi as listed in Table 1, for dye removal studies either in living or dead form (Kaushik and Malik 2009). Three principal mechanisms are involved during thedye removal process mediated by fungi; biosorption, bioaccumulation and biodegradation. Biosorption is a metabolically

Table 1 Application of various fungi in dye removal

Fungi	Dye	Mechanism	Reference
Agaricus bisporus	Reactive Red 2	Biosorption	Akar and Divriklioglu (2010)
Rhizopus arrhizus	Yellow RL	Biosorption	Aksu and Balibek (2010)
Bjerkandera adusta	Acid Blue 62	Biodegradation	Anastasi et al. (2010)
A. lentulus	Acid Navy Blue	Biosorption and Bioaccumulation	Kaushik and Malik (2010)
Trametes trogii T. Villosa Coriolus versicolor	Indigo carmine Anthraquinone Blue	Biodegradation	Levin et al. (2010)
Aspergillus filiculoides	Basic Orange	Biosorption	Tan et al. (2010)
A. niger	Reactive Black 5	Bioaccumulation	Taskin and Erdal (2010)
A. versicolor	Remazol Blue	Bioaccumulation	Tastan et al. (2010)
A. niger	Direct Blue 199	Biosorption	Xiong et al. (2010)
A. ochraceus	Methyl Orange	Biodegradation	Telke et al. (2010)
Penicillium oxalicum	Reactive Blue 21	Bioaccumulation	Xin et al. (2010)
Irpex lacteus	Isolan Dark Blue 2SGL	Biodegradation	Kalpana et al. (2010)
A. niger	Orange G	Biosorption	Sivasamy and Sundarabal (2011)
Trichoderma sp.	Acid Brilliant Red B	Bioaccumulation	Xin et al. (2012a)
Magnusiomyces ingens	Acid Red B	Biodegradation	Tan et al. (2014)

independent process which involves the binding of solutes to the fungal biomass and thus can occur in either living or dead biomass (Srinivasan and Viraraghavan 2010). Biodegradation is an energy intensive and metabolically dependent process, where the complex dye molecules are broken down into simpler molecules through the action of certain enzymes. Bioaccumulation is also energy and metabolically dependent process, where actively growing cells accumulate the pollutants inside their cytoplasm (Chojnacka 2010).

2.1 Dye Biosorption

Left over spent fungal biomass, which is a by-product of industrial fermentations, is a very good and cheap source to be used in extensive use for dye biosorption (Fomina and Gadd 2014). Various functional groups, that are present on the fungal

cell wall i.e. amino, carboxyl, thiol and phosphate groups, can bind dye molecules (Tan et al. 2011; Fan et al. 2012). Biosorption of dye molecules onto the surface of fungal cells is a quick process and often gets completed in a few hours. Dye biosorption process is also affected by various process parameters, such as pH, temperature, initial dye concentration and type of dye present in the solution (Srinivasan and Viraraghavan 2010). Therefore, to obtain efficient dye removal, it is necessary to optimize various process parameters. Moreover, selection of a fungal strain fordye biosorption should be made in a way that it is capable of removing wide variety of dyes belonging to different classes.

The biosorption capacity of biomass can be further increased by certain physical (drying, autoclaving) or chemical (organic, inorganic) pre-treatments (Viraraghavan and Srinivasan 2011). Immobilization in alginate beads (Prigione et al. 2008) or loofa-sponge (Iqbal and Saeed 2007) has been reported to enhance biosorption capacity. Biosorbent can be regenerated by treatment with certain chemicals, such as alkalis, chelating agents etc. Recovery of the adsorbent and dye makes the treatment process more economical. It is observed that most of the studies are performed with the help of dye solutions. However, mixed effluent from textile industries containing mixtures of dyes and certain other chemicals may interfere with the process of dye removal through biosorption. Therefore, more studies should be performed utilizing the mixtures of dyes and industrial effluent.

2.2 Dye Bioaccumulation

Majority of the reports, that report bioaccumulation as the principle mechanism for dye removal, are focused on the use of single cell fungi (Dias et al. 2010; Das et al. 2011) and cyanobacteria (Silva-Stenico et al. 2012). However, a few studies report bioaccumulation by mycelial fungi, such as *A. niger* (Taskin and Erdal 2010) and *P. oxalicum* (Xin et al. 2010). Bioaccumulation of dyes by fungi is mediated by initial biosorption to the fungal cell wall which is metabolism-independent and then accumulation into the cytoplasm which is metabolism-dependent (Wang and Hu 2008).

2.3 Dye Biodegradation

Biodegradation is described as the breakdown of chemical compounds which is mediated by the action of biological enzymes. Complete biodegradation is the total breakdown of organic molecules into water, carbon dioxide and/or any other inorganic end products which is known as mineralization. White-rot fungi secrete certain ligninolytic enzymes that bind non-specifically to the substrate. Therefore, they are capable of degrading a wide variety of recalcitrant compounds and complex mixtures of pollutants, such as dyes. Since these ligninolytic enzymes secreted

by fungi are extracellular, therefore, problem related to substrate diffusion limitation into the cell, which is commonly encountered in bacteria, is not observed in fungi. Also, enzyme secretion by white-rot fungi depends on nutrient limitation (nitrogen or carbon) and is not altered by the presence of pollutants. This implies that for dye removal through biodegradation as the principle mechanism, acclimatization of the fungi with the pollutants may be skipped (Ge et al. 2004). Laccase, Manganese Peroxidase (MnP), Manganese Independent Peroxidase (MIP), Lignin Peroxidase (LiP), Tyrosinase etc. are the various enzymes that are involved in the degradation of the dye.

For biodegradation, fungal cells have to be in their growing form. This limits their application in treatment of toxic dye effluents. Nevertheless, the enzyme activity is often not altered by the presence of other pollutants, but biosorption as a process is influenced by the factors, like ionic strength, ionic state of dye and biosorbent. Thus, biodegradation, as a means for dye removal, has its own advantages. The growth of the fungus, enzyme production and subsequent dye decolorizationare effected by the culture conditions, like initial dye concentration, pH, agitation, media components, presence of heavy metals etc. (Martorell et al. 2012; Daâssi et al. 2013; Jin and Ning 2013; Moreira-Neto et al. 2013). Growth of the fungus is also affected by the nutritional conditions of the environment. Table 2 summarizes the key differences among three principle mechanisms fordye removal process. It is important to note that during the process of dye removal through fungi, multiple mechanisms may be operative either simultaneously or sequentially.

Table 2 Comparison between biosorption, bioaccumulation and biodegradation process (modified after Chojnacka 2010)

Biosorption	Bioaccumulation	Biodegradation
Passive process	Active process	Active process
Biomass is not alive	Biomass is alive	Biomass is alive
Dye molecules are bound with cellular surface	Dye molecules are bound with cellular surface and interior	Dye molecules are degraded by enzymes
Adsorption	Absorption	Extracellular degradation/ adsorption followed by degradation
Reversible process	Partially reversible process	Irreversible process
Nutrients are not required	Nutrients are required	Nutrients are required (nitrogen limiting conditions favourable)
Single-stage process	Double-stage process	Double-stage process
The rate is quick	The rate is slow	The rate is slow
Not controlled by metabolism	Controlled by metabolism	Controlled by metabolism
No danger of toxic effect	Danger of toxic effects caused by contaminants	Interference by contaminants, byproducts may be toxic
No cellular growth	Cellular growth occurs	Cellular growth occurs

For example, removal of Acid Navy Blue by *A. lentulus* pre dominantly followed bioaccumulation mechanism, while biosorption played a minor role (Kaushik and Malik 2010, 2013). Also, each mechanism i.e. biosorption, bioaccumulation or biodegradation, has its own advantages and limitations. Therefore, it is always beneficial to use a consortium of microbes for such treatment processes over pure cultures, as it promotes the dye removal process through multiple mechanisms, making it more efficient in terms of percentage removal, time required and supplementation needs.

3 Analytical Tools to Study Dye Removal Process

Monitoring ofdye removal process is based on the study of dye concentration at a given time or by estimating the production ofmetabolites/intermediates or dye by products. Dye concentration can be estimated through various measurements, such as Total Organic Carbon (TOC), Chemical Oxygen Demand (COD) or by measuring absorbance of the solution at the absorption maxima of the dye with a UV-Vis spectrometer. However, these techniques are not selective in nature and affected by other contaminants that may be present in the solution. More specific analytical techniques have been used by the researchers to monitor thedye removal process as well as to determine the dye intermediates that are released as a result of degradation process. The choice of analytical technique depends on the type of dye removal process involved and type of the dye. These analytical techniques can be categorized into two types: separation techniques and spectroscopic techniques. Separation techniques include various chromatographic techniques, such as High Performance Liquid Chromatography (HPLC), Gas Chromatography Mass Spectrometry (GCMS), Liquid Chromatography Mass Spectrometry (LCMS), High Performance Thin Layer Chromatography (HPTLC) etc. which help in the identification of the intermediate compounds released during the dye removal process as well as the final degradation products formed as a result of biodegradation. Spectroscopic techniques, such as UV-Vis spectroscopy, have been widely used by the researchers to quantify the dye removal process in terms of reduction brought about in the absorbance value (at absorption maxima) of the dye. In addition to this, Fourier Transform Infra-Red Spectroscopy (FTIR) and Nuclear magnetic Resonance (NMR) have also been utilized as an essential tool for estimating the dye degradation pathway. A recent review by Fernández et al. (2010) has listed the advantages and limitations of these two types of analytical techniques in Table 3.

Apart from these techniques,enzymatic assays, that involve the detection of various dye degrading enzymes, have been used by the researchers to confirm biodegradation process during dye removal. Various white-rot fungi, such as *Trametes versicolor*, *Phanerochaete chrysosporium*, *Pleurotus ostreatus* etc., have been reported to produceextracellular ligninolytic enzymes, such as lignin peroxidase (LiP), laccase, manganese peroxidase (MnP) etc. which cause dye degradation. The assay to detect the presence of an enzyme is based on either of the

Table 3 Advantages and limitations of various analytical techniques (modified after Fernández et al. 2010)

Analytical techniques	Advantages	Limitations
Separation techniques (HPLC, GCMS etc.)	High selectivity	Analysis time can be high
	Degradation byproducts can be accurately identified with an MS detector	Samples normally need to be pretreated
		Organic solvents need to be used
Spectroscopic techniques (UV-vis, FTIR etc.)	Low analysis time	Low selectivity
	Sample pre-treatment is simple or not necessary	The presence of interferents (degradation byproducts) can lead to erroneous results
	Dyes can be determined on the catalyst surface (SERS)	Expensive deuterated solvents need to be used (NMR)
	Chemometrics can be used to simultaneously identify and quantify several dyes and intermediates	
Microscopic techniques	Short analysis time	Sample preparation required for electron microscopy
	Any morphological changes occurring on microbial biomass can be visually seen	Few techniques are only qualitative (except EDX)
Enzymatic techniques	Highly selective	Applicable in only live process
	Do not vary with microbe or substrate	Dependent on nutritional conditions
		Individual assay required for each enzyme

following two principles: disappearance of the substrate or the appearance of the new product, essentially a colored compound which can be quantified by spectroscopic techniques.

Table 4 shows various studies which adopted described analytical techniques in examining thedye removal process. Recently, use of high performance capillary electrophoresis has gained a lot of importance (Zhao et al. 2007; Lu et al. 2008) in determining the dye and their intermediates or by-products (which have ionic character). During dye degradation process, the dye peak undergoes a continuous change. Initially, it decreases and then certain new peaks appear which later on disappear due to the formation of small undetectable compounds. Use of a MS detector helps in a more accurate identification of such compounds. For the study of

Table 4 Different analytical techniques used to study dye removal process

Dye	Dye class	Microorganism	Removal process	Analytical technique to monitor dye removal process	Analytical technique to study intermediate formation	Degradation product	Reference
Disperse Orange 3	Azo	*Pleurotus ostreatus*	Biodegradation		GCMS, HPLC	4-nitroaniline, nitrobenzene, 4-nitrophenol, 4-nitroanisole	Zhao et al. (2006)
Orange G	Azo	*Trametes versicolor*	Biodegradation	UV-Vis	NMR		Casas et al. (2007)
Reactive Blue 25	Cu-phthalocyanin and monochlorotriazine	*Aspergillus ochraceus*	Biosorption and biodegradation	UV-Vis and enzyme assays (Lignin peroxidase, laccase, tyrosinase)	FTIR, GCMS, HPLC	Phthalimide, di-isobutyl phthalate	Parshetti et al. (2007)
Orange 3, Acid Orange 8, Food Yellow 3, 4-HABA	Sulfonated azo dyes	*Pleurotus ostreatus*	Biodegradation		Capillary electrophoresis—mass spectrometry (CE-MS)	4-hydroxy benzene sulfonic acid, 3-methyl 4-hydroxy benzenesulfonic acid, benzene sulfonic acid, 1-2, naphthoquinone 6-sulfonic acid and 3-methyl benzenesulfonic acid	Zhao et al. (2007)

(continued)

Table 4 (continued)

Dye	Dye class	Microorganism	Removal process	Analytical technique to monitor dye removal process	Analytical technique to study intermediate formation	Degradation product	Reference
Acid Orange 7, Acid Orange 8, Mordant Violet 5	Sulfonated phenylazo naphthol	*Pleurotus ostreatus*	Biodegradation		HPLC, capillary electrophoresis and CE-ESI-MS	Benzene sulfonic acid, 1,2 naphthoquinone	Lu et al. (2008)
Direct Violet 51	Azo dye	*Candida albicans*	Biodegradation	UV-Vis	FTIR		Vitor and Corso (2008)
Acid Blue 120	Azo	*Aspergillus lentulus*	Biosorption and Bioaccumulation	UV-Vis	FTIR		Kaushik and Malik (2010)
Amaranth	Azo	*Bjerkandera adusta*	Biodegradation	UV-Vis, enzymes assay for Peroxidase activity	HPLC, ESI-TOF-MS	Phenol, 4-nitrobenzene sulfonate	Gomi et al. (2011)
Rubine GFL	Azo	*Aspergillus ochraceus* and *Pseudomonas* sp.	Biodegradation	UV-Vis and enzyme assays (laccase, veratryl alcohol oxidase, azo reductase, NADH-DCIP reductase)	FTIR, GCMS, HPTLC		Lade et al. (2012)

(continued)

Table 4 (continued)

Dye	Dye class	Microorganism	Removal process	Analytical technique to monitor dye removal process	Analytical technique to study intermediate formation	Degradation product	Reference
Congo Red	Azo	*Alternaria alternate*	Biodegradation	UV-Vis, (SEM and light microscopy to study fungal morphology)	HPLC, FTIR		Chakraborty et al. (2013)

intermediate compounds from sulfonated azo dyes, electro spray ionization (ESI) source can be employed which causes minimal fragmentation of such dyes (Zhao et al. 2007; Lu et al. 2008; Gomi et al. 2011).

Apart from monitoring the dye removal process, the morphological changes in fungal biomass can also be a useful aid in establishing the correlation between dye removal process and the microbial agent present in the solution. Various microscopic techniques, such as light microscopy, scanning and transmission electron microscopy, may provide useful insights into the mechanism involved in the dye removal process. Biosorption or bioaccumulation process by fungal biomass can conclusively be explained through light microscopy techniques. Figure 2 shows the phase contrast micrographs of Acid Navy Blue laden pellets of *Aspergillus lentulus*. The size variation in the pellet structure, if any, resulting from various cultivation conditions, such as nutrient sources, can also be visualized through microscopy (Kaushik and Malik 2010b).

Chakraborty et al. (2013) used light microscopy to show the biosorption of Congo red dye on the biomass of *Alternaria alternate*. Also, SEM micrographs were used by Chakraborty et al. (2013) to describe the amorphous nature of the fungal biomass after dye removal process. Similarly, Transmission electron microscopy can also be utilized to examine the difference obtained in the morphological structure of the fungi prior to and after the biosorption of dye. Das et al. (2006) demonstrated with the help of TEM micrographs that the cells of *Rhizopus oryzae* in presence of Rhodamine B dye exhibit electron dense molecules mainly in

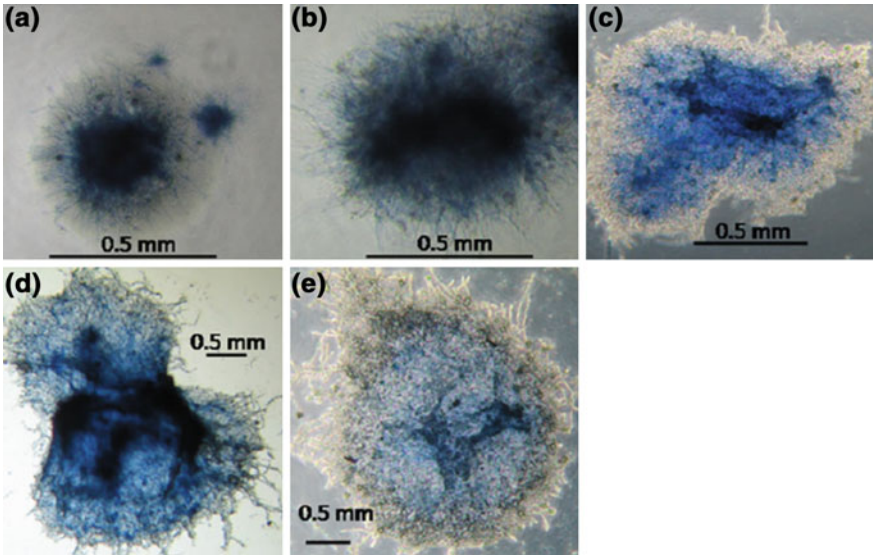

Fig. 2 Microscopic pictures of *A. lentulus* grown in different initial glucose concentrations: **a** 0 %, **b** 0.1 %, **c** 0.2 %, **d** 0.5 % and **e** 1 % on the pellet (Magnification: 10X) (Kaushik and Malik 2010b)

the region of cell surfaces, whereas these are absent in control cells. In living cells, the dye molecules accumulate in the cytoplasm as granules, whereas, in starved cells, dye molecules mainly bind on the cell surface and a very small amount is transported to the cytoplasm.

Moreover, any toxicity response, exhibited by the fungi towards the test dye, also becomes evident through these techniques. Figure 3 shows the difference in the mycelial structure of the fungus *Aspergillus lentulus* which was grown in the presence of various dyes (Kaushik and Malik 2013). The broad and flattened hyphae exhibited by *A. lentulus* in presence ofdye Methylene Blue as compared to that shown in the presence of Acid Navy Blue and in absence of any dye, shows the toxicity of Methylene Blue dye to the fungi. SEM or TEM technique coupled with Energy Dispersive X-Ray (SEM-EDX/TEM-EDX) can be a useful tool for estimating and quantifying the presence of dye molecules on/inside the fungal biomass after dye biosorption (Kaushik 2011).

Thus, it can be concluded that the analytical techniques are an important tool to study thedye removal process and utilization of these techniques in combination can provide a detailed insight into the process of dye removal, its mechanism, dye degradation pathway and study ofdegradation metabolites. This can further aid in the development of suitable reactor design/technology for the treatment dye effluents.

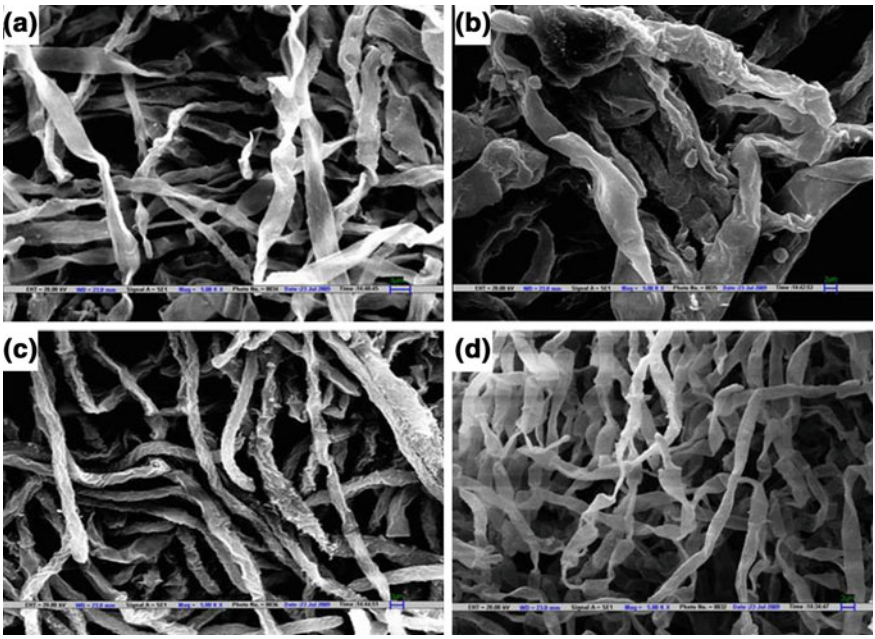

Fig. 3 SEM micrographs showing fungal pellets grown in different conditions **a** presence of Methylene Blue (10 mg l^{-1}), **b** presence of Methylene Blue (50 mg l^{-1}), **c** presence of Acid Navy Blue (100 mg l^{-1}) and **d** absence of dye (Magnification: X 5,000) (Kaushik and Malik 2013)

4 Statistical Tools Required for Process Optimization

Design of experiment (DOE) is an important tool to study any process involving multiple variables that affect it. Through this approach, a process under study is described through a mathematical model and an experimental design is created to obtain a set of experiments to collect the data to be analyzed using the model equations. DOE approach enables the researcher to optimize the conditions for maximizing the process and aids in selecting the principle factor affecting it. DOE methodologyis superior to the conventional approach of one variable at a time (OVAT) analysis, being less labour intensive and time consuming. It has an added advantage of providing the interaction studies of various variables affecting the process which is not possible through OVAT analysis. The two most widely applied tools of DOE utilized indye removal process are Taguchi method and response surface methodology (Fig. 4).

4.1 Response Surface Methodology (RSM)

RSM is a method that utilizes statistical and mathematical techniques to optimize a process in which the output or response is influenced by different factors or variables. RSM analyzes the effect of independent variables alone and in combination and generates a mathematical model (Bas and Boyaci 2007). The term independent variables refer to theexperimental variables that can be changed independently of each other. In a typical dye removal process, these variables can be pH, temperature, initial dye concentration, nutrient concentration, contact time etc. A response is defined as a dependent variable which is measured as an output of the

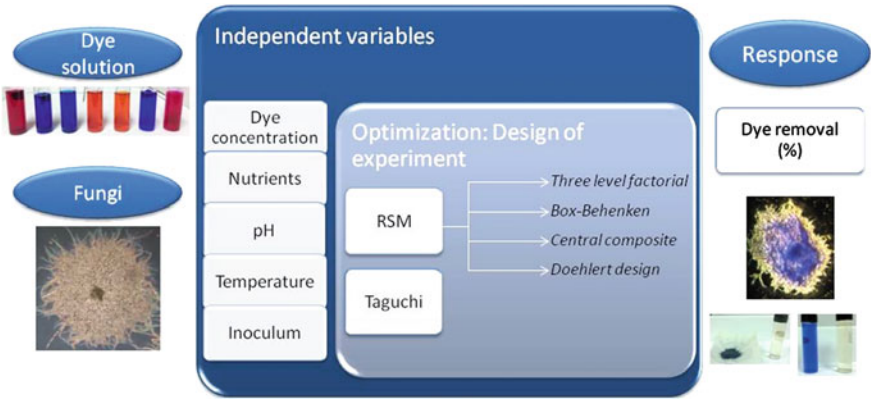

Fig. 4 Statistical approach involving design of experiment methodology for optimizing dye removal process

experiment. Percentage dye removal, residual dye concentration and biomass generated can be regarded as the response of a dye removal process. To obtain the best response value of dye removal process, the independent variables need to be optimized (Kaushik and Malik 2009). The optimization study using RSM can be divided into three stages. In the first stage, the independent variables and their levels are determined. Screening experiments are performed to determine the important parameters that influence the process of dye removal. Further, depending upon the direction in which these parameters affect the process, their levels (ranging from -1 to +1) are determined. In the second stage, the experimental design is selected and the model equation is predicted and verified. Generally a full quadratic equation (second order) is used in RSM.

$$y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum \sum_{i<j} \beta_{ij} X_i X_j$$

In the third stage, responses of surface plot and contour plot are obtained and their optimum points are determined (Bas and Boyaci 2007). Many researchers have employed RSM to optimize the dye removal process by fungi in growing mode in terms of initial dye concentration (Alam et al. 2009; Gönen and Aksu 2009a; Sharma et al. 2009; Srinivasan and Murthy 2009; Das et al. 2010), pH (Sharma et al. 2009; Qu et al. 2010), temperature (Sharma et al. 2009; Qu et al. 2010) and nutritional conditions (Gönen and Aksu 2009b; Kaushik and Malik 2011; Aghaie-Khouzani et al. 2012; Papadopoulou et al. 2013). However, certain factors must be considered while selecting RSM for biological processes. For example, it is not necessary that all the systems, that show curved graph, fit to second order polynomial and thus require to be converted to other forms, such as the log values or by changing the range of parameters (Bas and Boyaci 2007). This limits its use in those biological processes which cannot be described by a second order polynomial equation.

4.2 Taguchi Method

Taguchi method was initially developed as a tool for improving the quality inengineering methodology and obtaining a robust design (Wang et al. 2002). However, it has been also employed to optimize the condition ofdye removal process (Engin et al. 2008; Yildiz 2008). Daneshvar et al. (2007) applied Taguchi method in optimizing the process of biological degradation of Malachite Green with respect to temperature, initial pH, type of algae, dye concentration and time of reaction. Revankar and Lele (2007) optimized the fermentation medium for *Ganoderma* sp. to obtain the maximum removal of amaranth dye using Taguchi methodology.

Taguchi method of statistical optimization involves fractional factorial experimental design which is a part of total possible combinations which are required to estimate the important factors affecting the process and their interaction (Kim et al. 2004). Taguchi method utilizes orthogonal array method, the matrices of which vary with the number of factors and interactions. For instance, 8-trial orthogonal array (L-8 matrix) is used when the number of factors is less than 7 and 16-trial orthogonal array (L-16 matrix) is used when the number of factors is less than 15. Taguchi method takes into account the "signal (S)" and "noise (N)" ratio to measure the quality characteristic of the process or system which deviates from the desired value. The "signal" represents the desirable and "noise" represents the undesirable values for the output characteristics. This S/N ratio varies according to the type of characteristics and can be calculated as follow:
if nominal is the best characteristic;

$$\frac{S}{N} = 10 \log_{\overline{S_y^2}}$$

if smaller is the best characteristic;

$$\frac{S}{N} = -10 \log \frac{1}{n} \left(\sum y^2 \right)$$

and if larger is the best characteristic;

$$\frac{S}{N} = -\log \frac{1}{n} \left(\sum \frac{1}{y^2} \right)$$

where, 'Σ' is the average of observed data, 'S_y^2 ' is the variation of 'y', 'n' is the number of observations and 'y' is the observed data.

Although Taguchi method provides better graphic visualization in determining the optimal conditions, the extent of influence each factor exerts in the output of any process needs further analysis through ANOVA. Nevertheless, this method requires less data to find optimum conditions as compared to RSM. Also, since Taguchi method minimizes the experimental runs, it is recommended to use this method when the number of variables under study is large and also when the experimental time run is lengthy and costly.

Thus, it can be concluded that application of various optimization tools is desirable in a process like dye removal which is effected by multiple factors and their interaction. Thus, optimizing the conditions to obtain the best possible combination of the process parameters and nutrients, makes the process viable and economic in terms of cost, time and waste production. For example, a process optimization study as described by Kaushik and Malik (2011), resulted in 85 % cost reduction, wherein the yeast extract from the unoptimized media was replaced by

low cost nitrogen supplements (urea and ammonium chloride). Further, this resulted in higher uptake capacity of the fungal biomass (*A. lentulus*), decreasing the production of excess biomass and reducing the production of dye laden waste sludge.

5 Reactor Designs Based on Different Mechanisms

Reactor scale studies are often necessary to evaluate the efficiency of the developed technology at industrial scale. Different reactor designs have been proposed by various researchers, depending upon the principle mechanism involved in the process of dye removal. The design of the bioreactor for dye removal process basically depends on two major factors, first being the type of organism and its growth properties i.e. whether it is a unicellular or multicellular microbe; whether it exhibits mycelial or pelleted growth etc. and second being the mechanism i.e. biosorption (the microbe need not be provided with nutrient support but contact between the biosorbent and dye should be maximized), bioaccumulation (nutrient support is required along with aeration) and biodegradation (nutrient support along with optimum pH and temperature for maximal enzyme activity needs special attention). Figure 5 provides a schematic representation of packed bed reactor (often suitable for dye biosorption) developed for the removal of Violet 14R dye by the dried and powdered biomass of *A. lentulus* (Singh 2010). The height of biomass bed can be varied depending upon the contact time required for accomplishing efficient dye removal. Second most common reactor design fordye biosorption is based on the phenomenon of immobilization where the biosorbent is immobilized on an inert carrier. Gupta (2010) utilized the blocks of ceramic monoliths for the immobilization of *A. lentulus* which were then utilized for the biosorption of Acid Navy Blue dye (Fig. 6).

Fordye removal process requiring the use of living cells, Mishra and Malik (2013) have proposed the use of three most commonly used reactor designs; continuous stirred-tank reactor, expanded and packed bed reactors and airlift bioreactors (Fig. 7). All these three designs require a constant supply of nutrient and air so as to keep the cells in their live state. One such design has been demonstrated by Xin et al. (2012b) where they have utilized air lift column bioreactor to obtain the pelleted growth of the fungi for removing dye through bioaccumulation mechanism. The air lift bioreactors, promoting pelleted growth of the fungi, have an added advantage where problems related to biomass clogging are minimized resulting in higher mass transfer and dye removal. Moreover, pelleted biomass allows quick separation of dye laden biomass from the treated wastewater.

Reactor designs based on biodegradation process may utilize either the microbial biomass capable of producing the enzyme responsible for dye removal or the purified enzyme, such as LiP, laccase etc. The microbial cells may be immobilized in a suitable bioreactor design for the production of enzyme which then brings about thedecolorization. For example, Dominguez et al. (2001) studied degradation of Poly R-478 dye by three enzymes i.e. MnP, LiP and laccase produced by

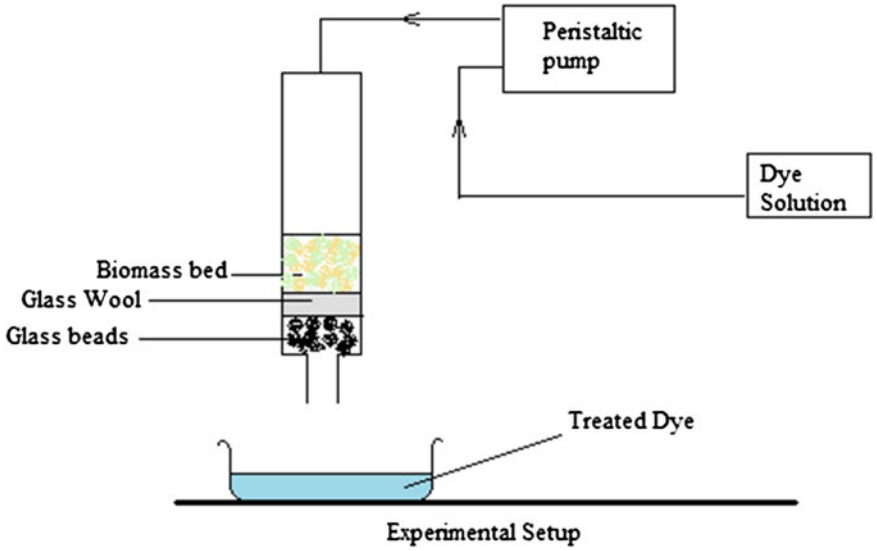

Fig. 5 Schematic representation of a packed bed bioreactor developed for the biosorption of Violet 14R dye by *A. lentulus* biomass (Singh 2010)

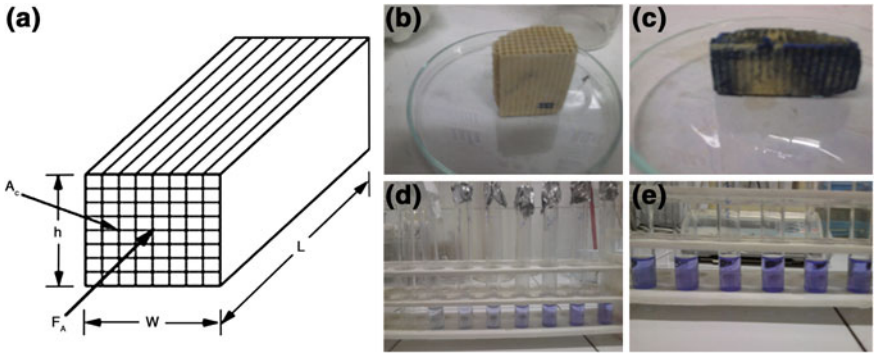

Fig. 6 Use of ceramic monoliths for the immobilization of fungi; **a** schematic representation of a monolith **b** monolith before biosorption **c** monolith with immobilized biomass of *A. lentulus* after biosorption of Acid Navy Blue **d** dye samples obtained from flask containing fungi immobilized monoliths **e** dye samples obtained from flask containing control monoliths; without immobilized biomass (Gupta 2010)

Phanerochaete chrysosporium immobilized on cubes of nylon sponge in a bioreactor based on a standard rotating drum configuration. Similar attempt was made by Kasinath et al. (2003), in which they immobilized the white rot fungus *Irpex lacteus* on polyurethane foam and pine wood to study the degradation of Remazol Brilliant Blue R dyein a packed bed bioreactor with the help of MnP and laccase enzyme.

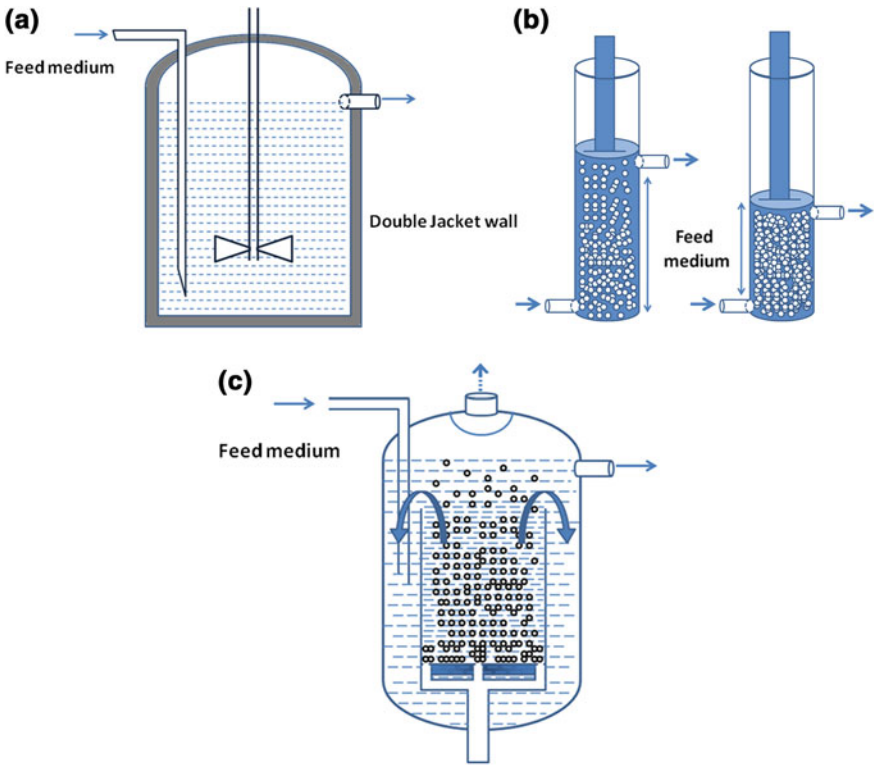

Fig. 7 Bioreactor designs: continuous stirred-tank reactor **a**; Expanded Bed and Packed Bed **b** and Airlift bioreactors **c** (Mishra and Malik 2013)

Recently, researchers have also attempted the immobilization of purified enzymes to carry out the degradation process, such as the immobilization of laccase enzyme obtained from *Myceliophthora thermophila* on Eupergit support in a packed bed bioreactor (Lloret et al. 2012).

Other than basic designs, sequential reactor configurations, employing anaerobic and aerobic conditions, have also been proposed by many researchers. Zee and Villaverde (2005), Khehra et al. (2006) have proposed the sequential bioreactors based on initial anaerobic treatment, followed by aerobic treatment, where the final degradation of the products of anaerobic cleavage occurs. However, carcinogenic aromatic amines, produced during the anaerobic degradation of azo dyes, are not effectively removed in the subsequent aerobic step, thus limiting the use of this type of sequential treatment (Mohanty et al. 2006). Reverse of this treatment process i.e. the aerobic degradation, followed by anaerobic treatment, has been proposed by Novotný et al. (2011) for wastewater containing high concentration of dye and organics. Aerobic step based on theenzymatic degradation by fungi *Irpex lacteus* in a fungal trickling filter (FTF) bioreactor, followed by anaerobic degradation in bacterial reactors, resulted in efficient decolorization in first step and marked TOC

reduction in second step. Thus, sequential bioreactors, based on combined aerobic-anaerobic treatment of dye wastewater, have a large potential. Another approach, which may be utilized for enhancing removal of dye, is the coupling of two mechanisms, such as chemical and biological method. Sudarjanto et al. (2006) integrated the chemical and biological degradation methods to degrade Reactive Azo Red 195A. For this, two reactors i.e. photoreactor and bioreactor were used in a series. Advanced oxidation of dye by UV/H_2O_2 was carried out in photoreactor, followed by aerobic biodegradation in bioreactor containing microbial biofilms. Shoabargh et al. (2013) have coupled photodegradation and enzymatic process of Acid Orange 7 dye degradation, using a rectangular recycling photo-bioreactor containing glucose oxidase (GOx) immobilized on TiO_2/polyurethane (PU).

Thus, it seems that recent technical advancements, employing combination of techniques, should be further explored so as to attain a design more suitable for dye containing effluents which can be acceptable in terms of inputs required, cost and time taken for the treatment.

6 Management of Dye Laden Fungal Biomass

The major hindrance faced after dye removal process is the disposal of dye laden microbial biomass. Only a few studies address to the problem posed by the generation of dye laden microbial slurry. Nigam et al. (2000) utilized different agricultural residues, such as wheat straw, wood chips and corn-cob shreds for the biosorption of mixture of dyes containing Cibacron Red, Remazol Navy Blue, Remazol Red, Cibacron Orange, Remazol Golden Yellow, Remazol Blue, Remazol Turquoise Blue and Remazol Black B dyes. After biosorption, the waste slurry is utilized as a substrate for solid state fermentation by two white-rot fungi; *Phanerochaete chrysosporium* and *Coriolus versicolor.* After theenzymatic degradation, the spent fermentation slurry was used as a soil conditioner. A variant approach was adopted by Kaushik et al. (2013), wherein the spent fermentation slurry, containing fungal biomass of *A. lentulus* and corn cob, was utilized for the biosorption of Acid Navy Blue dye and the disposal of dye laden slurry was accomplished through vermicomposting, resulting in the production of compost ideal for disposal in the soil. Although these studies provide encouraging results to overcome the waste management problem, still more studies are required in this regard to provide viable and sustainable options.

7 Future Perspectives

Currently biological removal of synthetic dyes is a widely researched topic. Yet, certain research gaps can still be identified which are important to be addressed for field application of the technology.

Majority of the reports available on this aspect indicate the optimum pH for fungal growth to be in acidic range as well as in mesophilic range. On the contrary, the pH of the dye effluent usually lies in the alkaline range and is released from the industry at high temperatures. Therefore, for a biological process to be effective at the industrial scale, it is important that the selected microbe should be able to grow in alkaline pH and to withstand high temperatures as well. Apart from containing a mixture of dyes, the effluent contains many other compounds, i.e. salts which can interfere with the process of dye removal. Thus, in addition to the studies with single dye solution, removal of dye mixtures and the effect of dye auxiliaries and validating the efficiency of the isolates in real effluent should be taken up.

Also, in order to ensure commercial application in remote industries, it is important to develop the seed culture in a form which can be easily transported and stored without requiring much energy inputs. Recently, attempts have been made to develop a carrier based microbial formulation for remediation of heavy metals and dyes from wastewater (Sharma 2009; Kaushik 2011; Mishra 2013).

8 Conclusion

Synthetic dyes are widely used in textile, dyeing, tanning, food and paper industries and are released into the environment through waste waters coming out from these industries. A lot of research has focused on mycoremediation of synthetic dyes which could offer an attractive decentralized system. A critical assessment of the recent studies indicates that a strong foundation of an efficient mycoremediation process can be laid by meticulously choosing fungal strains tolerant to high pH, temperature and salt concentration, and designing consortiums of such strains with variable mechanisms of dye sequestration. This could be the first step to ensure a reliable process performance. Further, the process cost, inputs, rates and dye uptake capacities can be optimized through statistical process optimization tools that help ascertain interactive effect of several process variables. Significant advancements have been made in the area of appropriate bioreactor designs which need to be testified at pilot scales. Further, integration of physico-chemical techniques with mycoremediation offers a novel approach for handling synthetic dyes. Nevertheless, more efforts must go in to address the need for user-friendly formulated products (of robust fungal strains/consortium) as well as fate of dye laden fungal sludge to establish the mycoremediation tool as eco-friendly process.

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Bacterial Enzymes and Multi-enzymatic Systems for Cleaning-up Dyes from the Environment

Sónia Mendes, Maria Paula Robalo and Lígia O. Martins

1 Introduction

Synthetic dyes are xenobiotic compounds that are being increasingly used in several industries, with special emphasis in the paper, textile and leather industries. Over 100,000 commercial dyes exist today and more than 7×10^5 tons of dyestuff is produced annually, of which $1–1.5 \times 10^5$ tons is released into the wastewaters (Rai et al. 2005). Among these, azo dyes, characterized by the presence of one or more azo groups (–N=N–), and anthraquinonic dyes represent the largest and most versatile groups. Synthetic dyes are highly visible pollutants and can hardly be removed from the effluents by conventional wastewater treatments. They are anthropogenic pollutants causing deterioration of water quality, affecting photosynthesis, decreasing dissolved oxygen levels and severely disturbing the aquatic ecosystems (Rai et al. 2005; van der Zee and Villaverde 2005). Moreover, dyes have become a health hazard as many of them and/or their breakdown products have been found to be toxic and potentially carcinogenic (Golka et al. 2004; Pinheiro et al. 2004; Schneider et al. 2004; van der Zee and Villaverde 2005; Chen 2006). Physico-chemical treatment processes, such as coagulation, precipitation, filtration, adsorption, photolysis and oxidation with hydrogen peroxide or ozone, can generate a large volume of sludge and usually require the addition of other

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environmental hazardous chemical additives (Forgacs et al. 2004; Chen 2006). Biological treatment technologies are attractive alternatives to the traditional physicochemical methods, as they are low-cost, environmentally friendly and can selectively provide a complete degradation of organic pollutants without collateral destruction of either the site's flora or fauna (Anjaneyulu et al. 2005; Chen 2006; Husain 2006; Rodriguez Couto 2009a). It has been demonstrated that microorganisms are able to degrade synthetic dyes to non-colored compounds or even mineralize them completely under certain environmental conditions (dos Santos et al. 2007; Saratale et al. 2011; Solís et al. 2012; Chengalroyen and Dabbs 2013; Khan et al. 2013). However, the fact that most of dye pollutants persist for long periods in the environment indicates the natural inadequacy of microbial activity to deal with these xenobiotic compounds. Biological systems need to exhibit not only a high catalytic versatility towards the degradation of a complex mixture of structurally different dyes, but also a superior robustness against the toxic effects of the dyes and their products, in addition to the salts, detergents, surfactants, and metals present in the dye-containing effluents, often at extreme pHs or high temperatures (Anjaneyulu et al. 2005; Chen 2006). Considering these requirements, there is currently no simple solution for the biological treatment of dye-containing effluents.

Enzymatic processes are particularly sought for the treatment of dye-containing effluents, mainly because of their specificity and relatively ease of engineering towards improved robustness; enzymes only "attack" the dye molecules, while valuable dyeing additives or fibers are kept intact and can potentially be re-used (Kandelbauer and Guebitz 2005). Likewise, new recycling technologies will allow a huge reduction in water consumption in the textile finishing industry. Although dye molecules display high structural diversity, they are only degraded by a few enzymes that share common mechanistic features as they all catalyze redox reactions and, exhibit relatively wide substrate specificities. The most important dye degrading enzymes are: azoreductases, laccases and peroxidases (Kandelbauer and Guebitz 2005). Azoreductases are oxidoreductases, which are particularly effective in the degradation of azo dyes through reduction of the azo linkage, the chromophoric group of azo dyes (Kandelbauer and Guebitz 2005; Rodriguez Couto 2009b). The majority of characterized azoreductases are FMN or FAD dependent enzymes that require the addition of NAD(P)H as electron donors for the reduction of azo dyes releasing aromatic amines as products (Stolz 2001; Deller et al. 2008). Laccases are multi-copper oxidases that couple the one-electron oxidation of four substrate molecules to the four electron reductive cleavage of the O–O bond of dioxygen to water. These enzymes have a great potential in various biotechnological processes mainly due to their high non-specific oxidation capacity, the lack of requirement for cofactors, and the use of the readily available molecular oxygen as an electron acceptor (Stoj and Kosman 2005; Morozova et al. 2007; Haritash and Kaushik 2009; Mikolasch and Schauer 2009). These include the detoxification of industrial effluents (Rodriguez Couto and Toca Herrera 2006), mostly from the paper and pulp, textile and petrochemical industries, and bioremediation to clean up herbicides, pesticides and certain explosives in soil (Morozova et al. 2007; Haritash

and Kaushik 2009). The capacity of laccases to produce polymeric products also makes them a useful tool for organic synthesis (Riva 2006; Madhavi and Lele 2009) and in addition, are also potential enzymes for biosensors or biofuel cells (Wheeldon et al. 2008; Willner et al. 2009). Peroxidases are heme-containing proteins that use hydrogen peroxide (H_2O_2) or organic hydroperoxides (R-OOH) as electron accepting co-substrates while oxidizing a variety of compounds. Due to their catalytic versatility and enzymatic stability, peroxidases are of particular interest for industrial redox conversion processes (Hofrichter et al. 2010). Among peroxidases, a new super family has arisen, the so-called dye-decolorizing peroxidases (DyPs) (Sugano 2009; Hofrichter et al. 2010; Colpa et al. 2013). These enzymes are known to successfully oxidize a wide range of substrates, but most importantly, they highly degrade high redox synthetic dyes, such as anthraquinone and azo dyes. In this paper, we have reviewed the enzymatic properties, mechanisms and toxicity of dye-degradation products of different bacterial enzymes and also the properties of in vitro and in vivo multi-enzymatic systems for the decolorization of synthetic dyes.

2 Biotransformation of Dyes Using Laccases

Laccases are a part of the large multi-copper oxidase family of enzymes that catalyze the four-electron reduction of oxygen to water (at the T2–T3 trinuclear Cu centre) by the sequential one-electron uptake from a suitable reducing substrate (at the T1 mononuclear copper centre) (Solomon et al. 1996; Stoj and Kosman 2005). Most of the known laccases have fungal (e.g. white-rot fungi) or plant origins. However, many laccases have been isolated from bacteria in the last decade (Claus 2003; Giardina et al. 2010). Fungal laccases are the enzymes used in the vast majority of the studies in the literature, but bacterial laccases show advantages for biotechnological processes due to the lack of post-translational modifications, their higher yields of production, easiness of manipulation and improvement by protein engineering approaches.

2.1 Decolorization Capacity of Bacterial CotA-Laccase

The first study, using bacterial laccases for synthetic dyes decolorization, was performed with recombinant CotA-laccase from *Bacillus subtilis*, which is a bacterial thermoactive and intrinsically thermostable enzyme (with half-life of 2 h at 80 °C), showing the predictable robustness for biotechnological applications (Pereira et al. 2009a, b). Twenty two synthetic dyes, both anthraquinonic and azo dyes, were found to be degraded to different extents, after 24 h of reaction by CotA-laccase (Fig. 1).

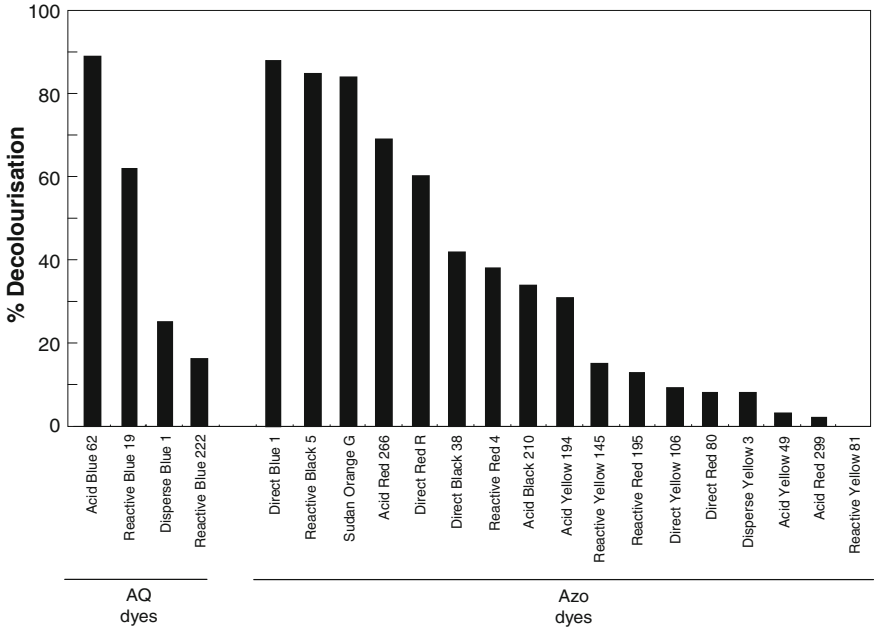

Fig. 1 Decolorization of several of anthraquinonic (AQ) and azo dyes after 24 h of reaction in the absence of redox mediators by using CotA-laccase (adapted from Pereira et al. 2009b)

Two major differences were observed when compared to dye degradation using fungal laccases: (1) the non requirement of redox mediators and (2) a maximal activity at the neutral to alkaline range of pH. The lack of a strict requirement for redox mediators exhibited by bacterial CotA-laccase constitutes a significant advantage over fungal enzymes from a technological perspective. Low-molecular weight compounds are expensive and a large quantity in relation to the substrate is often required. Moreover, some mediators give rise to highly unstable compounds that can lead to enzyme inactivation and are toxic upon release into natural environments. The requirement of redox mediators, acting as electron shuttles, is usually justified to overcome the steric hindrance of substrates that impairs its proper approach to the enzyme's catalytic center or the high redox potential of the substrates in comparison to the enzyme (Bourbonnais and Paice 1990). Interestingly, it was observed that CotA, a low redox laccase (E^o = 525 mV vs. NHE), is able to degrade high redox compounds, e.g. the azo dye reactive black 5 (E^o = 742 mV) to a higher extent in the absence of redox mediators, in contrast to what was observed with high-redox potential fungal laccases (E^o ~780 mV vs. NHE) which requires the presence of redox mediators (Abadulla et al. 2000; Zille et al. 2004; Camarero et al. 2005; Tauber et al. 2005). This indicates that redox potential is not the only or the most important parameter to be considered in what concerns substrate oxidation by laccases (Durão et al. 2006).

The optimal pH for dye-decolorization by CotA-laccase around 8–9 is a distinctive feature shared with other bacterial laccases from *Streptomyces ipomoea* (Molina-Guijarro et al. 2009), *Bacillus vallismortis* (Zhang et al. 2012) or *Bacillus subtilis* X1 (Guan et al. 2013) which is in contrast with the optimal pH values in the acidic range shown by laccases of fungal origin (Abadulla et al. 2000; Almansa et al. 2004; Maier et al. 2004; Camarero et al. 2005; Rodriguez Couto et al. 2005; Zille et al. 2005a, b; Pogni et al. 2007). In order to explore the enzymatic mechanism of azo dyes degradation, Sudan orange G (SOG) was selected for more detailed investigations. Two pKa values for SOG were measured using potentiometric measurements, 6.90 ± 0.02 and 11.74 ± 0.02, which were attributed to *ortho* and *para* hydroxyl groups of the azo dye (Pereira et al. 2009b). Based on this data, the oxidation of SOG is mostly dependent on the protonation-deprotonation equilibrium of the more acidic hydroxyl group of the substrate molecule, since maximal rates are found at pH 8, above the pKa value of the *ortho* hydroxyl group of SOG. This is in contrast with fungal laccases, which, in agreement with their optimal pH at acidic ranges, oxidise more easily the protonated form of the dye. The results obtained with CotA for the oxidation of SOG are consistent with data obtained using syringyl-type phenolic compounds, where maximal enzymatic rates were also observed at pH values above the pKa value of the compounds tested which confirmed the preference of CotA for deprotonated phenolic groups (Rosado et al. 2012). The differences in the optimal pH, as observed in bacterial and fungal laccases, are most probably related to the presence of a conserved negatively charged residue close to the substrate binding cavity of fungal laccases and absent in CotA or in any bacterial laccase identified so far and proposed to stabilize the formation of the phenoxy radical during the catalytic reaction of fungal laccases (Bertrand et al. 2002; Piontek et al. 2002; Madzak et al. 2006; Kallio et al. 2009; Rosado et al. 2012). Therefore, the oxidation of phenolic groups by bacterial laccases without any carboxylic acid residue in the substrate binding site is strictly dependent on the chemical nature of the substrates i.e. maximal rates are found at pH values above the pKa values, when phenolate anions, which are more prone to oxidation than the phenol form, are present at higher concentrations.

2.2 Azo Dyes Biotransformation

The transformation of SOG (Pereira et al. 2009a) resulted in a decrease in the intensity of the dye absorption band, at λ_{max} = 430 nm, with concomitant increase in absorption bands at 325 and 530 nm, indicating the generation of biotransformation products (Fig. 2a).

The time course of SOG biotransformation was additionally monitored by HPLC (Fig. 2b), where SOG was chromatographically separated from products of the enzymatic reaction. A major peak with R_t of 5 min, corresponding to the substrate which decreased over the time course of the reaction and disappeared after 7 h

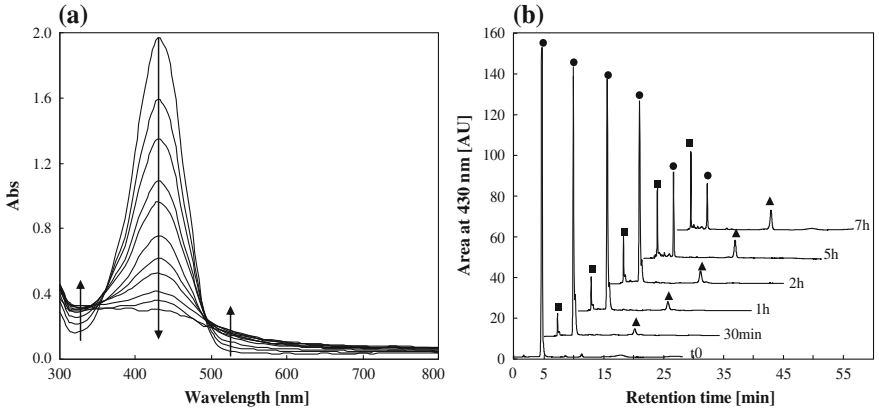

Fig. 2 Time course for Sudan orange G (SOG) biotransformation as monitored by absorbance (**a**) and by HPLC (**b**). [(**b**) *black circle* SOG and products: *black square* R_t 2.2 min and, *black triangle* R_t 15 min] (Pereira et al. 2009b)

(Fig. 2b). Two major products emerged at R_t of 2.2 and 15 min. The CotA efficiency of 8×10^3 M^{-1} s^{-1}, is in the same order of magnitude to those calculated for fungal laccases (Pereira et al. 2009b). The assay mixtures became browner in color over the course of reaction, presumably due to formation of products (Fig. 3). After centrifugation the final reaction mixture, the supernatant contained the compounds corresponding to the major peak with R_t of 2.2 min and the pellet contained the major product with R_t of 15 min. The full identification of this latter fraction was impaired by its low solubility in several solvents: acetone, ethanol, methanol, chloroform, dichloromethane, ethyl ether, toluene, hexane and tetrahydrofuran. A partial solubility (25 %) was found on acetonitrile and thus, the identification of products was performed only in the soluble part of acetonitrile-dissolved fraction. The structural identification of twelve SOG biotransformation products (Fig. 4b) was based on ESI-MS and MALDI-TOF MS data in combination with a putative degradation pathway (Fig. 4a) based on the accepted model for azo dye degradation by laccases (Chivukula and Renganathan 1995; Zille et al. 2005a, b).

Our results indicate that the enzymatic electron transfer occurs upon oxidation of SOG deprotonated hydroxyl group. The one-electron oxidation of SOG molecule by the enzyme results, therefore, in the formation of unstable radical molecules and in the concomitant destruction of dye chromophoric structure in accordance with previous reports (Chivukula and Renganathan 1995; Zille et al. 2005a, b). In addition, the presence of these products can undergo coupling reactions between themselves or with unreacted dye molecules, producing a large array of oligomeric products (Fig. 4b). The presence of these compounds is in accordance with the darkening of the enzymatic reactions, the high insolubility of products formed, and also with the reduced toxicity of the final reaction mixture as compared to solutions of intact SOG which was tested using a yeast-based bioassay (Pereira et al. 2009b).

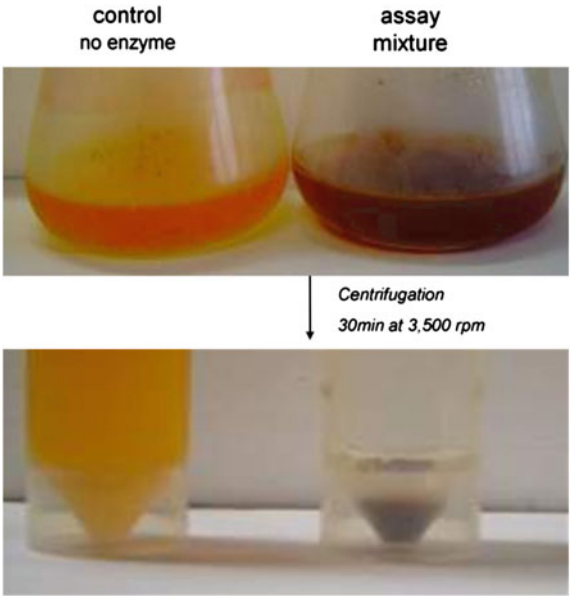

Fig. 3 Reaction mixtures: control and in the presence of enzyme after 24 h, showing the darkening of the enzymatic treated solution, most likely due to the high insolubility of the oligomeric products formed

2.3 Anthraquinonic Dyes Biotransformation

The transformation of the anthraquinonic model dye acid blue 62 (AB62) was carried out using CotA-laccase (Pereira et al. 2009b, Fig. 5a) or Lac3 from *Trametes* sp. C30 following the research initiated in Sophie Vanhulle group (Trovaslet et al. 2007; Vanhulle et al. 2008a, b). The degradation of AB62 resulted in a decrease in the intensity of the dye absorption bands, at λ_{max} = 600 and 630 nm, along with an increase in absorption around 500 nm due to the formation of reddish biotransformation products (Vanhulle et al. 2008a).

The time course of the biotransformation of AB62 was additionally monitored by HPLC (Fig. 5b). The AB62 biotransformation resulted in a product with a R_t of 13 min that appeared in the first minutes of reaction, although, as the reaction proceeded, it decreased concomitantly with the appearance of a new product with R_t of 50 min (Fig. 5b). The CotA steady-state the catalytic efficiency (k_{cat}/K_m) for AB62 oxidation is 5×10^3 M^{-1} s^{-1} around 2–3 fold lower when compared to other fungal laccases, including Lac3 from *Trametes* sp. C30 (Klonowska et al. 2002, 2005; Vanhulle et al. 2008a). The biotransformation products were identified after purification in the enzymatic reaction mixtures by NMR, MS/MS^n, LC-MS and GC-MS analysis. Using ^1H NMR and MS/MS^n was possible to identify the intermediate product DAAS (R_t = 13 min) and the final product of the reaction (4)

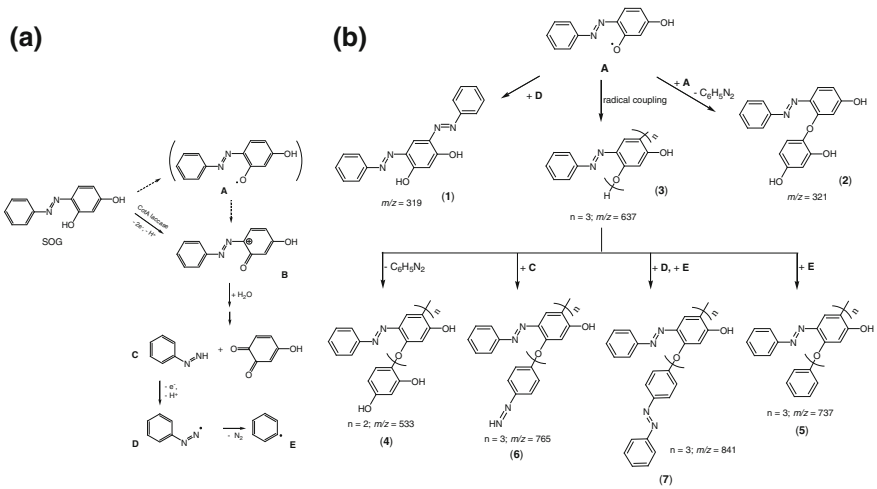

Fig. 4 Proposed mechanism for the biotransformation of SOG by CotA-laccase (**a**) and proposed structures (1)–(7) for the oxidation products (**b**). The oxidation of azo dyes occurs without the cleavage of the azo bond, through a highly non-specific free radical mechanism resulting in the formation of phenolic type compounds. Following this mechanism, CotA-laccase oxidizes one hydroxyl group of SOG generating the phenoxyl radical A, sequentially oxidized to a carbonium ion (B). The water nucleophilic attack on the phenolic carbonium, followed by N–C bond cleavage, produces diazenylbenzene (C) and the 4-hydroxy-1,2-benzoquinone. The diazenylbenzene (C) can lead to the radical (D) and then, to a benzene radical (E) upon loss of a nitrogen molecule. All these radicals were involved in further coupling reactions (adapted from Pereira et al. 2009b)

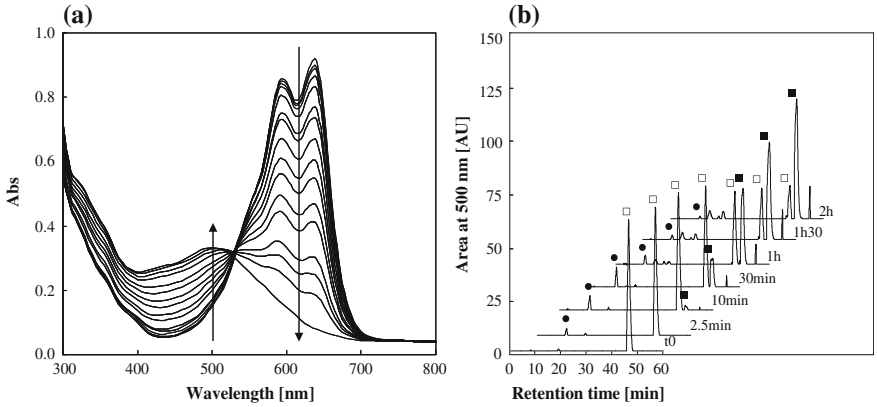

Fig. 5 Time course for acid blue 62 (AB62) biotransformation as monitored by absorbance (**a**) and by HPLC (**b**). [(**b**) *white square* AB62 and products: *black circle* R_t 13 min and, *black square* R_t 40 min] (adapted from Pereira et al. 2009a)

(R_t = 50 min) (Fig. 5b). The proposed mechanism of biotransformation of AB62 by laccases is illustrated in Fig. 6, showing the pathway for formation of an azo bound in (4) which is responsible for the color observed in the reaction mixtures.

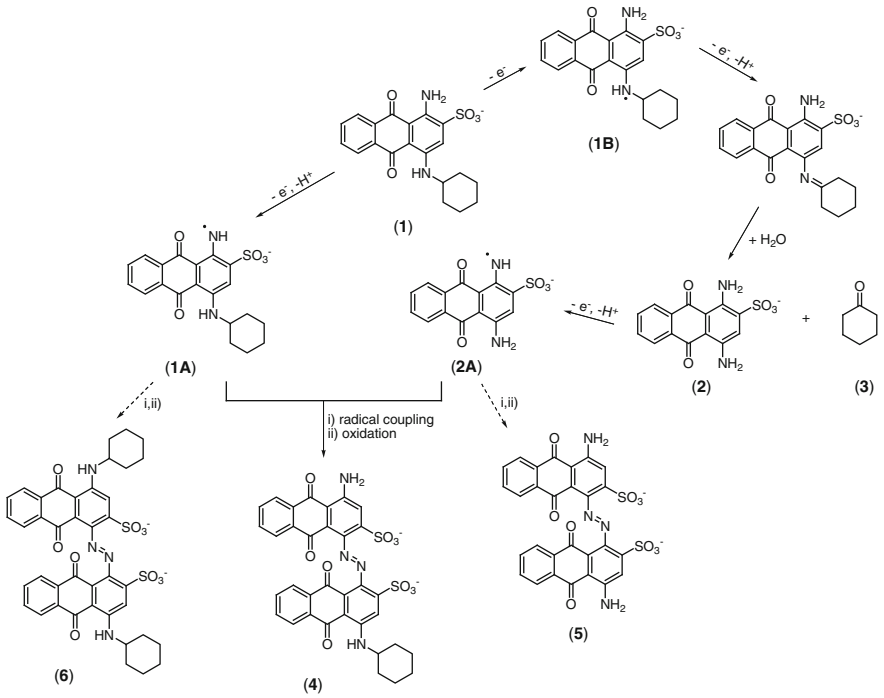

Fig. 6 Proposed mechanism of AB62 biotransformation by laccases. Two oxidative routes are possible, since laccases are able to catalyze a single-electron oxidation, either from the primary or the secondary amines of the compound 1 (AB62). The reactive radical species 1A and 1B are formed, and 1B was sequentially oxidized into an imine, which hydrolyzes leading to cyclohexanone (3) and the first intermediate 2 (DAAS). Compound 2 could be further oxidized and the resulted radical (2A) should lead to the formation of the main product of the reaction, the azo dimer (4) by cross-coupling reaction with 1A, followed by an oxidative step that could also be catalyzed by the enzyme. Similar dimerization processes of radicals 2A or 1A should end in the formation of compounds 5 and 6, but in a very low extent. The final product 4 was identified by both NMR and MS techniques and the formation of compounds 5 and 6 was supported by LC-MS^n analysis of the reaction mixtures (adapted from Pereira et al. 2009a)

The toxicity of synthetic dyes as well as of their bioconversion products presents a great environmental concern (O'Neil et al. 1999; Robinson et al. 2001). AB62 causes a significant inhibitory effect on yeast growth and values of LOEC and IC_{50} of around 7 and 420 μM (3 and 177 mg L^{-1}) respectively, were estimated. The IC_{50} is well above the expected dye concentrations in the environment, but is within the same order of magnitude of the typical dye concentration in spent dye baths (Robinson et al. 2001). The mixture, containing AB62 biotransformation products after 2 h of reaction with CotA-laccase, was significantly less toxic to the yeast cell population. Consistent with the reduced overall toxicity of AB62 solution, compound 4, the reddish azo product that accumulates during the biotransformation reaction, is significantly less toxic to the yeast (LOEC ~45 μM, $IC_{50>}$750 μM) than the parent molecule.

3 Biotransformation by Bacterial Azoreductases

Azoreductases is a generic name given to enzymes involved in the reduction of azo bonds (–N=N–) and azoreductase activity was identified in several organisms including algae, yeast and bacteria (Saratale et al. 2011; Solís et al. 2012; Chengalroyen and Dabbs 2013; Khan et al. 2013). These enzymes are flavin-independent or flavin-dependent oxidoreductases which utilize NADH and/or NADPH as an electron donor and catalyze the reductive cleavage of the azo bonds to produce colorless aromatic amine products under anaerobic or aerobic conditions. Flavin-dependent azoreductases share strong similarities with regard to sequence, structure, and reaction mechanism with the larger family of flavin-dependent quinone reductases that include Lot6p from *Saccharomyces cerevisiae* and the mammalian NQO1 (Deller et al. 2008). These enzymes are involved in the reduction of quinones, quinoneimines, azo dyes, and nitro groups to protect the cells against the toxic effects of free radicals and reactive oxygen species arising from electron reductions. They are assumed to take part in the organism's enzymatic detoxification systems; e.g., the azoreductases from *E. coli* and *B. subtilis* were recently implicated in the cellular response to thiol-specific stress (Towe et al. 2007; Leelakriangsak et al. 2008; Liu et al. 2009) and Lot6p, the azoreductase homologue in *S. cerevisiae* has been implicated in the response to oxidative stress (Sollner et al. 2007, 2009). Furthermore, as additional members of this family of enzymes are discovered, the list of transformed substrates continues to grow. Evolutionarily, these enzymes may provide a selective advantage to bacteria under various conditions of environmental stress (Khersonsky and Tawfik 2010).

3.1 Decolorization of Azo Dyes by PpAzoR from Pseudomona putida *MET94*

In an effort to find bacterial strains with a superior ability to degrade synthetic dyes, a collection of 48 bacterial strains was screened to select the strain *P. putida* MET94 for its superior ability to decolorize a diverse array of azo dyes to higher extent (Mendes et al. 2011b) (Fig. 7).

A BLAST search of the *P. putida* genome was performed and a 612-bp ORF encoding a 203 amino acid residue was identified containing all the conserved motif patterns of flavin-dependent azoreductases (Wang et al. 2007) and was, therefore, named PpAzoR (*Pseudomonas putida* azoreductase). The *ppAzoR* gene was cloned and expressed in *E. coli*. Subsequently, the recombinant FMN-dependent PpAzoR protein was purified and thoroughly characterized following kinetic, spectroscopic and biochemical and structural approaches (Correia et al. 2011; Mendes et al. 2011b; Gonçalves et al. 2013). It was observed that PpAzoR reduced several quinones (anthraquinone-2-sulfonic acid (AQS), 1,4-benzoquinone, catechol, 2-hydroxy-1, 4-naphtoquinone (Lawsone), 1,2-naphthoquinone) at rates 10–100 times higher than

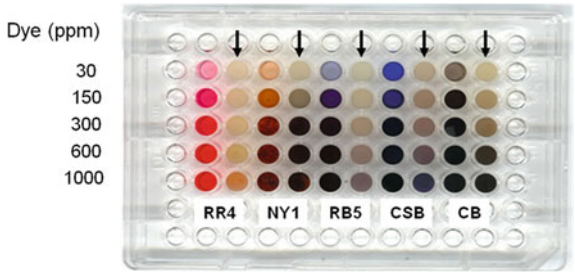

Fig. 7 Screening for decolorization of reactive red 4 (RR4), acid red 299 (NY1), reactive black 5 (RB5), direct blue 1 (CSB) and direct black 38 (CB) at increasing concentrations using growing cells of *P. putida* MET94

azo dyes (Mendes et al. 2011b; Brissos et al. 2014). The steady-state kinetic analysis, using 1,4-benzoquinone or reactive black 5 and NADPH, resulted in a family of parallel lines in a double reciprocal plot (Mendes et al. 2011b; Gonçalves et al. 2013) which is indicative of a ping-pong bi-bi kinetics as described for other flavin-dependent azoreductases. The efficiency for 1,4-benzoquinone is one and two orders of magnitude higher than azo dyes (V_{max} = 50 U mg^{-1}, $K_{m\ app}$ = 0.005 mM, $k_{cat\ app}$ = 49 min^{-1}, k_{cat}/K_m = 98 × 10^5), showing that quinones represent most probably the physiological substrates of this enzyme in *P. putida* cells.

PpAzoR (PDB code 4C0 W) is a homodimer and its tertiary structure adopts a flavodoxin-like fold characterized by a central twisted five parallel β-sheet connected by α-helices, which flank the sheet from the front and the back (Correia et al. 2011; Gonçalves et al. 2013). The arrangement of the α-helices and β-stands is identical to structures of azoreductases from *E. coli* (PDB code 2Z98), *Pseudomonas aeruginosa* (PDB code 2V9C), *Enterococcus feacalis* (PDB code 2HPV) and *Salmonella typhimurium* (PDB code 1T5B). Moreover, it contains the conserved motif patterns of flavin-dependent azoreductases, i.e. the sequence involved in the binding of FAD/FMN co-factors, the sequence involved in the dimerisation of the two monomers of the enzyme and the possible putative NAD(P)H binding motif. The crystal structures of native PpAzoR (1.6 Å) and PpAzoR complexed with anthraquinone-2-sulphonate (1.5 Å) or reactive black 5 (1.9 Å), were solved revealing the residues and subtle changes that accompany substrate binding and release. Such changes highlight the fine control of access to the catalytic site and tune the specificity offered by the enzyme towards different substrates. In particular, it helps to explain how PpAzoR allows for the accommodation of bulky substrates explaining its enlarged substrate utilization with similar catalytic efficiencies (Gonçalves et al. 2013).

The enzymatic activity of PpAzoR was tested using 18 structurally different synthetic dyes by measuring the decolorization levels after 24 h of incubation under anaerobic conditions. The results show that PpAzoR exhibits a broad substrate specificity with decolorization levels above 80 % for most of the dyes tested (Fig. 8).

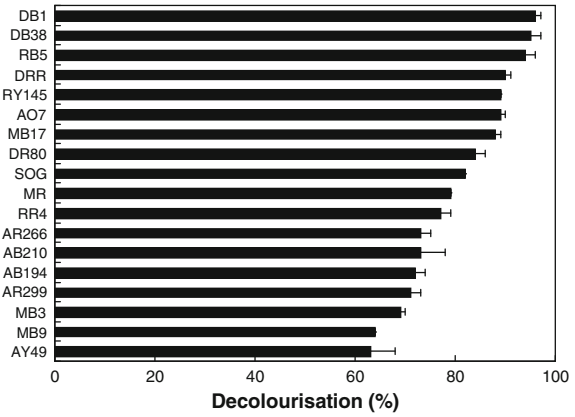

Fig. 8 Decolorization of several azo dyes after 24 h of reaction under anaerobic conditions using PpAzoR (Mendes et al. 2011a)

The specificity of PpAzoR was investigated by measuring the initial rates of reduction of a set of structurally different azo dyes under anaerobic conditions (Mendes et al. 2011b). PpAzoR uses either NADPH or NADH as electron donor, but the efficiency for NADPH is twice that of NADH (Gonçalves et al. 2013). The enzyme is particularly unspecific with regard to the azo dyes used, showing only smooth trends with methyl red and reactive black 5 representing the substrates reduced with higher specificity (around 1–2.5 × 10^3 M^{-1} s^{-1}) and mordant black 9 and acid orange 7 reduced at the lowest efficiency (around 0.3–0.4 × 10^3 M^{-1} s^{-1}). The affinity for dyes is reduced with K_m values between 0.1 and 4 mM indicating the need of adding 1–40 mM of dyes (10 × the K_m value) to the reaction mixtures in order to accurately measure the maximal rates of dye degradation (Mendes et al. 2011b). The high concentration of dyes leads to initial absorbance values out of the Lambert-Beer law's applicability range. Therefore, the reaction assays to determine the kinetic parameters for dye consumption need to be performed using a photometric discontinuous method, where samples are withdrawn from reactions at time intervals, diluted and the absorbance measured at the maximum wavelength for each substrate.

To further characterize the properties of PpAzoR, in particular the oxygen-sensitivity of PpAzoR to oxygen, the initial rate of reactive black 5 degradation was measured as a function of oxygen concentration (Fig. 9a). The results show that the rates of dye decolorization decreased with increased O_2 concentration. This is in conformity with the low levels of dye decolorization by growing or resting cells of *P. putida* MET94 cells under aerobic conditions. In order to test if oxygen is a non-competitive, competitive inhibitor or instead is substrate for the PpAzoR enzyme, oxygen consumption was measured in a reaction containing only enzyme, buffer and NADPH (Fig. 9b). The addition of catalase resulted in a 2-fold increase in the concentration of dioxygen in the mixture, showing that peroxide is in the solution most likely as a result of PpAzoR activity (Fig. 9b). When catalase was added at the beginning of the reaction, only half of the possible concentration of oxygen produced was attained (Fig. 9b). These results clearly show that oxygen is reduced to peroxide by PpAzoR.

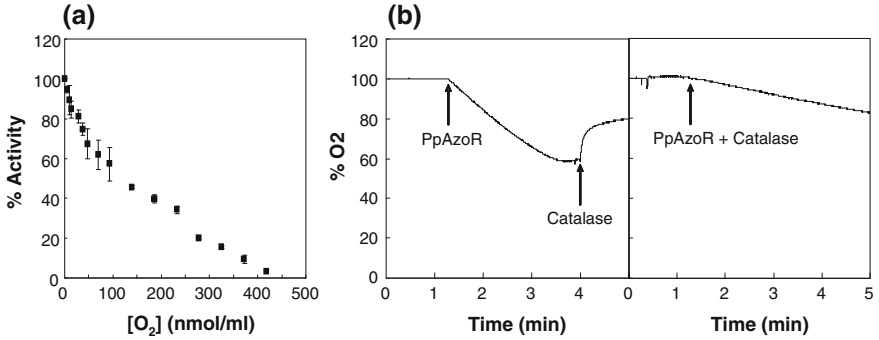

Fig. 9 **a** Inhibition of dye decolorization by increasing concentrations of dioxygen. **b** Consumption of oxygen with an "Oxygraph" equipped with a Clark oxygen electrode. The chamber volume (1 mL) contained 0.25 mM of NADPH in 20 mM Tris-HCl, pH 7.6 buffer. Reaction was initiated by the addition of 3.5 µM PpAzoR and 1,500 Units of catalase (Sigma) was added at different time periods, as indicated by the arrows

The steady state kinetics of PpAzoR for oxygen reduction (V_{max} = 5 U mg^{-1}, $K_{m\ app}$ = 0.1 mM, $k_{cat\ app}$ = 238 min^{-1}, k_{cat}/K_m = 7 × 10^4 M^{-1}.s^{-1}) showing higher affinity and one order of magnitude higher specifity as compared to dyes reduction which explains the competitive catalytic behaviour of oxygen and thus, the "oxygen sensitive" character of PpAzoR and the need to perform the decolorization of dyes under anaerobic conditions.

3.2 The Catalytic Mechanism of PpAzoR and the Toxicity of Dye Products

The ping-pong bi–bi mechanism is indicative that PpAzoR reduces the substrates in 2 distinct steps: first, a complete reduction of the FMN co-factor; and second, transfer of these electrons from the flavin to the substrate, resulting in the formation of the corresponding putative hydrazo derivatives. This reaction cycle proceeds a second time and delivers the necessary 4 electrons in order to obtain a complete reduction of the substrates into the final products. The mechanism of azo reduction by PpAzoR was supported by the detection of aromatic amines by HPLC (Fig. 10).

The reported high toxicity of the azoreductase products relates to the toxic nature of the aromatic amines formed (Pinheiro et al. 2004). Therefore, the toxicity of the azo dyes and PpAzoR enzymatic products was tested based on the inhibitory effects on the growth of *Saccharomyces cerevisiae* and on the reproduction inhibition of *Caenorhabditis elegans* (Mendes et al. 2011a). In general, the toxicity of intact dyes correlates well between the 2 model eukaryotic organisms tested (Fig. 11). Nevertheless, *C. elegans* seems more susceptible to the presence of intact dyes, since some dyes show more than 2-fold higher inhibition for the nematodes reproduction than for *S. cerevisiae* growth.

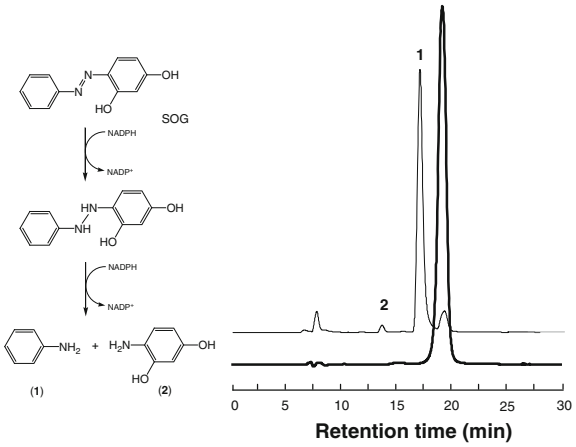

Fig. 10 Proposed mechanisms for the biotransformation of the azo dye Sudan orange G by PpAzoR and HPLC chromatograms of the reaction mixture after 24 h of reaction with (*thin line*) or without (*thick line*) PpAzoR. Products of the reaction were identified, in comparison to the standards: (1) aniline, (2) 4-aminoresorcinol

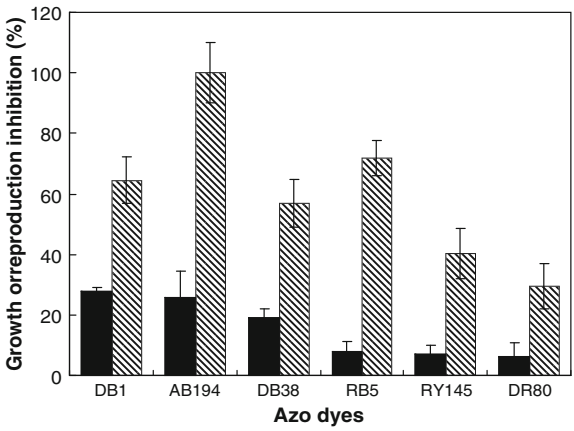

Fig. 11 Inhibitory effects of intact dyes over *Saccharomyces cerevisiae* (*dark bars*) and *Caenorhabditis elegans* (*dashed bars*) (adapted from Mendes et al. 2011a)

However, for the majority of the other dyes tested, the enzymatic products present a higher toxicity than intact dyes themselves, as assessed by the *S. cerevisiae* system, exhibiting 2 to 4-fold higher toxicity than intact dyes (Fig. 12) (Mendes et al. 2011a).

3.3 Engineering of PpAzoR for Improved Thermal Stability

PpAzoR broad substrate specificity makes it attractive for bioremediation processes, but its low thermal stability (half life of 13 min at 50 °C) impairs its full potential for environmental related applications. Thermal stability is a critical property, as it

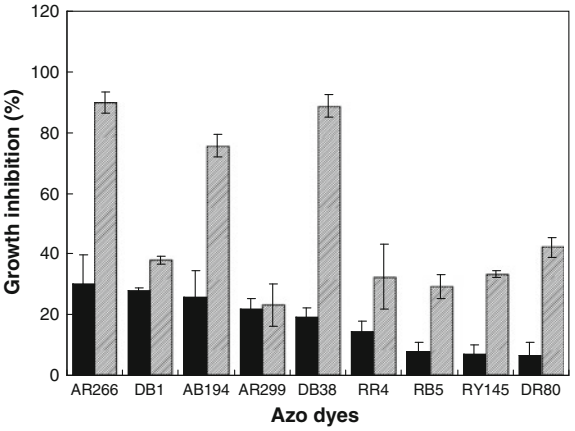

Fig. 12 Inhibitory effects of intact dyes on *Saccharomyces cerevisiae* (*dark bars*) and of 24 h-reaction mixtures treated with PpAzoR (*dashed bars*) (adapted from Mendes et al. 2011a)

correlates with longer life-times of enzymes and frequently relates also to higher tolerance to the presence of organic co-solvents, extreme pH values and high salt concentration or pressures, harsh conditions frequently found in industrial processes. Therefore, thermostable PpAzoR variants were generated by directed evolution (Brissos et al. 2014). Directed evolution is considered to be the most powerful approach for improving the thermostability of proteins. Different properties in various target enzymes have been successfully improved using directed evolution approaches (Kaur and Sharma 2006; Böttcher and Bornscheuer 2010; Wang et al. 2012). After five rounds of random mutagenesis, recombination and high-throughput screening, a thermostable 1B6 variant was identified. Noteworthy purified 1B6 variant enzyme maintains its full activity after incubation for 1 h at temperatures between 40 and 85 °C in clear contrast with the wild type enzyme that totally looses activity after 1 h at 50–55 °C (Fig. 13a). The kinetic or, the so called, long term stability was measured, showing that 1B6 is remarkably more stable than wild type with nearly a 300-fold higher half-life, i.e. retaining 50 % of activity after 58 h at 50 °C, while wild type enzyme takes 13 min to lose half of its initial activity (Fig. 13b). Therefore, a hit variant of PpAzoR was identified with increased resistance to inactivation, showing full reversibility of the unfolded state upon thermal inactivation i.e. it could be maintained at high temperatures for prolonged periods of time without losing its ability to be active at lower temperatures with an encouraging potential for biotechnological applications.

4 Biotransformation of Dyes Using Bacterial Dye-Decolorizing Peroxidases

Heme peroxidases catalyse the H_2O_2-dependent oxidation of a variety of substrates, most commonly small organic substrates, playing multiple physiological roles in a wide range of living organisms. Considering their broad specificity, these enzymes

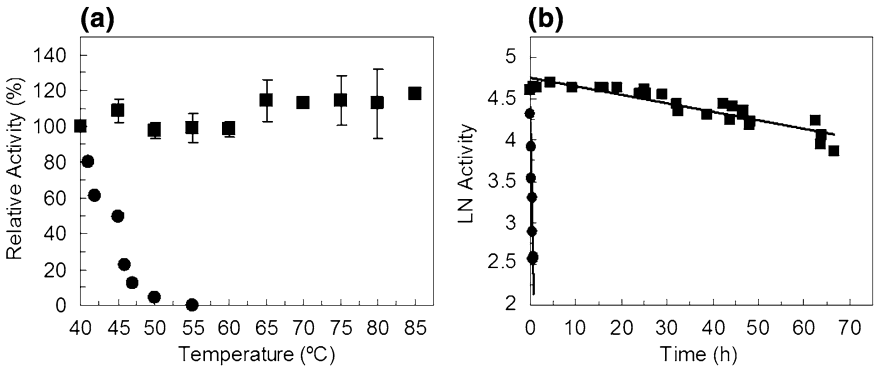

Fig. 13 **a** PpAzoR activity measured at 30 °C after incubation at different temperature (55–70 °C) for 1 h: wild type PpAzoR (*circles*) and 1B6 variant (*squares*). **b** Thermal inactivation of wild type PpAzoR (*circles*) and 1B6 variant (*squares*). Enzyme samples were incubated at 50 °C and catalytic activity was measured at known time intervals at 30 °C (adapted from Brissos et al. 2014)

have a considerable potential for application in many different areas. In particular, the interest in ligninolytic peroxidases, harbouring the highest redox potential among peroxidases, for biotechnological applications has increased rapidly in industrial areas related with the biorefineries, in particular for the selective delignification of lignocellulosic materials for production of biofuels (Martinez et al. 2009; Ruiz-Duenas and Martinez 2009). These enzymes are also suitable for environmental applications, including the treatment of toxic effluents, containing synthetic dyes, generated in various industrial processes (Wesenberg et al. 2003; Kandelbauer and Guebitz 2005; Husain 2006; Rodriguez Couto 2009b; Chacko and Subramaniam 2011; Khan et al. 2013). However, these enzymes are still not commercially available, in part due to constraints related to the genetic manipulation and relatively low levels of protein expression in both native and fungal host strains.

A new family of microbial peroxidases, known as dye decolorizing peroxidases (DyPs), was demonstrated to successfully degrade not only high redox anthraquinone-based, but also azo dyes, β-carotene (Scheibner et al. 2008), aromatic sulfides (van Bloois et al. 2010), phenolic or non-phenolic lignin compound units (Liers et al. 2010; van Bloois et al. 2010; Brown et al. 2012) and manganese (Roberts et al. 2011; Brown et al. 2012). The physiological function of these enzymes is at present unclear, but there are increasing evidences of their involvement in the degradation of lignin (Ahmad et al. 2011; Salvachua et al. 2013; Singh et al. 2013), and therefore, DyPs seem to have the potential to replace the high-redox fungal ligninolytic peroxidases in biotechnological applications. DyPs have primary sequence, structural and apparently, mechanistic features, unrelated to those of other known "classic" plant and animal peroxidases (Sugano et al. 2007; Liu et al. 2011; Yoshida et al. 2011; Singh et al. 2012; Strittmatter et al. 2012). The uniqueness of these enzymes is, therefore, interesting both at the fundamental and

applied perspectives. Importantly, DyPs, first discovered in fungi, were later identified in a wide range of bacterial strains. In fact, the increasing number of putative DyP-type peroxidases, identified in genomes and proteomes of bacteria, leads to the suggestion that this super family should be renamed into the super family of bacterial peroxidases (Colpa et al. 2013). DyPs have been classified into four phylogenetically distinct sub-families, with bacterial enzymes constituting A-C sub-families and fungal enzymes belonging to D sub-family (Ogola et al. 2009).

DyPs show characteristic conserved residues in the heme-binding site, in particular the characteristic GXXDG motif and an aspartate residue replacing the distal histidine, which acts as the acid-base catalyst in classical peroxidases (Sugano 2009; Hofrichter et al. 2010; Colpa et al. 2013). Structurally, these DyPs comprise two domains that contain α-helices and anti-parallel β-sheets, unlike plant and mammalian peroxidases, that are primarily α-helical proteins (Colpa et al. 2013). Both domains adopt a unique ferredoxin-like fold and form an active site crevice with the heme co-factor sandwiched in between (Colpa et al. 2013).

The cloning and characterization of two new DyPs, one from *Pseudomonas putida* MET94 designated as PpDyP (*P. putida* DyP) and another from *Bacillus subtilis*, called BsDyp (*B. subtilis* DyP) were recently described (Santos et al. 2014). The biochemical characterization of these bacterial enzymes allowed assessing their versatility and catalytic efficiency towards structurally different type of substrates as well as their stability properties. The constructed phylogenetic tree shows that BsDyP belongs to subfamily A and PpDyP belongs to subfamily B (Santos et al. 2014).

4.1 PpDyP and BsDyP Performance Towards Dye Decolorization

In order to characterize the catalytic specificity of PpDyP and BsDyP for synthetic dyes, an array of both anthraquinonic and azo dyes were tested as substrates (Table 1). All the dyes were degraded by both enzymes after 24 h of reaction to different extents (Fig. 14). However, no major differences were observed in the levels of decolorization of anthraquinonic as compared to azo dyes, in contrast to other DyPs that show typically lower activities for the azo dyes (Kim and Shoda 1999; Sugano et al. 2000; Ogola et al. 2009; Li et al. 2011).

The potential of these enzymes was well demonstrated by comparing the decolorization rates (V_{max}) of the studied DyPs with the azoreductase PpAzoR and the CotA laccase. Two to 40-fold higher activities were measured in DyPs (Table 1).

The *Bacillus* enzyme BsDyP is in general a less versatile and a weaker biocatalyst than PpDyP (Santos et al. 2014). Except for the oxidation of ABTS, *Pseudomonas* enzyme shows one to two orders of magnitude higher efficiency (k_{cat}/K_m) for the different synthetic dyes (Table 2 and data not shown), manganese

Table 1 Activities of PpDyP and BsDyP as compared to PpAzoR and CotA, using 2 mM of anthraquinonic (disperse blue 1, reactive blue 5, acid blue 62 and reactive blue 19) or azo (mordant black 9, acid black 194 and acid yellow 49) dyes as substrate (Santos et al. 2014)

Substrates	V_{max} (U mg^{-1})			
	PpDyP	BsDyP	PpAzoR	CotA
AQ dyes				
Disperse blue 1	10 ± 3	3 ± 0.1	nd	0.6 ± 0.04
Reactive blue 5	11 ± 1	9 ± 1	nd	0.3 ± 0.01
Acid blue 62	9 ± 1	10 ± 0.1	nd	1.3 ± 0.9
Reactive blue 19	9 ± 2	5 ± 0.2	nd	nd
Azo dyes				
Mordant black 9	26 ± 2	4 ± 0.1	2 ± 0.1	1 ± 0.3
Acid black 194	12 ± 2	2 ± 0.1	3 ± 0.4	0.9 ± 0.2
Acid yellow 49	10 ± 1	3 ± 0.2	2 ± 0.3	2 ± 0.2

nd not detected

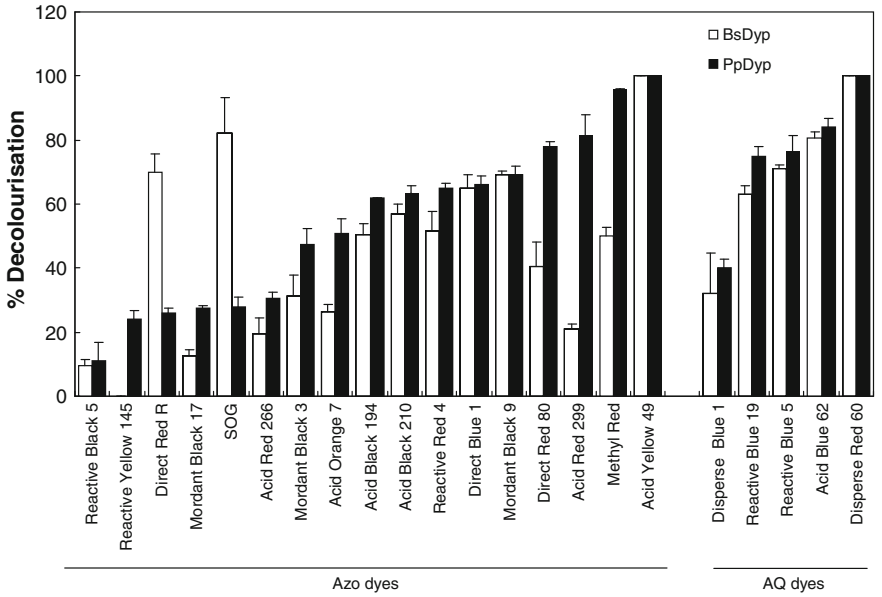

Fig. 14 Dye decolorization by the enzymes PpDyP (*black bars*) and BsDyP (*white bars*). Decolorization was measured by HPLC after 24 h of reaction (adapted from Santos et al. 2014)

or phenolic substrates tested, than the *Bacillus* enzyme. Moreover, PpDyP is able to oxidise the high redox non-phenolic veratryl alcohol compound (1.4 V) in the absence of redox mediators as DyPB from *R. jostii* and DyPs from C-D subfamilies

Table 2 Steady-state apparent catalytic constants of purified recombinant PpDyP and BsDyP (Santos et al. 2014)

Substrates	PpDyP			BsDyP		
	V_{max} (U mg^{-1})	$K_{m\ app}$ (μM)	k_{cat}/K_m (M^{-1} s^{-1})	V_{max} (U mg^{-1})	$K_{m\ app}$ (μM)	k_{cat}/K_m (M^{-1} s^{-1})
AQ dyes						
Reactive blue 5	15 ± 0.2	40 ± 3	2×10^5	11 ± 0.6	157 ± 46	5×10^4
Acid blue 62	14 ± 0.3	30 ± 4	2.4×10^5	12 ± 0.2	444 ± 45	2×10^4
Azo dyes						
Mordant black 9	32 ± 0.2	320 ± 47	5×10^4	5 ± 0.1	385 ± 46	1×10^4

of DyPs (Liers et al. 2010, 2011; Ahmad et al. 2011; Brown et al. 2012) and shows a reasonable metalloxidase activity towards ferrous ions, not detected in the *Bacillus* enzyme.

The different catalytic characteristics between members of the DyPs sub-families point to distinct heme micro-environments. The UV-visible absorption spectra of the Bs and Pp enzymes obtained upon addition of hydrogen peroxide reveal the accumulation of different catalytic intermediates. The accumulation of compound I in PpDyP is in accordance with results obtained for all other DyPs and the majority of classical peroxidases, while the accumulation of compound II intermediate in BsDyP was previously observed in A-type DypA from *R. jostii RHA1* (Roberts et al. 2011). The reasons behind the distinct spectral behaviour of BsDyP and PpDyP are possibly related to the higher redox potential of BsDyP which contributes to a relatively lower stability of Fe^{3+} and thus to a lower stability of compound I upon addition of hydrogen peroxide (Fig. 15). The poorer catalytic activity of BsDyP, as compared to the Pp enzyme, must rely to a highly abundant

$$P_{ox} + H_2O_2 \longrightarrow \text{Compound I} + H_2O \quad (1)$$

$$\text{Compound I} + AH \longrightarrow \text{Compound II} + A^{\bullet} \quad (2)$$

$$\text{Compound II} + AH \longrightarrow P_{ox} + A^{\bullet} + H_2O \quad (3)$$

Fig. 15 The three-step catalytic cycle in the classical peroxidation reaction catalyzed by peroxidases, where P_{ox} is the resting enzyme containing a ferric heme iron, Compound I is the first enzyme intermediate, which contains an oxyferryl iron center and a second oxidizing equivalent stored as a radical ($Fe(IV)=OR^{+\bullet}$) to give a formal oxidation state of +5, and Compound II is the second enzyme intermediate in which the radical is discharged leaving only the oxyferryl iron (formal oxidation state +4). AH represents the reducing substrate and $A^{\bullet}$ the radical product

catalytic incompetent 6-coordinated low spin state in the Bs enzyme, while the major population in PpDyP is the 5-co-ordinated quantum mechanically mixed spin state, as observed by resonance Raman (Sezer et al. 2013).

4.2 The Catalytic Pathway for Biotransformation of Anthraquinonic Dyes by DyPs

The transformation of the anthraquinonic dye reactive blue 5 was carried out using DyP from *Thanatephorus cucumeris* Dec 1 (Sugano et al. 2009). Changes in the visible spectrum of RB5 treated with DyP resulted in a decrease in the intensity of the dye absorption band, at λ_{max} = 600 nm, along with an increase in absorption at 400–500 nm as the color of the solution became red-brown.

Analysis of the final enzymatic reaction mixtures by NMR and MS techniques showed that the anthraquinone dye reactive blue 5 was transformed by DyP from *T. cucumeris* Dec 1 to three reaction products detected by their distinct molecular ion signals. The first product (**1**) was identified as phthalic acid by comparison with an authentic sample. The second one (**2**), with a molecular mass of 472 g mol^{-1}, can be attributed to a reactive blue 5 molecule without the anthraquinone frame (see Fig. 16). Finally, the third product (**3**) can be obtained from compound (**2**) which loss a 2,5-diaminobenzene sulfonic acid (ABS) molecule.

Based on these results, a reasonable degradation pathway of reactive blue 5 by DyP was proposed as shown in Fig. 16. The final red-brown color of the reaction mixture of reactive blue 5 biotransformation and the absence of *o*-ABS and *m*- or *p*-ABS as final products, suggest the presence of other products resulting from the dimerisation and polymerization reactions of ABS type substrates by DyP action. In fact, this was confirmed with the *o*-ABS reaction with DyP, leading to the formation of high weight colored products, from which compound **4,** containing an azo group, was identified.

4.3 Combined Sequential Enzymatic Treatment for Dye Degradation and Detoxification

In order to set-up enzymatic processes for maximal decolorization as well as detoxification, a sequential enzymatic procedure was performed combining the PpAzoR reduction of azo dyes to the oxidation of aromatic amines by CotA-laccase. It is know that laccases catalyze the oxidation of ortho- or para-substituted phenolic or aromatic amine substrates by one electron abstraction to form free radicals that undergo further coupling, polymerization, demethylation, or quinone formation (Abadulla et al. 2000; Kandelbauer and Guebitz 2005). In particular, we have shown recently that CotA-laccase catalyzed the homocoupling of primary

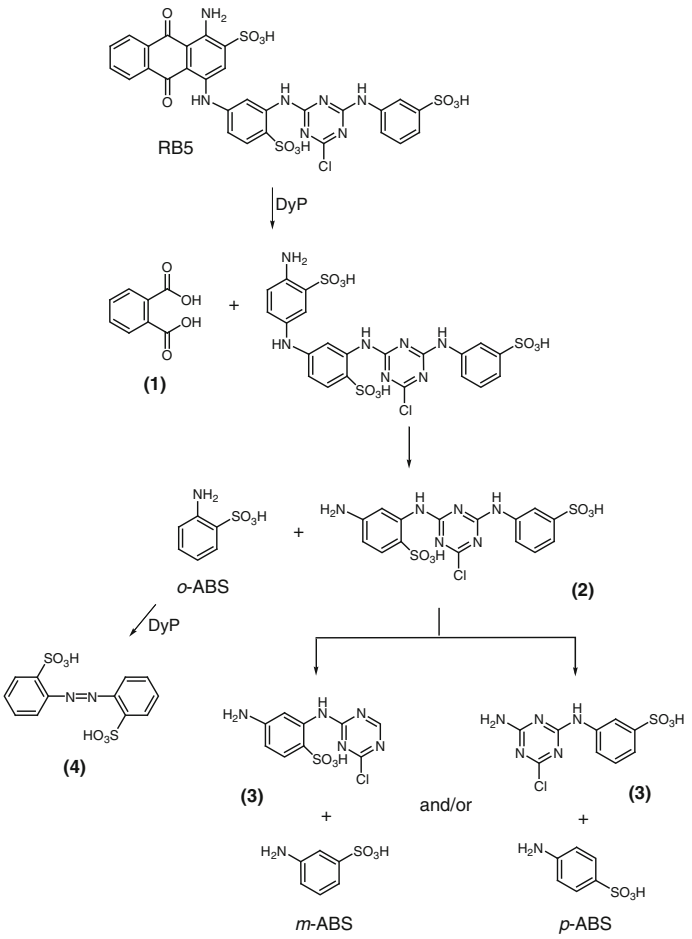

Fig. 16 Proposed pathway for the biotransformation of reactive blue 5 by DyPs. The presence of products (1) and (2) was consistent with an oxidative ring-opening of the anthraquinone frame generated by DyP, which appears in this case to behave as a hydrolase or oxygenase rather than a peroxidase, although H_2O_2 was indispensable for the reaction. The formation of compound (4) can be explained by a reaction mechanism of a typical peroxidase leading to the formation of a radical from *o*-ABS, which will be further involved in a spontaneous chemical reaction. Product (4) was characterized by NMR and ESI-MS techniques and the formation of products (1), (2) and (3) was supported by ESI-MS analysis of the final reaction mixtures (adapted from Sugano et al. 2009)

aromatic amines that represent good oxidative substrates (Sousa et al. 2013). Therefore, azo dyes were reduced by PpAzoR under anaerobic conditions and after 24 h of reaction, CotA-laccase was added with agitation. Interestingly, this sequential enzymatic procedure resulted not only in 100 % decolorization of all azo dyes tested, but also in 50–95 % detoxification of dye-products that exhibited the highest initial toxicity (Fig. 17) (Mendes et al. 2011a).

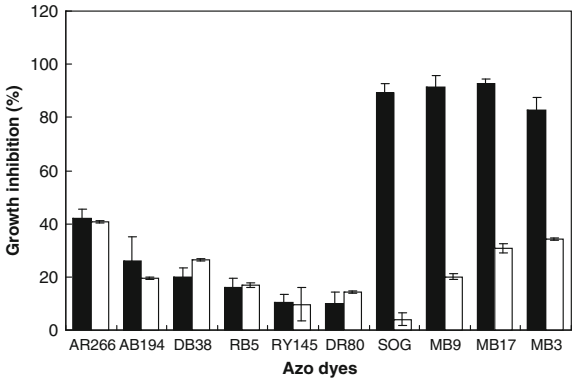

Fig. 17 Inhibitory effects of intact dyes over *Saccharomyces cerevisiae* (*black bars*) and upon the stepwise treatment with PpAzoR followed by CotA (*white bars*) (adapted from Mendes et al. 2011a)

4.4 Construction of an E. coli *Strain Producing Both Azoreductase and Laccase*

The use of whole cell catalysis is considered one of the most appropriate systems for biodegradative processes. It allows the lowering of the costs associated with enzyme purification and co-factors supply and also providing protection to the biocatalysts from harsh process environment. Therefore, a host strain co-expressing the genes coding for both enzymes of interest, PpAzoR and CotA, was constructed and a whole cell system was tested for the decolorization of dyes (Mendes et al. 2011a).

Three model dye-containing wastewaters were designed to mimic textile effluents produced during cotton or wool textile dyeing processes containing other additives and salts (30–90 % of the total weight) in addition to dyes (Prigione et al. 2008; Mendes et al. 2011b). These dyes are representative of different structural dye types and are widely applied in the textile industry. A step-wise sequential process was set-up, where the sequential action of PpAzoR and CotA enzymes could be tuned by aeration conditions. Whole cells remained in anaerobic conditions for 24 h, appropriate for PpAzoR degradation of azo dyes to aromatic amines, followed by a second 24 h period where with appropriate shaking, CotA aerobically oxidized the aromatic amines, and also the anthraquinonic dyes present in the model dyes (Mendes et al. 2011a). This procedure resulted in almost 100 % decolorization levels for the acid dye bath and around 80 % for both the reactive and direct dye baths (Fig. 18a). After this sequential treatment the toxicity levels of the final products was reduced for both *S. cerevisiae* model growth or for *C. elegans* reproduction (Fig. 18b).

Taken together, the results showed that the genetically engineered *E. coli* strain expressing the gene coding for azoreductase and laccase is able to decolorize and detoxify to a significant level the 3 model wastewaters tested, highlighting its

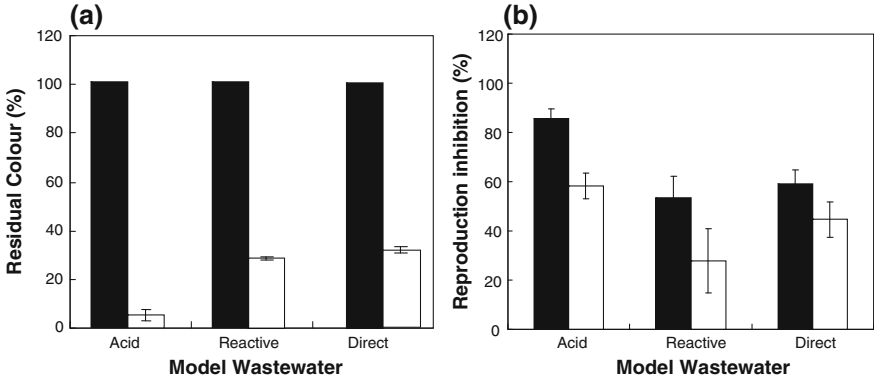

Fig. 18 **a** Residual color after the stepwise sequential process using whole cells co-producing PpAzoR and CotA (*white bars*). **b** Toxicity over *Caenorhabditis elegans* reproduction of intact model wastewater (*black bars*) and after the stepwise sequential process using whole cells co-producing PpAzoR and CotA (*white bars*) (Mendes et al. 2011a)

potential as a degradative and detoxifying bio-system for the treatment of real dye-containing effluents, without the costs associated with enzyme purification and cofactors addition.

5 Conclusions and Future Perspectives

Synthetic dyes impart an intense color to wastewater effluents from the textile, leather or other dye manipulating industries leading to environmental, medical and aesthetic problems. The diversity and complexity of dyes present in these effluents are designed to resist fading on exposure to light or chemical attack, pose serious problems on the design of technically feasible and cost-effective treatment methods. There are a relatively low number of known enzymes that are efficiently involved in the degradation of synthetic dyes in natural systems. Therefore, the characterization of enzymes, that make a discernible contribution to the degradation of synthetic dyes, paves the way for the improvement of multi-enzymatic systems, through protein engineering strategies, to maximize their biodegradation, biotransformation and valorization potential. In this review, we have described the properties, enzymatic mechanisms and products toxicity of three different types of bacterial enzymatic systems. The CotA-laccase from *B. subtilis* is a promising enzyme for the oxidative degradation of both antraquinonic and azo dyes in addition to their efficiency in the biotransformation of toxic aromatic amines (the degradation products of azoreductases). The azoreductase PpAzoR from *P. putida* MET94 is an enzyme that uses a broad range of azo dyes as substrates leading to high levels of decolorization. The major drawback associated with the use of

azoreductases is their requirement for expensive co-factors and the toxicity of the aromatic compounds produced. This can be overcome using whole cells systems of recombinant cells overproducing azoreductases and laccases as described for the decolorization and detoxification of model wastewater baths. The bacterial dye decolorizing peroxidases are new biocatalysts with a high potential for the set-up of bioprocesses considering the enlarged substrate spectrum exhibited, in particular their high efficiency for the biotransformation of anthraquinonic dyes. Both enzymes from *P. putida* and *B. subtilis* are interesting biocatalysts showing higher rates of decolorization as compared to azoreductases and the laccases tested. Moreover, we have shown the benefits of genetic engineering and evolutionary approaches to modify enzymes and microorganisms with enhanced stability and catalytic performance towards an efficient enzymatic treatment of dye-containing wastewaters.

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Bacterial Degradation of Azo Dye Containing Wastes

Shailesh R. Dave, Tallika L. Patel and Devayani R. Tipre

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 7×10^5–1×10^6 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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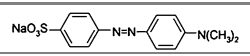
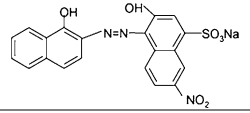
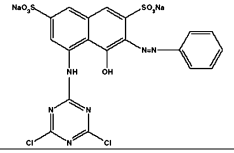
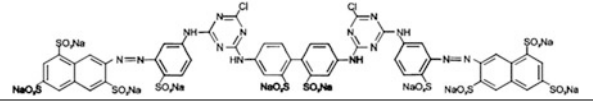
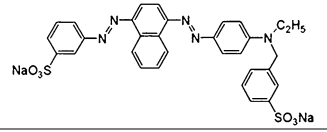
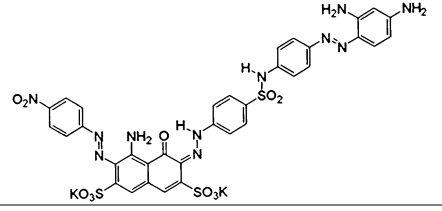
Table 1 Chemical classes of azo dyes with their different structures and molecular weights

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 0.85×10^{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 λ_{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:

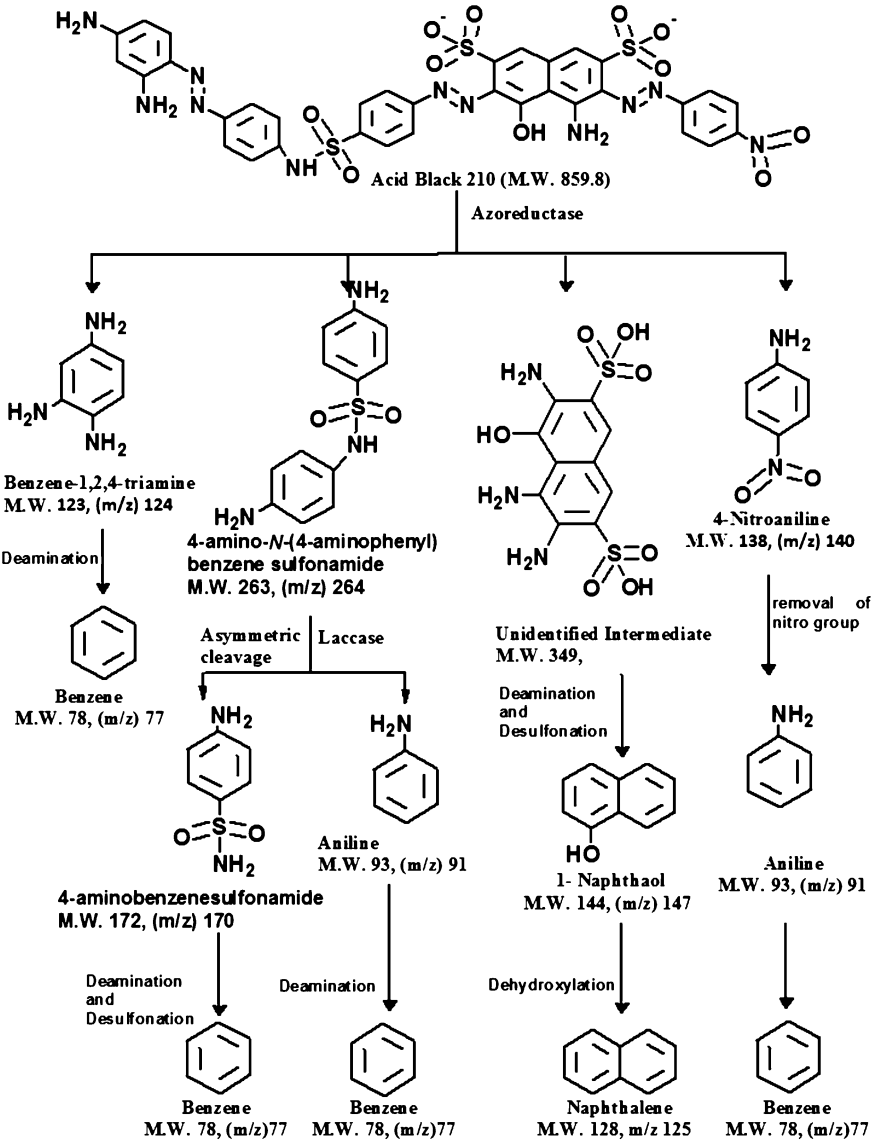


Fig. 1 Bacterial degradation pathway of azo dye Acid Black 210

- 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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Microbial Degradation of Basic Dyes in Wastewaters

C. Ganesh Kumar and Poornima Mongolla

1 Introduction

Dyes have been used since ages in textile dyeing, paper printing, pulp, plastics, food, cosmetics and tannery industries. The dye molecule comprises of two components: the chromophore (responsible for producing the color) and the auxochrome, which supplements the chromophore and renders its solubility in water and also provides enhanced affinity towards the fibers. They exhibit a wide structural diversity and based on the surface charge, are classified as anionic (direct, acid and reactive dyes), cationic (basic dyes) and non-ionic (disperse dyes) (Mishra and Tripathy 1993). Among the industrial wastewaters, wastewater from textile and dyestuff industries is the most difficult and poses a serious environmental problem. Unfortunately, the exact quantity of dyes produced across the world is not yet known. However, it is estimated that over 10,000 tons of dyes are produced per year. Similarly, the exact amount of dyes discharged into the environment is also not available. It is fairly estimated that losses during production is around 1–2 % and in usage, it is about 10–15 %. For basic dyes, this figure can be about 5–7 %. Dye wastewater has high alkalinity, biological oxidation demand, chemical oxidation demand and total dissolved solids with dye concentrations generally below 1 g dm^{-3} (Kaushik and Malik 2009). The synthetic nature and complex aromatic structures contribute to their stability and increased recalcitrance to biodegradation (Fewson 1998).

A release of large amounts of synthetic dyes in the wastewater causes aesthetic nuisance and toxicity which are major environmental concerns (Banat et al. 1996). The biomagnification of the residual dyes at every trophic level results in genotoxic and carcinogenic manifestations in higher animals (Culp and Beland 1996). They also significantly affect photosynthetic activity of aquatic plants due to reduced light

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penetration (Walsh et al. 1980). Some of the basic dyes, such as crystal violet, brilliant green, etc. are potent clastogens, which may promote tumor growth in some species of fishes (Cho et al. 2003). In view of the recalcitrant nature of the modern synthetic dyes there has been imposition of strict environmental legislation in many countries. Conventionally, various physical and chemical treatment methods are used for color removal from wastewater which includes chemical coagulation, flocculation, froth flotation, precipitation, adsorption, ozonation, photooxidation, irradiation, reverse osmosis, ion exchange and membrane filtration (Banat et al. 1996; Srinivasan and Viraraghavan 2010). However, these methods have limited use since they are very cost-intensive, face operational problems and generate a large amount of solid waste, resulting in higher pollution load than the effluents and produce toxic by-products (Dönmez and Aksu 2002). On the other hand, biological decolorization and degradation are environmentally friendly and cost-competitive alternative to chemical decomposition (Robinson et al. 2001a). One key step to efficient dye degradation is to use broad-spectrum and highly efficient dye-decolorizing microorganisms. Further, the effectiveness of these treatment systems depends on the survival and adaptability of microorganisms during the treatment processes. Over the past decade, many microorganisms capable of decolorizing basic dyes at laboratory scale level have been reported (McMullan et al. 2001; Srinivasan and Viraraghavan 2010), but only a few reports are available on their exploitation at treatment process level (Fu and Viraraghavan 2001; Kaushik and Malik 2009). Further, some studies also reveal that processes using immobilized cells are more promising than free cells, as immobilization allows repeated and continuous use of the microbial cells (Cassidy et al. 1996).

2 Microbial Treatment of Waste Water Containing Dyes

Phanerochaete chrysosporium is the most versatile, robust and model white-rot fungus reported in the literature for decolorizing various basic dye-based wastewaters (Knapp et al. 1995; Tatarko and Bumpus 1998; Gomaa et al. 2008; Faraco et al. 2009). Earlier studies indicated that a few white rot fungi, like *Phanerochaete chrysosporium* (Bumpus and Brock 1988) and *Cyathus bulleri* 195062 (Vasdev et al. 1995), degraded Methyl violet, while *Trametes hirsuta*, *Trametes gibbosa* and *Trichaptum biforme* (Eshghi et al. 2011) metabolized Methylene blue and *Irpex lacteus* (Novotny et al. 2001) degraded Victoria blue. Jayasinghe et al. (2008) reported that white rot fungi, like *P. cinnabarinus* and *G. lucidum*, had the ability to degrade approximately 80 % of the Methylene blue within 20 days, while *C. versicolor*, *F. fomentarius*, *T. suaveolens*, *S. ostrea* and *P. coccineus* were able to degrade only 40 % of the Methylene blue during same incubation period. Some white rot fungi, like *Phanerochaete chrysosporium, Pleurotus ostreatus*, *Coriolus versicolor* (Knapp et al. 1995), *Pycnoporus sanguineus* (Pointing and Vrijmoed 2000), *Dichomitus squalens, Phlebia fascicularia*, *Phlebia floridensis* (Gill et al. 2002), *Fomes sclerodermeus* (Papinutti et al. 2006), *Grammothele subargentea*

LPSC no. 436 (Saparrat et al. 2008), *Trametes trogii* and *Trametes versicolor* (Levin et al. 2004; Casas et al. 2009) have been reported to degrade Brilliant green. In addition to white rot fungi, other fungi, which were reported to decolorize and/or adsorb basic dyes are *Cunninghamella elegans* (Cha et al. 2001), *Aspergillus niger* (Fu and Viraraghavan 2002a), *Acremonium kiliense* (Youssef et al. 2008) and *Rhizopus arrhizus* (Gül 2013). Apart from fungi, different bacteria, such as *Bacillus subtilis* IFO 13719 (Yatome et al. 1991), *Enterobacter cloacae* MG82 (Jeong et al. 1998), *Pseudomonas mendocina* MCM B-402 (Sarnaik and Kanekar 1999), *Stenotrophomonas maltophila* LK-24 (Kim et al. 2002a), *Sphingomonas paucimobilis* (Cheriaa and Bakhrouf 2009), *Rhizobium radiobacter* (Parshetti et al. 2009) and *Agrobacterium radiobacter* (Parshetti et al. 2011) were also reported to degrade Crystal violet, while *Kurthia* sp. (Sani and Banerjee 1999), *Citrobacter* sp. strain KCTC 18061P (An et al. 2002) and *Sphingomonas paucimobilis* (Cheriaa and Bakhrouf 2009) degraded Brilliant green (Table 1).

Algae are photosynthetic organisms, which grow in both fresh and salt water habitats, and also identified as promising and potential biosorbent materials for wastewater treatment, can very well compete with commercial biosorbents due to their low cost, easy to culture, ready availability in large quantities and also display a high adsorption capacity. The biosorption ability of algae is mainly attributed to their high surface area and high binding affinity (Dönmez and Aksu 2002). The algal cell wall properties contribute to the biosorption process mainly due to electrostatic attraction and complexation (Aksu and Tezer 2005). The biosorption process is influenced by various factors, such as initial pH, temperature, contact time, initial dye concentration, biosorbent dosage, biosorbent particle size, biosorption kinetics and isotherms. The initial pH influences the biosorption process, since it affects the adsorbate solubility and ionizing functional groups of algal cell walls. It has been reported that alkaline pH is suitable for Malachite green removal by microalga, *Cosmarium* sp. (Daneshvar et al. 2007). The isoelectric point (pI) of the cell wall surface determines the adsorption ability. If the pH value is lower than the pI value, the algal cell wall functional groups get protonated favoring anionic dye removal and when the pI value becomes more negatively charged, it favors adsorption of cationic dyes, due to electrostatic forces of attraction (Khataee et al. 2013). The algal cell wall matrix contains different functional groups, such as hydroxyl, carboxyl, sulphate and other charged groups, which are generated by complex heteropolysaccharides and lipid components which favor sequestration of positively charged molecules, such as cationic dyes from wastewater (Mohan et al. 2002; Daneshvar et al. 2012). The dye removal by algae is due to the accumulation of dye ions on the surface of algal biopolymers and further diffusion of the dye molecules from aqueous phase onto the solid phase of the biopolymer (Özer et al. 2006). Extracellular polymers consist of surface functional groups, which enhance sorption of the dye molecules onto the surface of the polymer (floc) and settle during the dye removal process. This phenomenon is known as biocoagulation which is mainly due to the release of metabolic intermediates (long chain biopolymers) by algae having good coagulation ability towards the dye remaining in the wastewater (Mohan et al. 2002) (Table 2).

Table 1 Different microbial strains used in the decolorization and degradation of basic dyes

Microbial strain	Dye	Experimental conditions	Mechanism	Percent dye removal (contact time)	Reference
Fungi					
Aspergillus niger (live biomass)	Basic blue 9 (Thiazine)	Initial dye conc. 50 mg l^{-1}; initial pH 5.1; biosorbent dose 0.2 g/75 ml	Biosorption	10 (2 days)	Fu and Viraraghavan (2000)
Aspergillus niger (immobilized) (dead biomass)	Basic blue 9 (Thiazine)	Initial dye conc. 8.3 mg g^{-1}; 4.5 g of beads; column dia 1.27 cm, height 40 cm; flow rate 6 ml min^{-1}	Biosorption	90 (5.2 min)	Fu and Viraraghavan (2003)
Aspergillus sp. strain CB-TKL-1 (live biomass)	Methyl violet (Triphenylmethane)	Initial dye conc. 20 mg l^{-1}; initial pH 5, 30 °C; 200 rpm; glucose (2 %); sodium nitrate (0.2 %)	Biosorption	>99.7 (24 h)	Kumar et al. (2011)
Aspergillus sp. strain CB-TKL-1 (live biomass)	Brilliant green (Triphenylmethane)	Initial dye conc. 10 mg l^{-1}; initial pH 5.5; 35 °C; 200 rpm; glucose (2 %); sodium nitrate (0.2 %)	Biosorption	99.3 (72 h)	Kumar et al. (2012)
Penicillium chrysogenum	Bromophenol blue (Triphenylmethane)	Initial dye conc. 800 mg l^{-1}; initial pH 2.0; Biosorbent dose 1 g l^{-1}	Biosorption	88 (20 h)	Zeroual et al. (2006)
Penicillium ochrochloron MTCC 517	Malachite green (Triphenylmethane)	Initial dye conc. 50 mg l^{-1}; initial pH 7; 30 °C; sucrose (10 %)	Peroxidase-mediated	93 (14 h)	Shedbalkar and Jadhav (2011)
Rhizopus arrhizus	Methylene blue (cationic thiazine)	Initial dye conc. 100 mg l^{-1}; initial pH 6; 30 °C; glucose (1 %); yeast extract (0.2 %)	Biosorption	92.5 (8 days)	Gül (2013)
Myrothecium roridum IM 6482	Malachite green (Triphenylmethane)	Initial dye conc. 50 mg l^{-1}; initial pH 6.5; 28 °C	Biosorption	94.5 (180 min)	Jasinska et al. (2013)

(continued)

Table 1 (continued)

Microbial strain	Dye	Experimental conditions	Mechanism	Percent dye removal (contact time)	Reference
White rot fungi					
Trametes versicolor	Crystal violet (Triphenylmethane)	Initial dye conc. 25 mg l^{-1}; 28 °C; 200 rpm;	Peroxidase-mediated	99 (7 days)	Liu et al. (2004)
Sporotrichum pulverulentum	Malachite green (Triphenylmethane)	Initial dye conc. 5 mg l^{-1}; initial pH 4.5; 30 °C; 200 rpm; glucose (0.1 %); $(NH_4)_2NO_3$ (0.5 %)	Biosorption	99 (48 h)	Vaidya and Konde (2008)
Pleurotus florida LCJ 65	Brilliant green (Triphenylmethane)	Initial dye conc. 10 mg l^{-1}; initial pH 5–6; 30 °C; 120 rpm; glucose (1 %); yeast extract (0.1 %)	Biosorption	80 (6 h)	Radhika et al. (2014)
Bacteria					
Bacillus subtilis IFO 13719	Crystal violet (Triphenylmethane)	Initial dye conc. 2.85 mg l^{-1}	Biodegradation	90 (24 h)	Yatome et al. (1991)
Pseudomonas mendocina MCM B-402	Methyl violet (Triphenylmethane)	Initial dye conc. 980 mg l^{-1}; initial pH 7; 28 °C	Biodegradation	99 (27 h)	Sarnaik and Kanekar (1999)
Stenotrophomonas maltophila LK-24	Crystal violet (Triphenylmethane)	Initial dye conc. 100 mg l^{-1}; initial pH 7; 37 °C; 150 rpm	Biodegradation	99 (30 h)	Kim et al. (2002a)
Clostridium perfringens AB&J	Bromophenol blue (Triphenylmethane)	Initial dye conc. 50 mg l^{-1}; initial pH 7; 40 °C	Biosorption	97.7 (27 h)	Kim et al. (2002b)
Brevibacillus sp.	Toludine blue (cationic thiazine)	Initial dye conc. 50 mg l^{-1}; initial pH 7.3; 37 °C; 200 rpm; beef extract	Biosorption	80 (72 h)	Alhassani et al. (2007)
Bacillus sp.	Crystal violet (Triphenylmethane)	Initial dye conc. 500 ppm; initial pH 7; 30 °C; 150 rpm	Biodegradation	100 (2.5 h)	Ayed et al. (2009)
Staphylococcus epidermidis	Malachite green (Triphenylmethane)	Initial dye conc. 750 ppm; initial pH 7.5; 25 °C; glucose (7 mM); yeast extract (0.1 %)	Biodegradation	90 (14 h)	Ayed et al. (2010)

(continued)

Table 1 (continued)

Microbial strain	Dye	Experimental conditions	Mechanism	Percent dye removal (contact time)	Reference
Shewanella decolorationis NTOU1	Crystal violet (Triphenylmethane)	Initial dye conc. 200 mg l^{-1}; 35 °C	Anaerobic Biodegradation	99 (2 h)	Chen et al. (2010)
Pseudomonas aeruginosa BCH	Acid violet 19 (Triphenylmethane)	Initial dye conc. 250 mg l^{-1}; initial pH 7; 30 °C; 200 rpm; mannitol (0.5 %)	Enzymes and biodegradation	98 (30 min)	Jadhav et al. (2012)
Bacillus subtilis ETL-2211	Crystal violet (Triphenylmethane)	Initial dye conc. 100 mg l^{-1}; initial pH 8; 35 °C; 200 rpm; dextrose (0.5 %); peptone	Biodegradation	92 (24 h)	Shah et al. (2013)
Actinomycetes					
Nocardia corallina IAM 12121	Crystal violet (Triphenylmethane)	Initial dye conc. 23 mg l^{-1}; initial pH 7; 25 °C	Biodegradation	80 (90 min)	Yatome et al. (1993)
Streptomyces rimosus (dead biomass)	Methylene blue (cationic thiazine)	Initial dye conc. 50 mg l^{-1}	Biosorption	86 (5 min)	Nacera and Aicha (2006)
Yeasts					
Rhodotorula sp. and *Rhodotorula rubra*	Crystal violet (Triphenylmethane)	Initial dye conc. 10 ppm	Enzymes	>99 (4 days)	Kwasniewska (1985)
Saccharomyces cerevisiae MTCC 463	Malachite green (Triphenylmethane)	Initial dye conc. 250 mg l^{-1}; initial pH 7; 33 °C; 150 rpm; glucose (1 %); peptone (0.1 %), yeast extract (0.1 %)	Enzymatic biodegradation	97.7 (7 h)	Jadhav and Govindwar (2006)

Table 2 Different algal strains used in the decolorization and degradation of basic dyes

Algal culture	Dye (class), dye concentration	Mechanism	Dye removal (%)	Contact time	Reference
Sargassum muticum (seaweed)	Methylene blue (cationic thiazine), 279.2 mg g^{-1}	Biosorption	90	34 min	Rubin et al. (2005)
Cosmarium sp.	Malachite green (Triarylmethane), 10 ppm	Biodegradation, pH 9	74	210 min	Daneshvar et al. (2007)
Ulva lactuca and *Sargassum*	Methylene blue (cationic thiazine), 40.2 mg g^{-1}	Biosorption	96	25 min	Tahir et al. (2008)
Caulerpa lentillifera	Methylene blue (Thiazine), 49.26 mg g^{-1}	Biosorption	n.s.	30 min	Marungrueng and Pavasant (2007)
Caulerpa racemosa var. *cylindracea*	Methylene blue (cationic thiazine), 66.7 g l^{-1}	Biosorption	95	90 min	Cengiz and Cavas (2008)
Cystoseira barbatula Kützing	Methylene blue (cationic thiazine), 38.61 mg g^{-1}	Biosorption	n.s.	210 min	Caparkaya and Cavas (2008)
Azolla filiculoides	Methylene blue (cationic thiazine), 166.7 mg g^{-1}	Biosorption	84.9	76.2 h	Padmesh et al. (2008)
Azolla filiculoides	Basic orange (cationic), 833.33 mg g^{-1}	Biosorption	93	4 h	Tan et al. (2011)
Chlamydomonas sp.	Basic green 4 (Triphenylmethane), 10 mg l^{-1}	Biosorption	80	180 min	Khataee et al. (2009)
Hydrilla verticillata	Methylene blue (cationic thiazine), 200 mg l^{-1}	Biosorption	99	150 min	Kannan et al. (2010)
Cladospora sp.	Malachite green (Triarylmethane), 10 mg l^{-1}	Biodegradation	71	75 min	Khataee and Dehghan (2011)
Dunaliella salina	Methylene blue (Thiazine), 5 ppm	Biosorption	97	7 days	Adb-Al-Kareem and Taha (2012)
Spirulina platensis	Acid blue 9 (Triphenylmethane), 1,653 mg g^{-1}	Biosorption	95	100 min	Dotto et al. (2012)

n.s.—not specified

3 Factors Effecting Dye Degradation Process

Microbial growth and enzyme production for decolorization and degradation are influenced by different factors, such as medium composition, pH, temperature, aeration and agitation and initial dye concentration. These factors effects have been briefly discussed and presented, as below.

3.1 Medium Composition

Each microbial strain has its own idiosyncratic, physiochemical and nutritional requirement for optimal growth and enzyme production to perform the dye degradation process. It is well documented that medium components greatly influence the microbial growth and their interaction plays an important role in the enzyme synthesis required for dye degradation. The industrial dye effluents have varied complex composition with a lack of nutrients as compared to the well defined media spiked with the dye used under lab conditions. Therefore, it is necessary that carbon and nitrogen sources be supplemented along with mineral salts and other additives (Hao et al. 2000).

3.2 Carbon Source

In general, the carbon source supplementation is required for microbial growth and also to meet the suitable oxidant supply for dye decolorization by the fungus. Majority of the studies have used glucose at a concentration of 5–10 g l^{-1}. Fructose, maltose, sucrose, xylose, cellobiose, arabinose, mannitol, glycerol, starch and carboxymethyl cellulose were also used as alternate carbon sources in different decolorization studies (Kumar et al. 2011). However, cellulose and its derivatives were not found effective. Nevertheless, effluents from dyeing operations or dye production units usually do not contain carbon substrates that are suitable for the microbial assimilation. Hence, there is a necessity to add one to two carbon sources which influences the microbial strain performance to treat the specific dye.

3.3 Nitrogen Source

The microbes demand nitrogen source supplementation for their growth; however, the requirement of a specific nitrogen source differs from organism to organism. White-rot fungi can use both inorganic and organic nitrogen sources. Several reports have indicated that in most cases, the rapidly metabolizable inorganic

nitrogen sources, like sodium or ammonium salts, are used for microbial growth and dye decolorization, since the use of organic nitrogen supplementation is cost-intensive (Radha et al. 2005; Kumar et al. 2012). An increased dose of nitrogen source inhibited decolorization of Congo red (Tatarko and Bumpus 1998), while nitrogen supplementation had no effect on dye decolorization by *Cyathus bulleri* (Vasdev et al. 1995). It is well established in *P. chrysosporium* that under conditions of carbon or nitrogen limitation, the ligninolytic enzyme production for dye decolorization is much more effective (Zhen and Yu 1998), while *Bjerkandera adusta* produced more lignin peroxidase and manganese peroxidase in nitrogen-sufficient media (Heinfling et al. 1998). In the case of effluents, the presence of usable nitrogen sources may cause rapid dye degradation.

3.4 Other Media Components

Many research studies have used various growth factors. From an economic perspective, it is not imperative to use them for developing decolorization technologies. All microbes have certain requirements of mineral nutrients for growth and decolorization. For example, the white-rot fungi need trace metal salts of iron, copper and manganese. They can be present in the effluent or must be supplemented to the medium to affect better dye biotransformation. Different oxidizing mediators, like veratryl alcohol, tryptophan and aromatics, e.g. phenol and aniline, can act as low molecular mass redox mediators of ligninolytic activities and therefore, promote the decolorization process (Rodriguez Couto and Toca-Herrera 2007).

3.5 pH

Most of the research studies on growth and decolorization have been performed in batch cultures, usually without the pH control during the cultivation. Indeed, culture pH mainly influences many enzymatic processes and transport of various components across the cell membrane. Depending on the type of substrates used the pH changes during cultivation. The growth on carbohydrate supplemented media generally causes acidification of the media, which depends on the carbon source and therefore requires buffering. However, majority of filamentous fungi along with white-rots grow optimally at acidic pH. It was earlier reported that different fungi favoured textile dye removal within an optimum pH ranging between pH 4 and 6 which depends on the medium composition and the dye present in the decolorization medium (Jadhav and Govindwar 2006; Parshetti et al. 2006; Asgher et al. 2008). Since Malachite green is a cationic dye, the optimal initial pH was alkaline which favored adsorption of dye on to the algal surface, which increased decolorization efficiency (Kumar et al. 2005; Daneshvar et al. 2007; Khataee et al. 2009). The medium pH mainly influences the solubility of the dye and the ionization state

of the functional groups like carboxyl, hydroxyl and amino groups of the fungal or bacterial cell wall (Fu and Viraraghavan 2002b; Won et al. 2009). At low pH, the biosorbent is rich in positive dye binding sites which attract the anionic dye molecule, while at higher pH, the biosorbent carries a net negative charge, resulting in electrostatic repulsion with the dye molecule. Lower biosorption at higher pH values may be due to the presence of excess hydroxyl ions competing with the negatively charged dye for the biosorption sites (Won et al. 2009). The decolorization process can be conducted with a whole fermentation broth (mycelium and enzymes) or with isolated enzymes. There needs to be a clear distinction among the optimum pH required for growth and enzyme production, the action of isolated enzymes and dye degradation. Therefore, optimum pH depends on the medium, microbial strain and its enzyme system, as well as on the decolorization or degradation process.

3.6 Temperature

Temperature is another critical parameter which needs to be optimized from organism to organism. Temperature has its influence on the growth, enzyme production, decolorization rate and the temperature of the waste stream. Different fungi and most white-rot fungi are mesophiles and exhibit optimal growth and dye decolorization at temperatures ranging between 25 and 35 °C (Fu and Viraraghavan 2001; Parshetti et al. 2007). This is mainly due to the increased surface activity and kinetic energy of each dye molecule (Kaushik and Malik 2009). The optimal temperatures for enzymatic reactions are usually higher, however, at temperatures of >65 °C may cause enzyme instability and degradation. Under dyeing operation conditions, various textile and dye effluents are generated at a temperature range of 50–60 °C. Hence, optimal temperature for decolorization process varies from case to case.

3.7 Agitation and Aeration

Ligninolytic fungi are obligate aerobes and therefore, require oxygen for growth and maintenance of their viability. In addition, dye degradation also requires oxygen, either for the mycelial generation of H_2O_2 for peroxidases or for the direct action of oxidases. The oxygen demand mainly depends on the fungus and its ligninolytic system. The major limitation of oxygen is its low water solubility, which is only 8 mg l^{-1} at 20 °C. Aeration and agitation are necessary to meet the microbial oxygen demand during the cultivation so as to increase better oxygen transfer and nutrient distribution under shaking conditions which enhance dye decolorization rates as compared to the stationary cultures (Swamy and Ramsay 1999). However, this may affect the morphology of filamentous fungi leading to the

decreased rate of enzyme synthesis. On the contrary; it was observed in a study that under both stationary and shaking culture conditions, *Phanerochaete chrysosporium* effected equal and efficient degradation of Malachite green. However, the degradation of Crystal violet by the same culture was five times more efficient under shaking conditions as compared to the stationary culture (Sani et al. 1998). Therefore, various types of bioreactors with static and agitated configurations were developed to provide sufficient oxygen. The choice of the reactor depends on the particular requirement, although lower agitation rate ranging between 100 and 150 rpm is found beneficial in achieving better decolorization results with fungi as compared to static conditions (Fu and Viraraghavan 2001; Parshetti et al. 2007).

3.8 Initial Dye Concentration

Dyes at higher concentrations are usually toxic to microorganisms, but toxicity also depends on the nature of dye. In general, most of the research studies have indicated a range of initial dye concentration between 50–1,000 mg l^{-1}. Hence, it is important to optimize the initial dye concentration for color removal, which depends on both the microbial strain and the type of dye used.

3.9 Statistical Design-Based Optimization

Many dye decolorization studies, as reported in the literature, practiced the classical optimization method which involved the alteration of "one factor at a time" (OFAT), while keeping all other factors at a predetermined level. In this approach, a series of experiments are carried out using a large number of variables which need to be tested to determine the optimum level. This process is very time-consuming, labour-intensive, open-ended, expensive and the interaction between the variables is ignored (Haaland 1989). On the other hand, for improving the decolorization efficiency, an experimental design approach may be followed which requires both a design and an optimization method. The design specifies the variables to test within the experiment, including the number of replicates and the arrangement of tests into homogenous 'blocks'. Further, a mathematical model is employed to predict the optimized conditions of the process, based on which minimum experiments are conducted to validate the predicted optimized conditions. The statistical approaches are preferred in process optimization, because it is economical, considers interactions between medium components and allows rapid optimization of the process (Mason et al. 1989). An artificial neural network model was developed to predict the biosorptive decolorization of Basic green 4 solution by microalga *Chlamydomonas* sp. With the optimum conditions of initial pH 9, 45 °C, dye concentration of 10 mg l^{-1} and reaction time of 180 min, 80 % decolorization efficiency was observed (Khataee et al. 2009). In one study, a central composite design, based on

response surface methodology was applied to optimize the key variables for biological treatment of Malachite green by *Cladospora* sp. The optimization results showed that maximum decolorization efficiency was achieved at the optimal conditions of initial pH 8, initial dye concentration 10 mg l^{-1}, algae amount 4 g and reaction time 75 min (Khataee and Dehghan 2011). An orthogonal design (L_4) 2^3 was used to decolorize a dye industry effluent by *Aspergillus fumigatus* XC6 under optimum conditions of pH 3.0, nitrogen sources [0.2 % NH_4Cl or $(NH_4)_2SO_4$] or carbon sources (1.0 % sucrose or potato starch) for 72 h (Jin et al. 2007). In another study, a Taguchi design was adopted to determine the optimum conditions of *Pseudomonas* sp. DY1 immobilization with *Aspergillus oryzae* for maximum biodegradation of Malachite green, which was obtained at an initial pH 6.5, 37 °C, inoculation size of *Pseudomonas* sp. DY1 (dry cell mass) 0.01 g and of *A. oryzae* (spore number) 1.0×10^9. Decolorization and biodegradation of Malachite green by immobilized pellets were 99.5 and 93.3 %, respectively, under optimum conditions (Yang et al. 2011).

4 Involvement of Oxidoreductive Enzymes in Degradation Process

White-rot fungi (WRF) represent a diverse ecophysiological group comprising mostly basidiomycetes and to a lesser extent, litter-decomposing fungi, having an extracellular ligninolytic enzyme system capable of extensive depolymerization and mineralization of a wide variety of recalcitrant compounds, such as xenobiotics, lignin, and various types of dyes (Paszczynski and Crawford 1995). This feature is based on the WRF's capacity to produce one or more extracellular lignin-modifying enzymes (LME) that are substrate non-specific and also able to tolerate high concentrations of pollutants (Reddy 1995). The important LME produced by WRF are oxidoreductases which include two types of peroxidases, lignin peroxidase (LiP, E. C. 1.11.1.14) and manganese-dependent peroxidase (MnP, E.C. 1.11.1.13) and a phenol oxidase (laccase, Lac, E.C. 1.10.3.2). These enzymes play an important role in the dye degradation. The LME are also essential for lignin mineralization which occurs through a combination of other processes involving other auxiliary enzymes (by themselves unable to degrade lignin), such as glyoxal oxidase and superoxide dismutase for intracellular production of H_2O_2, which acts as a co-substrate of LiP and MnP, as well as glucose oxidase, aryl alcohol oxidase and cellobiose dehydrogenase which are involved in feedback circuits, linking the ligninolysis with cellulose and hemicellulose degradation (Leonowicz et al. 1999).

Different WRF produce one or more of these LME, and based on the types of enzymes produced by them, they are divided into four groups, namely LiP-MnP-laccase-producing fungi; MnP-laccase-producing, LiP-MnP-producing and laccase-producing fungi. WRF produce LME during their secondary metabolism since lignin oxidation provides no net energy to the fungus; the synthesis and secretion of

these enzymes is often induced by limited nutrient levels (mostly C or N). Under submerged fermentation, the production of LiP and MnP is generally optimal at high aeration levels, but repressed by agitation. The laccase production is often enhanced by agitation, aromatic compounds and organic solvent (Galhaup et al. 2002), and normally more than one isoforms of LME are expressed by different taxa and culture conditions. These features are important from a process design perspective and for optimization of fungal treatment of dye-containing effluents. In reality, many WRF produced these oxidoreductases (LiP, MnP) in low quantities which are dependent on metal ions and the co-substrate hydrogen peroxide, and thus have not been able to reach large-scale commercial applications (Wesenberg et al. 2003). Table 3 describes the details on oxidoreductase enzymes produced by different white rot fungi and other fungi involved in decolorization and/or biodegradation of basic dyes.

Phanerochaete chrysosporium was the first widely investigated WRF capable of biodegradation of various pollutants and industrial dye effluents (Glenn and Gold 1983) and was considered as a model WRF in many research studies carried out till now (Wesenberg et al. 2003). This fungus produces lignin peroxidase (LiP) and manganese peroxidase (MnP) (Faraco et al. 2009). Later, several white rot fungi, such as *Pleurotus ostreatus* (Shin and Kim 1998), *Pleurotus calyptratus* (Eichlerova et al. 2006a), *Trametes versicolor* (Heinfling et al. 1997; Casas et al. 2009; Pazarlioglu et al. 2010), *Ischnoderma resinosum* (Eichlerova et al. 2006b), *Bjerkandera adusta* (Robinson et al. 2001b; Eichlerova et al. 2007), *Irpex lacteus* (Novotny et al. 2000) and *Dichomites squalens* (Eichlerova et al. 2006c) were investigated in details for dye degradation. Particularly, laccases secreted by *Pycnoporus sanguineus* (Pointing and Vrijmoed 2000) and *Trametes* sp. SQ01 (Yang et al. 2009) demonstrated their ability to decolorize azo, triphenylmethane and anthraquinonic dyes. Novotny et al. (2004) studied the biodecolorization of many synthetic dyes by using a white rot fungus, *Irpex lacteus* and 96 % of decolorization of Bromophenol blue was observed within 2 weeks. Papinutti et al. (2006) reported that *Fomes sclerodermeus* could decolorize Malachite green-adsorbed wheat bran during solid state fermentation which may be due to the effect of laccase or Malachite green reductase activity. Jasinska et al. (2012) demonstrated that Malachite green decolorization by the submerged culture of *Myrothecium roridum* IM 6482 was due to stimulation of laccase production. In addition, lignin peroxidase from different bacterial sources, such as *Kocuria rosea* MTCC 1532 (Parshetti et al. 2006), *Pseudomonas desmolyticum* NCIM 2112 (Kalme et al. 2007), *Rhizobium radiobacter* MTCC 8161 (Parshetti et al. 2009) and *Acinetobacter calcoaceticus* NCIM 2890 (Ghodake et al. 2009) has been reported to be involved in dye decolorization. Several anaerobic intestinal microflora (Henderson et al. 1997) and waterborne pathogenic mycobacterial strains (Jone and Flakinham 2003) were found to perform decolorization of Malachite green and Crystal violet to their respective leuco derivatives through enzymatic reduction. However, the enzymes involved in the reduction process have not yet been characterized. A triphenylmethane reductase (TMR), that catalyzed the reduction of triphenylmethane dyes, was synthesized by *Citrobacter* sp. strain KCTC 18061P which was purified,

Table 3 Oxidoreductase enzymes of white rot fungi and other fungi involved in decolorization and/or biodegradation of basic dyes

WRF strain	Dye (class)	Enzymes	Decolorization rate (%)	Reference
Flavodon flavus (Klotzsch) Ryvarden (strain 312)	Brilliant green (triarylmethane)	Laccase	75 (9 days)	Raghukumar et al. (1999)
Lentinula (*Lentinus*) *edodes*	Bromophenol blue (triphenylmethane)	Laccase (5 U ml^{-1})	92 (90 min)	Nagai et al. (2002)
Lentinula (*Lentinus*) *edodes*	Brilliant Cresyl blue and Methylene blue	Manganese peroxidase	n.s.	Boer et al. (2004)
Coriolus versicolor f. *antarcticus*	Malachite green (triarylmethane)	Laccase	88.10	Levin et al. (2004)
Pycnoporus cinnabarinus and *Trametes villosa*	Aniline Blue (triphenylmethane)	Laccase + acetosyringone and syringaldehyde	80 (5 min)	Camarero et al. (2005)
Ischnoderma resinosum	Crystal violet (triphenylmethane)	Laccase + MnP	99.6 (20 days)	Eichlerova et al. (2006b)
Ischnoderma resinosum	Malachite green (triarylmethane)	Laccase + MnP	97 (20 days)	Eichlerova et al. (2006b)
Grammothele subargentea LPSC no. 436	Brilliant green (triarylmethane)	Laccase	90 (21 days)	Saparrat et al. (2008)
Fome lignosus	Coomassie brilliant blue G-250 (triphenylmethane)	Laccase	72	Hu et al. (2009)
Ganoderma lucidum	Malachite green (triarylmethane)	Laccase + syringaldehyde	95 (12 h)	Murugesan et al. (2009)
Trichoderma harzianum ZF-2	Basic violet (triphenylmethane)	Laccase	n.s.	Gao et al. (2013)
Alternaria alternata	Crystal violet (triphenylmethane)	Laccase	69.35 (30 days)	El Aty and Mostafa (2013)

n.s.—not specified

biochemically characterized and the gene encoding the enzyme was cloned (Jang et al. 2005). Further, the TMR crystal structure was worked out at a resolution of 2.0 Å (Kim et al. 2008).

5 Mechanism and Pathways of Degradation

Many studies have revealed that a two-step mechanism, namely physical adsorption and enzymatic degradation, are involved in dye decolorization by white rot fungi and other fungal species. Both live and dead forms of diverse fungal genera were reported to be employed in the decolorization process of a wide variety of dyes (Fu and Viraraghavan 2001). Adsorption is considered as an effective process for color removal from dye wastewater, which is dependent on the dye properties, such as molecular structure and type, number and position of substituents in the dye molecule (Reife and Freeman 1996). Limited information is available on the interactions between microbial biomass (living and dead cells) and dyes to enable decolorization which occurs through several complex mechanisms such as surface adsorption, ion-exchange, complexation (coordination), chelation and micro-precipitation (Crini 2006). The bacterial cell wall comprises of polysaccharides, proteins and lipids offering varied functional groups. The dyes can interact with these active groups on the cell surface through adsorption process. The presence of hydroxyl, nitro and azo groups in the dye molecule enables increased adsorption, while decreased adsorption is attributed to the sulfonic acid groups (Reife and Freeman 1996). It is now widely accepted that ion-exchange mechanisms are involved in the efficiency and the selectivity of adsorption by microbial biomass. The efficiency of dye treatment is dependent on various environmental conditions and variables used for the adsorption process, such as pH, ionic strength, temperature, contact time and adsorbent concentration as well as the properties of the adsorbent and adsorbate (Crini 2006).

Limited information is available on the interactions between dead fungal biomass and different types of dyes with complex molecular structures. Different functional groups, such as carboxyl, amino, phosphate and lipid fractions present in the fungal biomass from *A. niger* played an important role in the biosorption of four different dyes (Fu and Viraraghavan 2002b). Carboxyl and amino groups were the main binding sites involved in the biosorption of Basic blue 9 by *A. niger*, while in the biosorption of Congo red, the amino, carboxylic acid, phosphate groups and lipid fractions were found to be important binding sites. In addition to electrostatic attraction, other mechanisms were also responsible to be involved in biosorption. Some studies have demonstrated effective dye removal properties with dead *Rhizopus arrhizus* biomass (Aksu and Tezer 2000; Aksu and Karabayir 2008).

In *P. chrysosporium*, different isozymes of LiP have been shown to decolorize azo, triphenylmethane and heterocyclic dyes in the presence of veratryl alcohol and H_2O_2 (Ollika et al. 1993). Complete decolorization of two triphenylmethane dyes (Bromophenol blue and Malachite green) was achieved by submerged cultures

producing laccase (Eggert et al. 1996). Crude cultures of *P. chrysosporium* and purified LiP were able to degrade Methyl violet and six other triphenylmethane dyes by sequential N-demethylation. Methyl violet (*N*,*N*,*N*′,*N*′,*N*″,*N*″-hexamethyl pararosaniline) on biodegradation showed only three degradation products, viz., *N*,*N*,*N*′,*N*′,*N*″-penta-, *N*,*N*,*N*′,*N*″-tetra-, and *N*,*N*′,*N*″-tri-methylpararosaniline when cultured in a nutrient with nitrogen-limiting conditions (Bumpus and Brock 1988). In case of *Bacillus subtilis* IFO 13719 (Yatome et al. 1991) and *Nocardia corallina* IAM 12121 (Yatome et al. 1993), the major Methyl violet biodegradation products were identified as 4,4-*bis*-dimethylamine benzophenone (Michler's ketone) and α-dimethylaminophenol. Later, detailed investigations were performed on Methyl violet (Kumar et al. 2011) and Brilliant green (Kumar et al. 2012) degradation using *Aspergillus* sp. strain CB-TKL-1. This process followed a stepwise *N*-demethylation pattern and *N*-demethylated intermediates were observed before its final mineralization. The proposed *N*-demethylation pathway for decolorization of Methyl violet and Brilliant green by *Aspergillus* sp. strain CB-TKL-1 is shown in Figs. 1 and 2, respectively.

6 Microbial Toxicity of Dyes and Their Degradation Products

Many treatments can be efficient in decolorization and degradation of dyes, however, it is prudent to evaluate whether there is formation of toxic products during the treatment process. The efficiency of a degradation process can be evaluated on bioindicators, such as *Artemia salina* and *Daphnia magna* (Matthews 1995; de Souza et al. 2007). The Ames mutagenicity test is evaluated against *Salmonella typhimurium* strains (Azizan and Blevins 1995; Schneider et al. 2004). The common phytotoxicity tests includes seed germination and plant growth bioassays (Kapanen and Itavaara 2001), as newly formed degradation products must be nontoxic for use of treated wastewater for irrigation purpose.

7 Future Prospectives

Biodegradation of synthetic dyes using different fungi, bacteria, yeasts, and algae is becoming a promising approach for the treatment of dye wastewaters. In order to establish efficient biological decolorization and biodegradation systems, concerted efforts are still required. The biodegradation abilities of microorganisms can be enhanced by gradually exposing them to higher concentrations of dyestuffs. The adaptation of a microbial community toward toxic or recalcitrant dyestuffs is viewed as a useful approach in improving the rate of decolorization process. The adaptation of microorganisms to higher concentrations of pollutants is called acclimatization and leads to forced or directed evolution. Microorganisms, thus

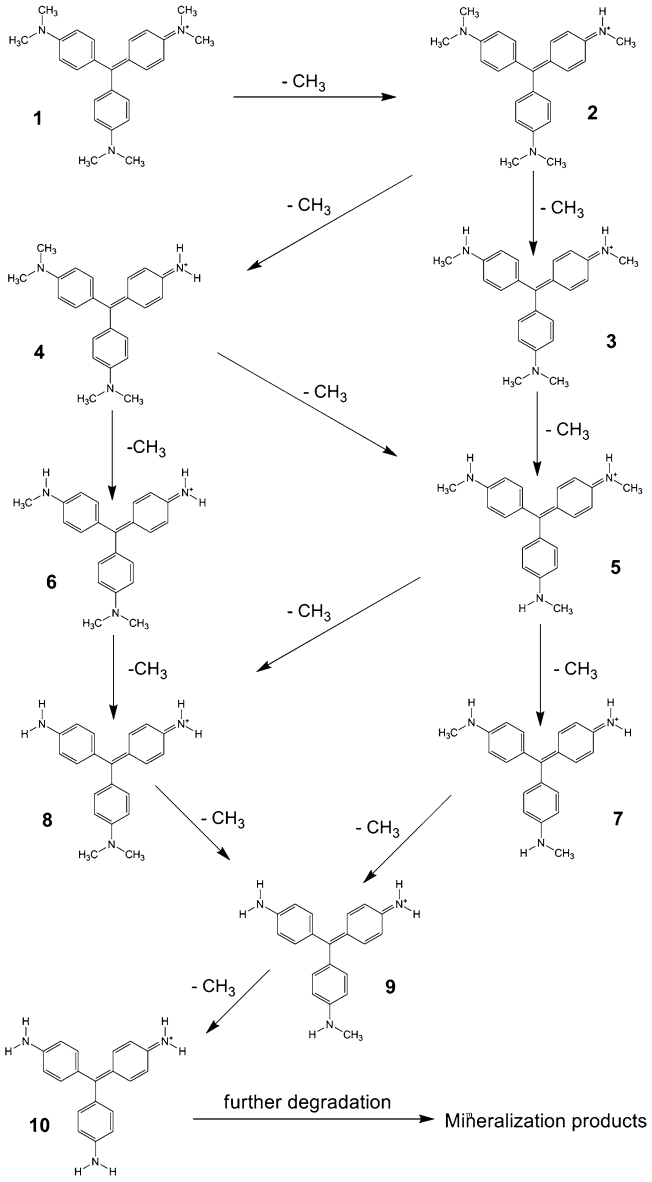
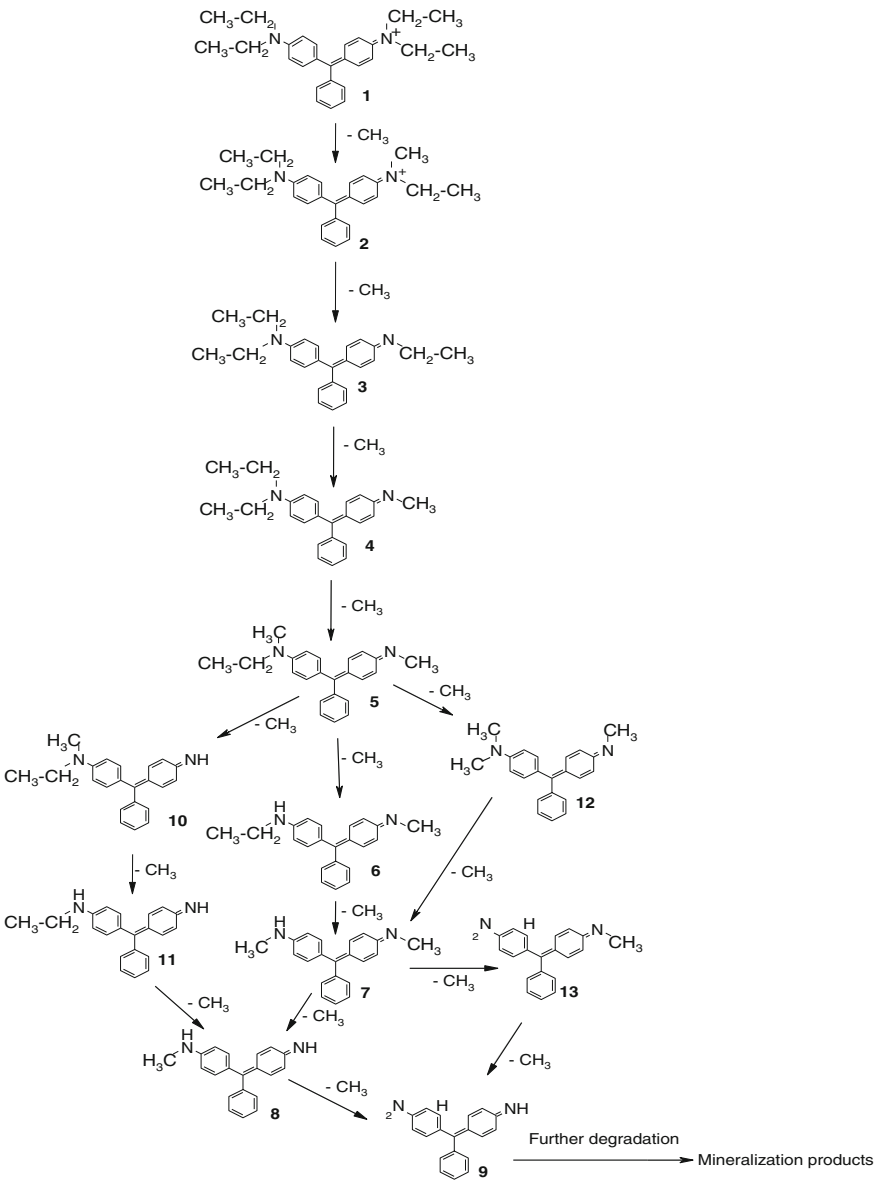


Fig. 1 Proposed *N*-demethylation pathway for decolorization of Methyl violet by *Aspergillus* sp. strain CB-TKL-1 (Kumar et al. 2011). The different degradation intermediates of Methyl violet (1, *m/z* = 371.6) = *N*,*N*,*N*′,*N*′,*N*″,*N*″-hexamethylpararosaniline identified by LC-ESI-MS were DDMPR (2, *m/z* = 358.2) = *N*,*N*-dimethyl-*N*′,*N*′-dimethyl-*N*″-methyl pararosaniline; DMMPR (3, *m/z* = 344.1) = *N*,*N*-dimethyl-*N*′-methyl-*N*″-methyl pararosaniline; DDPR (4, *m/z* = 344.1) = *N*,*N*-dimethyl-*N*′,*N*′-dimethyl pararosaniline; MMMPR (5, *m/z* = 330.1) = *N*-methyl-*N*′-methyl-*N*″-methyl pararosaniline; DMPR (6, *m/z* = 330.2) = *N*,*N*-dimethyl-*N*′-methyl pararosaniline; MMPR (7, *m/z* = 316.1) = *N*-methyl-*N*′-methyl pararosaniline; DPR (8, *m/z* = 318.3) = *N*,*N*-dimethyl pararosaniline; MPR (9, *m/z* = 305.2) = *N*-methyl pararosaniline; PR (10, *m/z* = 287.7) = pararosaniline]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Further degradation
Mineralization products

◀ **Fig. 2** Proposed *N*-demethylation pathway for decolorization of Brilliant green by *Aspergillus* sp. strain CB-TKL-1 (Kumar et al. 2012). The different degradation intermediates of Brilliant green (1, *m/z* = 385.1) identified by LC-ESI-MS were DAPPMCDME (2, *m/z* = 372.5) *N*-(diethylamino) phenyl) (phenyl) methylene) cyclohexadienylidine) *N*-methylethanaminium; DAPPMCDE (3, *m/z* = 356) N-(diethylamino) phenyl) (phenyl) methylene) cyclohexadienylidene) ethanaminium; DEAPPMCDM, (4, *m/z* = 342) *N*-(diethylamino) phenyl) (phenyl) methylene) cyclohexadienylidene) methanaminium; EMAPPMCDM (5, *m/z* = 328) *N*-(ethyl(methyl)amino)phenyl)(phenyl) methylene) cyclohexadienylidine) methanaminium; EAPPMCDM (6, *m/z* = 314) *N*-((ethylamino) phenyl) (phenyl)methylene) cyclohexadienylidine) methanaminium; MAPPMCDM (7, *m/z* = 300) *N*-(methylamino) phenyl) (phenyl) methylene) cyclohexadienylidine) methanaminium; MAPPCD (8, *m/z* = 286) (methylamino) phenyl) (phenyl) methylene) cyclohexadieniminium; APPMCD (9, *m/z* = 275) (aminophenyl) (phenyl) methylene) cyclohexadieniminium; EMAPPMCD (10, *m/z* = 314) (ethyl(methyl)amino) phenyl) (phenyl) methylene) cyclohexadieniminium; EAPPMCD (11, *m/z* = 300) (ethylamino) phenyl) (phenyl) methylene) cyclohexadieniminium; DMAPPMCDM (12, *m/z* = 314) *N*-(dimethylamino) phenyl (phenyl) methylene) cyclohexadienylidine methanaminium and MAPPMCD (13, *m/z* = 286) (methylamino)phenyl) (phenyl) methylene) cyclohexadieniminium

exposed to higher concentrations, evolve mechanisms and pathways for degrading them. This happens through expression of genes encoding for enzymes responsible for degradation. Alternatively, the identification, isolation, and transfer of genes encoding for degradative enzymes can greatly help in designing microbes with enhanced degradation capabilities. Thus, acclimatization and genetic engineering both can be helpful in designing superbugs with enhanced degrading ability. Another aspect, that needs to be explored, is the use of thermotolerant or thermophilic microorganisms in decolorization systems. This would be of advantage as many textile and other dye effluents are produced at relatively high temperatures (50–60 °C), even after a cooling or heat-exchange step. The availability of such thermotolerant microbes for decolorization may consequently reduce the treatment cost significantly. The enzymatic approach has also attracted much interest in the recent years for the bioremediation or decolorization of various dyes present in wastewater or industrial effluent. The enzymatic treatment has its own inherent problems, such as the feedback inactivation of enzyme by its own product/products and recalcitrant nature of the dyestuffs. The addition of some suitable redox mediators can help oxidoreductive enzymes in enhancing the decolorization ability of recalcitrant dyes. However, immobilized enzymes have been proven to be superior to free enzymes and can be used successfully in the reactors for continuous remediation of synthetic dyes from wastewater. Treatment of recalcitrant dyes by using enzyme-redox mediator system will be helpful for targeting a number of dyes with diversified structures. The viability of developing commercial scale treatment processes lies in using oxidoreductive enzymes. A two reactors system approach can be useful for the decolorization/degradation of dyes wherein the first reactor has an immobilized enzyme, while the second reactor contains an adsorbent. The immobilized enzyme would catalyze and breakdown the dye resulting in activated by-products which would bind to the adsorbent in the second reactor and finally pollutant free water is released. Indeed to develop such a system which becomes commercially viable necessitates the identification of a cheaper biocatalyst and

adsorbent. Further, the availability of biofilms for immobilization of lignin degrading fungi or their enzymes for continuous use in wastewater treatment is yet another challenge for the future.

8 Conclusions

Dyestuffs and dye wastewater accumulation create not only environmental pollution, but also medical and aesthetic problems. Regulations are becoming more stringent day-by-day and hence there is an urgent need to develop technically feasible and cost-effective treatment methods. Different physical and chemical methods have been employed for the treatment of synthetic dye wastewaters. These methods mostly suffer from serious limitations like high cost, low efficiency, limited versatility, and production of secondary pollution (sludge), etc. In contrast, bioremediation is an efficient, eco-friendly, cost-effective and environmentally benign approach for the removal of dyes from industrial wastewaters. A large number of lab-scale studies have demonstrated the decolorization of dye solutions using pure and mixed cultures; however, there is still a need to generate comparative performance data on the decolorization of industrial dye effluents. Further, to ensure the safety of the decolorized wastewater, studies should be conducted on the toxicity of the treated effluent/dye solution. Based on the successful laboratory results, efforts should then be made to scale-up further at commercial scale and apply bio-based decolorization techniques in treating real industrial effluents. Moreover, the techniques of molecular biology and biochemistry coupled with the latest advances in genomics and proteomics revolutionizing various aspects of fundamental biological science offers a wide range of possibilities for enhancing the performance of microbial treatment of dye-containing wastewater.

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Reductive Decolorization of Azo Dye by Bacteria

Guangfei Liu, Jiti Zhou, Jing Wang, Xin Zhang, Bin Dong and Ning Wang

1 Introduction

Archaeological studies have found the application of colorants in cliff and cave paintings by prehistoric human ancestors. Inorganic pigments include soot, ochre, manganese oxide and hematite, while organic colorants contain kermes from *Kermes vermilio*, alizarin from madder and indigo from natural origins. Then in 1856 William Henry Perkin, an 18 year old English chemist, accidentally discovered the world's first synthetic dye in his attempt to synthesize the antimalarial drug quinine. The bluish substance with excellent dyeing properties was later known as mauveine or aniline purple. Since then, more than ten thousand synthetic dyes were developed and the use of synthetic dyestuffs has now far exceeded natural dyestuffs by the end of 19th century (Robinson et al. 2001). It was suggested that more than 10^5 different commercial dyes and over 7×10^5 metric tons of dyestuffs are produced every year worldwide (Supaka et al. 2004).

The textile industry is the largest consumer of synthetic dye and uses high quantities of water. Hence, it generates the highest amount of dye wastewater (Ali 2010). It was estimated that around 2.8×10^5 tons of textile dyes were discharged in textile industry effluent annually around the world (Jin et al. 2007). Depending on the class of dye, its loss in effluents during application could vary from 2 % to as high as 50 %, which led to severe contamination of surface and ground waters in the vicinity of dyeing industries (O'Neill et al. 1999). The dye concentration in textile wastewater generally ranges between 10 and 200 mg l^{-1}. Many dyes are visible in water at a concentration as low as 1 mg l^{-1} (Pandey et al. 2007).

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The improper discharge of colored dye effluents into natural water bodies undoubtedly causes aesthetical unpleasantness. In addition, it leads to a reduction in sunlight penetration, which, in turn, decreases photosynthetic activity and dissolved oxygen amount in the water, and affects aquatic flora and fauna (Saratale et al. 2011). However, due to increasing stringent government legislation against dye pollution all over the world, serious attention has been paid for the treatment of dye wastewater. We would like to focus here on the bacterial decolorization of azo dyes. Recent studies on factors affecting the decolorization performance, the involvement of azoreductase in decolorization, potential mechanisms of azo dye decolorization, and toxicity evaluation of azo dyes and their decolorized metabolites are presented here.

2 Bacterial Decolorization of Wastewater Containing Azo Dye

Based on their chemical structures and chromophores, synthetic dyes could be classified into 20–30 different groups. More than 2–3 × 10^3 azo dyes, containing one or more –N=N– groups, constitute the largest commercially available class of synthetic dyes (Stolz 2001; Pandey et al. 2007; Vijaykumar et al. 2007; Saratale et al. 2011). It was estimated that azo dyes make up around 70 % of all dyestuffs used worldwide by weight (Zollinger 1987). Azo dyes are easy to be synthesized, encompass all colors, attach well to fabrics and have good fastness properties (Chengalroyen and Dabbs 2013). Because of their high chemical, biological and photocatalytic stability and resistance to breakdown caused by time, water and soap, microbes, and sunlight exposure, azo dyes are widely used in many different industries including textile dyeing, paper printing, leather, photography, food, pharmaceuticals and cosmetics etc. (Pandey et al. 2007; Solís et al. 2012). Due to the wide use and release of azo dye into the environment, many studies on the treatment of azo dye wastewater have been carried out during the past decades.

Physicochemical methods, like adsorption, coagulation/flocculation, advanced oxidation, and membrane filtration etc., have generally inherent drawbacks including intensive energy and cost input, generation of large amounts of sludge that may cause secondary pollution, and complicate operation procedures (Saratale et al. 2011). Therefore, more environment-friendly and cost-competitive alternatives are needed. To date, only one naturally occurring azo compound (4, 4′-dihydroxy azo benzene) has been reported (Gill and Strauch 1984). Thus, all the other azo compounds are xenobiotic and expected to be recalcitrant to biodegradation. It was observed that azo dyes generally resist biodegradation in conventional activated sludge treatment units (Stolz 2001). The adaptability and activity of selected microbes determine their effectiveness in microbial decolorization.

Consequently, a great number of studies have been carried out to test different species for decolorization and mineralization of azo dyes. It was observed that many microbes, including bacteria, fungi, yeasts and algae could decolorize or even completely mineralize various azo dyes under certain environmental conditions.

A great diversity of bacteria species have been isolated and identified to be capable of removing different azo dyes under varied conditions. The complete removal of azo dye by bacteria generally takes two steps, i.e. the initial reductive decolorization of azo dye into corresponding aromatic amines, followed by mineralization of the aromatic amines under aerobic conditions. The reductive cleavage of the azo bond was believed to be the rate-limiting step and hence it has attracted more interests. Table 1 summarized some typical bacteria species reported for azo dye decolorization and their specific decolorization performance under different conditions. The diversity of these strains suggested a wide distribution of decolorization capacity in bacteria and offered candidates for the biological treatment of azo dye wastewater.

3 Factors Affecting Bacterial Decolorization

Like many other biological treatment processes, various physico-chemical operational parameters, such as pH, temperature, dissolved oxygen/agitation, salinity, dye structure/concentration, carbon/nitrogen sources etc., might directly affect the decolorization capacity of different bacterial strains. Therefore, optimization of these factors is essential to obtain good treatment results.

3.1 Effects of pH

The pH of the medium not only affects the adsorption capacity anddecolorization activity of cells, but also impacts the solubility of the dye and color of the solution. The tolerance to high pH is essential as many processes using azo dyes were operated under alkaline conditions. The optimal pH for bacterial decolorization is often between 6.0 and 10.0 (Saratale et al. 2011; Khan et al. 2013). A pH-independent decolorization performance was found for the Brilliant Blue G by a combination of *Galactomyces geotrichum* and *Bacillus* sp. (Jadhav et al. 2008a). Wang et al. (2009a) reported the effective decolorization of Reactive Red 190 by *Citrobacter* sp. CK3 under strongly acidic (pH 4) and alkaline (pH 12) conditions. Tolerance to extreme pH values makes the bacterial strain suitable for practical application.

Table 1 Decolorization of azo dyes by bacterial strains

Name of strain	Dye name and concentration	Condition [pH, temp. (°C), agitation]	Time (h) and Decolorization percentage (%)	Carbon sources	References
Alishewanella sp. KMK6	Mixture of textile dyes (Raspberry red, Orange 2R, Orange M2R, Golden Yellow HER each at 0.5 g l^{-1})	7.0, 37, Static Anoxic	8, 95–98	Yeast extract (1 %), Peptone (1 %), Starch (1 %)	Kolekar et al. (2013)
Alishewanella sp. strain KMK6	Reactive Blue 59 (2.5 g l^{-1})	7.0, 37, Static	6, 95	Yeast extract (5.0 g l^{-1})	Kolekar and Kodam (2012)
Aeromonas hydrophila	Red RBN; (0–3,000 mg l^{-1})	7.0, 30, Anoxic	192, >90	Yeast extract (10 g l^{-1})	Chen et al. (2003)
Bacillus sp.	Congo Red; (700–1,000 mg l^{-1})	7.0, 37, NA	37–48,100	No additional carbon source	Gopinath et al. (2009)
Bacillus fusiformis KMK5	Disperse Blue 79 and Acid Orange 10; (1.5 g l^{-1} each)	9, 37, Anoxic	48,100	NA	Kolekar et al. (2008)
Bacillus sp. strain AK1	Metanil Yellow (200 mg l^{-1})	7.2, 37, Static	12, 100	Yeast extract (2.5 g l^{-1})	Anjaneya et al. (2011)
Bacillus sp. VUS	Navy Blue 2GL (50 mg l^{-1})	NA, 40, Static Anoxic	48,94	Yeast extract (5 g l^{-1})	Dawkar et al. (2009)
Bacillus cereus strain HJ-1	Reactive Black B (20 mg l^{-1})	8.0, 25, Static	NA, NA	Yeast extract (0.15 g l^{-1}), Glucose (0.125 g l^{-1})	Liao et al. (2013)
Bacillus cereus M1	Reactive Red 195 (200 mg l^{-1})	7.0, 37, Static	20, > 97	Maltose (1 %)	Modi et al. (2010)
Bacillus lentus BI377	Reactive Red 141 (500 mg l^{-1}) Reactive Red 2 (500 mg l^{-1})	8.0, 40, Static	6, 98.22–99.11	Peptone (5 g l^{-1}), Yeast extract (5 g l^{-1})	Oturkar et al. (2013)

(continued)

Table 1 (continued)

Name of strain	Dye name and concentration	Condition [pH, temp. (°C), agitation]	Time (h) and Decolorization percentage (%)	Carbon sources	References
Bacillus thuringiensis RUN1	Congo Red (100 mg l^{-1})	7.2, 30, Static Anoxic	12, 72.84 ± 3.25	Beef extract (1 g l^{-1}), Yeast extract (2 g l^{-1}), Peptone (5 g l^{-1})	Olukanni et al. (2013)
Brevibacillus laterosporus	Remazol Red (50 mg l^{-1})	9.0, 40, Static	30, 100	Bacteriological peptone (10 g l^{-1}), Yeast extract (1.5 g l^{-1}), Beef extract (1.5 g l^{-1})	Kurade et al. (2013)
	Rubine GFL(50 mg l^{-1})	7.0, 40, Static	48, 95		
Brevibacillus laterosporus MTCC 2298	Methyl Red; (200 mg l^{-1})	7.0, 30, 150 rpm	12,93	NA	Gomare and Govindwar (2009)
Comamonas sp. UVS	Direct Red 5B; (1.1 g l^{-1})	6.5, 40, Static	125,100	Peptone (5 g l^{-1}); Beef extract (3 g l^{-1})	Jadhav et al. (2008b)
Citrobacter sp. CK3	Reactive Red 180; (200 mg l^{-1})	7.0, 32, Anaerobic	36,95	Glucose (4 g l^{-1})	Wang et al. (2009a)
Citrobacter sp.	Crystal Violet (100 μM)	7.0–9.0, 20–40, Static	1, >90	LB	An et al. (2002)
Dyella ginsengisoli LA-4	Acid Red GR (200 mg l^{-1})	7.06, 29, Anaerobic	NA, 98.36	LB	Zhao et al. (2010)
Enterobacter sp. EC3	Reactive Black 5; (1 g l^{-1})	7.0, 37, Anaerobic	120,92.56	Glucose (8 g l^{-1})	Wang et al. (2009b)

(continued)

Table 1 (continued)

Name of strain	Dye name and concentration	Condition [pH, temp. (°C), agitation]	Time (h) and Decolorization percentage (%)	Carbon sources	References
Enterococcus faecalis strain ZL	Acid Orange 7 (100 mg l^{-1})	NA, 37, Static	5, 98	Glycerol (0.1 %v/v)	Lim et al. (2013)
Enterococcus gallinarum	Direct Black 38 (100 mg l^{-1})	7.0, NA, Static	NA, NA	LB	Bafana et al. (2009)
Escherichia coli K12	Amaranth (80 μM)	8.0, 37, 150 rpm	NA, NA	Glucose (2 g l^{-1})	Lu et al. (2010)
Escherichia coli NO3	Reactive Red 22; (200 mg l^{-1})	7.0, 28, Static	139,50	Yeast extract (0.5 %)	Chang et al. (2004)
Geobacter metallireducens	Amaranth (25–800 μM)	7.0, 30, Anaerobic	40, 93.7	Pyruvate (20 mM)	Liu et al. (2013a)
Klebsiella oxytoca GS-4-08	Disperse Orange 3 (24.2 mg l^{-1}) /Methyl Red (269 mg l^{-1})	7.0, 30, Anaerobic, Static	7, 100/100	Sucrose (20 mM)	Yu et al. (2012)
K1ebsi1e11a pneumoniae RS-13	Methy1 Red; (100 mg l^{-1})	6.0–8.0, 23–37, Static	168,100	Glucose (0.1 g l^{-1})	Wong and Yuen (1996)
Lactobacillus acidophilus and *Lactobacillus fermentum*	Sudan III; (1.5 mg l^{-1}) Sudan IV; (1.5 mg l^{-1})	NA, 37, Anaerobic	36,100 36,100	MRS broth or MRS agar medium	Chen et al. (2009)
Lysinibacillus sp. strain AK2	Metanil Yellow (200 mg l^{-1})	7.2, 37, Static	24, 100	Yeast extract (2.5 g l^{-1})	Anjaneya et al. (2011)
Micrococcus glutamicus NCIM 2168	Reactive Green 19 A; (50 mg l^{-1})	8.0, 37, Static	42,100	Peptone (1 %)	Saratale et al. (2009)

(continued)

Table 1 (continued)

Name of strain	Dye name and concentration	Condition [pH, temp. (°C), agitation]	Time (h) and Decolorization percentage (%)	Carbon sources	References
Pseudomonas sp.	Orange I, Orange II; (1 g l^{-1})	7.0, 30, 110 rpm	35,90	4-Hydroxybenzoate, Glycerol or Acetate	Kulla et al. (1983)
Pseudomonas sp. SUK1	Reactive Red 2; (5 g l^{-1})	6.2–7.5, 30, Static	114, 80	Peptone (10 g l^{-1}), Yeast extract (2 g l^{-1}), Beef extract (1 g l^{-1})	Kalyani et al. (2009)
Pseudomonas aeruginosa	Remazol Orange; (200 mg l^{-1})	7.0, 30, Static	24,94	Glucose (1.2 g l^{-1}), Peptone (0.2 g l^{-1})	Sarayu and Sandhya (2010)
Pseudomonas aeruginosa BCH	Amaranth (50 mg l^{-1})	7.0, 30, NA	6, 97	NA (no additional carbon source)	Jadhav et al. (2013)
Pseudomonas aeruginosa BCH	Remazol Orange (400 mg l^{-1})	8.0, 30, Static	6.5, 92	Yeast extract (2 g l^{-1})	Jadhav et al. (2012)
Pseudomonas aeruginosa strain BCH	Direct Orange 39 (1,500 mg l^{-1})	7.0, 30, Static	48, 60	Yeast extract (0.3 %)	Jadhav et al. (2010)
Proteus mirabilis LAG	Reactive Blue 13 (100 mg l^{-1})	7.0, 35, Static	5, 100	Beef extract (1 g l^{-1}), Yeast extract (2 g l^{-1}), Peptone (5 g l^{-1})	Olukanni et al. (2010)
Pseudomonas aeruginosa NBAR12	Reactive Blue 172; (500 mg l^{-1})	7.0–8.0, 37, Static	42,83	Dextrose (3.0 g l^{-1}), Yeast extract (2.5 g l^{-1})	Bhatt et al. (2005)
*Psychrobacter alimentarius*KS23	Reactive Black 5 (100 mg l^{-1})/Reactive Golden Ovifix (100 mg l^{-1})/Reactive Blue BRS (100 mg l^{-1})	7.0, 30, Static	8, 100/8, 100/3, 100	Yeast extract (4 g l^{-1})	Khalid et al.(2012)
Rhizobium radiobacter MTCC 8161	Reactive Red 141; (50 mg l^{-1})	7.0, 30, Static	48,90	Urea (0.5 %), Yeast extract (0.5 %)	Telke et al. (2008)

(continued)

Table 1 (continued)

Name of strain	Dye name and concentration	Condition [pH, temp. (°C), agitation]	Time (h) and Decolorization percentage (%)	Carbon sources	References
Rhodopseudomonas palustris AS1.2352	Reactive Brilliant Red X-3B; (70 mg l^{-1})	8.0, 30, Anaerobic	10,90	Peptone (NA)	Liu et al. (2006)
Shewanella algae	Amaranth (200 μM)/Acid Orange 52 (200 μM)	7.0, 30, Anaerobic, NaCl (50 g l^{-1})	6, 97.5/49.1	Lactate (20 mM)	Liu et al. (2013b)
Shewanella decolorationis S12	Fast Acid Red GR; (150 μM)	NA, 30, Microaerophilic	68,100	Yeast extract (2 g l^{-1}), Lactate (20 mM)	Xu et al. (2007)
Shewanella marisflavi	Amaranth (200 μM)/Acid Orange 52 (200 μM)	7.0, 30, Anaerobic, NaCl (50 g l^{-1})	6, 53.4/32.7	Lactate (20 mM)	Liu et al. (2013b)
Shewanella oneidensis MR-1	Dye mixture (Methyl Orange and Naphthol Green B each at 0.1 mM)	8.0, 35, Anaerobic	NA, NA	Lactate (18 mM)	Cao et al. (2013)
Shewanella oneidensis MR-1	Methyl Orange (100 mg l^{-1})	6.5, 30, Anaerobic	NA, NA	Lactate (18 mM)	Cai et al. (2012)
Shewanella oneidensis MR-1	Acid Red 27 (200 μM)	7.0, 30, Anaerobic	6, 78.4	Lactate (20 mM)	Liu et al. (2011)
Shewanella putrefaciens AS96	Reactive Black-5 (100 mg l^{-1})/Direct Red 81 (100 mg l^{-1})/Acid Red 88 (100 mg l^{-1})/Disperse Orange 3 (100 mg l^{-1})	7.0, 30, Static, Anaerobic	8, 100/12, 87/24, 53/24, 58	Yeast extract (4 g l^{-1})	Khalid et al. (2008)
Shewanella xiamenensis BC01	Methyl Orange (100 mg l^{-1}), Reactive Red 198 (200 mg l^{-1}), Congo Red (200 mg l^{-1})	7.0, 30, Static	6, 96.2/93.0/87.5	LB	Ng et al. (2013)

(continued)

Table 1 (continued)

Name of strain	Dye name and concentration	Condition [pH, temp. (°C), agitation]	Time (h) and Decolorization percentage (%)	Carbon sources	References
Sphingobacterium sp.	Direct Red 5B (DR5B) (500 mg l^{-1})	7.0, 30, Static	24, 100	Molasses (1 or 2 %)	Tamboli et al. (2010a)
Sphingobacterium sp. ATM	Direct Blue GLL (300 mg l^{-1})	7.0, 30, Static	24, 100	Molasses (1–2 %)	Tamboli et al. (2010b)
Staphylococcus equorum KS26	Reactive Black 5 (100 mg l^{-1}) /Reactive Golden Ovifix (100 mg l^{-1}) /Reactive Blue BRS (100 mg l^{-1})	7.0, 30, Static	8, 100/8, 100/, 3, 100	Yeast extract (4 g l^{-1})	Khalid et al. (2012)

NA, not available

3.2 Effects of Temperature

Temperature is another important environmental factor affecting various processes associated with microbial activity. There exists certain temperature range for the growth and reproduction of microbe cells and the effective function of enzymes. The decolorization performance of bacteria was generally observed to increase up to the optimum temperature, and then decreased. A decline performance after the optimum temperature was usually attributed to the loss of cell viability or the denaturation of enzyme responsible for decolorization (Saratale et al. 2011; Solís et al. 2012).

3.3 Effects of Dissolved Oxygen/Agitation

Although there are few studies which suggested that agitation could improve the decolorization process by increasing mass transfer and nutrient distribution, we generally believe that the presence of oxygen will compete with azo dye for electrons and inhibit most bacterial decolorization performance. It should be noted that in some studies of azo decolorization under agitation conditions, the dissolved oxygen could be rapidly depleted by high amounts of cellular biomass in the nutrient media. Thus, the decolorization reaction was actually performed under anoxic conditions. Nowadays many studies suggested to incubate specific strains under aerobic conditions first to obtain the needed biomass rapidly, and then to use the harvested cells to decolorize azo dyes under static or anaerobic conditions. Of course, the aerobic conditions are required for further mineralization after color removal. Anaerobic decolorization process combined with subsequent aerobic treatment was typically applied for the complete treatment of azo dye wastewater.

3.4 Effects of Salinity

In textile dyeing, various salts were utilized to separate organic contaminants (brine rinse), to help precipitation of dyestuff (salting out), and to mix with concentrated dyes to standardize them (Dennis 1996). Sodium level could also be elevated by addition of sodium hydroxide into dye bath to increase the pH (Khalid et al. 2008). Besides azo dye residues, textile effluents also contain substantial amounts of various salts. High salt concentrations may cause plasmolysis and reduce biological activity (Kargi 2002). Azo dye removal efficiencies under saline conditions were usually decreased due to inhibitory effects of salt on microbial flora (Khalid et al. 2008). Dilution of effluents before entering into traditional activated sludge systems might alleviate salt stresses but this may produce a large volume of wastewaters which could enhance the treatment burden (Khalid et al. 2008). In recent studies,

the researchers have isolated and characterized several halophilic and halotolerant microorganisms, which belonged to genus *Halomonas* and could decolorize azo dyes under high salt conditions (Asad et al. 2007; Guo et al. 2008; Amoozegar et al. 2010). Moreover, Khalid et al. (2008) isolated *Shewanella* sp. AS96 from activated sludge and studied its impact on azo dye decolorization under saline conditions. We recently have systematically investigated the decolorization capacities of different *Shewanella* strains and found that strains, isolated from marine sources including *Shewanella algae*, *Shewanella aquimarina* and *Shewanella marisflavi,* demonstrated a high ability in color removal in the presence of up to 100 g l^{-1} NaCl or Na_2SO_4 (Meng et al. 2012; Liu et al. 2013b). These salt-tolerant microbial strains would facilitate treatment of saline dye wastewater.

3.5 Effects of Carbon/Nitrogen Sources

Efficient decolorization of azo dye by bacteria generally requires the supplement of carbon and nitrogen sources. Glucose has been frequently demonstrated to improve the decolorization efficacy (Khan et al. 2013). However, higher concentration of glucose could also decrease the decolorization performance, probably due to acidification of the medium. Other organic substances, such as acetate, glycerin, sucrose, salicylate and citrate etc. could also act as carbon source for azo dye decolorization. However, the most effective carbon source is strain-specific and needs detailed investigation for different bacterial strains. The addition of organic nitrogen sources including peptone, beef extract, yeast extract etc. could help the regeneration of NAD(P)H, which donates electrons for bacterial azo dye reduction (Saratale et al. 2011). There have been some reports indicating that certain bacterial species could utilize azo dye itself or its reduction products as carbon/nitrogen sources, but such microorganisms were very specific to their substrates (Pandey et al. 2007; Ali 2010). However, their effectiveness for practical azo dye wastewater treatment needs further investigation.

3.6 Effects of Dye Structure/Concentration

Besides the common chromophore azo bond, azo dyes have diverse structures, which significantly affects the decolorization performance of bacteria. Dyes with simple structures and low molecular weights are generally easier to be decolorized. Substitution of electron withdrawing groups (e.g. $–SO_3H$, $–SO_2NH_2$) in the para-position of phenyl ring relative to the azo bond makes dyes difficult to be reduced. It is well known that the decolorization rates of monoazo dyes are generally higher than those of diazo and triazo dyes (Saratale et al. 2011). However, sometimes the above-mentioned rules do not work. Besides molecular structure and molecular weight, the decolorization of an azo dye is also dependent on its redox potential.

An increase of dye concentration generally resulted in decrease of decolorization percentage (Saratale et al. 2011). However, when the specific decolorization rate was considered, the relationship between decolorization rate and dye concentration could be well described with Michaelis-Menten equation. Therefore, before the level off of the decolorization rate, it increases with the increase of dye concentration. The kinetic parameters could be used to compare the decolorization capacity of different bacterial strains (Liu et al. 2007b, 2013a,b).

4 Involvement of Bacterial Azoreductase in Azo Dye Decolorization

Azoreductases are enzymes catalyzing the reductive cleavage of azo bonds. Enzymes with azo-bond reducing activities were initially found from rat or rabbit liver (Autrup and Warwick 1975; Stoddart and Levine 1992). In early 1980s, Zimmermann et al. (1982, 1984) purified and compared two azoreductases from *Pseudomonas* KF46 (later known as *Xenophilus azovorans* KF46) and *Pseudomonas* K24 (later known as *Pigmentiphaga kullae* K24), which promoted great interests in studies on bacterial azoreductase. The two azoreductases demonstrated different substrate specificities. The Organe II azoreductase from KF46 required the presence of a hydroxyl group in the ortho-position of the aromatic ring of the dye. In contrast, the Orange I azoreductase from K24 required a hydroxyl group in the para-position of the aromatic ring of the dye (Zimmermann et al. 1982, 1984). During the past three decades, many azoreductases were purified from different bacterial strains (Table 2). Based on their functions, azoreductases are categorized as flavin (FMN)-dependent and flavin-independent azoreductase. The former could be further classified on the basis of its co-factor (NADH, NADPH) preference (Bürger and Stolz 2010). Azoreductases show low level of nucleotide or amino acid sequence similarity. However, when their tertiary structures were superimposed and compared, azoreductases could be classified into two families (Abraham and John 2007). The monomer of almost all the obtained flavin-dependent azoreductase possesses flavodoxin-like structure (Liu et al. 2007a, 2008, 2009).

The involvement of intracellular azoreductase in bacterial decolorization has been in doubt recently. On one hand, azo dye molecules generally have high molecular weight, high polarity and complex structure, which make it difficult for them to enter the cells and react with intracellular enzymes. On the other hand, as mentioned above, there is almost no azo compound in the natural environment, which means intracellular proteins with azoreductase activity should be prepared for other functions. To deduce the physiological role of azoreductase, we first carried out the three-dimensional structure modeling studies of bacterial azoreductase. Based on structure analysis and experimental test, we found the nitroreductase and quinone reductase activities of bacterial azoreductase, respectively (Liu et al. 2007a, 2008). Then based on gene knock-out and mutant studies, we

Table 2 Decolorization of azo dyes by bacterial azoreductase

Species	Molecular mass	Cofactor	Electron donor	O_2	Optimum pH	Optimum Temp.	K_m (μM)	V_{max} (U)	References
Bacillus sp. Strain SF	62 kDa (monomer)	NA	NADH	S	8.0–9.0	80	NA	NA	Maier et al. (2004)
Bacillus velezensis Strain AB	60 kDa	NADH	NADH	I	NA	NA	NA	NA	Bafana et al. (2008)
Caulobacter subvibrioides strain C7-D	30 kDa	NA	NADH NADPH	I	6.0–7.5	NA	1	NA	Mazumder et al. (1999)
Xenophilus azovorans KF46	30 kDa (monomer)	NA	NAD(P)H	I	5.0–8.0	45	1.5	17.8	Zimmermann et al. (1982), Blümel et al. (2002), Bürger and Stolz (2010)
Pigmentiphaga kullae K24	21 kDa (monomer)	NA	NADPH NADH	I	6.2–6.8	41	1.1	NA	Zimmermann et al. (1984), Blümel and Stolz (2003)
Pseudomonas aeruginosa	29 kDa (monomer)	NA	NADH	I	7	35	62.5	16.67	Nachiyar and Rajakumar (2005)
Bacillus badius	43 kDa (monomer)	NA	NADH	I	7.4	60	1330	100	Misal et al. (2011)
Geobacillus stearothermophilus	46 kDa (homodimer)	FMN	NADH	I	NA	85	NA	NA	Matsumoto et al. (2010)
Bacillus sp. B29	48 kDa (homodimer)	FMN	NADH	I	6–10	60–80	NA	NA	Ooi et al. (2007)
Bacillus sp. B29	48 kDa (homodimer)	FMN	NADH	NA	5.0–11	70	37	NA	Ooi et al. (2009)
	48 kDa (homodimer)	FMN	NADH	NA	6.6–10	55	61.1	NA	
Lysinibacillus sphaericus	29 kDa (monomer)	NA	NADH	NA	7.4	75	34	67	Misal et al. (2014)

(continued)

Table 2 (continued)

Species	Molecular mass	Cofactor	Electron donor	O_2	Optimum pH	Optimum Temp.	K_m (μM)	V_{max} (U)	References
Staphylococcus aureus	85 kDa (tetramer)	FMN	NADPH	I	6.0–6.6	35–40	57	0.41	Chen et al. (2005)
Rhodobacter sphaeroides AS1.1737	18.7 kDa	NA	NADH	I	8	50	420	65.2	Yan et al. (2004)
Clostridium perfringens	90.4 kDa (tetramer)	NA	NADH	S	9	30–37	0.005	0.42	Morrison et al. (2012)
Bacillus sp. OY1-2	20 kDa	NA	NADPH	I	NA	70	NA	NA	Suzuki et al. (2001)
Pseudomonas aeruginosa PAO1	110 kDa (homotetrame)	FMN	NAD(P)H	I	NA	NA	76	0.34	Wang et al. (2007)
Enterococcus faecalis	NA	FMN	NAD(P)H	NA	NA	NA	11	29	Punj and John (2009)
Enterococcus faecalis	43 kDa (homodimer)	FMN	NADH	I	6.6–7.0	35–40	24	86.2	Chen et al. (2004)
Pseudomonas putida MET94	40 kDa (homodimer)	FMN	NAD(P)H	S	7	32	400	1	Mendes et al. (2011)
Enterobacter agglomerans	28 kDa (monomer)	NA	NADH	I	7	35	29.5	9.21	Moutaouakkil et al. (2003)
Escherichia coli	46 kDa (homodimer)	FMN	NADH	NA	NA	NA	17.9	NA	Nakanishi et al. (2001), Ito et al. (2006)
Aquiflexum sp. DL6	80 kDa (monomer)	FMN	NAD(P)H	I	7.4	60	1110	30.77	Misal et al. (2013)
Brevibacillus laterosporus TISTR1911	23 kDa (monomer)	FMN	NADH	I	6	30	8	167	Lang et al. (2013)

NA not available, *S* sensitive, *I* insensitive

ultimately concluded that bacterial azoreductase is indeed involved in resistance to thiol-specific stress caused by electrophilic quinones (Liu et al. 2009).

More and more evidences suggested that intracellular azoreductase might not play a significant role in bacterial decolorization of azo dye. Although bacterial azoreductases could efficiently decolorize azo dyes during in vitro tests, the application of these enzymes for practical azo dye wastewater treatment seems to be difficult.

5 Mechanisms and Pathways of Bacterial Decolorization

The decolorization of azo dye is realized through the cleavage of azo bond. At present, three mechanisms might be responsible for bacterial decolorization.

5.1 Direct Bacterial Decolorization

As mentioned in previous section, the participation of intracellular azoreductase in bacterial decolorization still lacks evidence. However, the intense studies on azo dye decolorization by *Shewanella oneidensis* MR-1 in recent years have provided new insights of direct bacterial decolorization. The Mtr pathway of MR-1 is well-known for the strain's excellent capabilities of extracellular electron transfer and diverse respiration forms.

Brigé et al. (2008) utilized random transposon mutagenesis and targeted insertional mutagenesis to find important genes involved in bacterial decolorization of *S. oneidensis* MR-1. It was found that the MTRA mutant was not completely deficient in dye decolorization, suggesting that additional proteins are possibly involved in the inter membrane electron transfer required for extracellular dye reduction. Although only a small decrease in azo dye decolorization rate was observed with *omcA* mutant, the reduction rates of *omcA*/*omcB* double mutants were significantly decreased compared with wild-type MR-1. The multi haem cytochromes MtrF, OmcA, and OmcB may fulfill a function as terminal dye reductase. Furthermore, azo dye decolorization studies have been carried out with MR-1 gene knock-out mutant strains by Yu's group in details recently (Cai et al. 2012; Cao et al. 2013). It was found that the block of the Mtr pathway resulted in 80 % decrease of the decolorization rate. Knockout of *cymA* resulted in a substantial loss of azo-reduction ability, which suggested that CymA is a key c-type cytochrome in the electron transfer chain to azo dye. Therefore, MtrA-MtrB-MtrC respiratory pathway was proposed to be mainly responsible for the anaerobic decolorization of azo dyes by *S. oneidensis* MR-1. The potential involvement of membrane proteins of other strains in azo dye decolorization remains yet to be studied.

5.2 Indirect/Mediated Bacterial Decolorization

During the past decades, the use of redox mediator or electron shuttle to stimulate bacterial decolorization of azo dye has attracted many interests. Redox mediator itself can rapidly get transformed between oxidized and reduced forms which helps the transfer of electron or reducing power from bacterial cells to extracellular azo dyes. Keck et al. (1997) for the first time reported that redox intermediates generated during aerobic degradation of a xenobiotic compound could cause the enhancement of azo dye decolorization under anaerobic conditions. Chang et al. (2004) also showed that the addition of culture supernatants containing metabolites of a dye-decolorization strain *E. coli* NO_3 enhanced azo dye decolorization rate. Field and Brady (2003) found that riboflavin in catalytic amounts significantly improved the decolorization performance of anaerobic granular sludge. Recent studies indicated that *S. oneidensis* MR-1 could self-excrete flavins to improve extracellular electron transfer (von Canstein et al. 2008). The addition of synthetic electron carriers, such as anthraquinone-2-sulfonate, could also greatly enhance azo dye decolorization (van der Zee et al. 2001). More recent studies tested the effectiveness of many different quinone compounds as redox mediators to stimulate bacterial decolorization. Besides, various immobilization methods were developed to prevent the loss and repeated dosage of quinone compounds. A detailed summary of this study and other investigations on redox mediator have been discussed (van der Zee and Cervantes 2009).

The use of synthetic quinones as redox mediators could significantly speed up the decolorization process; however, the high cost of quinone addition limited its practical use. Recently, we found that natural humic acids containing lots of quinone groups could also stimulate the decolorization of azo dyes by *S. oneidensis* MR-1 (Liu et al. 2011). On the other hand, carbon material, such as activated carbon, carbon black and carbon nano tube, were also found capable of acting as redox mediators for dye wastewater treatment (Pereira et al. 2014). These studies offered practical application to bacteria-mediated decolorization.

5.3 Azo Dye Decolorization by Biogenic Inorganic Compounds

Inorganic compounds, such as sulfide and ferrous ion generated during metabolic reactions under anaerobic conditions, could also decolorize azo dyes via purely chemical reactions. However, this way of azo dye decolorization is less important compared to the above two ways (Pandey et al. 2007).

6 Toxicity of Azo Dyes and Their Decolorization Products

It has been found that the purified forms of many azo dyes are directly mutagenic and carcinogenic (Chen 2002). In addition, the transformed intermediates of azo dyes are also highly toxic and mutagenic in nature (An et al. 2007; Pandey et al. 2007). Thus, merely showing decolorization of azo dyes is not enough for the publication of a study, but verification of detoxification after decolorization is also essentially required in recent years. The toxicity study of azo dyes and their metabolic intermediates was usually carried out by assessing the phytotoxicity or microbial toxicity of the sample before and after bacterial decolorization. In phytotoxicity, the seeds of model plant were treated with certain concentration of dye and its decolorization metabolites and incubated for a given period. Then germination percentage, lengths of plumule and radicle of the treated seeds were three most investigated parameters for the phytotoxicity assessment (Khan et al. 2013). For microbial toxicity test, the zones of inhibition in the presence of azo dye or its decolorization metabolites were usually measured and compared (Khan et al. 2013).

7 Future Prospectives

Based on the development of bacterial decolorization during the past two decades, now we have to focus on: (i) Mineralization and detoxification besides decolorization. As the list of dye decolorizing bacteria continues to extend, more attention should be paid to the isolation of strains capable of removing aromatic amine metabolites. The combination of certain physicochemical method with bacterial decolorization could also be a good effort. (ii) Adaption better than genetic modification. Considering the present situation of ineffective intracellular azoreductase and strict regulation on gene-modified organisms, we believed that the acclimatization of natural microbes to practical operation conditions should be the first choice of dye decolorizer. (iii) Design of more biodegradable azo dye. The knowledge of effects of dye structure on its biodegradability should be provided to dye chemists to direct the synthesis of more environment-friendly dye molecules. (iv) New process and technology for dye production and application industries. The development of novel process and technology, which can bring down water use and recycle the treated wastewater, would reduce azo dye pollution from the source. This is more important than treatment at the final stage.

8 Conclusion

Pollution caused by azo dye wastewater not only creates aesthetic problems, but may also affect the equilibrium of natural ecosystem and human health. Regulations on azo dye use and discharge are becoming more and more stringent. Hence,

efficient and cost-effective treatment methods are urgently needed. Various bacterial strains, capable of decolorizing azo dyes under different conditions, have been isolated and characterized during the past decades. Moreover, in addition to purification of intracellular azoreductase, the continuous endeavors on revealing the real decolorization mechanisms helped researchers to approach the essence of bacterial decolorization. With the help of latest advances in studies like omics, nanotechnology and microbial fuel cell etc., more achievements in understanding bacterial decolorization process and more efficient decolorization technology are expected in coming years.

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Congo Red Decolorizing Bacteria from Paper Factory Effluent

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1 Introduction

Synthetic dyes usually consist of aromatic rings, methyl, methoxy, sulfo or nitro group. They are not readily degradable and can not be removed from water by conventional wastewater treatment systems (Anliker 1979).

Synthetic dyes are frequently found in effluents from the textile and paper industries. These dyes are generally very toxic. One example of these synthetic dyes is the azo dyes (e.g. monoazo, diazo, triazo and polyazo). Azo dyes represent the largest class of organic colorants listed in the Color Index (i.e. 60–70 % of the total). They constitute a vast majority of the dyes which are discharged in the wastewaters (van der Zee 2002).

Azo dyes are synthetic dyes that have a azo group of two nitrogen atoms (N = N) connecting aromatic ring compounds. Azo dyes may be direct, acid or basic. Direct dyes are relatively large molecules with high affinity especially for fibers. Acid dyes are anionic compounds that are mainly used for dyeing nitrogen-containing fabrics

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like wood, polyamide, silk and modified aryl. Basic dyes are cationic compounds that are used for dyeing acid-group containing fibers, usually synthetic fibers like modified poly aryl (van der Zee 2002).

Dyes are usually aromatic and heterocyclic compounds and are often recalcitrant in nature. A few of them are very toxic and even carcinogenic in nature (Vyas and Mollitoris 1995). They include a broad spectrum of different chemical structures, primarily based on substituted aromatic and heterocyclic groups, such as the aromatic amine (C_6H_5–NH_2), which is a suspected carcinogen, phenyl (C_6H_5–CH_2) and naphthyl (NO_2–OH). Common to all is their ability to absorb light in the visible region (Rajamohan and Karthikeyan 2006). Direct dyes lack fastness during washing and so, they are popular for items which are unlikely to require fastness during washing like paper. A direct diazo dye commonly used in the paper industry is Congo red, which is intended primarily for the coloration of paper products. It is a recalcitrant and also a well known carcinogen due to the presence of the aromatic amine group (Cripps et al. 1990; van der Zee 2002; Rajamohan and Karthikeyan 2006). Congo red is the sodium salt of benzidinediazo-bis-1-naphtylamine-4-sulfonic acid ($C_{32}H_{22}N_6Na_2O_6S_2$; mw: 696.66 g mol^{-1}). It is a secondary diazo dye.

2 Microbial Treatment

Various physical, chemical and biological pre-treatment, as well as main treatment techniques have been reported to remove color from dye-containing wastewater (van der Zee 2002). Biological techniques include bacterial and fungal biosorption and biodegradation in aerobic or anaerobic treatment processes. Over the last two decades, a lot of work has been done with the purpose of using microorganisms as bioremediation agents in the treatment of wastewater-containing textile dyes (Ramalho et al. 2004).

Numerous studies have demonstrated the ability of bacteria in monoculture to degrade dyes anaerobically and aerobically (Banat et al. 1996). Dye decolorization by bacteria has been observed under different conditions. Reductive cleavage of the azo bond by a wide variety of microorganisms, sediments and sludges occurs readily under anaerobic conditions (Stolz 2001; Field and Brady 2003). The effectiveness of microbial decolorization depends on the adaptability and the activity of selected microorganisms. Many microbes including bacteria, fungi and yeast are capable of degrading azo dyes (Chen et al. 2003). A highly alkali thermostable *Bacillus* sp. strain SF isolated from the wastewater drain of a textile finishing company was found capable of degrading an azo dye (Maier et al. 2004). An azo dye-reducing and endospore forming bacterium was isolated from textile industry wastewater that can decolorize the azo dye Remazol Black B. The bacterium was found to be 92.1–95 % similar to *Paenibacillus* sp. based on 16S rDNA sequence homology (Meehan et al. 2001). Transformation of six industrial dyes by manganese peroxidase from *Bjerkandera adusta* and *Pleurotus eryngii* was also investigated (Heinfling et al. 1998).

In an earlier study, two types of bacteria in monoculture and two consortia have been isolated and found to decolorize Congo red under fermentative, nitrate reducing and denitrifying condition (Decena and Barraquio 2004; Jalandoni-Buan et al. 2010). Samples used for isolation included sediment from polluted canal receiving the effluents containing Congo red and other dyes, soil from non-polluted source, rhizospheric soil and roots of *Imperata cylindrica* growing in the vicinity of the Congo red-polluted canal. For the enrichment of the Congo Red Decolorizing Bacteria (CRDB), minimal salts medium with glucose, ammonium sulfate, potassium nitrate and Congo red were used in both aerobic and anaerobic conditions. Putatively decolorizing cultures were further enriched by transferring aliquots of the enriched cultures into fresh media. The final enriched culture medium was analyzed for Congo red and nitrate colorimetrically.

Potentially decolorizing cultures were plated onto same medium for monoculture isolation. Pure cultures and consortia were confirmed for decolorization activities. Our study showed that in the presence of oxygen as electron acceptor, no decolorization of the dye occurred. The rate of decolorization of the monocultures after purification was observed to be slower than that of the consortia. The monocultures decolorized Congo red after 2 weeks of incubation (SB13B, SB12D and IRRI-1C) and after 1 month of incubation in the case of S22B. However, the consortia, on the other hand, decolorized Congo red after only a week of incubation. Blackening of the rubber stopper was observed in consortium S22 together with rotten egg odor which indicated the possible presence of a sulfate reducing organism in the mixed bacterial culture. The presence of sulfate reducing bacteria in the consortium could have hastened the rate of decolorization of the dye. Van der Zee et al. (2003) reported that the sulphide produced by a sulfate reducing bacteria can reduce azo bonds.

The monocultures, SB13B and S22B decolorized Congo red concurrently with nitrate reduction. The 16S rRNA sequencing identified SB13B as *E. coli*, SB12D as *Enterobacter dissolvens* and S22B as *Pseudomonas citronellolis*. API 20E identified IRRI-1C as *Klebsiella oxytoca*.

3 Factors Affecting Degradation Process

We conducted a microcosm experiment wherein both polluted and unpolluted waters were inoculated with the monocultures and consortia and incubated with Congo red statically at room temperature and at 28–30 °C (Jalandoni-Buan et al. 2009). Polluted water was collected from a portion of the Laguna de Bay near a duck farm in Victoria, Laguna (Fig. 1). That part of the bay was polluted with waste products coming from the ducks as well as other waste products coming from different sites. The pH of the water was found to be 7.37. The water appeared brown to black with odor of manure. Clean water was collected near the middle of Lake Caliraya also in Laguna (Fig. 2). The water appeared clear with a pH of 7.16. Both the monocultures and consortia were able to decolorize the polluted water with the

Fig. 1 A portion of Laguna de bay near a duck farm in Victoria, Laguna

Fig. 2 A portion of Lake Caliraya, Laguna where the clean water was sampled

dye (Fig. 3). The uninoculated polluted water with Congo red was not decolorized, even if resident organisms in the polluted water were present.

No decolorization by both monocultures and consortia was observed in the inoculated and uninoculated clean water with Congo red (Fig. 4). However, CRDB and consortia enriched in polluted water with Congo red were able to decolorize the dye. The presence of other organic matter in the polluted water could have helped in the degradation of the dye. Agricultural waste from poultry farming is the source of organic and inorganic pollutants in surface water and ground water (Krantz and Kifferstein 2007). Presence of resident microorganisms in the polluted water could also have helped in the degradation of the dye, but these organisms were not able to decolorize the dye by themselves since the uninoculated polluted water with Congo red was not decolorized. This could be possible because the microorganisms present in the polluted water do not produce the enzyme that could reduce azo dyes.

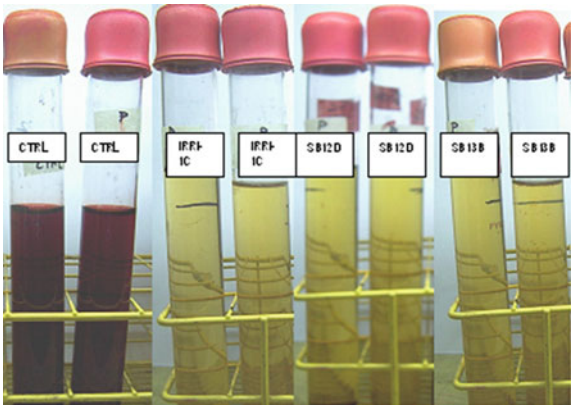

Fig. 3 Decolorization of polluted water by monocultures and consortia. *Left to right* Uninoculated polluted water with Congo red as negative control (CTRL), IRRI-1C, SB12D and SB13B

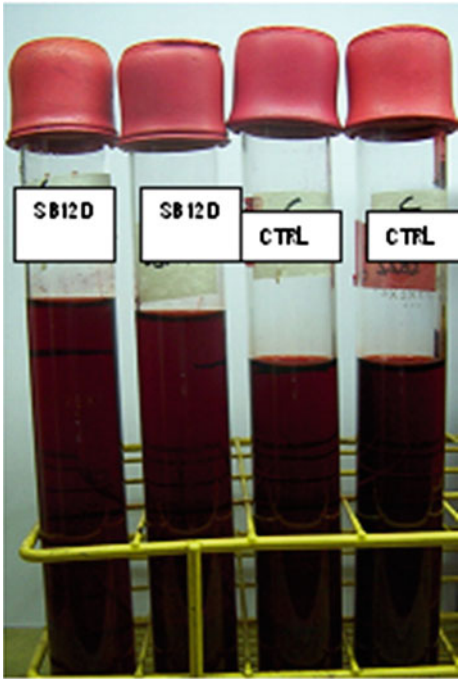

Fig. 4 No decolorization of clean water by monocultures and consortia. *Left to right* SB12D and uninoculated clean water with Congo red as negative control (CTRL)

Another explanation could be that they were not exposed to azo dyes prior to the experiment and hence, they were not adapted to the presence of the dye. Bacteria need to be adapted to the pollutant before they can degrade it (Baker and Herson 1994). In the clean water with less or no organic matter present, decolorization of Congo red by the CRDB and consortia did not occur. One explanation could be that the CRDB and consortia were not able to grow due to less or absence of organic matter that provide nutrients to the organisms. The environmental condition must be

favorable for the degradation of a certain compound (Mueller 1992). Presence of toxic materials, type and amount of organic material (carbon) present, pH, electron acceptor and inorganic nutrients such as nitrogen and phosphorus are the most important abiotic factors limiting bacterial population from the perspective of bioremediation (Baker and Herson 1994).

4 Involvement of Oxidoreductive Enzymes in Degradation Process

Various bacterial strains reduce azo dyes under anaerobic condition. The most accepted hypothesis for this phenomenon is that many bacterial strains possess rather unspecific cytoplasmic enzymes which act as "azo reductases". It was earlier suggested that under anaerobic condition, unspecific cytoplasmic azo reductases act via the intermediate formation of free reduced flavin (Walker 1970). Previous reports on azo dye degradation by bacteria focused on a certain enzyme which is the azo reductase responsible for the reduction of azo dye. According to Russ et al. (2000), enzymatic reduction can occur both intracellularly and extracellularly. Rafii et al. (1990) isolated anaerobic bacteria that produced azo reductases constitutively and released extracellularly. An azo reductase was purified form *E. faecalis* by hydrophobic, anion exchange and affinity chromatography (Punj and John 2008). Maier et al. (2004) have shown that a NADPH-dependent azoreductase is responsible for azo dye reduction by *Bacillus* strain SF. In a study of the anaerobic reduction by whole cells, cell extracts and cell membranes of *Sphingomonas* sp. strain BN6, enzymatic azo dye reduction activity was found to be located in the cytoplasm as well as in the membrane fraction. It was also suggested that azo dye reduction by whole cells is mainly related to the membrane fraction (Kudlich et al. 1997).

5 Mechanism and Pathways of Degradation

In our study, we conducted test to determine if the azo dye degrading enzyme is located intracellularly or extracellularly. To test if the enzyme is located extracellularly, we used the decolorized culture broth of monocultures and centrifuged at 10,000 rpm for 10 min at 5 °C. The supernatant was collected and then filter sterilized using a 2 μm sterile syringe filter. The sterile supernatant was incubated at 30 °C with Congo red and decolorization was observed. To test for the intracellular location of the enzyme, we prepared the cell free extract by growing whole cells in nutrient broth up to its logarithmic phase based on the growth curve. Samples were taken and then centrifuged at 10,000 rpm and the pellet was suspended in 30 ml phosphate buffer. The pellet suspension was transferred to a 50 ml flask containing

0.3 ml phenylmethylsulfonyl fluoride (PMSF) and then the cells were sonicated in ice for 20 min at rest intervals of 1 min. The sonicated cells were centrifuged for 10 min at 10,000 rpm at 5 °C and after which the supernatant or cell-free extract (CFE) was collected (Barraquio 1992) and tested for decolorization. Qualitative analysis of dye decolorization was done using the spectrophotometer and based on the disappearance of the color.

It was observed that dye degradation occurred in the supernatant of the sonicated cells of the monocultures incubated with Congo red. In the case of the filter sterilized culture broth with Congo red, no decolorization occurred even after several months of incubation, indicating that the enzyme responsible for the reduction of Congo red is located intracellularly. This could also explain why the number of cells decreased as Congo red decolorization progressed over time (Fig. 5). Over time, the cells become aged and lyze and the enzyme inside the cell goes out into the medium which interacts with the dye to reduce it. This was found to be true for all the four (4) Congo red decolorizing bacteria. The cell membrane of bacteria is not permeable to sulfonated azo dyes, such as Congo red. This is why reduction of highly polar dyes is usually postulated to take place outside the cell (Stolz 2001).

Most investigators, who studied the reduction of azo dyes by bacteria, observed the extreme lack of specificity of the azo reductase system and showed that cell extract or aged (damaged) cells reduced azo dyes more efficiently and metabolized a wider range of azo dyes than intact cells (Dickhues 1960; Wurhmann et al. 1980; Mechsner and Wuhrmann 1982). The azo reductase from *Clostridium perfringens*

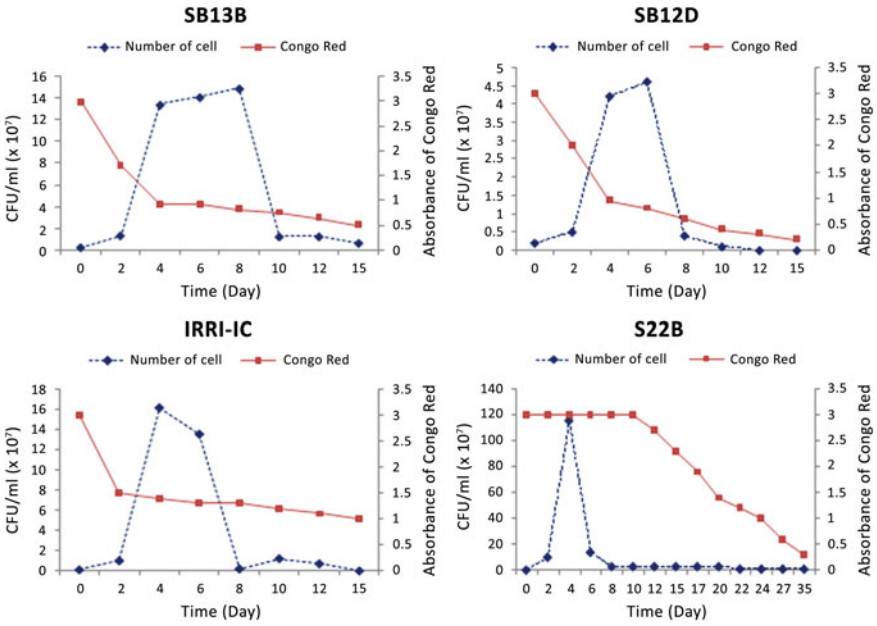

Fig. 5 Rate of Congo red decolorization and growth of cells over time

was detected by immune electron microscopy in the vicinity of the cells which suggests that the protein is not a typical extracellular enzyme, but presumably released from the lysed cells (Russ et al. 2000). Most probably, anaerobic biological azo dye reduction occurring outside the cells is catalyzed directly by periplasmic enzymes or indirectly in a reaction with reduced electron carriers that are generated by periplasmic enzymes (Kudlich et al. 1997).

However, Rafii et al. (1990) noted that the azo reductase enzyme was secreted extracellularly in all of the bacteria isolated from the intestines as evidenced by the presence of more enzymes in the culture supernatant. To further determine the exact location of the dye degrading enzyme inside the cell, the azo reductase should be purified and characterized using SDS-PAGE and HPLC (Punj and John 2008).

A recent report described a high in vitro azo reductase activity of a cytoplasmic flavin reductase, a part of the ribonucleotide reductase complex in *E. coli*, but its overexpression in *Sphingomonas* sp. strain failed to significantly increase the in vivo reducing activity of the bacteria (Russ et al. 2000). An intracellular azo reductase is responsible for decolorization activity as previously shown for other azo dye degrading microorganisms (Roxon et al. 1967; Gingell and Walker 1971). In general, azo reductase activity was only measured in the absence of oxygen (Maier et al. 2004). It appears that the enzyme involved in azo dye degradation is irreversibly inactivated in the presence of oxygen (Rafii et al. 1990).

The term "anaerobic dye reduction" comprises different mechanisms. A distinction can be made between direct enzymatic azo dye reduction and indirect azo dye reduction catalyzed by enzymatically (re)generated redox mediating compounds. It is also possible that azo dyes are chemically reduced by biogenic bulk reductases like sulphide (van der Zee 2002).

6 Microbial Toxicity of Dyes and Their Degradation Products

In our study, toxicity of decolorized culture medium was tested on prokaryotic cells (*Pseudomonas aeruginosa* UPCC 1244 and *Staphylococcus aureus* UPCC 1143, Gram negative and Gram positive bacteria, respectively) and eukaryotic cells (*Saccharomyces cerevisiae* UPCC 2113, a yeast; *Oryza sativa* NSIC RC112, rice; and *Vigna radiata*, mung bean). For microbial toxicity test, four treatments were tested: decolorized medium on the 7th day of incubation, decolorized medium on the 14th day of incubation, negative control and positive control (with Congo red). For the plant toxicity test, the roots of selected rice and mung bean seedlings were immersed overnight in the following treatments: decolorized medium on the 7th day of incubation, decolorized medium on the 14th day of incubation, negative control and 1 % Congo red solution (positive control). After incubation, the plants were carefully uprooted from the media and the heights and dry weights were determined. Heights were measured from the point separating the root and shoot to

the highest point or apex of the plant shoot. Dry weights, were determined by drying whole plants in an oven at 50 °C until constant weight was obtained.

The growth of bacteria and yeast was not inhibited by both the consortium-decolorized medium and monoculture decolorized medium as shown by the absence of zones of inhibition on 24–48 h-old lawn of microorganisms. This indicates that bacterial decolorization of Congo red did not result in metabolic products which were more toxic than the dye. The undegraded Congo red (positive control) also did not affect the growth of the microbes. Thus, these microbes have an inherent resistance to the toxic effects of the undegraded Congo red. This inherent resistance to diazo dye may not hold true for all microbes since some might be sensitive to this toxic dye.

However, the effects of undegraded Congo red and the decolorized culture medium on the plants were different from that on bacteria and yeast. Figure 6 shows that rice seedlings, soaked in denitrifying consortium-decolorized medium obtained on the 14th day of incubation (T2), have higher plant shoots as compared to seedlings exposed to decolorized filtrate on the 7th day of incubation (T1), in Fahreus medium (T3), and 1 % Congo red solution (T4). Therefore, higher dry weight of rice seedlings was obtained.

Overnight soaking of selected rice seedlings in nitrate reducing monoculture decolorized medium obtained on the 7th day of incubation, resulted in the highest plant shoots as compared to seedlings soaked in decolorized medium derived on the 14th day of incubation, Fahreus medium and 1 % Congo red solution. Rice seedlings soaked in 1 % Congo red solution resulted in the lowest shoot heights (Fig. 7)

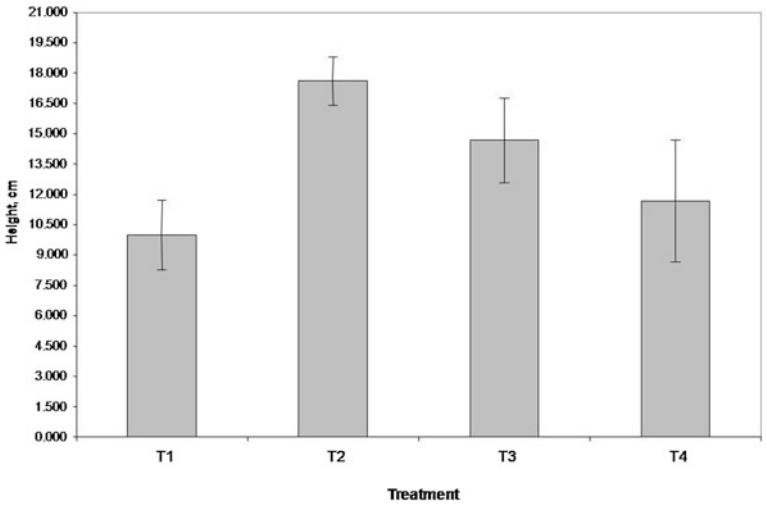

Fig. 6 Effect of denitrifying consortium-decolorized medium on growth of rice shoots. The bars are the averages of three replicates from each treatment, and error bars indicate ± standard deviations from the means. Treatments used were *T1* Denitrifying consortium-decolorized medium on 7th day of incubation, *T2* Denitrifying consortium decolorized medium on 14th day of incubation, *T3* Fahreus medium (negative control) and *T4* 1 % Congo red solution (positive control)

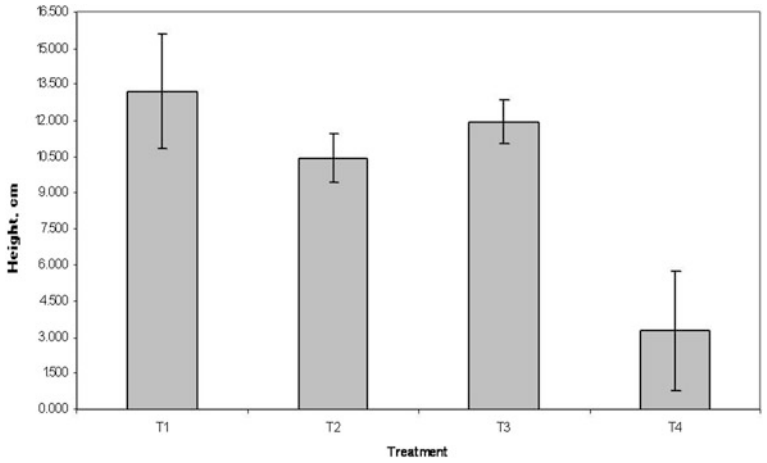

Fig. 7 Effect of nitrate-reducing-decolorized medium on growth of rice shoots. The bars are the averages of three replicates from each treatment, and error bars indicate ± standard deviations from the means. Treatments used were *T1* Nitrate-reducing monoculture-decolorized medium on 7th day of incubation, *T2* Nitrate-reducing monoculture-decolorized medium on 14th day of incubation, *T3* Fahreus medium (negative control) and *T4* 1 % Congo red solution (positive control)

and lowest dry weights. However, the height of mung bean shoots exposed to denitrifying consortium-decolorized medium (Fig. 8) and nitrate-reducing monocultures-decolorized medium (Fig. 9) obtained on the 7th day and on the 14th day

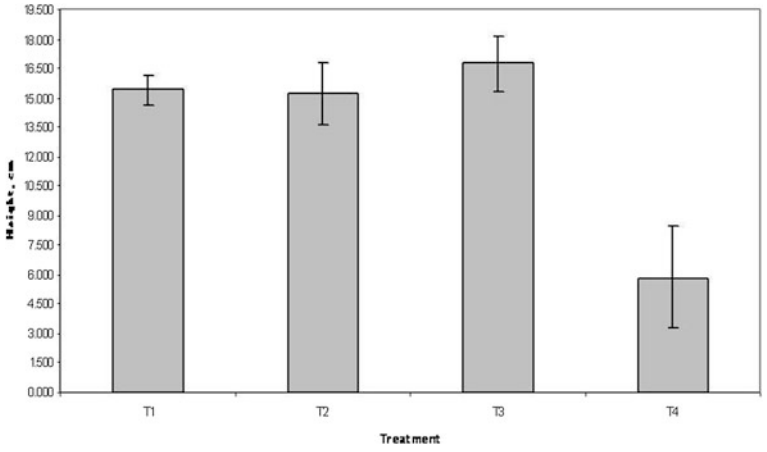

Fig. 8 Effect of denitrifying consortium-decolorized medium on growth of mung bean shoots. The bars are the averages of three replicates from each treatment, and error bars indicate ± standard deviations from the means. Treatments used were *T1* Denitrifying consortium-decolorized medium on 7th day of incubation, *T2* Denitrifying consortium decolorized medium on 14th day of incubation, *T3* Fahreus medium (negative control) and *T4* 1 % Congo red solution (positive control)

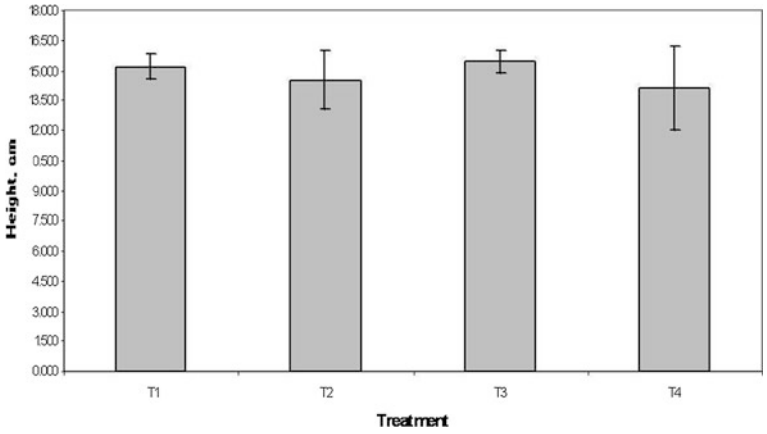

Fig. 9 Effect of nitrate-reducing monocultures-decolorized medium on growth of mung bean shoots. The bars are the averages of three replicates from each treatment, and error bars indicate ± standard deviations from the means. Treatments used were *T1* Nitrate-reducing monoculture-decolorized medium on 7th day of incubation, *T2* Nitrate-reducing monoculture-decolorized medium on 14th day of incubation, *T3* Fahreus medium (negative control) and *T4* 1 % Congo red solution (positive control)

of incubation were similar to that of seedlings exposed to Fahreus medium, but higher than that of seedlings soaked in 1 % Congo red solution.

Based on the results of the toxicity of the decolorized culture medium and Congo red towards plants, it can be deduced that the undegraded Congo red, is toxic to both rice and mung beans as evidenced by the stunting of plant growth and low dry weights. The effects of the denitrifying consortium decolorized medium and nitrate reducing monoculture decolorized medium on plant growth may be attributed to the presence of small amounts of still undegraded Congo red and also bacterial decolorization metabolites being slightly toxic to both rice and mung beans. Many bacteria reduce azo bonds to the corresponding amines. Several aromatic amines can be degraded under the aerobic condition (van der Zee 2002). The higher plant shoots than the negative control may be attributed to the promotion of plant growth by the nutrients formed during bacterial decolorization which were imbibed by the seedlings. Therefore, the decolorization of Congo red by the bacteria diminished the severe toxic effect of the dye to the rice and mung bean plants.

7 Future Prospectives

The Congo Red Decolorizing Bacteria (CRDB), isolated from the paper factory effluent, are very promising as bioremediation agents as they have shown high decolorizing potential in the microcosm study. The CRDB can be further developed

as inoculants to degrade the Congo red dyes that are being used in some paper factories or other factories using this kind of dye.

However, there is a need for further studies to confirm other aspects of this investigation. It has to be worked out whether the Congo red was completely degraded and the degradation products be identified. If these microbes are used as bioremediation inoculants, the amount of glucose utilized while degrading Congo red should be determined and how its metabolism caused dye degradation in order to optimize the decolorization process.

The exact location of the dye degrading enzyme inside the cell should be determined using SDS-PAGE and the gel should be run with Congo red to determine the ability of the enzyme to degrade the said dye. Further this enzyme should be purified and characterized for potential commercial applications.

Field application of the CRDB and consortia in dye polluted areas should be conducted to test their efficiency as bioremediation inoculants for dye degradation/decolorization.

8 Conclusion

Congo red is a carcinogenic direct diazo dye used for the coloration of paper products. It is recalcitrant and usually found in effluents of paper factories. Bacteria in consortia and monocultures capable of decolorizing Congo red were also isolated earlier. The consortia were labeled as IRRI-1 and S22. The monocultures were labeled as SB13B, SB12D, IRRI-1C and S22B. The rate of decolorization by the consortia was faster (1 week) than that of the monocultures (2 weeks to 1 month). The 16S rRNA sequencing revealed identities of SB13B as *E. coli*, SB12D as *Enterobacter dissolvens* and S22B as *Pseudomonas citronellolis*. API 20E identified IRRI-1C as *Klebsiella oxytoca*.

Dye degradation occurred in the supernatant of sonicated cells, indicating that the dye degrading enzyme was located intracellularly. The enzyme capable of reducing azo dyes was found to be present inside the cell of the four CRDB and was released upon cell lyses, thus decreasing the absorbance of Congo red (decolorization) as cell number increased.

The monocultures and consortia were able to decolorize Congo red in polluted water, while no decolorization of the dye was observed in clean water. The resident microorganisms together with other organic matter in the polluted water could have caused dye degradation.

Toxicity tests of consortium- and monoculture-decolorized media and undegraded Congo red revealed that the test microorganisms used as bacteria and yeast, were resistant to the toxic effects of Congo red. Toxicity tests on plants, however, showed that undegraded Congo red was toxic to both rice and mung bean seedlings and that the toxic effect on plants was reduced by decolorization. The monocultures SB13B and S22B decolorized Congo red with concomitant nitrate reduction.

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Bacterial Enzymes and Their Role in Decolorization of Azo Dyes

Amar A. Telke, Avinash A. Kadam and Sanjay P. Govindwar

1 Introduction

Azo dyes are one of the largest groups of textile dyes, extensively used for dyeing purposes. A significant proportion of these dyes were entering into the surrounding environment in the form of wastewater. Discharge of such wastewater into natural water resources has created an aesthetic problem to aquatic life and human being. Several physico-chemical methods have been employed for azo dye decolorization, but they have facing several problems, such as generation of toxic by-products and economical unfeasibility. Several reports have been stated that bacteria, fungi and plants have ability to decolorize and detoxify azo dyes. However, ubiquitous nature of bacteria makes them as invaluable tool for the textile dye decolorization. Bacterial genera, such as *Pseudomonas*, *Bacillus*, *Rhodococcus*, were reported for rapid azo dye decolorizing biological agents (Kalyani et al. 2008; Telke et al. 2008). The azo dye decolorization by bacteria has been associated with the production of oxidoreductive enzymes, such as lignin peroxidase (Ghodake et al. 2008), laccase (Telke et al. 2009), azoreductases and other non specific reductases (Chen et al. 2005; Dhanve et al. 2008a, b). Azo dye decolorization by purified bacterial enzymes was demonstrated in several reports. Previous reviews have indicated that azo dye decolorization was associated with reductive cleavage of azo bond by

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reductase (Dos Santos et al. 2007; Pandey et al. 2007). However, this chapter has focused on the various oxidoreductive enzymes involved in decolorization and detoxification of textile azo dyes and their mechanism of azo dye decolorization.

2 Bacterial Decolorization of Textile Azo Dyes

Bacteria degrade the textile dyes under both aerobic and anaerobic conditions. The optimum pH for decolorization of textile dyes varied from acidic to basic range. The bacteria, used for decolorization of textile dyes, were isolated from textile dye contaminated soil by enrichment techniques and identified on the basis of 16s rRNA sequence and biochemical characteristics (Kalyani et al. 2008; Dawkar et al. 2008; Dhanve et al. 2008a, b; Telke et al. 2008). However, certain microorganism, which was not isolated from textile dye-contaminated soil, but showed excellent dye decolorization property.

3 Decolorization of Azo Dye Under Anaerobic Condition

At present, only mechanism of azo dye decolorization under anaerobic condition is well known. Anaerobic azo dye reduction involves the reduction of azo bond by the transfer of reducing equivalents resulting in the formation of aromatic amines (Chung and Cerniglia 1992). As removal of a large amount of azo dyes from wastewater is a major concern, research on bacterial azo dye reduction has mainly focused on the activity of (facultative) anaerobic bacteria. Various anaerobic bacteria were used in bioreactor for treatment of textile effluent containing azo dyes (Işık and Sponza 2005; Maas and Chaudhari 2005; Mezohegyi et al. 2008).

4 Decolorization of Azo Dyes under Anoxic Condition

Pure bacterial strains, such as *Pseudomonas luteola*, *Aeromonas hydrophila*, *Bacillus subtilis*, *Pseudomonas* sp. and *Proteus mirabilis* were utilized for azo dyes decolorization under anoxic condition (Chen et al. 1999, 2003; Chang et al. 2001; Yu et al. 2001). Although, most of these cultures were able to grow aerobically, but decolorization were achieved only under anaerobic conditions. Anoxic decolorization of various azo dyes by mixed aerobic and facultative anaerobic microbial consortia has been reported by many researchers (Nigam et al. 1996; Kapdan et al. 2000; Padmavathy et al. 2003; Khehra et al. 2005; Moosvi et al. 2005). Azo dye decolorization by pure as well as mixed cultures have required complex organic carbon sources, such as, yeast extract, peptone, or a combination of complex organic source and carbohydrate (Chen et al. 2003; Khehra et al. 2005). The decolorization

of azo dyes in anoxic condition was influenced by various substrates used in cell growth medium. Although, azo dye decolorization under anoxic condition was non-specific, limitation of this method was requirement of yeast extract or peptone. This makes the process economically in viable for industrial-scale application unless alternate cheaper sources are identified (Nigam et al. 1996; Chen et al. 2003; Moosvi et al. 2005).

5 Decolorization of Azo Dyes Under Aerobic Condition

5.1 Decolorization of Azo Dyes Under Static Condition

The optimum pH for decolorization of azo dyesat static condition is 7.0–8.0. The pure bacterial cultures isolated from textile dye contaminated soil, such as *Pseudomonas* sp. SUK 1, *Kocuria rosea*, *Rhizobium radiobacter*, *Bacillus* sp.VUS, *Commamonas* sp. UVS,*Exiguobacterium* sp. RD3, *Proteus* sp. SUK 7; *Bacillus* sp. ADR; and *Pseudomonas* sp. SU-EBT were efficiently decolorized azo dyes at static condition than shaking condition (Kalme et al. 2007; Parshetti et al. 2007; Dawkar et al. 2008; Dhanve et al. 2008a, b; Jadhav et al. 2008; Kalyani et al. 2008; Patil et al. 2008; Telke et al. 2008, 2009). The pure bacterial cultures of *Pseudomonas* sp. SUK1 and *Rhizobium radiobacter* MTCC 8161 were able to tolerate and degrade the higher concentration (more than 1 g l^{-1}) of azo dyes (Kalyani et al. 2008; Telke et al. 2008). The recent reports showed that the combination of yeast extract with urea and agricultural wastes (bagasse powder, wheat bran, rice bran and wood shaving) were effective medium for the decolorization of azo dyes (Jadhav et al. 2008; Telke et al. 2008) at static condition.

5.2 Decolorization of Azo Dyes Under Shaking Condition

For a long time it was thought that azo dyes were recalcitrant under aerobic conditions. A bacterial strain S5, derived from *Hydrogenophaga palleronii* S1, mineralized sulfonated azo dyes by utilizing them as carbon and nitrogen source for cell growth (Blumel et al. 1998). The recent studies have shown that the pure bacterial culture (Unidentified KMK 4, strain S5, *E. coli* NO_3 and *Flavobacterium* sp. ATCC39723) and mixed bacterial culture (unidentified BF1, BF2 and *Pseudomonas putida* MTCC 1194) have ability to degrade higher concentration (1 g $^{-1}$) of azo dyes (Cao et al. 1993; Chang and Kuo 2000; Senan and Abraham 2004; Kodam et al. 2005). Aerobic bacterial cultures were reduced the azo linkage by the involvement of azo reductases and oxidases or combination of both enzymes. Azo reductases isolated from aerobic bacteria have a narrow substrate range (Kulla 1981; Zimmermann et al. 1982, 1984; Kulla et al. 1983). The involvement of peroxidase was reported in azo dye oxidation by Cao et al. (1993).

6 Mechanism of Dye Decolorization

Although for a long time, it was thought that the bacterial degradation of azo dyes in either anaerobic or aerobic conditions were associated with symmetric cleavage of the azo group (–N=N). This cleavage may be mediated through different mechanisms, such as enzymes, low molecular weight redox mediators, chemical reduction by biogenic reductants like sulfide, or a combination of both. However, the location of such type of reactions could be either intracellular or extracellular. The recent reports on azo dye decolorization have indicated involvement of peroxidase, laccase, tyrosinase, NADH-DCIP reductase, and MG reductase in decolorization of azo dyes.

6.1 Enzymatic Dye Decolorization

6.1.1 Azo Reductase

The presence of extracellular oxygen sensitive azo reductases in anaerobic bacteria, such as *Clostridium* and *Eubacterium,* were decolorized sulfonated azo dyes during their growth on solid or complex media (Rafii et al. 1990). Anaerobic azo reductases were flavoprotiens (NAD(P)H:flavin oxidoreductase), localized at intracellular or extracellular site of cell membrane, required NADH, NADPH and FADH as electron donors for reduction of azo bonds (Rafii and Cerniglia 1995; Russ et al. 2000). Co-factors like $FADH_2$, $FMNH_2$, NADH and NADPH, which provide 'H' for reduction, were located at cytoplasm (Russ et al. 2000). Lysis of cells would release co-factors at extracellular environment could be the possible reason for higher azo dye reduction rates using cell extracts or starving or lysed cells than intact or resting cells (Roxon et al. 1967; Walker 1970; Dubin and Wright 1975; Wuhrmann et al. 1980; Mechsner and Wuhrmann 1982; Russ et al. 2000).

Intracellular sulfonated azo dye reduction required the specific transport system that allows the uptake of the sulfonated azo dye into the cells (Russ et al. 2000). The role of flavin dependent azo reductase was shown by adding of flavins to resting cells of strain *Sphingomonas* strain BN6, which resulted in no significant increase in azo dye reduction. These findings suggest that in living cells with intact cell membranes, other enzyme systems and/or other redox mediators are responsible for reduction of azo dyes. In bacteria that possess electron transport systems in their membranes, as in the case of aerobic or facultative anaerobic bacteria, such as *Sphingomonas* strain BN6, the transfer of electrons from the respiratory chain to appropriate redox mediators could take place directly. If intracellular reductases are involved in the process, it is assumed that mediators, different from flavin cofactors with a higher ability to pass through the membranes, must be involved. Although

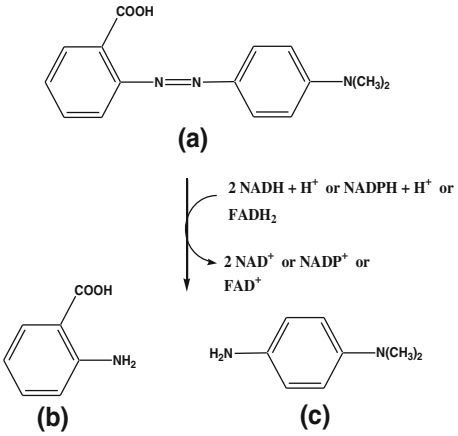

Fig. 1 Reaction catalyzed by azo reductases. **a** Methyl red; **b** 2-Amino benzoic acid; **c** *p*-dimethyl amino anilline

there are a few reports on systems which are involved in the transport into bacterial cells of other sulfonated substrates, such as *p*-toluene sulfonate, taurine and alkane sulfonates (Locher et al. 1993; Eichhorn et al. 2000). The 2-ABS degrading *Alcaligenes* sp. strain O-1 can utilize two other aromatic sulfonates, benzene and toluene sulfonate, for growth. However, cell extracts of this strain can desulfonate at least six substrates (Thurnheer et al. 1986). This suggests that the presence of highly specific transport systems for the uptake of aromatic sulfonates in these cultures. Azo reductase and flavin reductase are the potent enzymes involved in the decolorization of azo dyes, but generate the toxic amines after reduction of azo bond. The reaction catalyzed by azo reductase has been shown in (Fig. 1).

6.1.2 NADH-DCIP Reductase and Malachite Green Reductase

Significant induction of non-specific reductase, such as NADH-DCIP reductase and Malachite green reductase during decolorization of azo dyes suggests their possible participation in decolorization (Kalyani et al. 2008; Telke et al. 2008). The function of non-specific reductases is still unknown. NADH-DCIP reductase belongs to the bacterial mixed function oxidase system may takes part in the detoxification of xenobiotic compounds (Salokhe and Govindwar 1999). NADH-DCIP reductase enzyme reduced DCIP substrate using NADH as electron donor. DCIP is a blue in its oxidized form and it becomes colorless after reduction. The significant induction of non-specific reductase in the biodegradation of Malachite green was observed and termed as MG reductase (Parshetti et al. 2006). MG-reductase enzyme reduced Malachite green into leucomalachite green using NADH as electron donor. The reactions catalyzed by DCIP-reductase and MG- reductase have been reflected in Figs. 2 and 3.

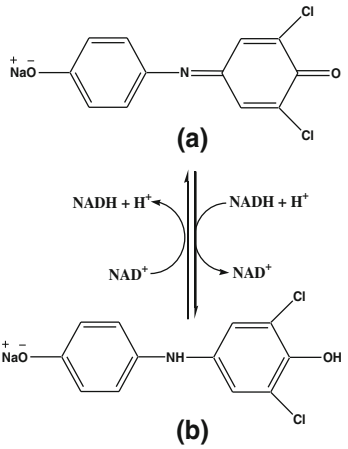

Fig. 2 Reaction catalyzed by NADH-DCIP reductase. **a** Oxidized form of DCIP; **b** Reduced form of DCIP

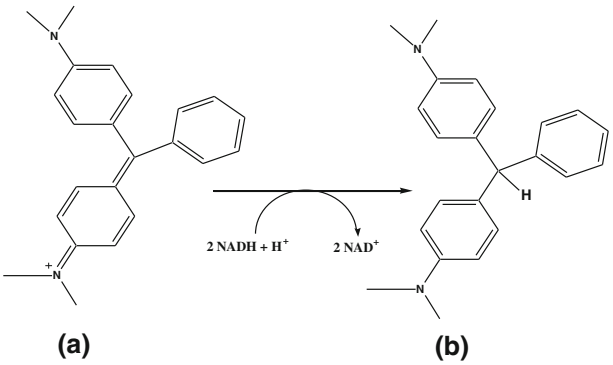

Fig. 3 Reaction catalyzed by MG reductase. **a** Malachite green; **b** Leucomalachite green

6.1.3 Lignin Peroxidase (LiP)

This enzyme belongs to the family of oxidoreductases, specifically those acting on peroxide as electron acceptor (peroxidases). The systematic name of this class enzyme was 1,2-bis(3,4-dimethoxyphenyl) propane-1,3-diol:hydrogen-peroxide oxidoreductase. LiP was *N*-glycosylated protein with molecular weight between 38 and 47 kDa. It contains heme in the active site and has shown a classical peroxidase mechanism (Tien et al. 1986). LiP catalyzed several oxidation reactions in the side chains of lignin and related compounds by one-electron abstraction to form reactive radicals (Tien and Kirk 1983; Kersten et al. 1985). The cleavage of an aromatic ring structure is also reported (Umezawa and Higuchi 1987). Several studies have found a significant induction in lignin peroxidase activity during decolorization of azo dyes by bacterial strains, suggesting their possible function in dye decolorization.

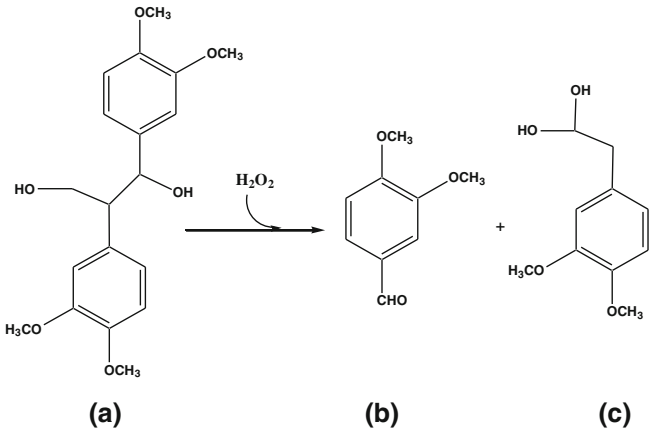

Fig. 4 Reaction catalyzed by lignin peroxidase. **a** 1, 2-bis (3, 4-dimethoxyphenyl) propane-1, 3-diol; **b** 3, 4-dimethoxybenzaldehyde; **c** 1-(3, 4-dimethoxyphenyl) ethane-1,2-diol

The role of Lip in dye decolorizationwas proved by in vitro dye decolorization assay with purified protein (Gomare et al. 2008). Reaction catalyzed by lignin peroxidase is shown in Fig. 4.

6.1.4 Laccase

Laccases (EC1.10.3.2) were mostly members of multi-copper oxidase protein family. It was catalyzes the oxidation of substituted phenolic and non-phenolic compounds in the presence of oxygen as an electron acceptor (Fig. 5). Phylogenetically, these enzymes have developed from small sized prokaryotic azurins toeukaryotic plasma proteins ceruloplasmin (Claus 2003, 2004). They contain four histidine-rich copper binding domains, which coordinate types 1–3 copper atoms that differ in their environment and spectroscopic properties (Givaudan et al. 1993). They are classified into two categories viz. blue laccase, had a blue color and characteristic absorption and EPR spectra and white or yellow-brown laccase, had no typical blue oxidase spectra and also showed atypical EPR spectra. According to Solano and Sanchez-Amat (1999), a prokaryotic melanogenic marine bacterium *Marinomonas mediterranea* containing two different polyphenol oxidases (PPO) have characteristics of both tyrosinase and laccase activity. The other PPO is a multi-potent enzyme, which is able to oxidize a wide range of substrate characteristics for both tyrosinases and laccases. The reaction catalyzed by laccase reflected in Fig. 6. The molecular weight of laccases varies from 60 to 390 kDa (Call and Mucke 1997). First prokaryotic laccase was reported in rhizospheric bacterium *Azospirillum lipoferum* (Solano et al. 1997). Laccase like activity has been found also in *Pseudomonas syringae* and *Pedomicrobium* sp. (Ridge et al. 2007). Laccase was decolorized azo dyes by non-specific free radical mechanism,

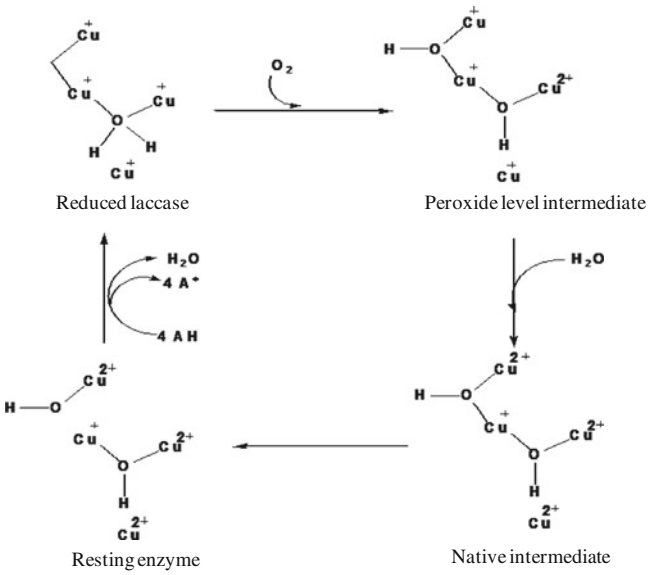

Fig. 5 Catalytic cycle of laccases

thereby avoiding the formation of toxic aromatic amines. The purified laccase from *Pseudomonas desmolyticum* NCIM 2112 and *Bacillus* sp. ADR have ability to decolorize the several textile dyes withdecolorization efficiency varying from 65 to 90 % (Kalme et al. 2009; Telke et al. 2011).

6.1.5 Polyphenol Oxidase (Tyrosinase)

Polyphenol oxidases (PPOs) were catalyze the *o*-hydroxylation of monophenols (phenol molecules in which the benzene ring contains a single hydroxyl substituent) to *o*-diphenols (phenol molecules containing two hydroxyl substituents). They can also further catalyze the oxidation of *o*-diphenols to produce *o*-quinones. The amino acid tyrosine contains a single phenolic ring that may be oxidised by the action of PPOs to form *o*-quinone (Fig. 7). Hence, PPOs may also be referred as tyrosinases. Polyphenol oxidases are enzymes that catalyze oxidation of certain phenolic substrates to quinones in the presence of molecular oxygen. Polyphenol oxidases have been reported in the bacteria viz. *Streptomyces glaucescens*, *Streptomyces antibioticus*, *Bacillus licheniformis*, *Bacillus natto* and *Bacillus sphaericus* (Whitaker 1994; Echiqo and Ritsuko 2001). The purified polyphenol oxidase was used for the oxidation of colored and phenolic substances.

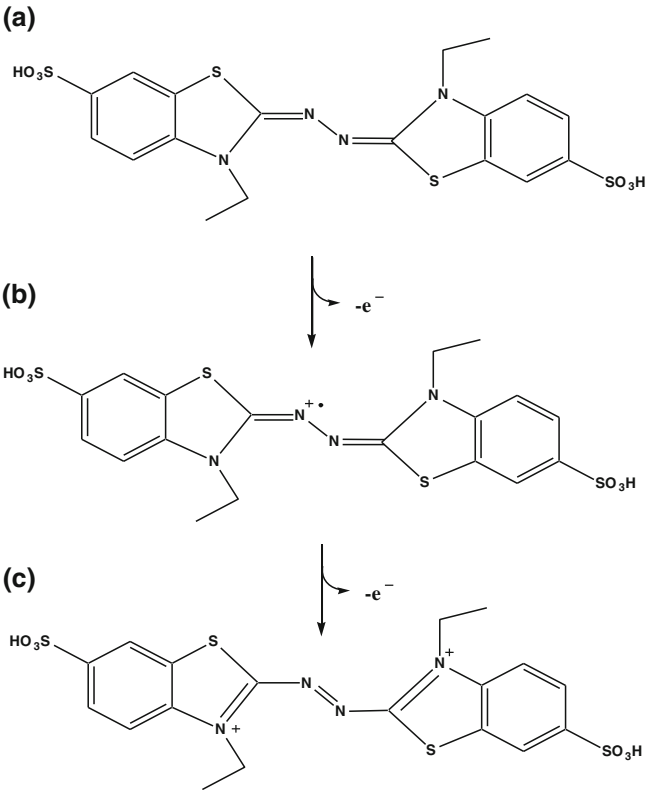

Fig. 6 Reaction catalyzed by laccases. **a** ABTS; **b** Intermediate ABTS radical; **c** Oxidized form of ABTS

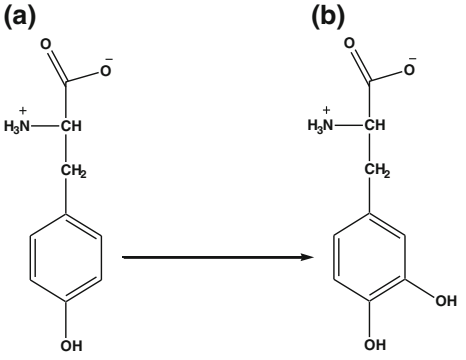

Fig. 7 Reaction catalyzed by polyphenol oxidase. **a** L-tyrosine; **b** *o*-quinone

6.1.6 Veratrol Alcohol Oxidase (VAO)

The enzyme veratrol-alcohol oxidase was catalyzes the chemical reaction as:

$$\text{Aromatic primary alcohol} + O_2 \rightleftharpoons \text{Aromatic aldehyde} + H_2O_2$$

Hence, this enzyme has two substrates i.e. aromatic primary alcohol and O_2 and their products are aromatic aldehyde and H_2O_2. This enzyme belongs to the family of oxidoreductases. VAO catalyzed the oxidation of aryl α- and α-β- unsaturated γ-alcohols to the corresponding aldehydes with concomitant reduction of O_2 to H_2O_2. The formation of H_2O_2 can be determined by the method as suggested by Tamboli et al. (2011).

6.2 Mediated Biological Azo Dye Reduction

The high molecular weight sulfonated azo dyes are unable to pass through the cell membrane (Levine 1991). It was suggested that the reduction of these dyes could occur through the mechanism that are not dependent on transport into the cell membrane. Many reports were available on the role of redox mediators in azo bond reduction by bacteria under anaerobic conditions (Keck et al. 1997; van der Zee et al. 2001a; Dos Santos et al. 2004). Riboflavin significantly enhanced the reduction of mordant yellow 10 by anaerobic granular sludge (Field and Brady 2003). 1-amino 2-napthol, one of the constituent amines of the azo dye, AO7, increased its decolorization rate, possibly by mediating the transfer of reducing equivalents (Mendez-Paz et al. 2005). The addition of synthetic electron carriers, such as anthraquinone-2,6-disulphonate, could also greatly enhance the decolorization of many azo dyes (van der Zee et al. 2001b). Keck et al. (1997) were first example of the anaerobic cleavage of azo dyes by redox mediators formed during the aerobic degradation of a xenobiotics compound. Cell suspensions of *Sphingomonas* sp. strain BN6, grown aerobically in the presence of 2-naphthyl sulfonate (NS), shown 10–20 fold increase in decolorization rate of an azo dye under anaerobic conditions. Even the addition of culture filtrates from these cells could enhance anaerobic decolorization by cell suspensions grown in the absence of NS. Redox intermediates generated during the aerobic degradation of aromatic compounds were act as enhancer in dye decolorization reactions under anaerobic condition (Keck et al. 1997; Chang et al. 2004).

6.3 Azo Dye Decolorization by Organic and Inorganic Compounds

Azo dye decolorization can occur from purely chemical reactions with inorganic compounds, such as sulfide and ferrous ion which are formed as end products of metabolic reactions under anaerobic condition. It has been shown that H_2S generation by SRB resulted in the extracellular decolorization of azo dyes (Yoo et al. 2000; Diniz et al. 2002). Sulfate-influenced dye reduction was correlated with biogenic sulfide formation under methanogenic conditions. In the absence of sulfur compounds, dye decolorization readily occurred in the presence of granular sludge, demonstrating the importance of enzymatic mechanisms. An analysis of decolorization kinetics, in batch reactor and in laboratory scale anaerobic sludge bed reactors, indicated that the relative importance of chemical dye reduction mechanisms in high rate anaerobic bioreactors was small due to the high biomass in the reactors (van der Zee et al. 2003). The various inducers of oxidative enzymes, such as $CaCO_3$, indole, *o*-tolidine, veratrole, and vanillin, enhanced the dye decolorization (Dawkar et al. 2008). The electron donors, viz. sodium acetate, sodium formate, sodium succinate, sodium citrate, and sodium pyruvate enhanced the azo dye decolorization rate by *Bacillus* sp. ADR. Sodium acetate, sodium formate, sodium citrate, and sodium pyruvate might act as artificial electron donors for reductases involved in azo dye decolorization. 3,4-dimethoxy benzyl alcohol and *o*-tolidine increased the azo dye decolorization rate of *Bacillus* sp. ADR by stabilizing the extracellular phenol oxidase enzyme (Telke et al. 2009).

7 Decolorization of Textile Dyes by Purified Bacterial Oxidoreductase Enzymes

7.1 Azoreductase Mediated Dye Decolorization

The azoreductase enzyme was purified from *Exigubacterium* sp. RD3 which had molecular weight of 95 Kda (Dhanve et al. 2008a, b). The purified azoreductase was studied for its ability for azo dye decolorization. Azoreductase mediated azo bond cleavage in Methyl Red produced intermediates i.e., *N*, *N*-dimethyl benzene-1,4 diamine and 2-amino benzoic acid (Fig. 8) whereas, Congo red produced diamino naphthyl sulfonic acid and benzedine by azoreductase action (Fig. 9).

7.2 Laccase Mediated Dye Decolorization

Laccase was purified from *Bacillus* sp. ADR (Telke et al. 2009). Purified laccase was studied for decolorization of Methyl Red and Methyl Orange.

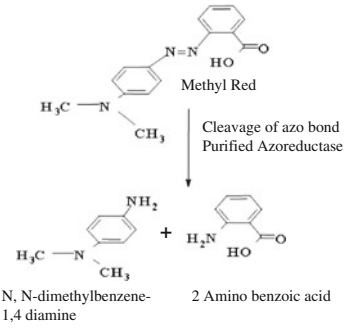

Fig. 8 Proposed degradation pathway for Methyl Red by purified azoreductase

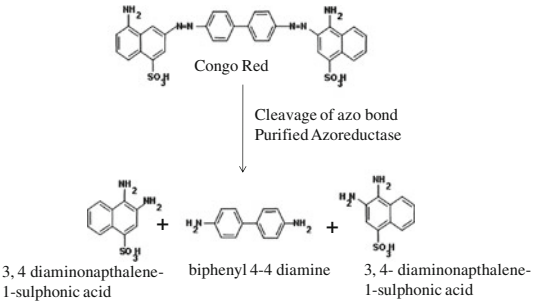

Fig. 9 Proposed degradation pathway for Congo Red by purified azoreductase

The formation of *p*-hydroperoxy dimethyl amino benzene suggests asymmetric cleavage of an azo bond in Methyl Red (Telke 2009) (Fig. 10). The formation of *p*-phenyldizine sulfonic acid and*p*-hydroxy N,N′-dimethyl amino benzene indicates asymmetric cleavage of an azo bond in Methyl Orange (Telke 2009) (Fig. 11). The biodegradation of dyes by purified laccase confirms their role in decolorization process.

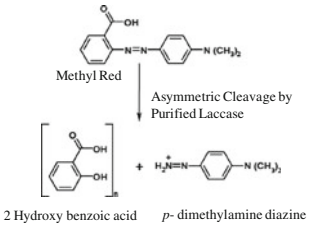

Fig. 10 Proposed degradation pathway for Methyl Red by purified laccase

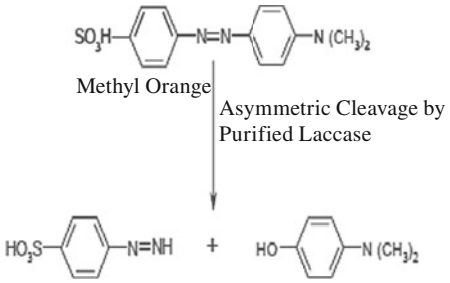

Fig. 11 Proposed degradation pathway for Methyl Orange by purified laccase

7.3 Lignin Peroxidase Mediated Dye Decolorization

The lignin peroxidase purified from *Bacillus laterosporus* was decolorizes Methyl Orange (Gomare et al. 2008). The biodegradation pathway of Methyl Orange was shown in Fig. 12.

In degradation pathway, Methyl Orange firstly forms cyclo-2, 5-ene-1-one radicals with the removal of sulfate ion, N, N di methylamine and a nitrogen molecule, where these two radicals condense to produce 1, 1′-dicyclohex-2, 5-ene-4-one moiety. Its one of the cyclohexene rings undergoes α-cleavage to form 4-substituted hexanoic acid, which on the removal of methyl radical undergoes cyclization to produce 4-cyclohexenone lactone cation. This substituted lactone cation finally forms a stable molecule of *p*-isopropanal-2,5-cyclohexa-dienone with the elimination of iminium moiety (Gomare et al. 2008).

7.4 Veratrol Alcohol Oxidase Mediated Dye Decolorization

The veratrole alcohol oxidase was purified from *Comomonas* sp. UVS and assessed fordye decolorization (Jadhav et al. 2009). Oxidative cleavage of Red HE7B (RRHE7B) yielded an unknown product. This product further undergoes desulfonation to give naphthalene-1,2,5-triol (Fig. 13). The use of purified enzyme suggested their direct involvement for dye decolorization.

8 Toxicity of Azo Dyes and Its Degradation Metabolites

The wastewater released from the textile industries after the treatment is sometime used for irrigation purpose in the agricultural fields (Paul et al. 2012). Hence, the toxicity assessment for the seed germination and plant growth of treated textile

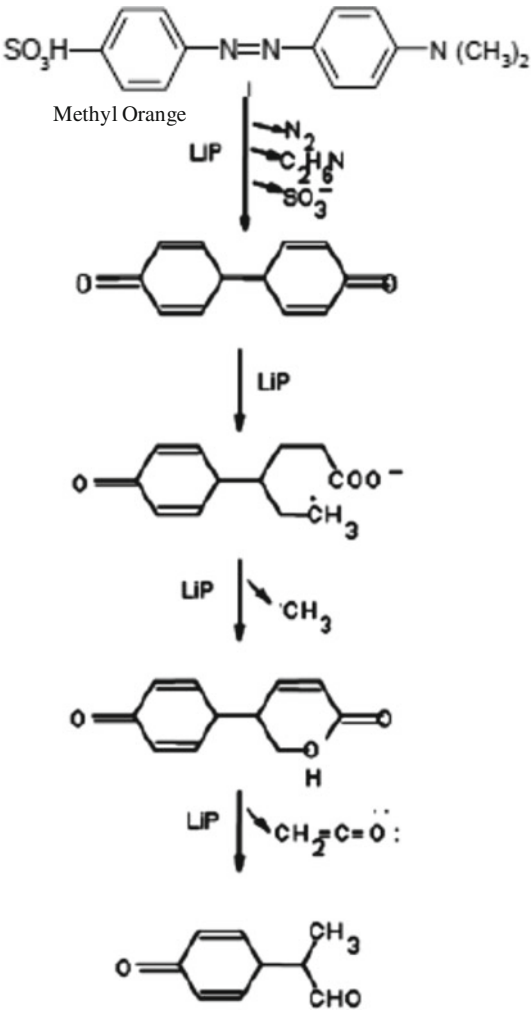

Fig. 12 Proposed degradation pathway for Methyl Orange by Lignin peroxidase

effluent is considered one of the important parameter (Paul et al. 2012). Use of plant bioassays for monitoring toxic effect of azo dyes as well as metabolites on plants was suggested by many researchers (Paul et al. 2012; Kadam et al. 2013a, b). Phytotoxicity and microbial toxicity studies revealed that the metabolites formed after azo dye decolorization were less toxic than original azo dye (Dawkar et al. 2008; Dhanve et al. 2008a, b; Jadhav et al. 2008; Kalyani et al. 2008; Telke et al. 2008). Ames test revealed that the mutagenicity of AV7 even with or without the S9 metabolizing system increased significantly after static biodegradation and totally disappeared after aerobic condition (Mansour et al. 2008).

Bacteria mediated decolorization of azo dyes under both static anoxic as well as anaerobic conditions, produces toxic aromatic amines due to enzymatic action of

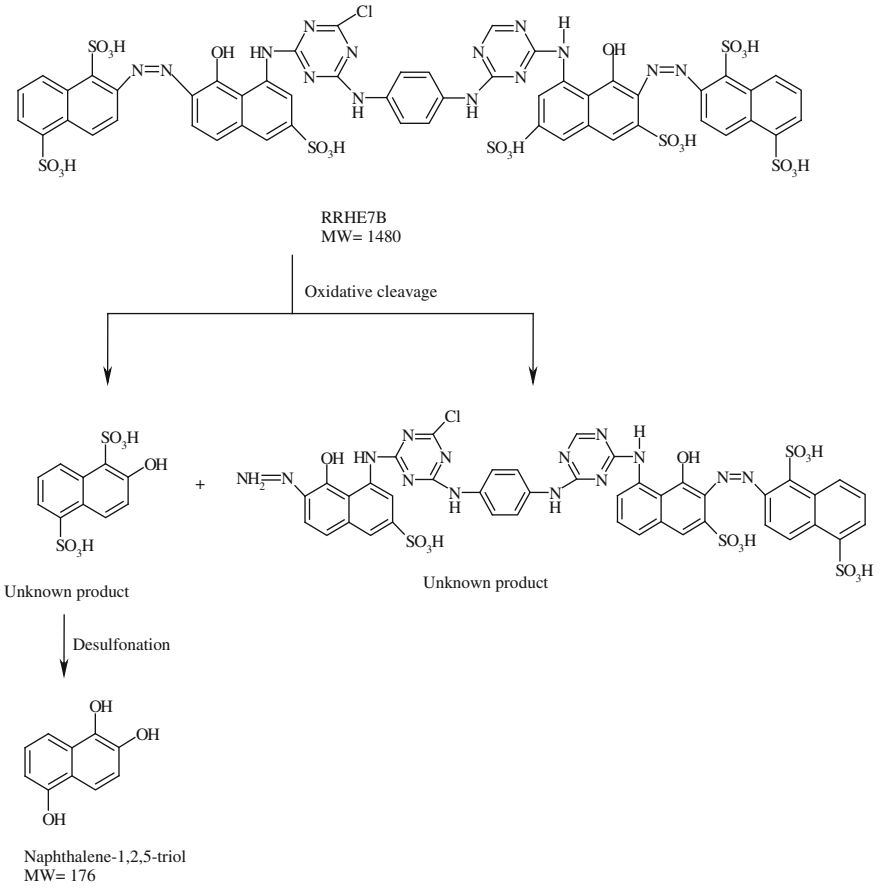

Fig. 13 Proposed degradation pathway for RRHE7B by Veratryl alcohol oxidase

azoreductase. Aromatic amines are highly toxic and carcinogenic in nature. On the other hand, decolorization of azo dyes by fungal species involves the role of oxidases which don't produce the toxic aromatic amines. Therefore, a consortium of bacteria and fungus used for decolorization of azo dyesproduces metabolite which was free from carcinogenic aromatic amines. Metabolites analyzed after decolorization of azo dye disperse Rubine GFL by consortium of *Pseudomonas* sp. SUK1 and *Aspergillus orchaceus* NCIM 1146, showed absence of the aromatic amines. However, *Pseudomonas* sp. SUK1 showed 0.14 mM concentration of aromatic amines (Lade et al. 2012). At the same time, *Aspergillus orchaceus* NCIM 1146 showed no presence of the aromatic amines. Therefore, use of enzymatic systems from fungal-bacterial co-cultures for decolorization of azo dyes enhances detoxification of azo dyes with enhanced biodegradationrate (Lade et al. 2012).

9 Conclusion

Different oxidoreductive enzymes, such as azoreductase, NADH-DCIP reductase, Riboflavin reductase, laccase, lignin peroxidase, tyrosinase and veratrol alcohol oxidase are involved in dye decolorization. The reductive enzyme system, such as azoreductase, generates toxic aromatic amines, but a combined reductive and oxidative system works better for detoxification of azo dyes. A combination of both oxidative as well as reductive enzymes removes azo dyes toxicity.

10 Futures Prospectives

Different oxidoreductive enzymes had shown their direct involvement indye decolorization. Findings of efficient enzymes, which are involved indecolorization of azo dyes, are to be explored using technologies like proteomics and genomics. Further, already knowndegradative enzymes can be cloned into single microorganism using genetic engineering technology to enhance the decolorization efficiency of synthetic dyes by microbes.

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Microbial Decolorization of Triphenylmethane Dyes

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1 Introduction

Synthetic dyes belong to important class of organic compounds extensively used in textile, paper printing, color photography, leather, plastics, food, cosmetic, and pharmaceutical industries (Ali 2010; Rauf and Ashraf 2012). According to Global Industry Analysts, the production of worldwide synthetic dyes and pigments industries is predicted to reach almost 10 million tons by 2017 which will be worth close to $27 billion (http://www.reportlinker.com/ci02367/Synthetic-Dye-and-Pigment.html). More than 11 % of these compounds produced annually is lost to effluents during application processes. The presence of even small amounts of dyes in water (<1 ppm) is visible. It affects the aesthetic value, causes loss in luminosity, decreases oxygen solubility in water and blocks the passage of light to the lower depths of aquatic systems. It results in the perturbation and even inhibition of photosynthesis of algae and phytoplanktons in water reservoirs. Synthetic dyes, like most xenobiotics, are photolytically and chemically stable as well as persistent to biodegradation. Some of them are toxic, carcinogenic and mutagenic (dos Santos et al. 2007; Srinivasan and Viraraghavan 2010; Verma et al. 2012).

Dyes are classified according to their origin, application and chemical structure. Based on the dye chemical structure and the type of the chromophore present in the molecule, there are currently known 20–30 dye classes. The most important of them are azo, triphenylmethane (TPM), anthraquinone, nitro, nitroso, indigoid and others dyes (Khan et al. 2013). TPM dyes, due to the presence of three aryl rings, belong to triarylmethane compounds and are characterized by outstanding intensity of color (red, blue, violet or green), brilliant shade and high tinctorial strength. They are relatively inexpensive and suitable for dyeing a wide range of textile substrates,

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such as wool, silk and polyamide. However, these pigments are seriously deficient in fastness properties, especially in relation to washing and light. Consequently, the use of TPM dyes in the textile industry has decreased significantly, since dye from other classes became available. At present, the photographical, food, cosmetic, and stationary industries seem to be the major consumers of this group of chemicals (Thetford 2000). TPM dyes are also extensively used in medicine and experimental biology (e.g. in cytology, histology, microbiology) to stain cells and proteins (Altikatoglu and Celebi 2011). Due to antibacterial, antifungal and antiprotozoal properties, several TPM dyes serve as medical disinfectants (Srivastava et al. 2004; Casas et al. 2009; Hashimoto et al. 2011). For example, Malachite green has been used since 1933 as a medicine for fish infected by fungi (*Saprolegnia* sp., *Aphanomyces* sp., *Haliphthoros* sp.) and protozoa (*Trichodina* sp., *Trichodinella* sp., *Chilodonella* sp.) (Srivastava et al. 2004; Hashimoto et al. 2011). Nevertheless, Malachite green is pointed out as a highly controversial compound due to the risk it poses to the consumers of the treated fishes. Malachite green and some of its metabolites have been reported to induce human carcinogenesis and mutagenesis (Sudova et al. 2007). Several studies have shown that exposure to this dye increases the risk of chromosomal fractures, reduces fertility and may also cause inhibition of respiratory enzymes (Srivastava et al. 2004; Stammati et al. 2005; Culp et al. 2006). Therefore, the use of Malachite green in aquaculture has been banned since 2000 in the European Union and in the United States of America and Canada since 2005 (Andersen et al. 2009). Similarly, Crystal violet, known as Gentian violet or Methyl violet 10B, is applied in human and veterinary medication as an antifungal agent for dermatological infections, treatment of pinworms and some tropical diseases (Casas et al. 2009). It has been also found to bind to DNA and thus causes replication errors (Ayed et al. 2009). The characteristics of TPM dyes, most frequently used in recent biological studies, are presented in Table 1.

2 Microbial Treatment of Waste Water Containing Dyes

As a consequence of many applications and accumulation ability, TPM dyes are commonly present in wastewater and hence pollute aquatic environments. For the removal of synthetic dyes from water bodies a wide range of physical, chemical and biological methods have been developed (Crini 2006; Ali 2010; Verma et al. 2012). Although chemical and physical decolorization techniques (e.g. adsorption, precipitation, coagulation, filtration, electrolysis, photodegradation and chemical oxidation) are effective for color removal, they have serious constraints, such as high cost, low efficiency, limited versatility, interference with other wastewater constituents, formation of hazardous metabolites and intensive energy requirements (Kaushik and Malik 2009). Therefore, there is an increasing interest in exploring the mechanisms of bioremoval of TPM dyes from wastewater which will be proven very effective and environment friendly methods (dos Santos et al. 2007; Sarayu and Sandhya 2012).

Table 1 TPM dyes most frequently used in decolorization studies

Dye	C.I. Name	C.I. Number	λ_{max} (nm)	References
Acid fuchsin	Basic Red 9	42500	544	Ren et al. (2006), Casas et al. (2009), El-Sheekh et al. (2009), Jing et al. (2009)
Brilliant green	Basic Green 1	42040	623	Ren et al. (2006), Casas et al. (2009), El-Sheekh et al. (2009), Jing et al. (2009), Kumar et al. (2012)
Bromophenol blue	Not available	Not available	590	Ren et al. (2006), Gomaa et al. (2008), Saparrat et al. (2008), Casas et al. (2009), Kumar et al. (2012), Przystaś et al. (2013)
Cotton blue	Acid Blue 93	42780	620	Jadhav and Govindwar (2006), Saratale et al. (2006), Shedbalkar et al. (2008)
Crystal violet	Basic Violet 3	42555	530	Jang et al. (2005), Šafaříkova et al. (2005), Eichlerová et al. (2006a), Jadhav and Govindwar (2006), Chen et al. (2007, 2008), Šafařík et al. (2007), Abedin (2008), Youssef et al. (2008), Moturi and Singara Charya (2009), Yan et al. (2009), Ayed et al. (2010), Ghasemzadeh et al. (2011), Pandey et al. (2011), Parshetti et al. (2011), Torres et al. (2011), Yang et al. (2011a, 2013), Cheriaa et al. (2012)
Malachite green	Basic Green 4	42000	615	Jang et al. (2004), Levin et al. (2004), Oranusi and Mbah (2005), Eichlerová et al. (2006a,b), Jadhav and Govindwar (2006), Papinutti et al. (2006), Parshetti et al. (2006), Ren et al. (2006), Saratale et al. (2006), Daneshvar et al. (2007), Abedin (2008), Deng et al. (2008), Jadhav et al. (2008), Youssef et al. (2008), Ayed et al. (2009, 2010), Jing et al. (2009), Moturi and Singara Charya (2009), Chen et al. (2010), Du et al. (2011), Shedbalkar and Jadhav (2011), Vasdev et al. (2011), Yang et al. (2011a, 2013), Cheriaa et al. (2012), Jasińska et al. (2012, 2013), Karimi et al. (2012), Lv et al. (2013), Mukherjee and Das (2013), Yan et al. (2014)

Bioelimination of TPM dyes can take place in two ways: by biomass sorption/accumulation (when the original structure of the dyes remains intact) or by biodegradation/biotransformation processes (Ali 2010). The process of biosorption can occur in either living or dead biomass. However, during accumulation, dyes are deposited inside actively growing cells (dos Santos et al. 2007). The efficiency of these processes depends on the chemical structure and the concentration of

particular dyes, the specific chemistry and dosage of sorbents as well as some physicochemical parameters such as pH, temperature and agitation. Biosorption is effective, especially when the dye-containing effluent is very toxic and when conditions are not favorable for the growth and maintenance of organisms. It tends to occur reasonably fast and various waste materials including spent microbial biomass can be employed as dyes adsorbents (Sharma et al. 2011). By developing the methods of adsorbents regeneration, dyes recovery makes these processes more economical (Won et al. 2006; Jasińska et al. 2013). However, biosorption and sole bioaccumulation do not eradicate the problem of dye pollution, because removed compounds are not destroyed, but only entrapped by the adsorbent. Thus, biodegradation (biologically mediated breakdown of the chemical structure) seems to be more suitable for dye removal (Kaushik and Malik 2009). Biodegradation processes cause not only visible decolorization, but also result in the production of intermediates usually not or less harmful as compared to parent compounds. Sometimes, complete mineralization of dyes (conversion into CO_2, H_2O, and/or any other inorganic end products) can be achieved. Dyes biodegradation may be mediated both by extracellular and intracellular enzymes. In the case of intracellular biodegradation, the bioaccumulation is a primary mechanism of decolorization. Recent findings also indicate that in the TPM dyes elimination, non enzymatic, low molecular weight compounds resistant to high temperature may also participate (Gomaa et al. 2008, Yan et al. 2009; Wang et al. 2011; Gomaa 2012). Also, hydroxyl radicals produced by white and brown rot fungi during the Fenton-like reaction $\left(Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{-}OH + {}^{\bullet}OH\right)$ are indicated as potential factors involved in TPM dyes decolorization (Karimi et al. 2012; Moldes et al. 2012).

In recent years, a lot of studies have been focused on various organisms (especially bacteria and fungi) capable of dyes degradation (Forgacs et al. 2004; Kaushik and Malik 2009; Rodríguez-Couto 2009). The most widely studied TPM dye-decolorizing microorganisms are white-rot fungi belonging to *Basidomycota* (Levin et al. 2004). The strains of *Phanerochaete chrysosporium* are the most popular basidiomycete models in the studies of synthetic dyes degradation. Members of this genus are also known as good decolorizers of various TPM dyes, such as Crystal violet, Pararosaniline, Cresol red, Bromophenol blue, Ethyl violet, Malachite green, and Brilliant green (Radha et al. 2005; Gomaa et al. 2008; Gomaa 2012). However, members of other basidiomycete fungi have been also found to remove TPM dyes. For example, *Dichomitus squalens* CCBAS 750 was proved to be a good decolorizer of several chemically different synthetic dyes, including Malachite green and Crystal violet (Eichlerová et al. 2006a). Casas et al. (2009) described effective elimination of Acid fuchsin, Brilliant green 1, Basic fuchsin, Methyl green and Acid green 16 by *Trametes versicolor* ATCC 42530. Saparrat et al. (2008) reported effective azo, heterocyclic and TPM dyes elimination by *Grammothele subargentea* LPSC no. 436, a white-rot fungus from temperate and tropical regions of America and East Africa. Despite a strong inhibition of growth caused by TPM dyes, this strain eliminated Brilliant green, Crystal violet and Fuchsin with high efficiency. According to Vasdev (2011), effective decolorization

of Crystal violet, Bromophenol blue and Malachite green was performed by six white rot fungi isolated from nature.

Also ascomycete fungi, exhibiting expansive growth, great biomass production and a high cell surface-to-volume ratio resulting in strong physical contact with the environment, are reported to be useful in the removal of various xenobiotics, including TPM dyes (Haritash and Kaushik 2009; Ali 2010). Many of them have origin from environments (soil, effluents and sludges) contaminated with dyes. For example, fungi derived from seawater, marine sediments and seagrass, identified as *Phialophora* sp., *Penicillium* sp. and *Cladosporium* sp. were shown to completely decolorize Crystal violet (Torres et al. 2011). *Aspergillus* sp. CB-TKL-1, isolated from a water sample, was found to be capable of quick decolorization of several structurally different dyes, especially Methyl violet (Kumar et al. 2011) and Brilliant green (Kumar et al. 2012). Mycelium of *Aspergillus ochraceus* NCIM-1146, maintained in water, decolorized Malachite green, Cotton blue, Crystal violet and Methyl violet with the efficiency of 98, 92, 61 and 57 %, respectively. This process involved microbial metabolism, not biosorption (Saratale et al. 2006). *Fusarium solani* isolated from dye containing effluents was able to decolorize with a high efficiency Malachite green and Crystal violet via biosorption, followed by intracellular degradation to colorless metabolites (Abedin 2008). A similar pattern of Malachite green removal was described for *Penicillium pinophilum* IM 6480 and *Myrothecium roridum* IM 6482, fungi isolated from soil around a textile dyeing factory (Jasińska et al. 2012). So far, very little work has been done to establish the TPM dyes decolorization ability of yeast. Strains of *Saccharomyces cerevisiae*, *Kluyveromyces fragilis*, *Candida krusei* and *Galactomyces geotrichum* were found to be efficient in decolorization of Malachite green, Crystal violet, Methyl violet, Cotton blue, Aniline blue and Basic violet 3 (Šafařiková et al. 2005; Šafařik et al. 2007; Jadhav et al. 2008; Deivasigamani and Das 2011).

Besides, several bacterial strains are able to degrade and even completely mineralize synthetic dyes (Chen et al. 2011; Saratale et al. 2011). As compared to fungi, bacteria are generally easier to culture, grow more quickly and are more amenable to genetic manipulations. These properties make bacteria a desired object of research concerning biodegradation of TPM dyes. Members of *Pseudomonas*, *Bacillus*, *Citrobacter*, *Desulfovibrio, Nocardia* and *Mycobacterium* genera are often recognized as good TPM dyes decolorizers (Oranusi and Mbah 2005; Guerra-Lopez et al. 2007; Deng et al. 2008; Wu et al. 2009). However, in recent years, new effective decolorant species: *Shewanella decolorationis, Acinetobacter calcoaceticus, Aeromonas hydrophila, Achromobacter xylosoxidans, Sphingomonas* sp., *Deinococcus radiodurans, Enterobacter asburiae* and *Staphylococcus epidermidis* have also been described (Ren et al. 2006; Ayed et al. 2010; Chen et al. 2011; Wang et al. 2011; Wu et al. 2011; Lv et al. 2013; Mukherjee and Das 2013; Pan et al. 2013). Bacteria, examined in the above-mentioned studies, were mostly isolated from environments contaminated with dyes and usually single strains were used for TPM decolorization. However, bacterial consortia also occurred to be very effective in the treatment of effluents originating from dyeing processes. Cheriaa et al. (2012) reported that a mixed culture containing cells of *A. radiobacter*,

Bacillus sp., *Sphingomonas paucimobilis* and *A. hydrophila* strains was found more efficient for faster decolorization of Malachite green and Crystal violet in comparison to individual cultures. Similarly, Acid violet 17 was decolorized more effectively by a consortium including the cells of *Bacillus* sp., *Alcaligenes* sp. and *Aeromonas* sp. than by the single bacterium (Sharma et al. 2004). It has been suggested that a microbial consortium may increase the rate of dyes decolorization due to synergistic actions among microorganisms.

3 Factors Affecting Bioelimination of TPM Dyes

TPM dyes bioelimination is strongly influenced by numerous operational parameters. Due to an enormous effect on organism growth and metabolism, media composition (especially compounds used as carbon and nitrogen sources) strongly affects synthetic dyes decolorization. Glucose and sucrose appeared to be the most suitable carbon sources for microbial removal of such TPM dyes as Basic violet 3 and Malachite green (Jadhav and Govindwar 2006; Deivasigamani and Das 2011). Acceleration of dye removal in culture media containing glucose, often results from the promotion of biomass production. However, due to the high cost of glucose, various cheaper compounds have been used as carbon sources in decolorization processes (Kaushik and Malik 2009). Among four different carbon sources (glucose, sucrose, starch and sodium citrate), starch was proved to be the best for Crystal violet decolorization by a strain of *F. solani* (Abedin 2008). Also Parshetti et al. (2006) successfully applied molasses (instead of glucose or sucrose) for Malachite green decolorization by *K. rosea* MTCC 1532.

Compounds, utilized as nitrogen sources, may also affect dyes biodegradation due to their influence on the synthesis of ligninolytic enzymes such as laccases and peroxidases. Generally, production of fungal ligninolytic enzymes is often stimulated by nitrogen deficiency depletion. For example, Ghasemzadeh et al. (2011) reported high production of LiP and MnP by *P. chrysosporium* in nitrogen-limited conditions and was correlated with effective elimination of Crystal violet. Dyes decolorization may be affected not only by the concentration, but also by the type of nitrogen source. It is postulated that in comparison to inorganic sources, organic compounds, such as peptone or yeast extract, frequently accelerate decolorization processes. Indeed, yeast extract was reported as the best nitrogen source for efficient decolorization of various TPM dyes (Cheriaa and Bakhrouf 2009; Parshetti et al. 2011). Recognition of yeast extract as an essential culture media supplement is probably related to its suitability for the regeneration of NADH acting as an electron donor for the enzymatic reduction of TPM dyes (Jang et al. 2005; Ogugbue and Sawidis 2011).

pH value of the decolorization environment influences the transport of dye molecules across cell membranes as well as extracellular biotransformation of TPM dyes by ligninolytic enzymes. Nozaki et al. (2008), while studying the decolorization of TPM dyes by 21 fungi of *Basidiomycota*, found that the optimum pH for

dyes removal ranged from 3 to 5. Similarly, according to Sarnthima et al. (2009), the highest percentage of Bromophenol blue removal by a strain of *Lentinus polychrous* occurred at pH 4. Generally fungi and yeast prefer acidic pH; however, Malachite green was effectively decolorized at pH 7 by the mycelium of *P. ochrochloron* (Shedbalkar and Jadhav 2011). Similarly, Deivasigamani and Das (2011) reported complete Basic Violet 3 elimination by a *C. krusei* strain cultured at pH 7. Yang et al. (2011a) estimated that growth medium at pH 6 accelerated Malachite green decolorization by the strain *Penicillium* sp. YW01. Bacteria more often decolorize dyes at neutral and alkaline pH. Du et al. (2011) observed that the proper pH for Malachite green decolorization by *Pseudomonas* sp. DY1 was between 5.5 and 8, and the highest decolorization was recorded at 6.6. Decolorization of Victoria blue R by *A. calcoaceticus* YC210 was found to be the most effective within a range of pH 5–7 (Chen et al. 2011). *P. aeruginosa* BCH decolorized Acid violet 19 up to 98 % within 30 min with optimum of pH 7 (Jadhav et al. 2012).

In most cases, the rate of TPM dyes bioremoval increases up to the temperature which is optimal for the growth of organisms and also the rate of dyes removal often rises with an increase in the temperature due to respiration and substrate metabolism acceleration. It is worth mentioning that some TPM dyes degrading enzymes are active and stable even at high temperatures. For example, Malachite green was decolorized by *Trametes trogii* Berk S0301 effectively in the range of 30–80 °C with an optimum decolorization at 50–70 °C (Yan et al. 2014). According to Zhang et al. (2012), spore laccase from *Bacillus vallismortis* fmb-103 was quite stable at a temperature range between 25 and 90 °C. TPM reductase from *Citrobacter* sp. KCTC catalyzing the NADH-dependent reduction of Crystal violet to its leuco dyes, exhibited maximum activity at 60 °C (Jang et al. 2005).

Biodegradation of TPM dyes may occur under aerobic as well as anaerobic conditions depending on metabolic features of particular microorganisms. Most fungi are obligate aerobes and they need oxygen for their growth and maintenance of viability. In order to meet their oxygen requirements and to enhance the oxygen gas-liquid mass transfer, the agitation is necessary. Most literature data indicate that fungi eliminate TPM dyes faster under shaking conditions (Saratale et al. 2006; Przystaś et al. 2013). Usually TPM dyes elimination by bacterial strains is also improved in the shaking conditions (Ali 2010; Du et al. 2011). In contrast, Malachite green decolorization by *B. cereus* DC11 required anaerobic or microaerophilic conditions and was strongly inhibited in an aerobic culture (Deng et al. 2008). It is suggested that the lack of decolorization in shaking conditions could result from the fact that dissolved oxygen often inhibits reductase-driven decolorization. Under aerobic conditions, oxygen can compete with the dye for the reduced electron carriers (Moosvi et al. 2005).

Generally, the decolorization efficiency decreases with an increase in the concentration of TPM dyes, which mainly results from the rising toxicity of dyes. When the microbial growth is reduced, secretion of enzymes can be also limited, especially in solid media (Eichlerová et al. 2006a; Parshetti et al. 2006). However, the initial concentration of the dye can possess a strong driving force to overcome

the mass transfer resistance between a dye and cells. For example, decolorization of Victoria blue R by *A. calcoaceticus* increased with increasing the dye concentration up to 450 mg 1^{-1} (Chen et al. 2011). Likewise, Victoria blue decolorization by *P. chrysosporium* ATCC 34541 was proved to be nearly independent of the dye concentration over the range from 50 to 350 mg 1^{-1} (Gomaa et al. 2008).

4 Role of Oxidoreductive Enzymes in the Degradation Process

Detoxification of synthetic dyes by various bacteria and fungi is often mediated by oxidoreductases. The main oxidoreductive enzymes playing a role in TPM dyes elimination are lignin peroxidases (LiP), E.C. 1.11.1.14, manganese peroxidases (MnP), EC 1.11.1.13 and laccases (Lac), EC 1.10.3.2 (Kandelbauer and Guebitz 2004).

Laccases are copper-containing enzymes which catalyze oxidation of phenolic compounds with a simultaneous reduction of one dioxygen molecule to two molecules of water. Laccases can act in the presence of redox mediators, such as 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), N-hydroxybenzotriazole (HBT) and 3-hydroxyanthranilic acid, which help them in breaking down several difficult to degrade compounds including synthetic dyes (Kunamneni et al. 2007). Based on the experimental evidence, a laccase-mediated dye decolorization mechanism was proposed in Fig. 1. MnP and LiP were also shown to take part in the degradation of various polycyclic aromatic and phenolic compounds, including synthetic dyes (Champagne and Ramsay 2005; Yang et al. 2011a, b, 2013). Generally, peroxidases are hemoproteins that catalyze reactions in the presence of hydrogen peroxide. MnP catalyzes transformation of phenolic compounds, which is associated with Mn (II) to Mn (III) oxidation (Shin et al. 2005). Similarly, LiP

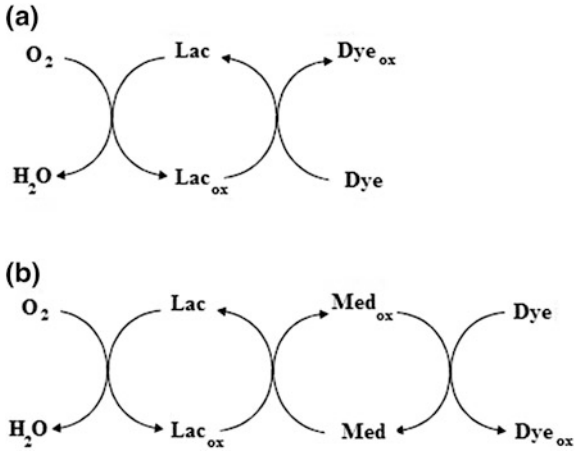

Fig. 1 Mechanisms of dyes decolorization catalyzed by laccase (**a**) without and (**b**) with the mediator engagement

performs one-electron oxidation of phenolic compounds, but also reactions not normally associated with other peroxidases, i.e. oxidation of non-phenolic aromatic substrates (Husain 2006). LiP decolorization activity can be enhanced in the presence of some compounds, such as veratryl alcohol, which acts as a redox mediator between an oxidized LiP and a dye (Alam et al. 2009). According to the data obtained by Gomaa et al. (2008), laccase, oxygenase/oxidase and/or a heat-stable non-enzymatic factor were suggested as the most probable agents involved in Victoria blue elimination by *P. chrysosporium* ATCC 34541. High laccase and MnP activities were also correlated with Ethyl violet decolorization by *Pleurotus pulmonarius* (FR) (dos Santos Bazanella et al. 2013). A white-rot fungus *Ischnoderma resinosum* was reported to be capable of almost complete decolorization of Malachite green and Crystal violet mainly by laccase and MnP action within 20 days of culturing in liquid medium (Eichlerová et al. 2006b). Degradation of the Malachite green-loaded rapeseed press cake in a laccase containing culture of the *M. roridum* IM 6482 was also described by Jasińska et al. (2013). Crude laccase of *Coprinus comatus* was able to decolorize over 90 % of Malachite Green, and higher decolorization was obtained in the presence of redox mediators (especially in the presence of 1-hydroxybenzotriazol) (Jiang et al. 2013). The strain *Lentinula edodes* CCB-42 was able to eliminate Methyl violet, Ethyl violet and Methyl green (Boer et al. 2004). Because the process was strongly influenced by the Mn ions and H_2O_2 presence, Mn peroxidase action was suggested as the main mechanism of dyes elimination. Transformation of Cotton blue, as a result of *Penicillium ochrochloron* MTCC517 LiP induction, was reported by Shedbalkar et al. (2008). The same strain was also found to detoxify Malachite green via peroxidase-mediated reactions.

TPM dyes can be also decolorized via NADH/NADPH-dependent reduction under the control of TPM reductase (TMR). The first purified and characterized biochemically TMR was isolated from *Citrobacter* sp. KCTC 18061P (Jang et al. 2005). However, TMR was also identified in *Pseudomonas aeruginosa* NCIM 2074, *Exiguobacterium* sp. MG2 and *Mucor mucedo* cells (Moturi and Singara Charya 2009; Kalyani et al. 2012; Wang et al. 2012). It shows that TMR decolorization activity is dependent on the chemical structure of the dyes. The most efficient TMR substrate appeared to be Malachite green, while Crystal violet was less favorable, perhaps because of the additional dimethyl amino group (Jang et al. 2005). Kim et al. (2008) suggested that the structures of other TPM dyes, such as Brilliant green, Bromophenol blue, Methyl red, and Congo red, made them incompatible with the size and hydrophobic restrictions of the TMR substrate binding pocket. Based on structural inspection, the modeled ternary protein/cofactor/substrate complex structure and mechanism of Malachite green decolorization by TMR were also proposed (Fig. 2).

TPM dyes removal by microorganisms may also occur as a result of action of membrane associated oxidoreductive enzymes. For example, the membrane fraction of *Mycobacterium avium* A5 had an about 5-fold higher Malachite green specific decolorization rate than the crude extract which suggested the involvement of membrane associated proteins e.g. cytochrome P-450 (Jones and Falkinham 2003).

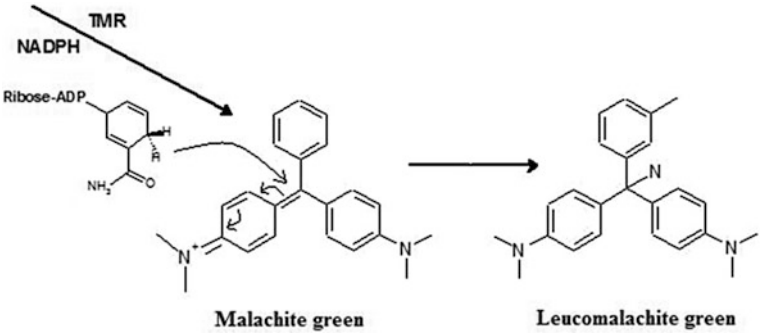

Fig. 2 Proposed mechanism of the Malachite green decolorization by triphenylmethane reductase (TMR) (Kim et al. 2008)

The involvement of cytochrome P-450 monooxygenases in Malachite green removal was also reported in *Cunninghamella elegans* ATCC 36112 cultures (Cha et al. 2001).

5 Mechanism and Pathways of Degradation

Many studies have been focused on involvement of possible enzymes as well as on the structures of formed TPM intermediates. Pandey et al. (2011), based on the UV, ^{1}H NMR and IR spectra, reported Crystal violet degradation by *Pseudomonas fluorescens* into three intermediates: 4-benzhydrylidene-cyclohexa-2,5-dienylamine hydrochloride; 4-benzhydrylidene-cyclohexa-2,5-dienol and 4-benzhydryl-cyclohexa-2,5-dienol. Michler's ketone (N,N'-bis[dimethylamino] benzophenone), a common TPM dyes intermediate, was detected in degradative pathways of Methyl violet B and Malachite green by *S. decolorationis* NTOU1 (Chen et al. 2010) and *Bacillus cereus* DC11, respectively (Deng et al. 2008). Generation of leucocrystal violet and five other intermediates: [N,N'-bis[dimethylamino] benzophenone]; [N,N-dimethylaminophenyl][N-methylaminophenyl]benzophenone; N,N-dimethylaminobenzaldehyde; N,N-dimethylaminophenol and 4-methylaminophenol during the degradation of Crystal violet by *Shewanella* sp. NTOU1 was described by Chen et al. (2008). The same intermediates of Crystal violet were produced in the liquid culture of *Agrobacterium radiobacter* MTCC 8161 (Parshetti et al. 2011). Stepwise demethylation leading to the yield of partially dealkylated dye molecules was the main mechanism of Victoria blue R removal by the strain of *A. calcoaceticus* (Chen et al. 2011). The authors also revealed that the studied bacteria could utilize the dye as a sole carbon source. The degradative mechanism involving production of N-demethylated intermediates was described by Chen et al. (2007), who studied Crystal violet elimination by *Pseudomonas putida.* Jadhav et al. (2012), based on the enzymatic studies and FTIR as well as GC-MS analysis, stated asymmetric cleavage of Acid violet 19 by *P. aeruginosa* BCH

laccase (resulting in 3-methylbenzenesulponic acid formation) and subsequent desulfonation by veratryl alcohol oxidase.

6 Microbial Toxicity of Dyes and Their Degradation Products

The majority of studies concerning biodegradation of TPM dyes are focused on mechanisms and factors involved in this process. In contrast, there are only a few reports which include the evaluation of the toxicity of the obtained TPM dyes metabolites. Such data are of great importance, because wastewaters decolorized by biodegradation methods should be safe for the environment. For practical and ethical reasons, mainly microorganisms, plants and invertebrates are used as model organisms in toxicity assays. They are characterized by quick development and an ability to produce resting forms. In microbial toxicity tests, the most often exploited strains belong to bacterial species as *K. rosea, P. aeruginosa, Azotobacter vinelandii, S. paucimobilis, Escherichia coli* and *B. subtilis* (Parshetti et al. 2006; Shedbalkar et al. 2008; Ayed et al. 2009). In these tests, the toxicity of the original dye and its by-products is usually expressed as a reduction in the number of microbial cells per milliliter (with the use of liquid cultures) or as a formation of inhibition zone surrounding the well filled with a dye solution (on solid media). Chen et al. (2008) tested toxicity of Crystal violet and its possible degradation products using the strain of *E. coli*. The parent dye occurred to be toxic, while the Crystal violet intermediates formed by *S. decolorationis* NTOU1 were not toxic to the test organism. While studying the biodegradation of Cotton blue by *P. ochrochloron* MTCC 517, the bacterial toxicity test (with the use of *A. vinelandii* strain) showed a growth inhibition zone (1.2 cm) around the well containing Cotton blue, in contrast to the products of the dye transformation, which did not express any inhibitory effect (Shedbalkar et al. 2008). Toxicity studies of Crystal violet and its metabolites formed by a strain of *A. radiobacter* proved that degradation products were less toxic to the strains of *A. radiobacter*, *P. aeruginosa* and *A. vinelandii* (Parshetti et al. 2011).

7 Future Prospectives

Biodegradation of synthetic dyes using fungi and bacteria has become a promising approach for the treatment of dye wastewaters. Now-a-days, molecular biology offers various tools to accelerate natural abilities of organisms and optimize degradation pathways. The principal approaches to develop genetically engineered microorganisms, useful mainly in bioremediation include: modification of specificity and affinity of microbial enzymes and/or bioprocess monitoring and regulation. Moreover,

several enzymes decolorizing TPM dyes were identified, cloned and expressed in diverse host organisms (Jang et al. 2005; Ren et al. 2006; Lu et al. 2009; Moturi and Singara Charya 2009). Recently, Chengalroyen and Dabbs (2013) identified in a strain of *Amycolatopsis* sp. four TPM dyes biodegradation genes encoding 3-deoxy-7-phosphoheptulonate synthase, N5,N10-methylenetetrahydro methanopterin reductase, polycystic kidney domain I and glucose/sorbose dehydrogenase. The synergistic action of these genes expressed in *Streptomyces lividans* TK23 led to complete decolorization of Crystal violet. Their activity was also tested in *Mycobacterium* sp. mc^2 155 and three strains of *Rhodococcus* sp. The range of decolorized dyes was extended in both species, showing that cloned genes had adopted novel functional potentials within the hosts. Also, a system of phytoremediation by *Arabidopsis* plants based on overexpression of TPM reductase from *Citrobacter* sp. was presented by Fu et al. (2013). The morphology and growth of transgenic *Arabidopsis* plants showed significantly enhanced tolerance to Crystal violet and Malachite green and ability to decolorize these dyes.

8 Conclusion

Due to the large-scale production and wide application, TPM dyes are serious health-risk factors and can cause considerable environmental damage. So far, a number of strategies for the treatment of dye containing wastewaters have been developed. However, the most promising method of the dyes removal seems to be bioremediation which is cost effective, ecologically suitable and involves the usage of various organisms especially bacteria, microscopic algae and fungi. The literature reviewed in this paper indicates that a large number of TPM dyes can be efficiently decolorized by bacterial (pure or mixed) and fungal cultures mainly due to the action of extra- and/or intracellular enzymes (e.g. laccases, peroxidases and reductases). Strains isolated from dye polluted environments (such as contaminated soil, dye effluents and sludges) are often well adapted to live in the presence of high concentrations of dyes and metabolize them to non-toxic intermediates. Some bacterial strains can even completely mineralize dyes, converting them into CO_2, H_2O, and/or any other inorganic end products. The biodegradation efficiency can be enhanced by exposing microorganisms to higher concentrations of synthetic dyes and using some molecular biology techniques. Biodegradation of TPM dyes is strongly influenced by various environmental factors (e.g. availability of nutrient compounds and oxygen, pH, temperature, initial dye concentration). Therefore, optimal operation parameters and toxicity of formed intermediates should be determined before practical utilization of biodegradation processes. Efforts should also be made to scale-up these processes before applying them for in real industrial effluents treatment.

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Textile Dyes Degradation: A Microbial Approach for Biodegradation of Pollutants

Lokendra Singh and Ved Pal Singh

1 Introduction

Rapid industrialization has given rise to various unwanted elements that accumulated in the biosphere up to toxic levels to degrade the natural environment. Scientific developments are considered as key factors for progress of both developing and under developed countries, but unfortunately, most of the industries in these countries do not have proper waste treatment facilities and releasing a large quantity of effluents. A majority of xenobiotics (either untreated or partially treated) released from industries are mixed up with the natural water bodies and to the soil of the biosphere. Untreated or partially treated textile effluents are highly toxic, as they contain a large number of toxic chemicals and heavy metals. The problem of water pollution due to the discharge of industrial wastewater into natural water bodies was witnessed by western countries in 19th century and also in India after independence.

Until the discovery of synthetic alternatives, most of dyes were derived from natural sources, such as plants and shellfish. These were only present in small amounts and their extraction was often inefficient, so they were usually expensive. In 19th century, there was a need to manufacture a large quantity of cheaper dyes and pigments for textile industries. As a result synthetic dye industry became a 'high-tech' industry of Victorian times, and its acknowledged founder was an English chemist, William Henry Perkin. In 1856, Perkin, in his experiment with aniline (one of the simplest chemical components of coal tar) obtained a black precipitate and discovered purple color, which readily dyed silk and was much more stable in sunlight than any other (natural) purple dye then in use. This first

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synthetic dye was patented by Perkin in August 1856, as dye aniline purple and after its success in France, it was renamed mauve (or mauveine), after the French word for the purple mallow flower (Susan 1982).

The coloring processes discharge huge quantities of dye effluents, which pollute local terrestrial habitat, rivers and others aquatic bodies. The untreated effluents and toxic chemicals, solid wastes find their way to the ground water and rivers, causing extensive damage to soil and water. In countries, such as India, China and Mauritius discharge from a large number of the textile industries go straight into the rivers. According to an annual report by Union Ministry of Environment and Forests (MoEF), about 4.4 million tons of hazardous wastes are generated by 13,011 units spread over 373 districts of the country (Ramaswamy 2003). The industrial process produces wastewater containing about one tone of dyes daily.

Dyes are natural or synthetic colored organic compounds having the property of imparting their color to the other substances, such as textile fibers. Synthetic dyes are used extensively for textile dyeing, paper printing, leather dyeing, color photography and as additives in petroleum products because of their ease and cost effectiveness in synthesis, firmness, high stability to light, temperature, detergent and microbial attack and variety in color as compared to natural dyes (Couto 2009). The dyestuff, textile, paper and leather industries, the major users of dyes, produce effluents that are usually very resistant to the biological treatment and hence, their industrial waste is a major problem to the environment. The released wastewater from textile industries is a complex mixture of many polluting substances, ranging from agro-based pesticides to heavy metals associated with dye or the dye process. Approximately, 10,000 different dyes and pigments are used in different industries and their production exceeds over 7×10^5 tonnes annually worldwide (Zollinger 1987). The textile industry generates a large volume of wastewater, which if not properly treated, can cause serious problems of environmental contamination (Kunz et al. 2002).

Many synthetic dyes are used in a number of industries, such as textiles, paper printing, color photography and the food industries (Meyer 1981) and as additive in petroleum products (Maynard 1983). The pigments used in printing ink, such as carbon black, titanium dioxide and others organic pigments, which are rendered insoluble by complexing with metal ions. Most of these pigments are prepared from azo, anthraquinone and triarylmethane dyes, and phthalocyanines. Since 50 % of dyes are used in the textile industry, azo dyes account for the largest proportion of all synthetic dyes in terms of number and amount. Approximately 70 % of all organic dyes that are currently available in the market are manufactured mainly in China, India, Korea, Taiwan and Argentina. Azo dyes, one of the largest classes of dyes used in textile industry, are released into the aquatic and terrestrial environments through the effluents emanating from textile and dyestuff industries and are normally not removed by conventional wastewater treatment system. The characteristic chemical structure (such as —C═C—, —N═N— and —C≡N—) of azo dyes makes them recalcitrant to biological break down.

Biodegradation refers to the breakdown of complex molecules to mostly smaller and simpler ones. The original complex molecules are often environmentally objectionable. The biodegradation is a biological process by which environmental

pollutants are eliminated or converted into less toxic (or even useful) substances. Natural biodegradation is often largely catalyzed by indigenous microbial or plant populations in soil or aquatic ecosystems. Biodegradation has at least three definitions (i) a minor change in an organic molecule leaving the main structure still intact, (ii) fragmentation of a complex organic molecule in such a way that the fragments could be reassembled to yield the original structure, and (iii) complete mineralization. Mineralization is the transformation of organic molecules to mineral forms, including carbon dioxide or methane, plus inorganic forms of other elements that might have been contained in the original structures. In recent years, several studies have focused on the use of microorganisms that are capable or potent to biodegrade and/or bio-accumulate toxic compounds (Aksu 2005). The bioremediation technology offers several advantages; it can be performed on site; generally has lower cost and minimum inconvenience in the process; eliminates the waste permanently; can be used in conjunction with methods of physical and chemical treatments; has minimal environmental impact and, therefore, has wide public acceptance and also encouraged by regulatory authorities (Boopathy 2000; Dias 2000).

2 Textile Effluents and Their Degradation

The wastewater treatment system is mainly based on physical and chemical procedures such as absorption, coagulation-flocculation, oxidation, filtration, and electrochemical. Although these methods are effective, but there are also some disadvantages and limitations, such as high cost, formation of hazardous by-products, operational problems and intensive energy requirements. Therefore, biological process is getting more and more attention, since it is cost-effective, environmental friendly and does not produce a large quantity of sludge (Seong et al. 1995). Microbial decolorization of dyes is currently in wide use in textile industry and also safe for environment due to non formation of toxic by-products during and after degradation. Several attempts have been made for dye degradation in the past, but yet complete dye degradation or decolorization is an important task to the researchers.

A wide variety of microorganisms (fungi, bacteria and actinomycetes) growing in biological treatment systems are the biological agents for biodegradation. Microbial degradation is usually only means for complete mineralization of organic molecules. Besides, microbes can concentrate, accumulate and absorb heavy metals inside cell or cell walls. Various microorganisms including, yeasts *Proteus* sp., *Enterococcus* sp., *Streptococcus* sp., *Bacillus subtillis* and *Streptococcus* sp. have been previously isolated to degrade azo compounds (Brown 1981). Immobilized microorganisms are also being used for water purification e.g. immobilized mycelium of *Coriolus versicolour* is being used for removing colors/pigments from Kraft mill wastes. In order to develop suitable technology to decolorize or degrade dyes discharged in the effluent and to convert them into beneficial products simultaneously, a well-planned scientifically acceptable technology is needed.

Use of biological methods for detoxification of hazardous waste is an emerging technology with great potential as an effective and inexpensive alternative to earlier methods for clean-up of polluted environments.

Among microorganisms, fungi possess some unique attributes that, in many ways, reflect their morphological and physiological diversity in different habitats. Fungal biosorption has been studied more extensively because of the availability of large amount of waste fungal biomass from fermentation industry and the amenability of the microorganisms to genetic and morphological manipulations. Paszczynski et al. (1992) have examined a new approach to increase the susceptibility of azo dyes to degradation by aerobic microorganisms, especially by *Streptomyces* spp. and *Phanerochaete chrysosporium*.

3 Fungal Dye Degradation

Dyes industries are one of the major sources of water pollution among different industrial effluents, which are directly poured into the water bodies. In India as well as in others developing countries, discharges/effluents from dye industries play a major role in deteriorating water quality. The wastes from these industries and dye process are not only responsible for water contamination, but also for many diseases as well as for the disturbance to the aquatic fauna and flora. The carcinogenic and persistence nature of azo and many other synthetic dyes, used in textile industry, have been reported extensively to be mutagenic in nature. Microbes have a great ability to degrade persistent organic compounds, which are used in dye industries. With earlier studies on microorganisms, a number of strategies have been applied to demonstrate or enhance the abilities of organisms to degrade various persistent and toxic xenobiotics. Fungi and bacteria, both are the principal degraders of organic matters, but fungi are better known for this purpose. There are about 72,000 species of fungi and the new species are being added at the rate of about 1,500 each year.

Decolorization of azo dyes by various fungi has been reported by Rafii et al. (1990). Fungal biosorption has been studied more extensively because of the availability of a large amount of waste fungal biomass from fermentation industry and the amenability of the microorganisms to genetic and morphological manipulations. About 99 % color removal was obtained by adsorption of dye to the cells of filamentous fungi. Heinfling et al. (1997) reported that *Bjerkandera adusta* and *T. versicolour* removed 95 % of HRB 8 dye within four days.

The basidiomycete's fungus *Phanerochaete chrysosporium* has unusual degradative capabilities and termed as "white rot fungus" because of its ability to degrade lignin, a randomly linked phenyl propane-based polymeric component of wood. This fungus possesses a great potential and has become a model example for its commercial and biotechnological use in bioremediation of dyes and lignin-cellulosic materials, present in the textile effluents. Bumpus and Aust (1986) reported the capability of this fungus, to degrade a wide variety of structurally diverse organopollutants through its non-specific H_2O_2-dependent extracellular lignin-degrading

enzyme system, produced during secondary metabolism in nitrogen-limited medium. This fungus has also been shown to degrade polymeric dyes (Glenn and Gold 1983), Crystal Violet (Bumpus and Brock 1988) azo and heterocyclic dyes. Cripps et al. (1990) reported the aerobic degradation of three azo dyes (Congo Red, Orange II and Tropaeolin O) by the fungus *P. chrysosporium*. The degradation of azo, anthraquinone, heterocyclic, triphenylmethane and polymeric dyes by *P. chrysosporium* has been intensively studied (Bumpus 1989; Cripps et al. 1990; Ollikka et al. 1993). Spadaro et al. (1992) established that *P. chrysosporium* was capable of mineralizing a variety of toxic azo dyes and was dependent on the nature of ring substituents. Another white-rot fungus, *Thelephora* sp. was also used for decolorization of azo dyes, such as Orange G, Congo Red, and Amido Black 10B.

Freitage and Morell (1992) reported the results of screening of 170 strains of white-, brown-, and soft-rot decay fungi and non-decaying xylophilous fungi for phenol oxidase activity with the polymeric dye Poly-478. This study also explored a relation between dye decolorization and ligninolytic activity and the presence of phenol oxidase and peroxidases. Yesilada (1995) reported decolorization of crystal violet by *Coriolus versicolour* and *Funalia trogii*. Wilkolazka et al. (2002) have studied the potential of 115 strains of fungi to decolorize two structurally different dyes (azo dye and anthraquinonic dye) and observed that the fungi, which have a great ability to degrade the azo and anthraquinonic dyes, are mainly white-rot fungi as listed in Table 1.

Some brown-rot fungi were also described by same authors for decolorization of some anthraquinonic and azo dyes i.e. *Coprinus micaceus*, *Fomtopsis pinicola*, and *Gloeophyllum odoratum*. Similarly, seven different fungi were isolated from the dye effluent sites and identified as, *Aserpgillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Trichoderma viride*, *Fusarium oxysporum*, *Penicillium chrysogenum* and *Mucor* sp. which were responsible for the degradation of a wide range of textile dyes (Saranraj et al. 2010). Machado et al. (2006) showed the potential of fungi *Trametes villosa* and *Pycnoporus sanguineus* to decolorize reactive textile dyes used for cotton manufacturing in the State of Minas Gerais, Brazil and found decolorization of 28 tested dyes by the fungus *T. villosa* and decolorization of 9 dyes by the fungus *P. sanguineus*. Higher decolorization of the synthetic effluent was also observed by mixed culture of these two fungi. The biodegradation of some

Table 1 Fungal strains mainly used for biodegradation of azo and anthraquinonic dyes

Fungi	
Strains	*Abortiporus biennis*, *Bjerkandera fumosa*, *Cerrana unicolour*, *Clitocybula dusenii*, *Dichomitus albiodoffuscus*, *Diplomitiporus crustulinus*, *Flammulina velutipes*, *Gonoderma lucidum*, *G. applanatum*, *Heterobasidion annosum*, *Keuhneromyces mutabilis*, *Lentinus edodes*, *Nematoloma frowardii*, *Panus tigrinus*, *Perenniporia subacida*, *Phanerocheate chrysosporium*, *Phlebia radiate*, *Pholiota glutinosa*, *Pleurotus pullmonarius*, *Pycnoporus coccineus*, *Stropharia rugosoannulata*, *Tremetes sanguinea*, *T. versicolour*, *Agrocybe cylidracea*, *Coprinus micaceas*, *Fomitopsis pinicola*, *Geotrichum sp. Gloeophyllum odoratum*, *Pestalotia sp. Pholiota glutanosa*

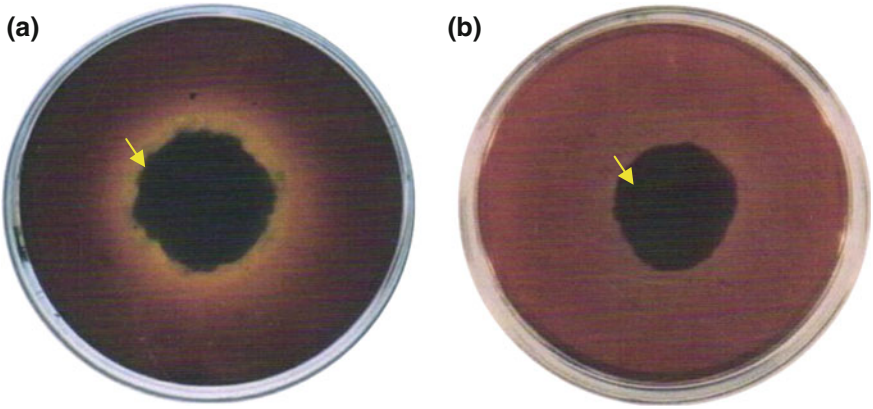

Fig. 1 Decolorization of Bromophenol blue (**a**) and Congo red (**b**) dyes by fungus *Aspergillus flavus*. *Arrows* indicating the zone of *yellow* color developed by the applied fungus during the dyes decolorization/degradation (Singh and Singh 2010b)

azo and anthraquinonic dyes by fungi *Aspergillus flavus* (Fig. 1) and *Trichoderma harzianum* was also recorded (Singh and Singh 2010a, b) which might be due to both; primary, by the adsorption and absorption of these dyes by fungal mycelia and secondary phenomenon, by producing extracellular enzymes during dye degradation (Singh and Singh 2012). However, bacteria and others microorganisms play an important role in the degradation of hazardous dyes, but among them, fungi have key role in biodegradation process.

4 Bacteria Used in Dyes Degradation

Apart from fungi, bacteria and actinomycetes also have ability to degrade the dyes and others pollutants. Various bacterial strains reduce azo dyes under both anaerobic and aerobic conditions to the corresponding amines (Meyer 1981). The bacteria *Sphingomonas xenophaga* BN6, *Agrobacterium tumefaciens*, *Ralstonia eutropha* 335, *Hydrogenophaga palleronii*, *Escherichia coli* K12 and *Flexibacter filiformis* (Gram negative), *Bacillus subtilis*, *Rhodococcus erythropolis* and *Lactobacillus plantarum*) (Gram negative) and Archea (*Halobacterium salinarum*) are reported to reduce azo dyes under anaerobic condition.

Neelambari et al. (2013) found the decolorization of azo dyes by some bacterial strains of *Pseudomonas aeruginosa*, *Alcaligenes faecalis*, *Proteus mirabilis*, *Serratia marcescens* and *Bacillus licheniformis* at static and shaking conditions. They also found that applied bacterial strains decolorized the dyes efficiently at about 3 % salt concentration and completed decolorization of dye in 48 h of incubation. The metabolites generated after the bacterial degradation were also found less toxic than original dye as confirmed by the phytotoxicity test. Jadhav et al. (2010) observed

that the *Pseudomonas aeruginosa* was able to detoxify the dye, Direct Orange 39 (1,000 ppm each day) effectively. Similarly, Roushdy and Abdel-Shakour (2011) observed zone of inhibition with control Malachite green with some microbial strains and found no growth inhibition by the degraded products. These findings clearly suggest non-toxic nature of the degradation products formed by the biological degradation.

The ability of actinomycetes to decolorize several anthraquinone and azo dyes has also been reported by several workers. The anthraquinonic dyes Remazol Brilliant Blue R, Poly B-411 and Poly R-478 were decolorized by *Streptomyces* sp. and *Thermomonospora* sp. Some species of *Streptomycetes* also decolorized azo dyes 4 (3-methoxy-4-hydroxy-phenylazo)-azobenzene-3,4′-disulfonic acid, 3-methoxy-4-hydroxy-azobenzene-4′-sulfonic acid and Orange I. Actinomycetes can catalyze hydroxylations; O, N, and S oxidations; and O- and N-dealkylation reactions against various xenobiotic compounds. *Streptomyces chromofuscus*, a member of actinomycetes group, was found capable to degrade azo dye in the presence of verytal alcohol (Paszczynski and Crowford 1991). The ability of *S. chromofuscus* to mineralize these dyes and guaiacol-substituted azo dye was of much significance, since azo dyes and some of its substitutes are resistant to aerobic degradation by most of soil bacteria. The genus *Streptomyces* has been reported to degrade benzene derivatives via classic aromatic catabolic pathways. A little research has been devoted to determining whether actinomycetes efficiently degrade condensed polycyclic aromatics, although some strains are able to metabolize naphthalene derivatives. *Streptomyces* spp. degrade some recalcitrant compounds, such as carbamates, diazinon, and bromoxynil. Actinomycetes were also found to degrade organo-chlorine compounds in spent sulfite bleach plant effluent. Further, evidences also indicate that *Streptomyces* sp. can act synergistically with other soil microorganisms to degrade recalcitrant compounds (Gunner and Zuckman 1968).

5 Mechanisms and Basic Steps During Biodegradation

The aerobic and anaerobic biological wastewater treatment has been the oldest known biodegradation based process technology. Several technologies have been evolved over the past decades since the development of conventional activated sludge plant in United Kingdom in 1914. Degradation of complex compounds takes place in several stages, for example, in the case of halogenated compounds, dehalogenation often occurs early in the over all processes. Dehalogenation of many compounds containing chlorine, bromine, or fluorine occurs faster under anaerobic than aerobic conditions. The study of reductive dehalogenation, especially for its commercial application, is gaining importance. Once the anaerobic dehalogenation steps are completed, degradation of the main structure of many xenobiotics often proceeds more rapidly in the presence of O_2. Observations of natural and synthetic organic compound accumulation in natural environment, however, began to raise questions about the ability of microorganisms to degrade these varied substances

and their role in environment protection. The chemical recalcitrance resulted from the inability of microorganisms to degrade some industrially synthesized chemical compounds.

White-rot fungi are unique among eukaryotes in their ability to cleave carbon-carbon bonds in polycyclic aromatic hydrocarbons (PAHs). In fact, biological degradation of these compounds was earlier considered as an exclusively bacterial process (Gibson and Subramanian 1984). The breakdown of most organo-pollutants by ligninolytic fungi is closely linked to ligninolytic metabolism. In this process, degradation is stimulated by nutrient limitation, and it is generally believed that enzymes, whose normal function is lignin degradation, also catalyze the highly non-specific xenobiotic oxidation. The biodegradation of these types of organo-pollutants has been shown to be dependent on the lignin-degrading system of the microorganism. Initial oxidation of several organo-pollutants has been reported to be catalyzed by ligninases isolated from *P. chrysosporium* (Hammel et al. 1992). The lignin-degrading system of this fungus includes a family of lignin peroxidases or ligninases which catalyze the initial oxidative depolymerization of lignin.

Several mechanisms used by the fungi to degrade chemicals have recently been elucidated. The first step in the degradation of many chemicals by white-rot fungi often involves formation of highly reactive free radical intermediates. These free radicals are formed any time; one electron is removed or added to the ground state of a chemical. Such free radicals are very reactive and will rapidly give up or withdraw an electron from another chemical. Free radical reaction often occurs as chains in which many different radicals are generated subsequent to formation of initial radical species and free radical reactions catalyzed by the peroxidases from white-rot fungi also appear to be involved in the degradation of many pollutants. The free radical process also provides some basis for the non-specific nature of white rot fungi.

Idaka et al. (1987) described reductive fission of azo bonds by *Pseudomonas cepacia*, followed by acetylation of the resulting amino benzenes. In a continuation of the study, they also reported an oxidative pathway for degradation of amino compounds which converted these compounds to amino hydroxy compounds. These metabolites are subsequently metabolized through the Kreb's cycle after opening of ring. However, their degradation by *Streptomyces* spp. depends upon the substitution pattern of the aromatic ring. Bacterial cytochrome P-450 is believed to catalyze most of the reactions.

6 Role of Fungal Enzymes in Degradation

Production of laccase by white-rot fungus *Phanerocheate chrysosporium*, *Neurospora crassa* and some other fungi has been extensively studied for removal of pigments and phenol from liquid waste. The production of extracellular enzymes by artificially captured cells of fungi (*Trichosporon cutaneum*, *Candida tropicals* and *Phanerocheate chrysosporium*) is possible. By using gel entrapment and adherence to a matrix, encouraging results can be obtained for the wastewater treatment.

Decolorization of dye is related to the presence of extracellular peroxidases, particularly manganese peroxidases (Gold et al. 1988). However, an evidence, relating degradation of polymeric dyes to phenol-oxidizing enzymes and to lignin degradation, is largely circumstantial. Paszczynski and Crawford (1991) reported involvement of veratryl alcohol during the degradation of some azo compounds by *P. Chrysosporium* ligninase. Veratryl alcohol stimulated azo dye oxidation by ligninase, acting as a third substrate (with H_2O_2 and the azo dye) to cycle the enzyme back to its native state. These studies report aerobic biodegradation of some of the commercial textile dyes by *P. Chrysosporium*. The fungal lignin-degrading system was implicated in the decolorization process, since crude lignin peroxidase was required for the initial step of decolorization of Orange II and Tropaeolin. Paszczynski and Crowford (1991) showed that while ligninase recognized Acid yellow 9 as a substrate, Mn(II) peroxidase was responsible for decolorization of other azo dyes. The extracellular ligninolytic enzyme systems of these fungi have been directly linked to the degradation of these compounds.

The other major group of extracellular oxidative enzymes, involved in the white-rot fungal lignin degradative process, is laccases. The laccases of *T. Versicolour* catalyze the initial oxidation step in the biotransformation of anthracene and benzo [a]pyrene. This process involves either a direct laccase oxidation mechanism or an indirect mechanism involving the participation of an oxidation mediator, such as putative present in the ultrafiltrate fraction. In some experiments with *P. Chrysosporium*, manganese peroxidases were found to play a major role in the initial breakdown and decolorization of high-molecular-weight chlorolignin in bleach plant effluents and also to transform other naturally occurring polymers, such as lignite and sub-bituminous coals. This fungus demonstrated a better ability than the actinomycetes to mineralize the azo dyes (Paszczynski et al. 1992).

The use of ligninolytic enzymes was investigated, but their production yields were too low for industrial applications (Bajpai 1999). Commercial enzymes, such as xylanases, are produced in the large quantities in *Trichoderma reesei* and hence used for pulp bleaching (Bajpai 1999). Application of xylanases was shown to decrease chlorine consumption, which is a environmental-friendly process on one hand and also increases the final brightness of the pulp. Other enzymes, such as laccases, have been studied for their in vivo capacity to degrade lignin (Mayer and Staples 2002). Laccases are commercially available and produced in fungal strains, such as *Aspergillus* sp. (Yever et al. 1991; Berka et al. 1997; Record et al. 2003). *Aspergillus niger* was used to produce the feruloyl esterase for pulp bleaching application (Record et al. 2003). A basidiomycetous fungus *Ganodermaa lucidum* has been found as a suitable organism for removal of Rhodamine-B and Sandolan rhodine. The decolorization of dyes, containing toxic chlorinated phenols used in Kraft bleach dyeing, has been observed by several workers. Bennett et al. (1971) reported that brownish color of the effluent in the textile industry was due to presence of chlorolignin. The ability of microfungi to degrade this component of the effluent has been studied by Cammarota and Santa-Anna (1992).

Adsorption of dyes to the microbial cell surface is the primary mechanism of decolorization (Knapp et al. 1995). Wong and Yu (1999) reported adsorption of

Acid green 27, Acid violet 7 and Indigo carmine dyes on living and dead mycelia of *Trametes versicolour*. Yong and Yu (1997) suggested the binding of dyes to the fungal hyphae, physical adsorption and enzymatic degradation by extracellular and intracellular enzymes as major mechanisms for the color removal. Besides, enzymes, such as lignin peroxidase (LiP), manganese-dependent peroxidase (MnP) and laccase, which are involved in lignin degradation, have been reported to decolorize dyes (Vyas and Molitoris 1995). Dyes with different structures are decolorized at different intrinsic enzymatic rates. Kim et al. (1996) demonstrated that presence of H_2O_2-dependent enzyme activity declourized Remozol Brilliant Blue R in the culture filtrate of *Pleurotus ostreatus* in a chemically defined medium.

Kim and Shoda (1999) have purified and characterized a novel peroxidase (Dyp) which is responsible for the dye-decolorizing activity of *Geotrichum candidum* Dec 1. Nine of the 21 types of dyes were decolorized by Dec 1, and in particular, anthroquinone dyes were effectively decolorized. Swamy and Ramsay (1999) reported that in fungus, *Trametes versicolour*, lignin peroxidase (Lip) was not detected during decolorization of the azo dye of Amaranth, while laccase and manganese peroxidase (MnP) were detected in the decolorizing cultures. A white-rot fungus, *Thelephora* sp. was isolated from the Western Ghats of South India and characterized for its lignolytic enzymes (Selvam 2000). Roushdy and Abdel-Shakour (2011) found that lignin peroxidase produced by *Cunninghamella elegans* was capable for 100 % decolorization of Malachite green dye under static condition, whereas no decolorization was observed in shaking condition. They also showed that Malachite green was degraded and decolorized under ligninolytic conditions. This indicated that ligninolytic enzymes were essential for the degradation of this dye by the fungal strain *Cunninghamella elegans*. Won et al. (2000) also stated that lignin degradation system of the fungi had been directly as well as indirectly linked to the degradation of various compound remains. Other studies reported that Malachite green was enzymatically reduced to leucomalachite green and also converted to N-demethylated and N-oxidized metabolites (Cha et al. 2001).

Traditionally, fungi have been classified as white-, brown-, or soft-rot fungi on the basis of technical decay descriptions (Nilsson 1988), regardless of their taxonomic position. Because the enzyme systems and metabolic pathways involved in the breakdown of carbohydrates and lignins are truly distinct in these fungi, rather than just modified in one or a few specific enzyme activities, decay type is of significant taxonomic importance. One important physiological characteristic of decay fungi in culture is production of extracellular phenoloxidases and peroxidases. Certain fungi produce brown diffusion zones in agar plates supplemented with 0.5 % (w/v) gallic or tannic acid, as a result of oxidation of the respective phenolic acid by extra- or intracellular phenoloxidases. Bavendamm (1928) suggested that the presence of phenoloxidases was correlated with fungi causing white-rot decay and that only these fungi were able to completely decompose lignin. Davidson et al. (1938) extended this method first time to 210 species of wood decaying fungi. Of all tested white-rot fungi, 96 % were positive on gallic acid agar, tannic acid agar, or both. Kaarik (1965) tested 173 wood-decaying species on the plates supplemented with 28 substances and found wide variations in the reactions of these strains to specific phenolic compounds.

7 Mechanism of Bacterial Dye Degradation

It has been demonstrated recently that the rate of anaerobic reduction of azo dyes by *Sphingomonas xenophaga* BN6 could be significantly increased by the addition of different quinines, such as anthraquinone-2-sulfonate or 2-hydroxy-1,4-naphthoquinone. Thus, it was demonstrated that the addition of naphtha quinone and natural organic matter could significantly enhance the reduction rate of nitro aromatic compounds and hexachloroethane in the presence of bulk reductant (e.g. H_2S). Furthermore, it has also been seen that strictly anaerobic Fe (III)-reducing bacteria use the reduction of quinine moieties of humic substances (and also sulfonated anthraquinones) to transfer the reduction equivalents released during the anaerobic oxidation of organic substances. Thus, it becomes evident that in the presence of redox mediators, many heterotrophic aerobic bacteria decolorize azo dyes under anaerobic conditions. Quinones may undergo one-electron reduction processes to the corresponding hydroquinone radicals or two-electron reduction to the corresponding hydroquinones. Therefore, one-electron potentials can be used to compare the relative tendency of different quinines to take up reduction equivalents. The understanding on microbial degradation and decolorization of azo and reactive dyes is still limited and has been studied by only a few workers.

Kulla et al. (1983) employed strains of *Pseudomonas* in chemostat culture for removal of dyes. Some anaerobic bacteria and *Streptomyces* have been characterized for decolorization of chromogenic dyes. A bacterium, *Proteus mirabilis*, isolated from acclimated sludge from a dyeing wastewater treatment plant, rapidly decolorized a deep Red azo dye solution (RED RBN). Features of decolorizing process related to biodegradation and biosorption were also studied. Although *P. mirabilis* displayed good growth in shaking culture, color removal was best in anoxic static cultures and found very effective for color removal under optimum conditions (pH 6.5–7.5 and temperature 30–35 °C). The organism showed a remarkable color removal capability, even at a high concentration of azo dye. More than 95 % of azo dye was reduced within 20 h at a dye concentration of 1.0 g l^{-1}. Decolorization appears to proceed primarily by enzymatic reduction associated with a minor portion (13–17 %) of biosorption to inactivated microbial cells.

8 Main Factors Effecting Dye Degradation

Biodegradation of xenobiotics depends upon the physical, chemical and biological processes which are also governed by some environmental factors. The fungal growth and enzyme production or secretion, and consequent decolorization and degradation are influenced by numerous factors, e.g. media composition, pH value, agitation and aeration, temperature and initial dye concentration. Thus, depending on the culture characteristics, the degradation potential for dyes also varies upon the environmental conditions (Robinson et al. 2001). The structure of dyes strongly

influences their degradability by pure cultures and enzymes produced. Variations regarding dye decolorization could be possible due to the complex chemical structure of dyes, e.g. diazo dyes have more complex structure than mono-azo dyes. With recent findings, it has also been demonstrated that chemical structure of dyes affects their decolorization process (Nozaki et al. 2008). Asgher et al. (2007) evaluated the dye decolorization efficiency of mixed microbial consortia from wastewater treatment plants of different textile units and concluded that the microbial consortium is a robust process for the bioremediation of textile dye effluents. The nature of substituents on the aromatic ring has been shown to influence the enzymatic oxidation. Electron donating methyl and methoxy substituents enhanced the enzymatic degradation of azo phenols, while electron withdrawing chloro, fluoro and nitro substituents inhibited oxidation by a laccase from *Pyricularia oryzae* and MnP from *P. chrysosporium* (Chivukula and Reganathan 1995; Pasti-Grigsby et al. 1992). Some studies have demonstrated that shaking conditions do not support decolorization. The reason for no decolorization at shaking condition might be due to the competition of oxygen and dye for the reduced electron carriers under aerobic condition. Similar studies were carried out by Parshetti et al. (2006) and they reported that Malachite green was completely decolorized under static condition within 5 h by a bacterium *Kocuria rosea* MTCC 1532. The nitrogen concentration in the culture medium also influences the growth of fungi. However, decolorization of some dyes, e.g. Drimaren brilliant blue by fungi was not found dependent on the initial nitrogen concentration (Machado et al. 2006). The fungi *T. villosa* and *P. sanguineus* belong to a group of fungi whose ligninolytic systems are not regulated by nitrogen concentration, and so was the case for *Pleurotus ostreatus* and *Ceriporiopsis subvermispora* also (Leatham and Kirk 1983; Niku-Paavola et al. 1990; Ruttimann-Johnson et al. 1993). In fact, the rates of pollutant degradation are proportional to the concentration of the chemical. The biodegradation of other commercially important classes of textile dyes, have also been addressed. Bakshi et al. (1999) found enhanced biodecoloration of synthetic commercial textile dyes by *P. chrysosporium* by improving Kirk's medium with respect to buffer, C:N ratio, Mg^{2+} and Zn^{2+}, temperature shifts, agitation, and sunflower oil addition.

9 Toxicity of Dyes and Their Effects on Fungi

Serious concern about textile dyes and intermediate compounds was first raised due to its toxicity and carcinogenicity that can cause damage to human health and environment (Banat et al. 1996). This is mainly due to the fact that many dyes are manufactured from known carcinogens, such as benzidine, naphthalene and other aromatic compounds (Nascimento et al. 2011). Many workers have studied the effects of dyes on microorganisms. Among them, Stearn and Stearn (1924), Dion and Lord (1944), Aiquel and Herraro (1948) have shown that basic dyes are toxic to some fungi, e.g. *Fusarium culmorum* and some Aspergilli. Further, similar studies

have also been done by many others to relate the toxicity of dyes with their structures (Mietzsch 1936; Goldacre and Phillips 1949; Fischer 1957). Michaelis and Granick (1945), Michaelis (1947, 1950) attempted to explain the toxicity by showing that basic dyes react irreversibly with nucleic acids. Similar studies have been also carried out by other workers (Neuberg and Roberts 1949; di Marco and Boretti 1950; Steiner and Beers 1958). Basu and Whitaker (1953) found that acid and basic dyes were inhibitory to the isolated cellulase of *Mlyrothecium verrucaria*. Conn (1935) disclosed the use of Crystal violet and malachite green for the preservation of cotton fish nets. Singh et al. (2007) also found an inhibition in the growth of fungus *Trichoderma harzianum* during the degradation of some hazardous dyes. In the same sequence, the toxicity of dyes and certain related compounds was also tested with six species of wood-destroying fungi, because of their economic importance to the wood-preserving industry (Weaver et al. 1959). The arylmethane basic dyes, particularly those containing three phenyl groups with two p-dialkylamino substituents, appear to be effective fungicides when tested on nutrient agar plates against six species of wood-destroying fungi. Brilliant green and Malachite green, both available commercially, are the most toxic dyes of the group, and it was concluded that alkyl substituents on the amino nitrogen atoms increase their toxicity. Malachite green is more toxic than Doebner's violet, while crystal violet is more toxic than para magenta (Weaver et al. 1959).

Most of the dyes are synthetic poly aromatic compounds and are difficult to be degraded in the environment. They are also potential to form carcinogenic breakdown products in the environment (Chung et al. 1992). Several amino-substituted azo dyes, including 4-phenylazoaniline and N-methyl- and N,N-dimethyl-4-phenylazoanilines, are mutagenic as well as carcinogenic (McCann and Ames 1975). The carcinogenicity of an azo dye may be due to the dye itself or to aryl amine derivatives generated during the reductive biotransformation of the azo linkage.

10 Future Prospectives

The work on microbial degradation/decolorization of synthetic dyes and other hazardous compounds or on the purification of wastewaters by using some ideal microbes is still not reached to development of a better alternate or superior technology. Presently, many laboratories in India and from abroad are involved in the same task and in the coming years, there would be a better option to use microbes for biodegradation. The use of microbes including fungi, bacteria, actinomycetes and algae in environmental biotechnology is still under investigation for their proper implementation. One main fact is clear that if any microbe has been shown ability to do work in vitro conditions, it doesn't mean that same work would be done in vivo conditions or vise versa. There is a need for further study on the microbes regarding the role of fungal genes to promote the degradation capacity of any microbe.

11 Conclusion

Degradation of hazardous dyes may be possible with three well known methods; physical, chemical and biological. The biological degradation or biodegradation of synthetic dyes using microbes is safe, economical and eco-friendly. A wide variety of microorganisms including, algae, bacteria, actinomycetes and fungi may be used for biodegradation process. Microbial degradation of hazardous dyes is well known and currently being adopted as a better alternative for degradation. The enzymes produced by a range of microbes, are commercially much important and are also used for the degradation of a large class of pollutants or xenobiotics. Some fungal strains, like *Phanerocheate chrysosporium*, *Aspergillus flavus*, *A. niger*, *Trichoderma harzianum* etc. are well known for the production of laccases, peroxidases and ligninases. These fungal enzymes are used for degradation of a range of organic toxic compounds including synthetic dies.

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Microbe-Mediated Degradation of Synthetic Dyes in Wastewater

Maulin P. Shah

1 Introduction

In many parts of the world, the availability of clean water is today a crucial issue. Moreover, water polluted by the textile industry discharges has received an increased attention for several decades (Robinson et al. 2001). Water pollution mainly arises from the discharge of untreated or poorly treated effluents during dyeing and finishing processes. Wastewater resulting from these processes has adverse impacts on the water quality in terms of total organic carbon (TOC), biological oxygen demand (BOD), chemical oxygen demand (COD), suspended solids, salinity, color, pH (5–12) and presence of recalcitrant synthetic compounds, such as azo dyes (Faryal and Hameed 2005; Savin and Butnaru 2008; Akan et al. 2009; Kuberan et al. 2011). The ratio of BOD/COD ranges from 0.2 to 0.5 which clearly indicates that these effluents contain a large proportion of non-biodegradable organic matter (Yusuff and Sonibare 2004; Savin and Butnaru 2008). Usually, 0.6–0.8 kg NaCl, 30–60 g dyestuff and 70–150 l water are necessary to dye 1 kg of cotton with reactive dyes which releases wastewater containing 20–30 % of the applied unfixed reactive dyes, with an average concentration of 2,000 ppm, high salt content and dyeing auxiliaries (Babu et al. 2007). Worldwide, 280,000 tons of textile dyes are discharged in industrial effluents every year (Jin et al. 2007), which have the adverse effects on the organisms in the environment, including their inhibitory effect on aquatic photosynthesis, ability to deplete dissolved oxygen, and toxicity to flora, fauna and humans. If the dyes are broken down anaerobically, aromatic amines are generated, which are very toxic, carcinogenic and mutagenic.

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Azo dyes are the most widely used dyes and represent over 60 % of the total dyes (Fu and Viraraghavan 2001). They are complex aromatic compounds with significant structural diversity. Their properties provide a high degree of chemical, biological and photocatalytic stability and therefore, resist breakdown over the time, exposure to sunlight, microorganisms, water and soap, in other words. Thus, they are very resistant to degradation process (Savin and Butnaru 2008). Over the years, a number of biological and physicochemical methods have been developed for the removal of industrial azo dyes (Forgacs et al. 2004; Saratale et al. 2011a). As the laws are becoming more and more stringent due to increasing environmental concern a large number of investigations have been recently carried out to find more efficient methodologies for the treatment of wastewater. Suitable microorganisms have been selected for the textile wastewater treatments. This review summarizes the recent achievements in the microbial technologies developed for the removal of azo dyes using simulated effluents and real textile industry effluents. The principal factors that affect dye removal were analyzed, and the microbial decoloration systems based on the use of algae, bacteria, filamentous fungi and yeast, genetically modified strains, microbial consortia, and microbial processes in combination with AOPs and MFCs are discussed in details with particular reference to the analysis of the toxicity of the metabolic products of azo dye decoloration. Concomitant with the in-house multi-dimensional pollution minimization efforts, a number of emerging material recovery/ reuse and end-of-pipe decolorization technologies are being proposed and tested at different stages of commercialization. However, due to their synthetic origin and complex structure deriving from the use of different chromophoric groups, dyes are extremely recalcitrant (Robinson et al. 2001). Along with the recalcitrant nature of dye wastewater, the frequent daily variability of characteristics of such wastewater adds to more difficulty to its treatment (Gürses et al. 2002). Despite the fact that virtually all the known non-biological and biological techniques have been explored for decolorization, (Hao et al. 2000), none has emerged as a panacea. While cost-competitive biological options are not very effective non-biological processes are restricted in scale of operation and pollution profile of the effluent. A list of advantages and disadvantages with different techniques has been reflected in Table 1. It appears that a single, universally applicable end-of-pipe solution is unrealistic, and only a combination of different techniques is required to devise a technically and economically feasible option for dyes wastewater. Besides a wide range of hybrid decolorization techniques have been also tried as depicted in Fig. 1.

2 Decolorization Process

In the following discussion, the biological and non-biological processes for dye decolorization have been discussed with focus on azo dyes, as they represent the largest class of dyes extensively and widely used in industries.

Table 1 Advantages and disadvantages of different dye wastewater treatment techniques

Sl. No.	Process	Advantages	Disadvantages	References
A.	Biological	Cost-competitive option. Direct, disperse and basic dyes have high level of adsorption on to activated sludge	Dyes are generally toxic and very resistant to biodegradation. Acid and reactive dyes are highly water-soluble and have poor adsorption on to sludge	Pagga and Taeger (1994)
B.	Coagulation	Economically feasible; satisfactory removal of disperse, sulphur and vat dyes	Removal is pH dependent; produces large quantity of sludge. May not remove highly soluble dyes; unsatisfactory result with azo, reactive, acid and basic dyes	Fu and Viraraghavan (2001), Hao et al. (2000), Robinson et al. (2001)
C.	Activated C adsorption	Good removal of wide variety of dyes, namely, azo, reactive and acid dyes; especially suitable for basic dye	Removal is pH dependent; unsatisfactory result for disperse, sulfur and vat dyes. Regeneration is expensive and involves adsorbent loss; necessitates costly disposal	Fu and Viraraghavan (2001), Hao et al. (2000), Robinson et al. (2001)
D.	Ion exchange	Adsorbent can be regenerated without loss, dye recovery conceptually possible	Ion exchange resins are dye-specific; regeneration is expensive; large-scale dye recovery cost-prohibitive	Robinson et al. (2001), Slocker and Marechal (1998)
E.	Chemical oxidation	Initiates and accelerates azo-bond cleavage	Thermodynamic and kinetic limitations along with secondary pollution are associated with different oxidants. Not applicable for disperse dyes. Negligible mineralization possible, release of aromatic amines and additional contamination with chlorine (in case of NaOCl) is suspected	Robinson et al. (2001), Slocker and Marechal (1998)

(continued)

Table 1 (continued)

Sl. No.	Process	Advantages	Disadvantages	References
F.	Advanced oxidation processes, AOPs	Generate a large number of highly reactive free radicals and by far surpass the conventional oxidants in decolorization	AOPs in general may produce further undesirable toxic by-products and complete mineralization may not be possible. Presences of radical scavengers reduce efficiency of the processes some of which are pH dependent. Cost-prohibitive at their present stage of development	Robinson et al. (2001), Slocker and Marechal (1998)
1.	UV/O_3	Applied in gaseous state, no alteration of volume. Good removal of almost all types of dyes; especially suitable for reactive dyes. Involves no sludge formation, necessitates short reaction times	Removal is pH dependent (neutral to slightly alkaline); poor removal of disperse dyes. Problematic handling, impose additional loading of water with ozone. Negligible or no COD removal. High cost of generation coupled with very short half-life and gas-liquid mass transfer limitation; suffer	Fu and Viraraghavan (2001), Gogate and Pandit (2004), Hao et al. (2000), Ince et al. (2002), Marachel et al. (1997), Robinson et al. (2001)
2.	UV/H_2O_2	Involves no sludge formation, necessitates short reaction times and reduction of COD to some extent may be possible	Not applicable for all dye types, requires separation of suspended solid and suffers from UV light penetration limitation. Lower pH required to nullify effect of radical scavengers	Gogate and Pandit (2004), Marachel et al. (1997)
3.	Fenton's reagent	Effective decolorization of both soluble and insoluble dyes; applicable	Effective within narrow pH range of < 3.5; and involves sludge	Hao et al. (2000), Marachel et al. (1997), Robinson et al. (2001)

(continued)

Table 1 (continued)

Sl. No.	Process	Advantages	Disadvantages	References
		even with high suspended solid concentration. Simple equipment and easy implementation. Reduction of COD (except with reactive dyes) possible	generation. Comparatively longer reaction time required	
4.	Photocatalysis	No sludge production, considerable reduction of COD, potential of solar light utilization	Light penetration limitation, fouling of catalysts, and problem of fine catalyst separation from the treated liquid (slurry reactors)	Konstantinous and Albanis (2004)
5.	Electrochemical	Effective decolorization of soluble/insoluble dyes; reduction of COD possible. Not affected by presence of salt in wastewater	Sludge production and secondary pollution (from chlorinated organics, heavy metals) are associated with electrocoagulation and indirect oxidation, respectively. Direct anodic oxidation requires further development for industrial acceptance. High cost of electricity is an impediment. Efficiency depends on dye nature	Chen (2004), Robinson et al. (2001)

2.1 Biological Color Removal by Aerobic Microorganisms

2.1.1 Bacteria

Aromatic compounds are susceptible to biological degradation under both aerobic and anaerobic conditions (Field et al. 1995). Under aerobic conditions, the enzymes mono and dioxygenase catalyze the incorporation of oxygen into the aromatic ring of organic compounds prior to ring fission (Madigan et al. 2003). In most mono oxygenases, the direct coupling to O_2 is through a flavin which gets reduced by the

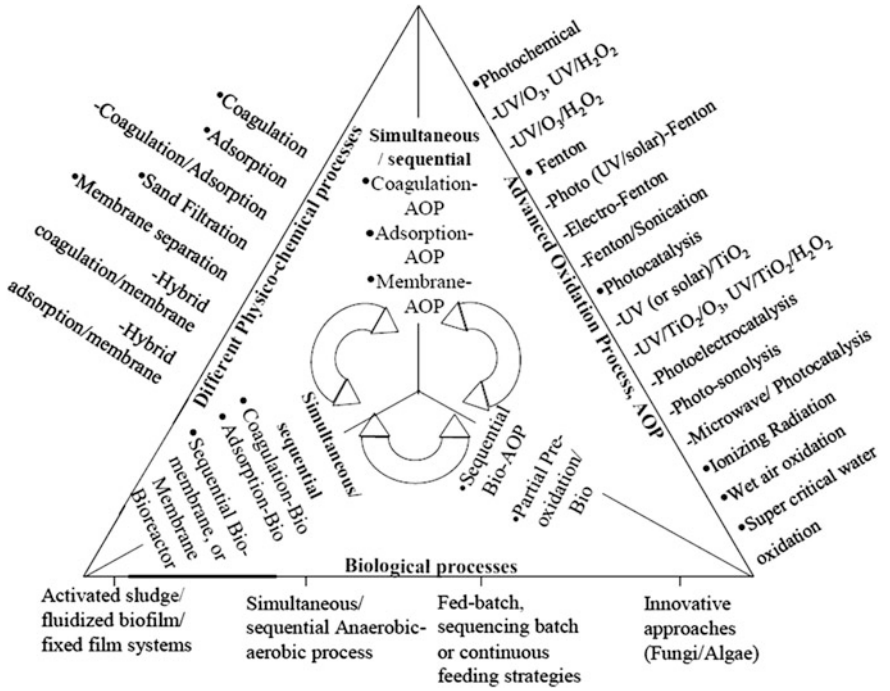

Fig. 1 Simplified representation of broad spectrum of combinations proposed by many workers

NADH or NAD(P)H donor (Madigan et al. 2003). Although azo dyes are aromatic compounds, but their substituents containing mainly nitro and sulfonic groups make them recalcitrant to aerobic bacterial degradation (Claus et al. 2002). This may be related either to the electron-withdrawing nature of the azo bond and their resistance to oxygenases attack, or oxygen is a more effective electron acceptor, as it has more preference for reducing equivalents than the azo dye (Chung et al. 1992; Knackmuss 1996). However, in the presence of specific oxygen-catalyzed enzymes, called azo reductases, some aerobic bacteria are able to reduce azo compounds and produce aromatic amines (Stolz 2001). Presence of aerobic azo reductases was found in *Pseudomonas* species strains K22 and KF46 (Zimmermann et al. 1982, 1984). These enzymes, after purification, characterization and comparison, were shown to be flavin-free. 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 sulfonated structural analogues. Recently, Blumel and Stolz (2003) cloned and characterized the genetic code of the aerobic azo reductase from *Pagmentiphaga kullae* K24. This strain was able to grow with the carboxylated azo compound 1-(4′-carboxyphenylazo)-4-naphtol as a sole source of carbon and energy. Furthermore, the gene encoded a protein with a molecular weight of 20,557 Da, having a conserved putative NAD(P)H-binding site in the amino-terminal region.

2.1.2 Fungi

The capacity of fungi to reduce azo dyes is related to the formation of exoenzymes such as peroxidases and phenol oxidases. Peroxidases are hemoproteins that catalyze reactions in the presence of hydrogen peroxide (Duran et al. 2002). Lignin and manganese peroxidases have a similar reaction mechanism which initiates with the enzyme oxidation by H_2O_2 to an oxidized state during their catalytic cycle. Afterwards, in a mechanism involving two successive electron transfers, substrates like azo dyes reduce the enzyme to its original form (Stolz 2001). Eighteen fungal strains, which were able to degrade lignocellulosic material or lignin derivatives, were tested with the azo dyes, Reactive Orange 96, Reactive Violet 5 and Reactive Black 5. Only strains of *Bjerkandera adusta*, *Trametes versicolor* and *Phanerochaete chrysosporium* were able to decolorize all azo dyes (Heinfling et al. 1997). Although lignin peroxidases are able to oxidize both phenolic and non-phenolic aromatic compounds, manganese peroxidases convert Mn^{2+} to Mn^{3+} in order to oxidize phenolic compounds (Glenn et al. 1986). Phenoloxidases, which can be divided into tyrosinases and laccases, are oxidoreductases that can catalyze the oxidation of phenolic and other aromatic compounds without the use of co-factors (Duran et al. 2002). Laccases are copper-containing enzymes which have a very broad substrate specificity with respect to electron donors, e.g. dyes (Abadulla et al. 2000). However, despite the fact that laccases from *T. versicolor*, *Polyporus pinisitus* and *Myceliophthora thermophila* were found to decolorize anthraquinone and indigoid-based dyes at high rates, the azo dye Direct Red 29 (Congo Red) was a very poor substrate for laccases (Claus et al. 2002). Chivukula and Renganathan (1995) stated that the azo dye must be electron-rich to be susceptible to oxidation by laccase of *Pyricularia oryzae*. This situation is suitable for the generation of a phenoxy radical, with consequent azo bond cleavage, and the release of molecular nitrogen (Fig. 2). However, an addition of redox mediators has been shown to further extend the substrate specificity of laccases to several dye classes. Redox mediators can also be formed from laccase oxidation of phenolic azo dyes (Li et al. 1999; Soares et al. 2001; Claus et al. 2002).

2.2 *Biological Color Removal by Strict Anaerobes or Facultative Microorganisms Under Anaerobic Condition*

Under anaerobic conditions, a low redox potential (< -50 mV) can be achieved, which is necessary for the effective decolorization of dyes (Beydilli et al. 1998; Bromley-Challenor et al. 2000). Color removal under anaerobic conditions is also referred as dye reduction. The azo bond cleavage –N=N– involves a transfer of four-electrons (reducing equivalents), which proceeds through two stages at the azo linkage. At each stage, two electrons are transferred to the azo dye, which acts as a final electron acceptor:

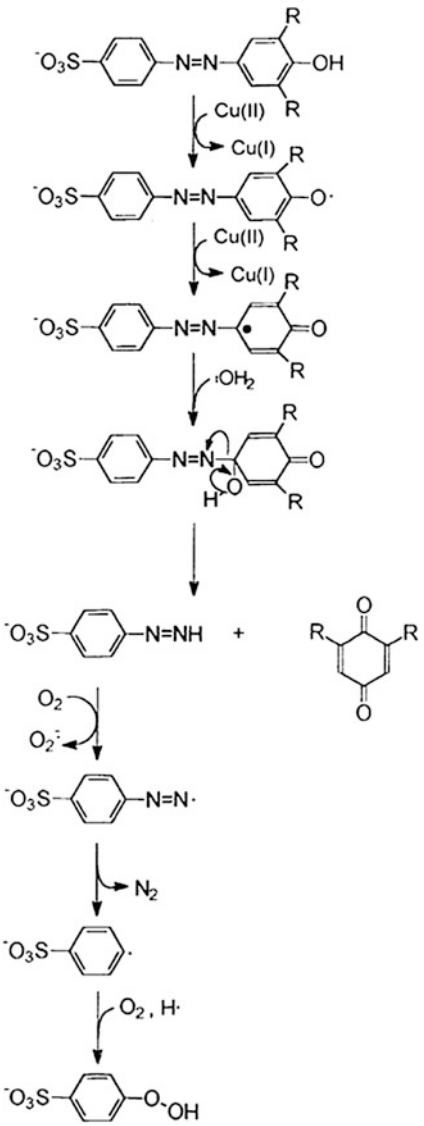

Fig. 2 Proposed pathway for peroxidases-catalyzed degradation of 4-(4'sulfophenylazo)-2,6-dimethylphenol by *Pyricularia aryzae* (Chivukula and Renganathan 1995)

2.2.1 Anaerobic Conditions

$(R_1–N=N=R_2) + 2e^- + 2H^+ \rightarrow R_1–NH–NH–R_2$ (Hydrazo intermediate)

$(R_1–HN–NH–R_2) + 2e^- + 2H^+ \rightarrow R_1–NH_2 + R_2–NH_2$ (Reductive cleavage of the azo bond)

The exact mechanism of azo dye reduction, whether occurring intracellularly or extracellularly, is still a subject of investigation, as it is the role of biogenic intracellular water-soluble electron carriers, such as flavins. Reduced flavins can act

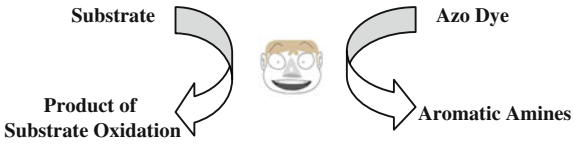

Fig. 3 Schematic for direct enzymatic azo dye reduction

as an electron shuttle from NADPH-dependent flavoproteins to azo dye as electron acceptor (Gingell and Walker 1971). Intracellular azo dye reduction cannot be responsible for the conversion of all types of azo dyes, especially for sulfonated azo dyes, which have limited membrane permeability (Stolz 2001). Kudlich et al. (1997) demonstrated an increase on color removal rates of sulfonated azo dyes by cell free-extracts, as well as after addition of toluene, i.e. a membrane-active compound which increases cell lysis, thus showing the limited membrane permeability of this type of dye. The current hypothesis is that azo dye reduction mostly occurs by extracellular or membrane-bound enzymes (Stolz 2001). Reduced cytoplasmic co-factors, such as reduced flavins, do not contribute to the chemical dye reduction due to their inability to cross living cell membranes (Russ et al. 2000). However, cell fractionation experiments demonstrated that a quinone reductase activity, located in the cell membranes, enhanced the reductive decolorization of a sulfonated azo compound. In such case, no dye cross-membrane transport was required (Kudlich et al. 1997). Recently, a NADH-dependent lawsone reductase activity, located in the cytosolic fraction of *Escherichia coli* also showed the capacity for azo dye reduction (Rau and Stolz 2003).

2.2.2 Biological and Chemical Reductive Decolorization

The reductive decolorization of azo dyes under anaerobic conditions is a combination of both biological and chemical mechanisms. The biological contribution can be attributed to specialized enzymes, called azo reductases, which are present in bacteria that are able to grow using only azo dye as a carbon and energy source. However, there is no clear evidence till date for anaerobic azo reductase; or non-specific enzymes that catalyze the reduction of a wide range of electron-withdrawing contaminants, including azo dyes (Stolz 2001). Thus, a co-metabolic reaction is probably the main mechanism of dye reduction (Fig. 3), in which the reducing equivalents or reduced co-factors like NADH, NAD(P)H, $FMNH_2$ and $FADH_2$ act as secondary electron donor and channel electrons to cleave the azo bond (Gingell and Walker 1971). The chemical contribution to the reductive decolorization of azo dyes under anaerobic conditions is linked to biogenic reductants like sulphide, cysteine, ascorbate or Fe^{2+} (Yoo 2002; Van der Zee et al. 2003). Among these sulphides can be formed by sulphate reduction in anaerobic bioreactors. Therefore, there will be a competition between sulphate and dye to become the terminal electron acceptor of the reducing equivalents. Van der Zee

et al. (2003) observed that different sulphate concentrations did not have an adverse effect on the reduction of RR2 in either batch assays or reactor experiments. Hence, they concluded that sulphate, even present at concentrations up to 60 mM, did not obstruct the transfer of electron to the azo dye. However, any noticeable effect of sulphate was verified on the dye removal effciency in the lab experiment. In another investigation on color removal, Albuquerque et al. (2005) used an anaerobic-aerobic sequencing batch reactor fed with sulphate (0.35 mM). The results indicated that the decolorization capacity was not improved while testing the dye Acid Orange 7, even though a sulphate reducing microbial population was used. Therefore, for a real perspective application, sulphide generated by sulphate reduction has no role to play and therefore, the color removal is mainly due to biological processes. Color removal by anaerobic granular sludge under mesophilic conditions has been reflected in Table 2. It is very difficult to compare the efficacy of different method because of the differences in type and concentration of dyes, sludge sources and concentrations, electron donor, the way of calculating the decolorization rates etc.

As evident from Fig. 4 that the electron flow preference in the presence of different redox couples involves biological processes. Thus, oxygen is a more effective electron acceptor than azo dyes, which justifies the low decolorization rates (10–30 %) under aerobic conditions.

2.2.3 Reductive Decolorization of Azo Dyes in the Presence of Redox Mediators

Redox mediators are compounds that accelerate the electron transfer from a primary electron donor to a terminal electron acceptor, which may increase the reaction rates by one to several orders of magnitude (Cervantes 2002; Dos Santos 2005). Redox mediators have shown to be effective not only for reductive decolorization, but also for the reductive transformation of many contaminants such as iron (Lovley et al. 1998), nitroaromatics (Dunnivant et al. 1992), polyhalogenated compounds (O'Loughlin et al. 1999) and radionuclides (Fredrickson et al. 2000). Recently, it was found that during the aerobic degradation of naphthalene-2-sulfonate (2NS) by *Sphingomonas xenophaga* strain BN6, quinoid redox mediators were produced, which mediated the reduction of azo dye under anaerobic conditions (Keck et al. 2002). Flavin-based compounds, like FAD, FMN and riboflavin, as well as quinone-based compounds, like AQS, AQDS and lawsone, have been extensively reported as redox mediators during azo dye reduction (Semde et al. 1998; Cervantes 2002; Rau et al. 2002a; Field and Brady 2003; Dos Santos et al. 2004a; Dos Santos 2005; Encinas-Yocupicio et al. 2006). As reflected in Fig. 5, reductive decolorization of azo dyes in the presence of redox mediators occurs in two distinct steps, the first step being a non-specific enzymatic mediator reduction, and the second step being a chemical re-oxidation of the mediator by the azo dyes (Keck et al. 1997).

Theoretically, feasible redox mediators for biological azo dye reduction must have redox potentials between the half reactions of the azo dye and the primary

Table 2 Color removal by anaerobic granular sludge under mesophilic conditions

Type	Names of dyes	Decoloration efficiency (%)	Decoloration rates	Concentrations	Reference
Anthraquinone	Reactive blue 19	70	–	About 50 mg l^{-1} of dye	Abadulla et al. (2000)
Anthraquinone	Reactive blue 4	73	4.3 mg l^{-1} h^{-1}	About 50 mg l^{-1} (0.08 mM) of dye	Albuquerque et al. (2005)
Anthraquinone	Reactive blue 19	90	13.0 mg l^{-1} h^{-1}	About 50 mg l^{-1} (0.08 mM) of dye	Albuquerque et al. (2005)
Anthraquinone	Disperse red 159	0	0		Beydilli et al. (1998)
Anthraquinone	Reactive Blue 4	>84	–	About 300 mg l^{-1} of dye	Blumel and Stolz (2003)
Anthraquinone	Reactive blue 19	>84	–	About 300 mg l^{-1} of dye	Blumel and Stolz (2003)
Azo dye	Direct blue 53	99	0.24 mg day^{-1}	About 0.3 mM of dye	Bragger et al. (1997)
Azo dye	Direct blue71	100	0.61 mg day^{-1}	About 0.3 mM of dye	Bragger et al. (1997)
Azo dye	Direct red 79	97	16.60 mg day^{-1}	About 0.3 mM of dye	Bragger et al. (1997)
Azo dye	Direct red 81	99	7.80 mg day^{-1}	About 0.3 mM of dye	Bragger et al. (1997)
Azo dye	Reactive red 235	100	4.42 mg day^{-1}	About 50 mg l^{-1}	Bromley-Challenor et al. (2000)
Azo dye	Reactive blue 235	100	23.5 mg day^{-1}	About 50 mg l^{-1}	Bromley-Challenor et al. (2000)
Azo dye	Reactive yellow 168	100	23.4 mg day^{-1}	About 50 mg l^{-1}	Bromley-Challenor et al. (2000)
Azo dye	Reactive red 198	95	11.6 mg l^{-1} h^{-1}	About 300 mg l^{-1} of dye	Brown (1981)
Azo dye	Mordant blue 13	83	–	About 50 mg l^{-1} of dye	Abadulla et al. (2000)
Azo dye	Mordant black 9	77	–	About 50 mg l^{-1} of dye	Abadulla et al. (2000)
Azo dye	Basic red 18	92	–	About 50 mg l^{-1} of dye	Abadulla et al. (2000)

(continued)

Table 2 (continued)

Type	Names of dyes	Decoloration efficiency (%)	Decoloration rates	Concentrations	Reference
Azo Dye	Acid yellow 151	88	–	About 50 mg l^{-1} of dye	Abadulla et al. (2000)
Methine	Basic yellow 22	35	–	About 50 mg l^{-1} of dye	Abadulla et al. (2000)
Nitro	Acid orange 3	62	–	About 50 mg l^{-1} of dye	Abadulla et al. (2000)
Oxazine	Basic blue 3	62	–	About 50 mg l^{-1} of dye	Abadulla et al. (2000)
Phthalocyanine	Reactive blue 21	80	8.6 mg l^{-1} h^{-1}	About 300 mg l^{-1} of dye	Brown (1981)
Phthalocyanine	Reactive blue 21	36	–	About 50 mg l^{-1} of dye	Abadulla et al. (2000)
Phthalocyanine	Reactive Blue 7	49-66		About 300 mg l^{-1} of dye	Blumel and Stolz (2003)
Phthalocyanine	Reactive blue 21	49-66		About 300 mg l^{-1} of dye	Blumel and Stolz (2003)

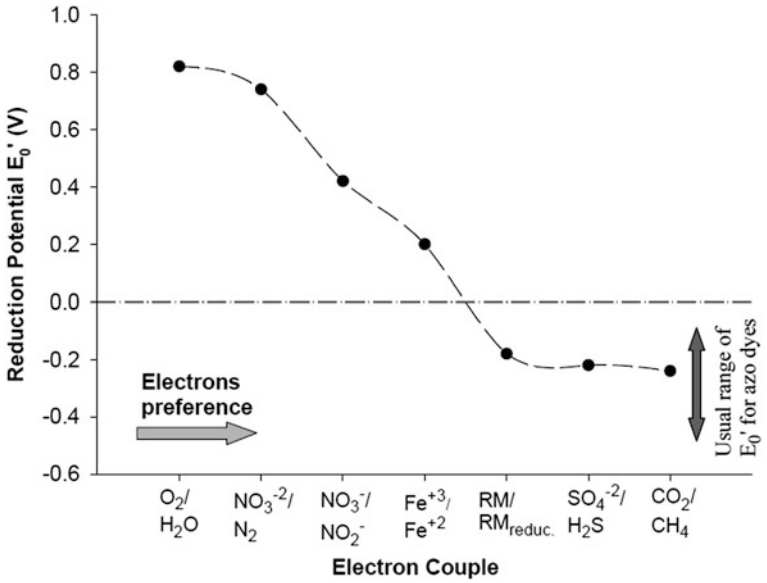

Fig. 4 Electron flow preference as a function of the different electron couples (Dubin and Wright 1975; Cervantes 2002; Madigan et al. 2003; Dos Santos 2005). RM and RM_{reduc} are the oxidized and the reduced forms of the redox mediator, respectively

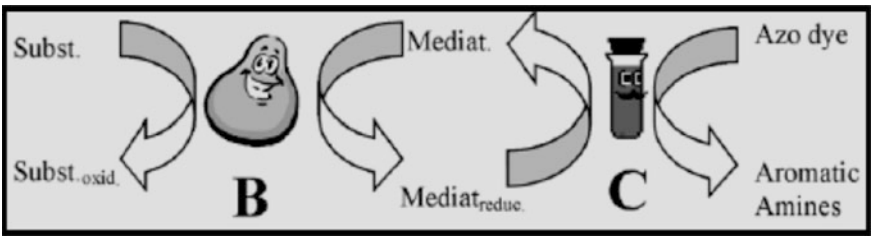

Fig. 5 Schematic for indirect azo dye reduction. B and C are the biological and chemical steps, respectively

electron donor (van der Zee et al. 2003). Unfortunately, the standard redox potential (E'_0) for most of azo dyes is unknown, however, this information can be obtained by using polarography. In a screening of redox potential values for different azo dyes, it was found that E0 values are generally between -0.430 and -0.180 V (Dubin and Wright 1975). Rau et al. (2002a, b) stated that the NAD(P)H co-factor, which had the lowest E'_0 value of −0.320 V, set the limits of redox mediators application. The reason for this is that mediators with a more negative E0 value will not be reduced by the cells, and mediators with $E'0$ greater than −0.05 V will not

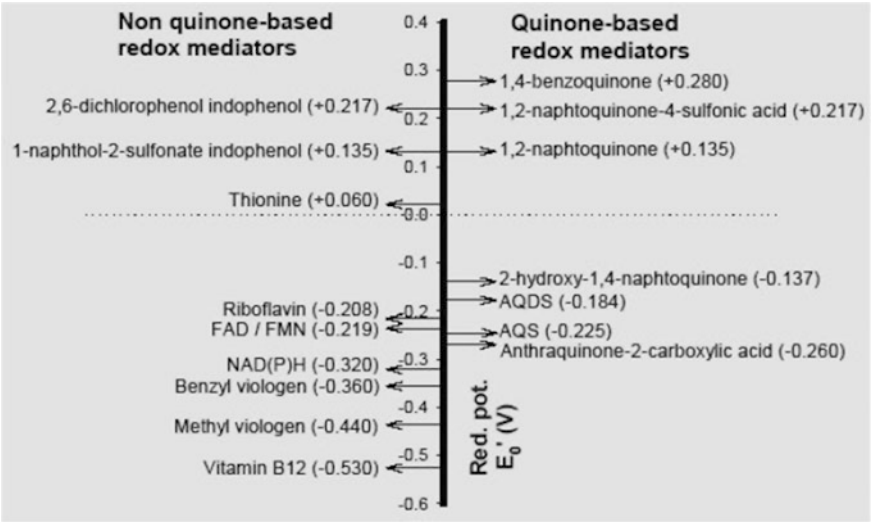

Fig. 6 E0' values for both quinone-based and non quinone-based redox mediators (Rau et al. 2002a)

efficiently reduce the azo bond at high rates. Figure 6 shows the E'_0 values for both quinone-based and non-quinone-based redox mediators. The standard redox potential value (E'_0) is a good indication of a compound capacity to function as a redox mediator. However, other factors are also of importance as well, since different decolorization rates in the presence of mediators with similar E'_0 values have been reported, and similar decolorization rates with mediators with different E'_0 values (Dos Santos et al. 2004a). For instance, Brown (1981) tested the polymeric nitro dye Poly Y-607 and found that methyl viologen and benzyl viologen increased the decolorization rates by 4.5-fold, even though the E'_0 of methyl viologen (−0.440 V) is much lower than that of benzyl viologen, i.e. −0.360 V (Fig. 6). Walker and Ryan (1971) postulated that decolorization rates were related to the electron density in the azo bond region. They suggested that color removal rates would increase by lowering the electron density in the azo linkage. Therefore, the use of redox mediators would not only tend to accelerate the transfer of reducing equivalents to the terminal electron acceptor, i.e. the azo dye, but also to minimize the steric hindrance of the dye molecule (Bragger et al. 1997; Moir et al. 2001) and to decrease the activation energy of the chemical reaction (Dos Santos 2005). Thus, in evaluation of theoretical decolorization rates by using specific redox mediators, differences in electrochemical factors between mediator and azo dye should also be considered.

2.2.4 Microbiological Aspects of the Reductive Decolorization of Azo Dyes

Pure Cultures

As reflected in Table 3, there are extensive reports for use of pure cultures, either whole cells or specific enzymes, for a better insight of the anaerobic azo dye reduction mechanisms, which are not fully understood yet (Stolz 2001; Dos Santos et al. 2004a,b). Microbial decolorization requires an unspecific enzymatic capacity ubiquitously found in a wide diversity of microorganisms (Chung and Stevens 1993). This has been mainly demonstrated with microorganisms present in the intestine, such as, *Clostridium, Salmonella, Bacillus, Eubacterium* and *Escherichia coli*, which are able to reduce the dyes ingested through food, drugs and cosmetics (Rau et al. 2002b; Chen et al. 2004). The understanding of azo dye reduction mechanisms is important not only for a biotechnological approach for decolorization, but also for a medical approach to have an insight into how the intestinal microflora metabolites ingested azo dyes (Semde et al. 1998). Azo dyes are converted into aromatic amines in the presence of microflora and the anaerobic condition in the human intestine. Aromatic amines are more mutagenic and carcinogenic than their precursor, azo dyes (Weisburger 2002). Therefore, a lot of effort has been made in the production of compounds, which are resistant to reductive transformations. Another approach has been looked into use of azo polymers that would be insoluble in the upper gastrointes-reduction.

Granular Sludge

Even though anaerobic azo dye reduction could be readily achieved with different microorganisms, there is no strain reported so far that is able to decolorize a broad range of azo dyes. Therefore, the use of a specific strain or enzymes for reductive decolorization does not make much sense in treating textile wastewater, which containing many kinds of dyes (Laszlo 2000). The use of mixed cultures, such as anaerobic granular sludge, which is composed of stable microbial pellets with a high activity, is probably a more logical alternative. However, a little is known about the microbiological aspects of the reductive decolorization of azo dyes with anaerobic consortia, commonly found in wastewater treatment plants, although the applicability of the cost-effective high-rate anaerobic reactors for azo dye reduction has been well demonstrated (Cervantes et al. 2001; Dos Santos et al. 2004b; Dos Santos 2005). As previously explained, the reductive decolorization of azo dyes by using methanogenic anaerobic granular sludge is likely to be controlled by a co-metabolic reaction in the presence of different electron donors, in which the azo dye is the terminal electron acceptor of the reduced co-factors (Dos Santos 2005). Thus, the reducing equivalents are formed during the conversion of the primary electron donor, i.e. the organic matter, during the different steps of carbon flow under

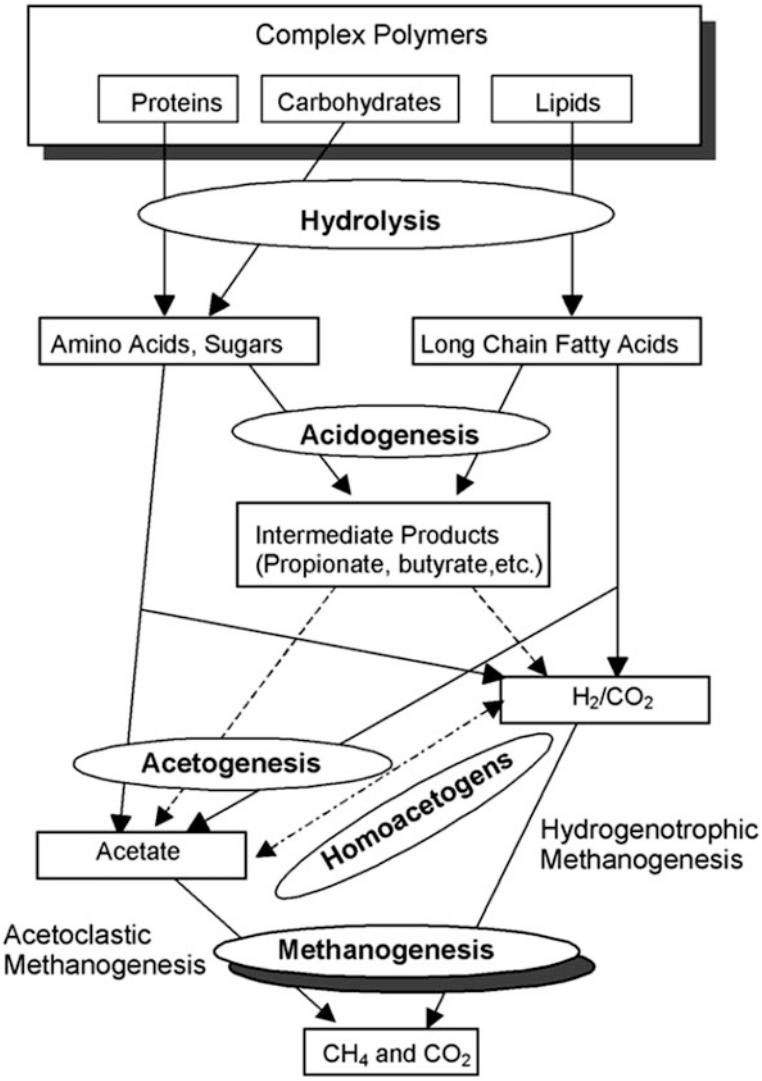

Fig. 7 Conversion of complex organic matter in methanogenic anaerobic reactors (Gujer and Zehnder 1983)

anaerobic conditions (Fig. 7). Dos Santos et al. (2004a) observed that the anthraquinone dye RB19 was mainly toxic to the acetate utilizing methanogens, whereas acidogens were not affected by its toxicity.

2.3 Non-biological Color Removal

While advanced oxidation processes (AOPs) have been studied extensively both for recalcitrant wastewater in general and dye wastewater in particular, their commercialization has yet not been realized because of certain barriers (Gogate and Pandit 2004). These processes are costly and complex also at the present level of their development (Papic 2004). Additional impediment exists in the treatment of dye wastewater with higher concentration of dyes, as AOPs are only effective for wastewater with very low concentrations of organic dyes. Thus, significant dilution is necessary for the wastewater treatment. For the AOPs, the basic reaction mechanism is the generation of free radicals and their subsequent attack on the organic pollutant species. Hence, it is strongly believed that their combination will result in more free radicals, thereby increasing the rates of reactions (Gogate and Pandit 2004). Moreover, some of the drawbacks of the individual AOPs may be overcome by the characteristics of other AOPs. The cost/energy efficiency, however, will be dependent on the operating conditions and the type of the wastewater.

2.3.1 Different Photochemical Processes

The photo-activated chemical reactions are characterized by a free radical mechanism initiated by the interaction of photons of a proper energy level with the chemical species present in the solution. Generation of radicals through UV radiation by the homogenous photochemical degradation of oxidizing compounds like hydrogen peroxide (Aleboyeh et al. 2003), ozone (Chen et al. 2004) or Fenton's reagent (Neamtu et al. 2002) has been frequently reported to be superior to sole UV radiation or sole utilization of such oxidants. The photocatalytic process can be carried out by simply using slurry of the fine catalyst particles dispersed in the liquid phase in a reactor or by using supported/immobilized catalysts. Fenton reagent (a mixture of H_2O_2 and Fe^{2+}) and its modifications, such as thermal Fenton process (Suty et al. 2004) or photo-Fenton reaction using Fe(II)/Fe(III) oxalate ion, H_2O_2 and UV light, have received a great attention as means for decolorization of synthetic dyes (Shah et al. 2003; Swaminathan et al. 2003). In the case of photo-Fenton technique, H_2O_2 is utilized more rapidly by three simultaneous reactions, namely direct Fenton action, photo-reduction of Fe (III) ions to Fe (II) and H_2O_2 photolysis. Thus, this process produces more hydroxyl radicals in comparison to the conventional Fenton method or the photolysis (Bandara et al. 1996; Gogate and Pandit 2004). Among the AOPs, the photo-Fenton reaction (Torrades et al. 2004) and TiO_2-mediated heterogeneous photocatalytic treatment (Chen et al. 2002) processes are capable of absorbing near-UV spectral region to initiate radical reactions. Their application would practically eliminate major operating costs when solar radiation is employed instead of artificial UV light. The ferrioxalate solution, which has long been used as chemical actinometer, may be used in photo-Fenton process to derive further benefit by replacing UV with solar radiation (Arslan et al.

Table 3 Facultative and strictly anaerobic bacteria cultures, which are able to decolorize azo dyes under anaerobic conditions

Organism	Dyes	Activity	Decoloration	Comments	References
Clostridium perfringens ATCC 3626	Amaranth Methyl orange Orange II Tartrazine	µmol ml^{-1} h^{-1} 0.74 0.62 0.70 0.67	– – – –	Dye concentration of 0.033 mM	Abadulla et al. (2000)
Pseudomonas GM3	Acid violet 7 Reactive blue 2 Acid green 27 Acid red 183 Indigo Carmine	– – – – –	97.4 18.3 75.6 20.1 69.0	After 72 h of incubation. Dye concentration of 100 mg l^{-1}	Albuquerque et al. (2005)
Enterococcus faecalis	Methyl red Orange II OrangeG Amaranth	AU × 10^{-2}/mg protein 1.81 1.39 1.20 1.37	99.84 95.1 64.1 99.5	After 20 h of incubation. Dye concentration of 0.2 mM	APHA (1998)
Eubacterium biforme	Tartrazine Sunset Yellow Methyl Orange Orange II Amaranth Allura red 40	– – – – – –	4.0 22.0 79.0 81.0 19.0 11.0	After 150 min of incubation. Dye concentration of 2 mM	Baughman and Weber (1994)

2000). Recently, several attempts have been made to increase the photocatalytic efficiency of TiO_2 which include noble metal deposition, ion doping, addition of inorganic co-adsorbent, coupling of catalysts, use of nanoporous films and so on. Apart from that, new catalysts, such as polymeric metalloporphyrins, have been reported to be easily excited by violet or visible light, whereas utilization of solar energy by commonly used TiO_2 is only about 3 % (Chen et al. 2004).

2.3.2 Photochemical/Electrochemical

In electrochemical treatments, oxidation is achieved by means of electrodes where a determined difference of potential is applied. On this principle, several different processes have been developed as cathodic and anodic processes-direct and indirect electrochemical oxidation, electrocoagulation, electrodialysis, electromembrane processes and electrochemical ion exchange (Chen 2004). Occasionally, a combination of electrochemical technology and photocatalysis has been adopted to yield some unique advantages. For instance, chemical synergism of photocatalysis and electrochemical processes may yield enhanced decoloration and COD removal (An et al. 2002) and added advantage may be derived from existence of salt in solution, which is detrimental for sole photocatalysis (Zhang et al. 2003). Conversely, electro-Fenton process requires no addition of chemical other than catalytic quantity of Fe^{+2}, since H_2O_2 is produced in situ, thereby avoiding transport of this hazardous oxidant (Guivarch et al. 2003; Neyens and Baeyens 2003).

2.4 Combination: AOPs and Other Physico-Chemical Processes

Many studies have focused on different combinations among physicochemical systems for treatment of textile and dye wastewaters. Combinations of conventional physicochemical techniques with the AOPs have been an attractive option.

2.4.1 Coagulation Based Combinations

Coagulation/flocculation/precipitation processes have been used intensively for decolorizing wastewater. For the pretreatment of raw wastewater before discharging to publicly owned treatment plants, these processes may be satisfactory with respect to COD reduction, and partial decolorization. Their alone application in treating textile/dye waste is, however, found to be relatively ineffective (Hao et al. 2000; Papic et al. 2004). For example, only 50 % removal was achieved using either alum or ferrous sulfate for an azo reactive yellow dye (Hao et al. 2000). In the coagulation process, it is difficult to remove highly water-soluble dyes, and even more

important, the process produces a large quantity of sludge (Robinson et al. 2001). Coagulation followed by adsorption was reported to produce effluent of reuse standard, apart from cutting down the coagulant consumption by 50 %. Hence, it lowers the volume of sludge formed, in comparison to coagulation process done (Papic et al. 2004). Coagulation in combination with advanced oxidation processes, either in sequential or in concurrent manner, has been reported for dye wastewater. Investigation on sequential use of coagulation and ozonation revealed the superiority of the scheme involving ozonation preceded by coagulation over the reversed scheme (Tzitzi et al. 1994). Multi-stage application of coagulation, followed by ozonation, was proved to be superior to their single pass sequential application (total ozonation time the same) (Hsu et al. 1998). The advantage of the multistage application was more convincing in case of wastewater with high recalcitrant composition.

2.4.2 Adsorption Based Combinations

Adsorption techniques, specially the excellent adsorption properties of carbon-based supports, have been utilized for the decolorization of dyes in the industrial effluents (Forgacs et al. 2004). Activated carbon, either in powder or granular form, is the most widely used as adsorbent for this purpose due to its extended surface area, micro porous structure, high adsorption capacity and high degree of surface reactivity (Malik 2003). It is very effective for adsorbing cationic, mordant, and acid dyes and to a slightly lesser extent for dispersed, direct, vat, pigment and reactive dyes (Robinson et al. 2001). However, the use of carbon adsorption for decolorization of the raw wastewater is impractical because of competition between colored molecules and other organic/ inorganic compounds. Hence, its use has been recommended as a polishing step or as an emergency unit at the end of treatment to meet the discharge color standards (Hao et al. 2000). There has been considerable interest in using low-cost adsorbents for decolorization of wastewater. These materials include chitosan, zeolite, clay; certain waste products from industrial operations, such as fly ash, coal, oxides; agricultural wastes and lignocellulosic wastes and so on (Naim and Abd 2002; Babel and Kurniwan 2003). Adsorption concurrent with ozonation (Lin and Lai 2000), UV-H_2O_2 (Ince et al. 2002) or microwave induced oxidation (Lin and Lai 2000) has been reported to yield mutual enhancements like catalysis of AOP by adsorbent and simultaneous regeneration of adsorbent. A rather elaborate method, involving solvent extraction and catalytic oxidation, has been documented in the literature (Hu et al. 2005; Muthuraman and Palanivelu 2005). The method consists of dye extraction, using an economical solvent followed by dye recovery through chemical stripping. In this way, the solvent is also regenerated. Finally, treatment of the extraction raffinate can be achieved by the catalytic oxidation.

3 Factors Controlling Microbial Decolorization Process

Microorganisms are sensitive to the presence of chemical substances, such as dyes, high salinity, variations in pH and high content of organic compounds (Dua et al. 2002; Ang et al. 2005; Megharaj et al. 2011). For bioremediation processes, the most useful microorganisms are those isolated from textile industry-contaminated environments, including soil, effluents and sludge from wastewater treatment plants, as they are adapted to grow in the extreme conditions (Yang et al. 2009; Ola et al. 2010; Ayed et al. 2011). The biodecoloration process is dependent on the following factors: the azo dye structure, carbon and nitrogen sources, salinity, pH, temperature, dye concentration and the presence or absence of oxygen.

3.1 Effects of the Azo Dye Structure

It has been observed that the enzymatic degradation of the dye is highly influenced by its structure (Pasti-Grigsby et al. 1992; Hsueh et al. 2009; Kuberan et al. 2011; Rajee and Patterson 2011). For example, azo dyes with electron-withdrawing groups, such as SO_3^-, are easier to degrade than those with electron-releasing groups, such as –NH-triazine. Therefore, azo dyes, that contain more electron withdrawing groups, show faster decoloration, and if these groups are in the para or ortho positions to the azo bond, the degradation proceeds faster than if they are in the meta position, because they provide a more effective resonance effect, causing azo dyes to be highly electrophilic and resulting in faster reductive decoloration (Pricelius et al. 2007; Tauber et al. 2008; Hseuh et al. 2009). Recent studies have revealed that the enzymatic activity is induced by the presence of dyes in such a way that this activity is significantly higher at the end of the decoloration process. For example, Reactive Black 5 induces MnP activity in *Debaryomyces polymorphus* (Yang et al. 2005) and *Trichosporon akiyoshidainum* and *Lac* activity in *Trametes versicolor* (Fernandez et al. 2009); azoreductase activity is improved in *Chlorella vulgaris* by the addition of G-Red, in *Nostoc linckia* by Methyl Red (El-Sheekh et al. 2009) and in *Scenedesmus bijugatus* by *Tartrazine* and *Ponceau* (Omar 2008); Reactive Blue 221 induces *Tyr* in *T. akiyoshidainum* (Pajot et al. 2011); Lac activity is enhanced in *Pleurotus sajorcaju* in the presence of Acid Blue 80, Acid Green 28 and Reactive Red 198 (Munari et al. 2008) and in *Galactomyces geotrichum* by a mixture of Remazol Red, Golden Yellow HER, Rubine GFL, Scarlet RR, Methyl Red, Brown 3 REL and Brilliant Blue (Waghmode et al. 2011). In the process of azo dye biotransformation, different types of enzymes, both oxidases and reductases, can be involved. During the decoloration of Reactive Orange 16 by *Irpex lacteus*, significant activity of Lac and MnP was found with the formation of three metabolites, but no polymerization products were detected by

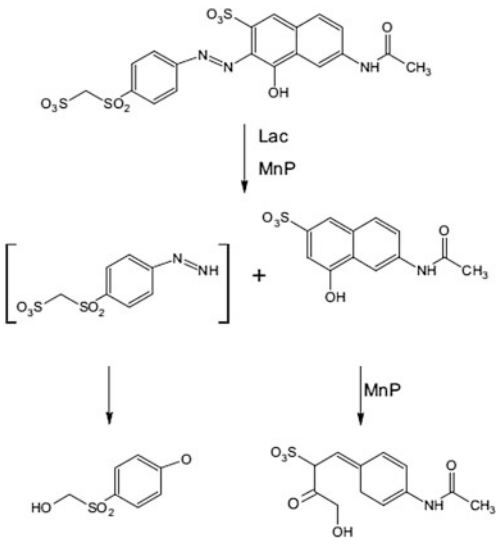

Fig. 8 Mechanism of reactive orange 16 degradation by the white rot fungus *I. lacteus,* the compound in bracket was not detected (Svobodova et al. 2007)

LC-MS analyses. The proposed degradation pathway has been elucidated in Fig. 8. However, other fungal Lac enzymes can cause polymerization. Different enzymes can be induced in a certain microorganism depending on the structure of the azo dyes (Fig. 9). In the presence of GYHER, an induction of *Lac* was observed in *Galactomyces geotricum* (Waghmode et al. 2011); whereas in the presence of Remazol Red, an induction of azoreductase and Lac was demonstrated. However, the proposed mechanisms for these dyes do differ. The degradation of GYHER starts with the asymmetric cleavage of the azo bond, which is catalysed by *Lac*, whereas the degradation of Remazol Red begins with the symmetric cleavage of the azo linkage, which is catalyzed by an azoreductase, followed by an asymmetric oxidative cleavage (Waghmode et al. 2011, 2012). The degradation of Remazol Red, Rubine and GYHER by *Brevibacillus laterosporus* is also dependent on the structure of the dye (Fig. 10). A significant increase was observed in the activity of azoreductase, NADH-DCIP reductase, veratryl alcohol oxidase and tyrosinase in cells obtained after the decoloration of Remazol Red and GYHER. However, the suggested degradation mechanisms for these two colorants are different. Furthermore, while NADH-DCIP reductase and riboflavin reductase were induced, a complete inhibition of azoreductase was observed during the decoloration of Rubine (Kurade et al. 2011; Waghmode et al. 2011). The use of microorganisms, that excrete various oxidases and reductases, can completely degrade azo dyes and detoxify contaminated water from the textile industry.

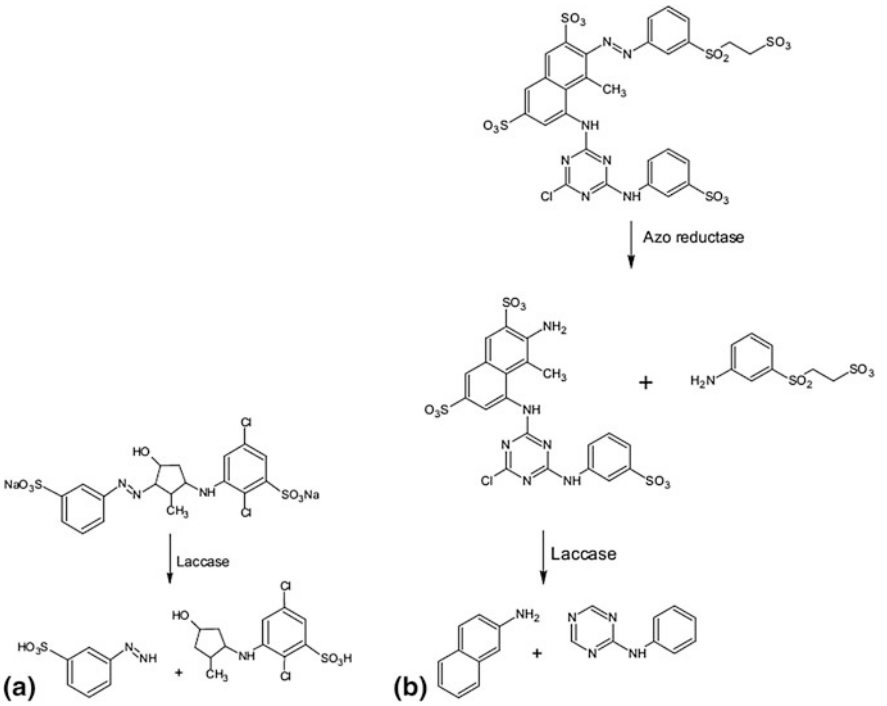

Fig. 9 Proposed mechanism for the degradation of **a** GYHER (adapted from Waghmode) and **b** remazol red (Waghmode et al. 2011, 2012) by *Galactomyces geotricum*

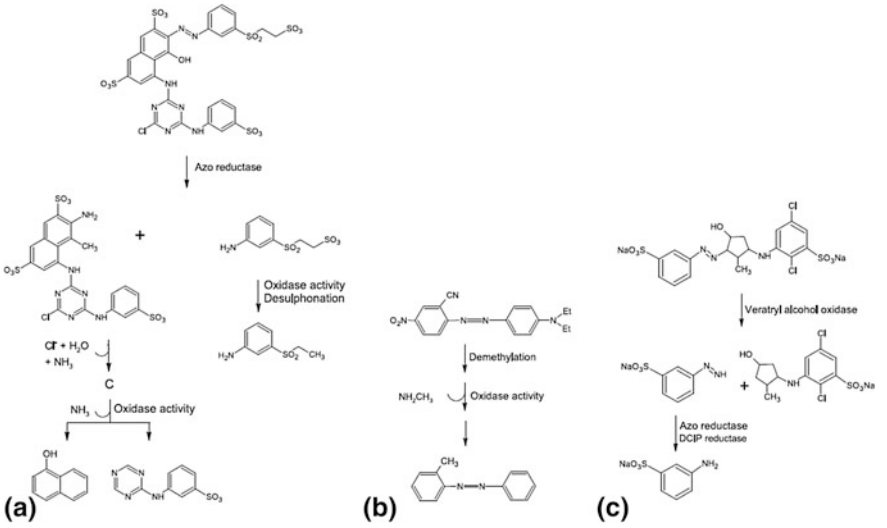

Fig. 10 Mechanism for the degradation of **a** remazol red, **b** rubine (adapted from Kurade) and **c** GYHER by *Bravibacillus laterosporous* (Waghmode et al. 2011, 2012)

3.2 Influence of Carbon and Nitrogen Sources in the Decoloration Process

Carbon and nitrogen sources have an important influence on the extent of decoloration using microorganisms. Different microbial metabolic characteristics cause to differential uptake of C and N sources, thus affecting azo dye decoloration. As dyes are deficient in carbon, biodegradation without an extra carbon source is very difficult (Asgher et al. 2009; Khelifi et al. 2009; Tony et al. 2009; Levin et al. 2010). Carbon sources serve two purposes: as a source of carbon and energy for the growth and survival of the microorganisms and also as an electron donor, which is necessary for the breakage of the azo bond (Perumal et al. 2007; Gonzalez-Gonzalez-Gutierrez and Escamilla-Silva 2009; Yang et al. 2009; Yemendzhiev et al. 2009). In several cases, the microbial decoloration of azo dyes or textile effluents was increased in the presence of glucose. In other cases, the presence of several carbon sources is necessary, as in the case of *Staphylococcus arlettae* which causes >90 % decoloration of Reactive Yellow 107 and Reactive Red in the presence of glucose and yeast extract, <50 % in the absence of yeast extract and no decoloration when yeast extract and glucose are substituted with sodium pyruvate (Elisangela et al. 2009). Starch is another common source of carbon that is frequently used as an additive in the textile finishing process. Therefore, the use of microorganisms, that can use starch as a co-substrate, would be beneficial for the treatment of wastewater from the textile industry (Babu et al. 2007). Furthermore, glucose also inhibits the discoloration rate of Golden Yellow by a consortium of *G. geotrichum* and *B. laterosporus* (Waghmode et al. 2011). In addition to the type of the carbon source, it is also important to consider the amount of the source which must be sufficient to meet microbial growth requirements and achieve decoloration. However, high carbon concentrations can also lead to low decoloration, because the microorganisms utilize the carbon source preferentially to the dye (Waghmode et al. 2011; Kumar et al. 2012). Additionally, the concentration must be sufficiently low to limit the growth of the biomass and allow metabolic activity without enhancing the biosorption process (Khouni et al. 2012). The goal for microorganisms is to use the dye as a carbon source or even as the sole source of carbon and nitrogen. This goal can be achieved in some cases, if the microorganisms are acclimatized by successively increasing the amount of dye and diminishing the carbon source until they can survive with the azo dye alone. Examples are the growth of *Saccharomyces cerevisiae* (Jadhav et al. 2007) and *Sphingomonas paucimobilis* (Ayed et al. 2011) with Methyl Red; *Pseudomonas*, *Arthrobacter* and *Rhizobium* consortium with Acid Orange 7 (Ruiz-Arias et al. 2010); *Pleurotus sanguineus* with Drimaren Brilliant Blue (Machado et al. 2006); *Aspergillus fumigatus* with a mixture of Reactive Black RC, Reactive Yellow HF2-GL, Reactive Blue BGFN, Reactive Black B-150 and Reactive Red A-6BF (Jin et al. 2007); *Micrococcus glutamicus* with Navy Blue HE2R and its reaction intermediates (Saratale et al. 2009); *Williopsis saturnus*, *Candida* sp. and *Trichosporon porosum* with Yellow 4R-HE, Black B–V, Blue RR-BB and Red 7B-HE (Martorell et al. 2012), *A. niger* with Direct

Violet, Direct Brown and Direct Green (Wafaa et al. 2010). *Oscillatoria curviceps* is able to use Acid Black 1 as a nitrogen source in an oligotrophic environment (Priya et al. 2011). Nitrogen sources are also important for microbial decolorization. The use of yeast extract by some of the microorganisms enhances dye removal, as observed in the consortium of *Paenibacillus polymyxa*, *Micrococcus luteus* and *Micrococcus* sp. during Reactive Violet 5R decoloration and *Aspergillus flavus* during True Blue dye decoloration (Ponraj et al. 2011). The addition of peptone, beef extract, or rice husk and rice straw extracts as nitrogen sources in the synthetic media, results in maximal decoloration of Brilliant Blue G by a consortium of *G. geotrichum* and *Bacillus* sp. (Jadhav et al. 2008). The decoloration of a mixture of reactive azo dyes by *A. fumigatus* and the decoloration of a mixture of Reactive Black RC, Reactive Yellow HF2-GL, Reactive Blue BGFN, Reactive Black B-150 and Reactive Red A-6BF by *A. fumigates* improved using ammonium sulphate and ammonium chloride as nitrogen sources (Saratale et al. 2010).

3.3 *Influence of Salinity, Dye Concentration, pH, Temperature and Oxygen in the Decoloration Process*

The operation conditions affect the efficiency of microorganisms to decolorate azo dyes, such as the presence of salts, concentration of the dyes, pH, temperature and oxygen. However, there are examples of halotolerant microorganisms that are able to decolorate azo dyes in the presence of salts (Meng et al. 2012). *Bacillus* sp. decolorates Navy blue 2GL in 48 h, but this process takes 18 h in presence of $CaCl_2$, as extracellular LiP and intracellular Lac activities are induced with $CaCl_2$ (Dawkar et al. 2009). *Exiguobacterium acetylicum, Exiguobacterium indicum* and *Staphylococcus gallinarum* are able to decolorate Reactive Black 5 even in cultures containing 60,000 ppm NaCl (Chen et al. 2011). The dye concentration also affects microbial azo dye decoloration. For example, *Lysinibacillus* sp. effectively decolorates 100 % of Metanil Yellow at 200 ppm, but was only able to decolorate 62 % of Metanil Yellow at 1,000 ppm (Anjaneya et al. 2011). *Sphingomonas paucimobilis* decolorated completely Methyl Red at 750 ppm, whereas only 38 % of a 1000 ppm dye solution was decolorated by this microbe (Ayed et al. 2011). The time required for complete decoloration of Reactive Blue 172Reactive Blue 172 at 50, 100, 150 and 200 ppm with *Proteus mirabilis* was 8, 12, 18 and 26 h, respectively (Saratale et al. 2011b). Bioaccumulation percentage is reduced with increasing dye concentration, and the specific growth rate of yeast is also decreased. For example, *C. tropicalis* adsorbed 100 % of Direct Red 28 at 10 ppm, 70 % at 30 ppm and only 60 % at a concentration of 50 ppm (Charumathi and Nilanjana 2010). Adsorption and enzymatic activity are dependent on the pH. As the extent of decoloration is influenced by the pH of the media, pH also affects the color of the solution and the solubility of the dye. *Candida tropicalis* adsorbs 45 % of Basic Violet 3 at pH 3, 85 % at pH 4 and 33 % at pH 9 (Das et al. 2010). *Micrococcus* sp. decolorates 65 % of 300 ppm of Orange MR at pH 4, 80 % at pH 6, and 40 % at pH

10 (Rajee and Patterson 2011). Temperature affects microbial growth and enzyme production and, consequently, the percentage of decoloration. For example, *Micrococcus* sp. decolorates 60 % of 300 ppm of Orange MR at 30 °C, 80 % at 35 °C and 42 % at 45°C (Rajee and Patterson 2011). *Pseudomonas aeruginosa* degrades 97 % of 50 ppm of Remazol Red at 40 °C, 72 % at 10 °C and 82 % at 30 °C (Jadhav et al. 2011). Temperature also affects biosorption as evident from the fact that *T. versicolor* absorbs 44 mg g^{-1} of Sirius Blue K-FCN at 7 °C, 58 mg g^{-1} at 26 °C and 48 mg g^{-1} at 45 °C, and this increase in biosorption could be due to the increased surface activity and kinetic energy of the dye molecules (Erden et al. 2011). The presence of oxygen can either favour or inhibit the microbial degradation of azo dyes. Shaking increases mass and oxygen transfer between cells and the medium, and enzyme activity can depend on the presence of oxygen, if the mechanism is aerobic. The time to decolorate Methyl Red using a *Micrococcus* strain was reduced from 24 to 6 h under a supply of oxygen (Olukanni et al. 2009). *Shewanella oneidensis* shows higher dye decoloration under static conditions (97 %) than in aeration (8 %), although cell growth is comparatively faster under shaking conditions (Wu et al. 2009). Orange II decoloration with *A. niger* improves with the shaking (84 %) compared to static growth (61 %) (Ali et al. 2009).

4 Involvement of Oxidoreductive Enzymes in Degradation Process

Azo dyes are electron-deficient xenobiotic compounds because of their azo linkage (–N N), and in many cases, they have sulphonic (SO_3^-) or other electron-withdrawing groups, which generate an electron deficiency and make the dye less susceptible to degradation by microorganisms (Hsueh et al. 2009; Enayatizamir et al. 2011; Kuberan et al. 2011; Kurade et al. 2011). The anaerobic mechanism of microbial degradation of azo dyes to their corresponding amines is initiated by the cleavage of the azo linkage with the aid of an anaerobic *azoreductase* and electron transfer by a redox mediator that acts as an electron shuttle between the extracellular dye and the intracellular reductase (Ramalho et al. 2002). The oxidative degradation of azo dyes is catalyzed by peroxidases and phenoloxidases, such as manganese peroxidase (MnP), lignin peroxidase (LiP), laccase (Lac), tyrosinase (Tyr), demethylase (Duran et al. 2002; Jadhav et al. 2011; Oturkar et al. 2011; Martorell et al. 2012).

5 Toxicity of Decoloration Products and Evaluation Methods

The metabolites produced from dye degradation are, in many cases, more toxic that the parent dye. For example, the products of the oxidation of indigo blue via electro incineration, coagulation with $Al_2(SO_4)_3$ or the use of Lac are more toxic than the

parent dye (Solis-Oba et al. 2009). Several azo dyes and the amines from their degradation have shown mutagenic responses in *Salmonella* and mammalian assay systems, and their toxicity depends on the nature and position of the substituents in the molecule. For example, the dyes Acid Red 18 and Acid Red 27 are non-mutagenic, whereas the structurally similar dye Acid Red 26 is carcinogenic because of the presence of a methyl group and the difference in the position of the sodium sulphonate. Similarly, 3-methoxy-4-aminoazobenzene is a potent hepatocarcinogen in rats and a strong mutagen in bacteria, whereas 2-methoxy-4-aminoazobenzene is apparently non-carcinogenic and an extremely weak mutagen in bacteria (Ferraz et al. 2011). Acid Violet 7 has a significant ability to induce chromosome aberrations, lipid peroxidation and the inhibition of acetylcholinesterase and the toxicity of this dye increases significantly after static biodegradation by *Pseudomonas putida* due to the corresponding azo reduction metabolites 4'-aminoacetanilide and 5-acetamido-2-amino-1-hydroxy-3, 6-naphtalene disulphonic acid (Mansour et al. 2010). Therefore, in general, it becomes very important for any bioremediation technology to assess the toxicity of the pollutants and metabolites formed after dye degradation in order to study the feasibility of the method (Jadhav et al. 2011). Toxicity has been evaluated using various methodologies with respect to phytotoxicity, ecotoxicity, genotoxicity, mutagenicity, acute toxicity, microbial toxicity and toxicity on invertebrates (Fig. 11). Phytotoxicity methodologies have become more prevalent, because they are less expensive and easier than other methods. According to the inhibition of the germination of selected seeds, the most-used species have been *Sorghum vulgare* (Parshetti et al. 2010; Jadhav et al. 2011; Kurade et al. 2011; Waghmode et al. 2011); *Phaseolus mungo* (Parshetti et al. 2010; Jadhav et al. 2011; Waghmode et al. 2011; Kumar et al. 2012; Kurade et al. 2012); *Triticum aestivum* (Parshetti et al. 2010; Ayed et al. 2011; Kumar et al. 2012); *Sorghum bicolor* (Parshetti et al. 2010; Ayed et al. 2011); *Oryza sativa* (Bohmer et al. 2010; Zhuo et al. 2011); *Lepidium sativum* (Jonstrup et al. 2011); *Cucumis sativus* (Anastasi et al. 2011); *Cajanus cajan* and *Cicer arietinum* (Anjaneya et al. 2011). Ecotoxicity has been measured in the terms of number of

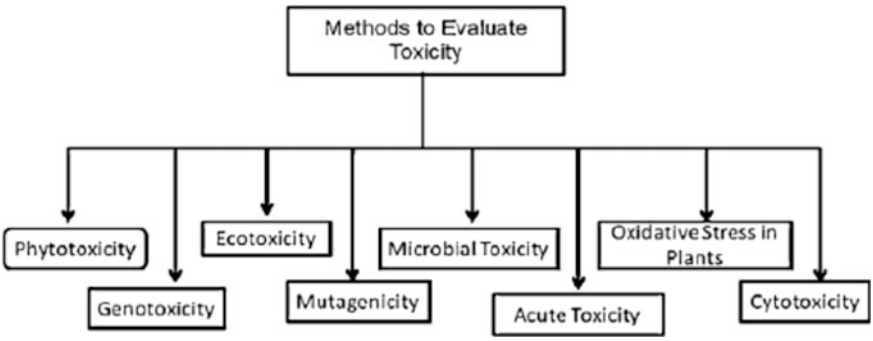

Fig. 11 Methods to evaluate the toxicity of azo dyes and their degradation products in treated effluents

fronds and dry mass of *Lemna minor* (Casieri et al. 2008). Cytotoxicity and genotoxicity have been evaluated using *Allium cepa* root cells (Carita and Marin-Morales 2008; Jadhav et al. 2011; Phugare et al. 2011). Microbial toxicity has been analysed using *Kocuria rosea, P. aerugenosa, Azotobacter vinelandii* (Parshetti et al. 2010), *Sphingomonas paucimobilis* (Ayed et al. 2011), *Vibrio fischeri* (Garcia-Montano et al. 2008; Anastasi et al. 2011), *Sinorhizobium meliloti* (Enayatizamir et al. 2011), *P. putida* (Bohmer et al. 2010), *Sinorhizobium meliloti* (Enayatizamir et al. 2011), *P. vulgaris, Rhizobium radiobacter, Acinetobacter* sp., *Pseudomonas desmolyticum*, *Cellulomonas biazotea, Escherichia coli* and *Micrococcus glutamicus* (Saratale et al. 2009, 2010) or by the inhibition of cellular growth of the green unicellular alga *Pseudokirchneriella subcapitata* (Anastasi et al. 2011). The oxidative stress response is a newly studied parameter for the assessment of the toxic effects caused by textile effluents. Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen, which include the superoxide anion (O_2^-), H_2O_2 and the hydroxyl radical (OH·). These molecules are by-products of the partial reduction of oxygen metabolism, and they play a crucial role in cell signaling and development in plants, including the plant defence response, cell death and oxidative stress. However, ROS can also cause protein, lipid and DNA damage. Plant systems possess defence mechanisms against such oxidative stress, which are composed of antioxidants or scavenging enzymes, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase and peroxiredoxins (Jadhav et al. 2011). Thus, the analysis of oxidative stress must include the analysis of these antioxidant enzymes, lipid peroxidation and protein oxidation. *Allium cepa* root cells have been used to carry out this type of analysis (Jadhav et al. 2011; Phugare et al. 2011). The same model has also been used to study chromosomal aberration and cell division (Phugare et al. 2011). The most employed, well-established and standardized acute lethality test uses *Daphnia magna* (Elisangela et al. 2009; Porri et al. 2011; Rizzo 2011).

6 Future Prospectives

The treatment of textile wastewater has become a great challenge over the past decades. Till recently, there is no single and economically attractive treatment method that can effectively decolorize and detoxify the textile wastewater. As regulations have become more stringent, companies are forced to use more technologically sophisticated methods. There is also a concomitant increase in cost for waste management that many companies may not be able to handle. Thus, effective wastewater treatment is not the only problem, but reductions in the waste and the reuse of water are also necessary. It is of the most importance to develop effective, accessible, cheap and environmentally friendly treatment processes. Taking into consideration the advances in this field, we believe that future research activities should focus on four principal areas: (a) achieve dye mineralization in addition to decoloration. This idea is very important because an additional problem with

wastewater treatment is the production of degradation products that are more toxic than the parent material. This challenge can be approached by three principal lines of attack: the combination of AOPs with biological processes to achieve the required degree of treatment; the application of molecular biology techniques to create recombinant strains with higher biodegradation capacities or that produce more active enzymes to mineralize dyes with less exposure time; and the use of MFCs to mineralize dyes and produce energy. (b) design novel dyes based on the introduction of substituents into the chemical structure to enhance their biodegradability. The effects of the dye structure on its degradation and the types of molecules that can be reduced/oxidized more easily are well known. (c) search for alternatives for dye removal from large volumes of effluents and get water into the appropriate condition so that it can be re-used in the same industry. The research in this area has only been performed at a small scale to date. (d) improve or modify the production process or implement new processes to reduce water use, eliminate or minimize the discharge of toxic chemicals, and recycle water as many times as possible to make companies more eco friendly at a competitive price.

7 Conclusions

The microbial degradation and biosorption of dyes have received much attention, as these are cost-effective methods for dye removal. The selection of the best treatment option for the bioremediation of a specific type of industrial wastewater is a difficult task, because of the complex composition of these effluents. The best option is often a combination of two or more systems, and the choice of such processes depends on the effluent composition, characteristics of the dye, cost, toxicity of the degradation products and future use of the treated water. However, the benefits of the different processes and the synergistic effect of the combination of such technologies must be studied carefully to create the best blend, taking advantage of the use of various strains and consortia isolated from dye-contaminated sites, the isolation of new microorganisms, such as thermotolerant or thermophilic microorganisms, or the adaptation of existing ones to consume dyes as their sole carbon and nitrogen sources in such a way that the effluents have low values for COD, TOC, color and toxicity. Through this review, it becomes evident that the development of new technologies to remove dyes, such as genetic engineering, which involves the creation of recombinant strains with higher degradation capacities, is important and should be applied in the future. The development of innovative methodologies, such as AOPs or MFCs combined with microbiological processes for treating wastewater containing azo dyes, and the addition of new efforts and approaches in this direction are mandatory in the future and will play a critical role in increasing environmental protection.

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Bacterial Degradation of Textile Dyes

Kisan M. Kodam and Yogesh M. Kolekar

1 Introduction

Color is a visible pollutant and its presence has always been undesirable in water used for either industrial or domestic needs. The exact period of the commencement of the art of dyeing in the world could not be ascertained correctly. However, archaeological evidence shows that dyeing was a wide-spread industrial enterprise in Egypt, India and Mesopotamia round third millennium B.C. (Ouzman 1998). Ever since the beginning of human civilization, people have been using colorants for painting and dyeing of their surroundings, skins and clothes. Until the mid 19th century, all colorants applied were from natural origin. These dyes were from plant sources, like roots, berries, bark, leaves, wood, fungi, and lichens. They fade early, because they were from natural origin and degradable. William Henry Perkin discovered the first synthetic dye stuff "Mauve" (aniline, a basic dye) while searching for a cure for malaria and thus, a new industry has opening. It was a brilliant fuchsia type color, but faded easily. Synthetic dyes were named after the chemical structure of the chromophoric group (azo dyes, anthraquinone dyes, xanthene dyes, triphenylmethane dyes, etc.) (Zollinger 2003). Amongst complex industrial wastewater with various types of coloring agents, dye wastes are predominant (Anjaneyulu et al. 2005).

Color is contributed by phenolic compounds, such as tannins, lignins (2–3 %) and organic colorants (3–4 %) (Clarke and Steinle 1995). However, synthetic dyes including dye intermediates i.e. sulfur, mordant, reactive, cationic, disperse, azo, acid, and vat dyes contribute maximum to waste waters (Raghavacharya 1997).

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Dyes are difficult to be decolorized due to their complex structure, synthetic origin and recalcitrant nature. Therefore, it is obligatory to remove them from industrial effluents before being disposed into water bodies. The color of water polluted with organic colorants gets reduced with the cleavage of the –C=C–, –N=N– bonds as well as heterocyclic and aromatic rings. The absorption of light by the associated molecules shifts from the visible to the ultraviolet or infrared region of the electromagnetic spectrum (Strickland and Perkins 1995). There are about 12 classes of chromogenic groups, among which, the most common are the azo dyes, which make up to 60–70 % of all textile and tannery dyestuffs produced (Carliell et al. 1996), followed by the anthraquinone type. Discharge of colorless effluents loaded with toxic and hazardous pollutants to water bodies is not usually objected. On the other hand, the discharge of colored effluents, though less toxic, is often objected by the public on the assumption that color is an indicator of pollution. It is, therefore, not surprising to note that color in wastewater has now been considered as a pollutant that needs to be treated before discharge. Wastewater treatment using biological process for decolorization of industrial effluents is still ambiguous and divergent.

2 Microbial Treatment of Waste Water Containing Dyes

Rapid industrialization releases an array of environmental pollutants which include several xenobiotics (Khan et al. 2013). These compounds are highly toxic and most of them are carcinogenic in nature. Therefore, they have been listed as highly toxic pollutants by the United States Environment Protection Agency (http://www.epa.gov). The synthetic dyes constitute the largest class and are widely used in the textile, cosmetic, printing, drug, and food processing industries. These dyes are toxic, mutagenic and carcinogenic in nature and have negative impact on the living organisms (Dos Santos et al. 2007). In the dyeing process, most of dyes remain unbound and get released into the environment with wastewater. This is an alarming situation. A dye house effluent typically contains 0.6–0.8 g dye l^{-1} (Gahr et al. 1994). Microbial degradation of textile dyes has become a key research area in environmental sciences. The microbes adapt to the toxic wastes and develop new resistant mechanism, they can biodegrade various toxic chemicals into less harmful forms. Many bacterial, fungal and algal species have developed the ability to absorb and/or degrade azo dyes (Pandey et al. 2007).

2.1 Bacterial Biodegradation

Microbial decolorization and degradation is an eco-friendly and cost-competitive alternative to the chemical decomposition process (McMullan et al. 2001). Most of studies on dye biodegradation have focused on bacteria and fungi, in which bacteria

are found to be more efficient and effective (Chang et al. 2001; Khehra et al. 2005). In the biodegradation, the original dye structure is cleaved into fragments by the microbial cells. Sometimes, bacterial degradation achieves complete mineralization, i.e. conversion of dyes into CO_2, biomass and inorganics (Kolekar et al. 2012). Thus, textile industries have to develop an effective biological effluent treatment method as an alternative to the conventional physico-chemical effluent treatment methods.

2.1.1 Decolorization Under Aerobic Conditions

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

2.1.2 Decolorization Under Anaerobic Conditions

Methanogenesis of complex organic compounds requires the co-ordinated participation of many different groups of bacteria, including acidogenic, acetogenic and methanogenic bacteria (Wuhrmann et al. 1980). Dye decolorization under these conditions requires an organic carbon/energy source. Simple substrates, like glucose, starch, acetate, ethanol and more complex ones, such as whey and tapioca, have been used for dye decolorization under methanogenic conditions (Yoo et al. 2001; van der Zee and Villaverde 2005). Extensive studies have been carried out to determine the role of the diverse groups of bacteria associated with the decolorization of azo dyes under methanogenesis (Carliell et al. 1996). However, a few

investigators have shown that acidogenic as well as methanogenic bacteria also contribute to dye decolorization. Reduction under anaerobic conditions appears to be non-specific, as a group of azo compounds are decolorized. In such situation, the rate of decolorization is dependent on the added organic carbon source, as well as the dye structure (Stolz 2001). Furthermore, there is no correlation between decolorization rate and molecular weight, indicating that decolorization is not a specific process and cell permeability is not important for decolorization. Thus, anaerobic azo dye decolorization is the process where dye might act as an acceptor of electrons supplied by carriers of the electron transport chain. Alternatively, decolorization might be also attributed to non-specific extracellular reactions occurring between reduced compounds generated by the anaerobic biomass (van der Zee et al. 2001).

2.1.3 Decolorization Under Anoxic Conditions

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

2.1.4 Anaerobic Azo Dye Reduction

Anaerobic azo dye reduction is the reductive cleavage of azo linkages resulting in the formation of aromatic amines. As aromatic amines are generally colorless, azo dye reduction is also referred to as azo dye decolorization. The first study on azo dye reduction was published as early as 1937, when the decolorization of food azo dyes by lactic acid bacteria isolated from the human gut was reported (Brohm and Frohwein 1937). Hence, as the formation of toxic aromatic amines in humans

was a matter of concern, research on bacterial azo dye reduction has focused on the activity of facultative anaerobic bacteria from mammalian intestines (Walker 1970; Chung et al. 1992). Later, when the removal of dyes from wastewater became a problem, bacteria from other origins were also used to investigate anaerobic azo dye reduction by pure cultures (Wuhrmann et al. 1980), mixed cultures (Haug et al. 1991), anaerobic sediments, digester sludge (Brown and Laboureur 1983), anaerobic granular sludge and activated sludge under anaerobic conditions. A large number of azo dyes that can be reduced by different bacteria indicate that azo dye reduction is a non-specific reaction and that the capability of reducing azo dye can be considered as a universal property of anaerobic bacteria.

2.1.5 Aerobic Oxidation of Aromatic Amines

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

2.1.6 Combined Anaerobic-Aerobic Treatment

The prerequisite for oxidative degradation of azo dye is reductive fission of the azo linkage under anaerobic condition, followed by aerobic degradation for complete removal of azo dyes (Field et al. 1995). Two different approaches can be discerned: sequential treatment in separate reactors and integrated treatment in a single reactor. The integrated approach is based on temporal separation of the anaerobic and the aerobic phase, as in sequencing batch reactors or on the principle that diffusion of oxygen in microbial biofilms is usually limited to 10–100 μm, so that anaerobic and aerobic conditions co-exist in a single environment (Lens et al. 1995). The removal of color in the anaerobic stage was generally high, mostly higher than 70 % and in several cases, even achieved almost 100 %. Color removal efficiencies differed widely in dyes, when the removal of different azo dyes was tested under similar conditions.

The reaction time is an important factor in the anaerobic removal of azo dyes. A decrease in the hydraulic retention time of the anaerobic stage was found to result in lower color removal efficiency and anaerobic azo dye reduction is also slow reaction (An et al. 1996). The biomass concentration also plays an important role in the anaerobic removal of azo dyes. Decrease in biomass and retention time of a sequencing batch reactor results in lower color removal efficiency (Lourenço et al. 2000). The percent recovery of aromatic amines ranged between <1 % to almost 100 %. A wide range in percent recovery may partly be explained by the difficulties encountered in analyzing these often chemically unstable compounds. Partial or complete removal of many aromatic amines can be suspected from the decrease or disappearance of the sometimes unidentified peaks in HPLC chromatograms (O'Neill et al. 1999; Lourenço et al. 2000) as well as from the decrease in UV absorbance. Moreover, a large decrease in toxicity to aerobic bacterial activity was measured between the effluents of anaerobic and aerobic stage (O'Neill et al. 1999). In summary, combined anaerobic-aerobic biological treatment holds to be a promising method to remove azo dyes from wastewater. However, there are two possible bottlenecks: (i) anaerobic azo dye reduction is a time-consuming process as reflected by the requirement of long reaction time and (ii) the fate of aromatic amines during aerobic treatment is not conclusively elucidated.

2.2 *Fungal Biodegradation*

Lignin-degrading fungi, white-rot fungi, can degrade a wide range of aromatics. In earlier reports, the dyes were degraded efficiently by wood-rotting fungi (e.g. *Phanerochaete chrysosporium*, *Trametes* sp.). Eventually, they were also found to be responsible for the degradation of lignin (David et al. 1994). In the fungal degradation, there are a few reports of adsorption of dyes on the fungal cells which forms sludge which requires further treatment. In this scenario, the fungal treatment of dye containing effluents is usually time consuming and difficult also (Erden el al. 2011). This property is mainly due to the relatively non-specific activity of fungal lignolytic enzymes, such as lignin peroxidase, manganese peroxidase and laccase. The reactions catalyzed by these extracellular enzymes are oxidation reactions, e.g. lignin peroxidase catalyses the oxidation of non-phenolic aromatics, whereas manganese peroxidase and laccase catalyze the oxidation of phenolic compounds (McMullan et al. 2001).

The degradation of dyes by white-rot fungi was first reported as early as in 1983 (Glenn and Gold 1983). Since then, it has been the subject of many research projects. Virtually all dyes from chemically distinct groups are prone to fungal oxidation, but there has been a wide difference among fungal species with respect to their catalyzing power and dye selectivity. Fungal degradation of aromatic structures is a secondary metabolic event which starts when nutrients (C, N and S) become limiting factors (Kirk and Farrell 1987). Therefore, the enzymes are optimally expressed under the starving conditions. However, supplementation of

energy substrates and nutrients are necessary for the propagation of cultures. Other important factors for the cultivation of white-rot fungi and expression of lignolytic activity are the availability of enzyme co-factors and the pH of the environment. Although stable operation of continuous fungal bioreactors for the treatment of synthetic dye solutions has been achieved (Mielgo et al. 2001), but application of white-rot fungi for the removal of dyes from textile wastewater faces many problems. As the wastewater is not natural, the enzyme production in white-rot fungi may be unreliable and the biomass growth and retention in bioreactors may be a matter of serious concern (Stolz 2001). Treatment of large volumes of wastewater may be very difficult, as extraction and concentration of dyes are necessary factors prior for the fungal treatment (Nigam et al. 2000). Furthermore, the low optimum pH for lignin peroxidase (4.5–5.0) requires extensive acidification of the usually highly alkaline textile wastewater which causes inhibition of other useful microorganisms, like bacteria. Moreover, other wastewater constituents, especially aromatics, may also interfere with fungal dye degradation (Stolz 2001).

2.3 Algal Biodegradation

Degradation of azo dyes by algae has been reported only in few studies. The degradation pathway is thought to involve reductive cleavage of the azo linkage, followed by further degradation (mineralization) of aromatic amines formed. Hence, algae have been demonstrated to degrade several aromatic amines, even sulfonated azo dyes. In open wastewater treatment systems, especially in (shallow) stabilization ponds, algae may, therefore, contribute to the removal of azo dyes and aromatic amines from the wastewater. However, algal biodegradation of dyes has some limitations: one of very common problem is the adsorption. It was reported that *Chlorella vulgaris* could absorb Supranol Red 3BW (35.62 mg dye g^{-1} biomass), Lanaset Red 2GA (44.98 mg dye g^{-1} biomass), Levafix Navy Blue EBNA (43.17 mg dye g^{-1} biomass), but the degradation from algal biomass is not competitive if we compare it with the bacterial degradation (Lim et al. 2010). *Oscillatoria curviceps* can alone degrade Acid Black (100 ppm) up to 84 % in 8 days (Priya et al. 2011). The efficiency of adsorption is highly influenced by the structure of the dye, species of algae and physical parameters, like pH, temperature, etc. (Solís et al. 2012).

2.4 Bioreactor for Effective Decolorization of Textile Dyes

Many bioreactors for the degradation of textile dyes have been developed by many researchers and a fair amount of research was conducted to assess their viability for biodegradation study. Different types of bioreactors were developed, such as upflow anaerobic sludge blanket reactor (UASB), activated sludge reactors, etc. The UASB

process was developed in the late 1970s (Lettinga et al. 1980). Initially the reactor was designed to treat concentrated industrial wastewater and its application was later extended to sewage treatment also. In the UASB reactor, wastewater flows upward through an anaerobic sludge bed, consisting of semi-immobilized microbial communities. The critical elements of the UASB reactor design are the influent distribution system, the gas-solid separator, and the effluent withdrawal system. The UASB process, the most popular among anaerobic treatment processes, relies on the establishment of a dense sludge bed or digestion zone at the bottom of the reactor, where the anaerobic degradation of the wastewater organics occurs and biogas is produced (Chinwetkitvanich et al. 2000).

Conventional activated sludge treatment of industrial wastes is an effective and highly economical system for the degradation of dyes and other organic pollutants from the wastewater. A lot of work has been done on the assessment of activated sludge system for the degradation of industrial effluents. The activated sludge treatment is the process in which the organic matter from wastewater is utilized as an energy source for the survival and multiplication of microorganisms in an aerobic environment. The microbes convert carbon into cell mass and oxidize the dyes finally to carbon dioxide and water. In addition, a limited number of microorganisms may exist in the activated sludge which get energy by oxidizing ammonia to nitrate, a process known as nitrification (Adav et al. 2009).

Aerobic sludge granulation is a biotechnological process used in biological wastewater treatment and treating chemical industrial influent (Adav et al. 2008). Now-a-days microbial granules play a major role in biological wastewater treatment due to several advantages over the conventional sludge flocks which include a denser and stronger aggregate structure, better settleability and ensured solid-effluent separation, higher biomass concentration and greater ability to withstand high organic loading, etc. (Lin et al. 2005; Adav et al. 2009).

The continuous aeration and feeding lead to an increase in bacterial cell mass and granulation was initiated at around 20 days and reached to mature granules in 60 days of reactor operation (Fig. 1a). High hydraulic selection pressure will retain denser, heavier, compact and smooth granules with improved metabolic activity (Chen et al. 2007). The SEM images of granules during the granulation process in the reactor depict average size of granules in the range of 5–8 mm (Fig. 1b).

Biodegradation can be coupled with other degradation methods like, physical or chemical treatments. A few investigations have shown that azo dyes can be completely decolorized and some intermediates, such as aromatic amines with side groups ($–SO_3$, –OH, –COOH, –Cl) containing metabolites were quantitatively detected (O'Neill et al. 1999). Actinomycetes were also tested for decolorization and degradation of textile dyes, as they produce peroxidases and also catalyses hydroxylation, oxidation, and dealkylation during various xenobiotic biotransformation (Goszczynski et al. 1994). McMullan et al. (2001) have reviewed the underlying mechanisms by which diverse categories of microorganisms from bacterial and fungal domains degrade dyestuffs. In nature, the biodegradation of pollutants (including textile dyes) was efficiently performed by a group of microorganisms in synergism. Hence, microbial consortium could be highly efficient than

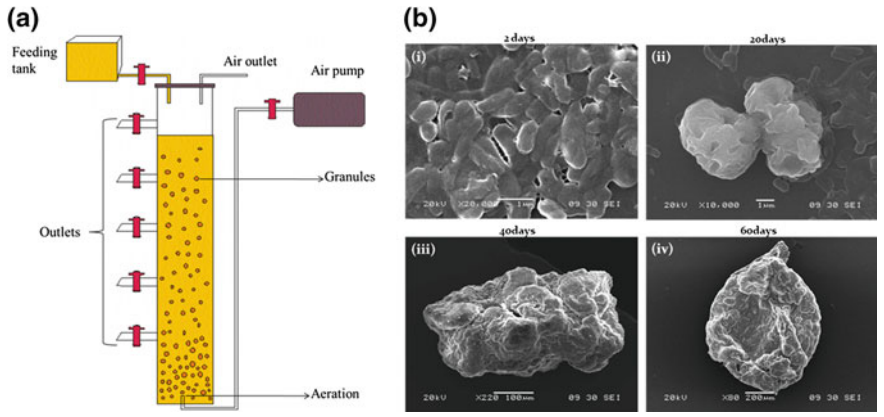

Fig. 1 **a** Systematic diagram of aerobic bioreactor for formation of bacterial granules. **b** SEM images of aerobic granules during the stages of granulation. (*i*) Cultures before granulation (2 days) and different stages of granulation (*ii*) 20 days, (*iii*) 40 days and (*iv*) 60 days (Kolekar 2010)

individual single microbe. Aerobic granules consist of dense microbial consortia packed with different microbial species and typically contain millions of organisms per gram of biomass. Thus, treating textile wastewater with environment friendly aerobic sludge granules could enhance decolorization and degradation of dyestuff, but it has been rarely documented. Recently, we have developed textile dyes degrading aerobic sludge granules in a batch reactor and evaluated their potential for textile dye removal and degradation. It was found that the dye degradation efficiency was very high as compared to individual cultures (Kolekar et al. 2012).

3 Factors Effecting Degradation Process

3.1 Microbial Growth and Energy Requirement for Degradation

The biodegradation efficiency depends on having the right microbes in the right place with the right environmental factors for degradation. The microbes (bacteria or fungi) have the physiological and metabolic potential to biodegrade the synthetic dyes (Boopathy 2000). The degradation process depends on biomass concentration (population diversity, enzyme activities), substrate (physico-chemical characteristics, molecular structure, and concentration) and a range of environmental factors like, dissolved oxygen, nitrate concentration, metals, salts, pH, temperature, moisture content, availability of electron acceptors and carbon and energy sources (Uddin et al. 2007; Phugare et al. 2010). Microorganisms are affected by changes in these parameters and consequently, their biodegradation capabilities are also

affected. In the biodegradation process, the contaminant serves as an effective energy source. The contaminant may take part as primary, secondary or as a co-metabolite in the bacterial metabolism (Kolekar et al. 2008). The structure of contaminant has a high impact on degradation process, followed by its purity and solubility (Khan et al. 2013).

3.2 Oxygen Availability

The decolorization of various textile dyes by mixed aerobic and facultative anaerobic microbial consortia has been also reported earlier (Nigam et al. 1996; Moosvi et al. 2005). Although many of these cultures could grow aerobically, decolorization was achieved only under anaerobic or static anoxic conditions (Kolekar and Kodam 2012). Pure bacterial strains, such as *Pseudomonas luteola*, *Aeromonas hydrophila*, *Bacillus subtilis*, *Pseudomonas* sp., *Proteus mirabilis*, *Bacillus fusiformis* and *Alishewanella* sp. decolorized dyes under both anoxic and static anoxic conditions (Chang et al. 2001; Kolekar et al. 2008; Kolekar and Kodam 2012). It was observed that under static anoxic conditions, the dye decolorization of different dyes was more than 75 % as compared to agitation, which caused less than 30 % decolorization (Kolekar et al. 2008; Chaudhari et al. 2013). This indicates that azo dye degradation primarily starts in anoxic conditions. The facultative anaerobes were able to grow under both aerobic and anaerobic conditions and capable of decolorizing dyes under anaerobic condition (Nigam et al. 1996). In the first step of biodegradation, a reductive cleavage of the azo bond gives rise to the production of colorless metabolites, mainly aromatic amines which are further degraded in aerobic conditions. The first step usually occurs in anaerobic/static anoxic conditions; however, it was observed by several researchers that aerobic microbes were able to cleave the azo group by synthesizing oxygen insensitive azoreductases in the presence of molecular oxygen (Nachiyar and Rajkumar 2005). Bacterial strains, that decolorize textile dyes aerobically, were reported for few years. Many of these cultures require external carbon sources, because they cannot utilize dye as terminal electron acceptor (Stolz 2001). Textile dye was decolorized by *P. aeruginosa* in the presence of glucose under aerobic conditions (Nachiyar and Rajkumar 2003). Azo dye decolorization by mixed, as well as pure cultures generally requires complex organic sources, such as yeast extract, peptone, or a combination of complex organic source and carbohydrate (Chen et al. 2003; Khehra et al. 2005).

3.3 Optimization of Biodegradation Conditions

Microbial degradation depends on various physico-chemical operational conditions like, pH, temperature, salinity, heavy metals, radioactive molecules, chlorinated compounds and other contaminants (Boopathy 2000). In this scenario, wastewater

contains many organic and inorganic pollutants. As textile wastewaters result from different classes of dyes, they vary in their composition. Every microorganism has some physico-chemical properties to work for biodegradation process, even if the microbes are in consortia these physico-chemical properties influence the biodegradation process (Mohana et al. 2008).

One of the factors, like pH has a major effect on the efficiency of textile dye degradation and the optimal pH for color removal in bacteria is often between pH 6.0 and 10.0 (Yoo et al. 2001; Kolekar et al. 2008; Kolekar and Kodam 2012). Most of fungi and yeast showed better decolorization at acidic or neutral pH (Mielgo et al. 2001). Many reports showed that the rate of dye degradation is higher at the optimal pH and gradually decreases at either side of optimum pH (Yoo et al. 2001; Saratale et al. 2009; Chaudhari et al. 2013). The effects of pH may be related to the transport of dye molecule across the cell membrane, which is considered as the rate limiting step for the decolorization (Kodam et al. 2005). The decolorization of Brilliant blue G by consortium of *Galactomyces geotrichum* and *Bacillus* sp. was found to have broad pH range from 5.0 to 9.0 (Jadhav et al. 2008).

During the biodegradation process, temperature also affects the biodegradation efficiency. The maximum rate of dye degradation by most of microbes depends on the optimum growth and temperature. It was observed that degradation potential was proportional to an increase of temperature, but within the optimum temperature range (Nachiyar and Rajkumar 2003; Kolekar et al. 2008). However, the rate of decolorization was reduced beyond optimum temperature. The reduction in decolorization can be attributed to the loss of cell viability or the denaturation of azoreductase enzyme which is a key enzyme for dye degradation (Saratale et al. 2009). It was also found that the dye degradation depended largely on the microbial optimum growth temperature (Stolz 2001; Khan et al. 2013). Recently, the degradation of reactive Orange M2R dye and chromate by *Lysinibacillus* sp. KMK-A was reported in the temperature range of 20–50 °C, but the degradation gradually decreased with increasing temperature (Chaudhari et al. 2013).

Microbial decolorization often gets affected in the presence of high amounts of salts (up to 10 g l^{-1} NaCl or Na_2SO_4) (Uddin et al. 2007). Many microbial species are able to decolorize azo dyes within a certain limit of salts. But in most of cases, they were unable to decolorize azo dyes at high salinity conditions. High salt concentration may cause plasmolysis or loss of activity of cells. It was observed that 85–95 % decolorization of acid orange 10 and disperse blue 79 by *Bacillus fusiformis* was found within 48 h for 0.5–3 % salt concentration, However, further increase in the salt (4 %) significantly decreased dye decolorization (Kolekar et al. 2008). There are only some bacteria which have the potential to grow, multiply in high salt concentrations and can degrade dye efficiently. *Gracilibacillus* sp. GTY strain, grown in the media containing 15 % (w/v) of NaCl, showed the best performance in dye decolorization (Uddin et al. 2007).

The dye decolorization affects with contaminants present in the wastewater. Many heavy metals are known to be toxic and can reduce the microbial growth which affects the biodegradation process. There are strict regulations to limit the amount of heavy metal present in the dye effluent in different countries, as some of

them are known to be carcinogenic. The emphasis is currently on the reduction of trace metals in textile processing effluent. It has been suggested that the heavy metals present in the dye bath would not affect the performance of bacteria during the degradation of the organic matters in the biochemical process (Boopathy 2000; Uddin et al. 2007).

4 Involvement of Oxidoreductive Enzymes in Degradation Process

In recent years, the enzymatic approach has attracted much interest in the biodegradation and decolorization of textile and other industrial wastewater. Enzymatic treatment is usually very effective in the degradation of pollutants. The potential of the enzymes like cytochrome P450, manganese peroxidases, lignin peroxidases, laccases, polyphenol oxidases, and azoreductases, has been studied in the decolorization and degradation of dyes. The expression of the enzymes involved in dye degradation may vary with time, but dependent on the growth phase of the microorganisms, and is also influenced by other compounds present in the effluent. There is an increasing industrial value for the alternative cost effective method for treatment of a large volume of wastewater. The application of enzyme-based systems in waste water treatment is highly valuable (Husain 2006).

4.1 Cytochrome P450

Cytochrome P450 (CYP) represents one of the largest and oldest gene super families coding for enzymes present in the genomes of all biological kingdoms. The terminology CYP is uncommon for enzymes, because it is not based on function, but describes originally the spectral properties of b-type heme containing red pigments, which display a typical absorption band at 450 nm of their reduced carbonmonoxide bound form (Omura and Sato 1962). In mammals, azo dyes are reduced to aryl amines by CYP and a flavin dependent cytosolic reductase. The reactions catalysed can be extremely diverse as hydroxylations, N–, O–, and S-dealkylations, sulfoxidations, epoxidations, deaminations, desulfurations, dehalogenations, peroxidations, and N-oxide reductions (Bernhardt 2006). Since many of the individual CYP catalyse multiple reactions, the usual method of naming enzymes is inadequate for this group of proteins, and a systematic nomenclature has been derived based on structural homology. The CYP has been exclusively studied for detoxification and degradation. The degradation of various textile dyes by *Alishewanella* sp. KMK6 showed an induction in CYP activities in the cells after dye decolorization indicating the role of these enzymes (Kolekar and Kodam 2012). Recently

Exiguobacterium sp. MG2 cytochrome P450 participated in the degradation of malachite green (N-methylated diaminotriphenylmethane), one of the most common dyes used in the textile industry (Wang et al. 2012).

4.2 Azoreductase

Azoreductase is also one of the biotransforming enzymes involved in decolorization of azo dyes by breaking azo bond. Azo dyes can be used by microorganisms as sole source of carbon, energy and nitrogen with the initial cleavage by azoreductase enzyme. Azoreductase requires NADH/NADPH as an electron donor to catalyze the reductive cleavage of azo bond. Recently, Nachiyar and Rajkumar (2005) have reported that purified azoreductase catalyzes the reductive cleavage of the azo bond of Navitan fast blue S5R in the presence of NADH as an electron donor, yielding metanilic acid and peri acid as degradation products (Fig. 2).

It has been known for a long time that metabolic processes, such as azo reduction, can activate and sometimes also detoxify azo compounds. The reduction of azo bonds in azo dyes is important in their toxicity, mutagenicity and carcinogenicity (Garner et al. 1984). It has been shown that intestinal bacteria carried out reduction of azo compounds to produce aromatic amines. Some 45 different intestinal bacteria have been found to express azoreductases in a diverse collection of bacteria, including both strictly anaerobic *Clostridium* species and facultative anaerobic Enterobacteriaceae. It is not yet clear that how many different types of azoreductase enzymes are found in the human gut, or which of them are relevant for metabolism of azo dyes.

During the aerobic, "semi-aerobic" (in static anoxic) or anaerobic incubation of bacteria with azo compounds, amines were often detected which originated from a reductive cleavage of the azo bond. The azoreductase from *Escherichia coli* CD-2

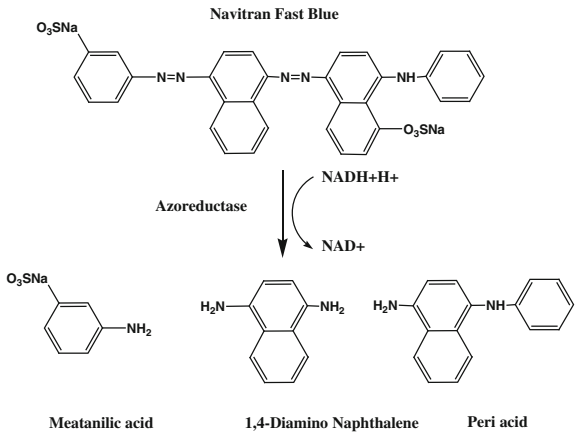

Fig. 2 Cleavage of azo bond by purified azoreductase from *Pseudomonas aeruginosa* (Nachiyar and Rajkumar 2005)

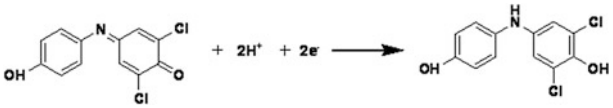

Fig. 3 DCIP reductase activity by using DCIP as a substrate (Bidoia et al. 2010)

was found to be responsible for the decolorization of azo dyes. The enzyme used methyl red as a substrate and the optimal pH value and temperature were pH 6.5 and 37 °C, respectively. The enzyme was stable under different physico-chemical conditions (Cui et al. 2012). Temperature stability studies showed that the enzyme was stable below 55 °C. However, azoreductase from *Enterobacter agglomerans* and *P. aeruginosa* lost most of their activities when the temperature reached to 50 °C (Nachiyar and Rajkumar 2005).

The aerobic reductive metabolism of azo dyes requires specific enzymes (aerobic azoreductases) that catalyze these reactions in the presence of molecular oxygen. In contrast to few reports of aerobic decolorization of azo dyes, a wide range of organisms are able to reduce azo compounds under anaerobic/anoxic conditions. This has been shown for purely anaerobic (e.g. *Bacteroides* sp., *Eubacterium* sp., *Clostridium* sp.), facultative anaerobic (e.g. *Proteus vulgaris*, *Streptococcus faecalis*), and aerobic (e.g. *Bacillus* sp., *Sphingomonas* sp.) bacteria, yeasts, and even tissues from higher organisms (Walker 1970). Cell extracts generally showed much higher rates of anaerobic reduction of azo dyes than of resting cells (Wuhrmann et al. 1980). Reductases for NADH-DCIP (NADH-dichlorophenol indophenols), azo and riboflavin were reported for the degradation of different dyes, but the actual role of different reductase is still unknown (Fig. 3). FMN-dependent NADH-azoreductase, found to degrade azo compounds, catalyses the reductive cleavage of an azo group by a ping pong mechanism (Ooi et al. 2009). The degradation of reactive blue 59 (RB59) by *Alishewanella* sp. KMK6 showed an increase in azoreductase and DCIP reductase activities by 10 fold in the cells after dye decolorization, indicating the role of these enzymes in the biodegradation (Kolekar et al. 2013).

4.3 Peroxidases

In recent years, research has developed processes in which peroxidases are employed to remove/transform phenolic compounds from polluted wastewater. The application of enzyme preparations shows considerable benefits over the use of microorganisms. Textile dyes can be efficiently degraded by various peroxidases as well. The enzymes, produced by the fungus, are lignin peroxidase (LiP) and manganese peroxidase (MnP). The lignin peroxidase and manganese peroxidase, produced by the white rot fungus *Phanerochaete chrysosporium*, have a potential to oxidize the substrates by an electron transfer process or by radicals generated during the enzyme catalytic cycle. There are various peroxidases which are commonly

used for biodegradation of textile wastewater. A common mechanism, in which the heme groups are the main constituents, catalyzes the reactions in the presence of hydrogen peroxide (Jamal et al. 2012; Zucca et al. 2012). The decolorization of dyes by white-rot fungi was first time reported by Glenn and Gold (1983) and been used by others to rapidly assess the biodegradative potential of a diverse group of white-rot fungi (Gao et al. 2006). Now, white-rot fungus has become a model for degradation research (Pazarlioglu et al. 2010).

The modified lignin has been used as a dispersing agent in synthesizing many textile dyes, Lignin peroxidase (LiP), also known as ligninase, was first discovered for partial decolorization of methylated lignin in vitro (Tien and Kirk 1983). The use of lignin degrading fungus has attracted increasing scientific interest, as these organisms are able to degrade a wide range of recalcitrant organic pollutants because they do not require pre-conditioning before the degradation of pollutants and the enzyme secretion depends on nutrient limitation rather than presence of pollutant (Wesenberg et al. 2003). The extracellular enzyme system from fungus also enables them to tolerate high concentration of pollutants.

Manganese peroxidase (MnP) from the lignin-degrading fungus *Phanerochaete chrysosporium* can oxidize a variety of organic compounds including dyes, but only in the presence of Mn(II). MnP catalysis occurs in a series of irreversible oxidation-reduction (redox) reactions which follow a ping-pong mechanism. MnP catalyzes the oxidation of several phenols and aromatic amine dyes (Mielgo et al. 2003). There are also reports of azo dye degradation by MnP from *Bjerkandera adusta* and *Pleurotus eryngii*. The reactions with the dyes were characterized by their apparent K_m values ranging from 4 to 16 μM and specific activities ranging from 3.2 to 10.9 U/mg (Heinfling et al. 1997). Recently, it has been reported that *Bjerkandera* sp. BOS55 (ATCC 90940) was able to decolorize high dye concentration (up to 1,500 mg l^{-1}) within 10 min reaction time. This is not because of change in pH, but has a specific oxidation capacity as high as 10 mg dye degraded per unit of MnP consumed which caused more than 90 % decolorization (Mielgo et al. 2003).

4.4 Laccases

Laccases are copper containing enzymes which catalyze the oxidation of several aromatic substances (phenols) with the preferable reduction of oxygen to water (Robles et al. 2000). Phenols are oxidized by laccases to generate phenoxy radicals, which couple to form oligomeric and polymeric products. The laccases produced by the white-rot fungus during its secondary metabolic stage of growth are known to oxidize a wide variety of organic compounds. Laccase decolorizes some textile dyes without direct cleavage of the azo bond through a highly non-specific free radical mechanism, thereby avoiding the formation of toxic aromatic amines. Because of its wide range of substrates laccases can be used for biodegradation of textile dyes. Industrial dye decolorization by laccases from ligninolytic fungi (*Pleurotus ostreatus, Pleurotus ostreiformis*, *Trametes versicolor*, etc.) has been

reported (Rodrıguez et al. 1999). Recently, laccase was isolated from the white-rot fungus *Lenzites gibbosa* and expressed in *Pichia pastoris*. This recombinant laccase efficiently decolorized alizarin red, neutral red, Congo red and crystal violet, without the addition of any redox mediators. This study strongly suggests that the decolorization capacity of recombinant laccase could be useful as biocatalyst for the treatment of dye-containing effluents (Zheng et al. 2014).

5 Mechanism and Pathways of Degradation

Biodegradation refers to chemical decomposition of organic substances by living organisms. In the decomposition or degradation, microbial enzyme systems act as biocatalysts to break down the original dye structure. There are several reports that microorganisms including bacteria, fungi, and yeasts can degrade textile dyes (Kolekar et al. 2008; Zucca et al. 2012). The reduction of azo linkage mediated is the initial step by the enzyme, azoreductase in the biodegradation of azo dyes (Levine 1991; Chung et al. 1992). The mineralization of dyes is usually achieved with two stage anaerobic/aerobic treatments. In this process, several environmental and physiological factors can influence the microbial activity and consequently the efficacy and effectiveness of complete biodegradation. The biodegradation process is depending on many factors, like concentration and structure of dyes, temperature, pH and the microorganisms involved in it. Considering all these factors, the degradation of dyes produced many small molecules which become very difficult to be degraded by the organism. These small molecules are aromatic amines and may cause more toxic and carcinogenic effect on the flora and fauna of water bodies, where aromatic amine containing waste water is getting discarded. In this scenario, it is very important to analyze the degradation of the dye as well as their intermediates, especially aromatic amines (Forss and Welander 2009). The study of the biodegradation products of synthetic dyes is very important in order to know about the environmental fate of these pollutants. Telke et al. (2010) reported the degradation of Congo red (1.0 g l^{-1}) within 12–60 h duration by an isolated bacterium *Pseudomonas* sp. SU-EBT. As shown in Fig. 4, the complete mineralization of azo dyes without the release of aromatic amines has also been observed in Reactive Blue 59 degradation by *Alishewanella* sp. KMK6, where the single microorganism was able to degrade 2.5 g l^{-1} dye within 6 h (Kolekar and Kodam 2012).

It is well known that several enzymes are present in the periplasmic and cytosolic fractions of the microorganisms where azoreductase cleaves an azo bond by reductive cleavage. The coordinated action of these enzymes give rise to low molecular weight molecules from complex dyes. The tracking of the various enzyme activities evidenced an involvement of various oxidative as well as reductive enzymes during the decolorization process. Induction of various enzymes during decolorization gives an additional insight of decolorization mechanism and also supports the active role of microorganisms in the biodegradation process (Phugare et al. 2010), mainly through oxidative enzymes, such as cytochrome

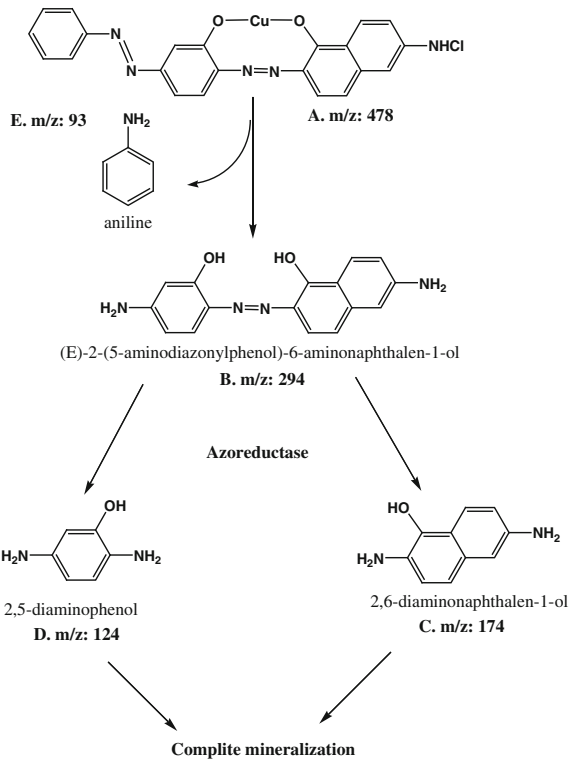

Fig. 4 Mechanism of degradation of reactive blue 59 by *Alishewanella* sp. strain KMK6 (Kolekar and Kodam 2012)

P-450 and also many reductases, such as azoreductase, NADH–DCIP reductase, and malachite green reductase influence the status of biotransformation enzymes (Wang et al. 2012). The role of cytosolic enzymes, like oxidase, DCIP reductase, and azoreductase in dye decolorization is well documented (Phugare et al. 2010; Kolekar and Kodam 2012).

6 Microbial Toxicity of Dyes and Their Degradation Products

The color in wastewater is highly visible and affects aesthetics, water transparency and gas solubility in water bodies, The toxicity (i.e. mortality, genotoxicity, mutagenicity and carcinogenicity) studies include the tests with aquatic organisms (fish, algae, bacteria, etc.), mammals and plants. The acute toxicity of dyestuffs is generally low. Algal growth in presence of different commercial dyestuffs was not inhibited at dye concentrations below 1 ppm, whereas the growth was severely inhibited at higher concentrations. The most acutely toxic dyes for algae are cationic basic dyes (Greene and Baughman 1996). Fish mortality tests showed that

2 % out of 3,000 commercial dyestuffs tested had LC50 values below 1 ppm. Basic dyes are found to be most acutely toxic to fish, especially those with a triphenylmethane structure. Fish seems to be relatively sensitive to many acid dyes. Mortality tests with rats showed that only 1 % out of 4,461 commercial dyestuffs tested had LD50 values below 250 mg kg^{-1} body weight (Clarke and Anliker 1980). Therefore, the chance of human mortality due to acute dyestuff toxicity is probably very low. However, acute sensitization reactions by humans to dyestuffs often occur. Especially, some disperse dyestuffs have been found to cause allergic reactions, i.e. eczema or contact dermatitis (Specht and Platzek 1995).

Chronic effects of dyestuffs, especially of azo dyes, have been studied for several decades. Researchers had traditionally focused on the effects of food colorants, usually azo compounds. Furthermore, the effects of occupational exposure of human workers to dyestuffs in dye manufacturing and dye utilizing industries have received little attention. Azo dyes in purified form are seldom mutagenic or carcinogenic, except for some azo dyes with free amino groups (Brown and Devito 1993). However, some of the degradation products of azo dyes are known to be mutagenic and/or carcinogenic. In mammals, metabolic activation (or reduction) of azo dyes is mainly due to bacterial activity in the anaerobic parts of the lower gastrointestinal tract. Various human other organs, especially liver and kidneys, can reduce azo dyes. After azo dye reduction in the intestinal tract, the released aromatic amines are absorbed by the intestine and excreted in the urine. The acute toxic hazard of aromatic amines is carcinogenesis, especially bladder cancer. The carcinogenicity mechanism probably includes the formation of acyloxy amines through N-hydroxylation and N-acetylation of the aromatic amines, followed by O-acylation. These acyloxy amines can be converted to nitrenium and carbonium ions that bind to DNA and RNA and induce mutations and tumor formation (Brown and Devito 1993). The mutagenic activity of aromatic amines is strongly related to molecular structure. Recently, mutagenicity and phytotoxicity tests were performed on different biological sequencing batch reactor (SBR) systems, which studied for efficiency of effectiveness in removing toxic substances from textile wastewaters and a comparison was made to evaluate the ecotoxicity results observed before and after wastewater treatment (Giorgetti et al. 2011). The biodegradation products of Congo red and textile industry effluent by *Pseudomonas* sp. SU-EBT were found to be non-phytotoxic to *Sorghum bicolor*, *Vigna radiata, Lens culinaris* and *Oryza sativa* plants as compared to Congo red and textile industry effluent (Telke et al. 2010).

7 Conclusions

Presence of color and its causative compounds has always been undesirable in water used for either industrial or domestic purposes. Amongst complex industrial wastewater with various types of coloring agents, dye wastes are predominant. The color in wastewater is highly visible and affects aesthetics, water transparency and

gas solubility in water bodies and their toxicity has been investigated in numerous researches. Dyes are difficult to be decolorized due to their complex structure, synthetic origin and recalcitrant nature. Hence, it becomes obligatory to remove them from industrial effluents before being disposed into water bodies. The degradation and decolorization of textile dyes within safer limits prior to release from treatment plant is essential for environmental safety. In this context, several workers have reported degradation and decolorization of dyes by using bacteria and fungus under different environmental conditions (pH, temperature, salinity, anaerobic/aerobic, nutrients, etc.). Besides, many researchers have studied the use of bacterial consortium for dye degradation. In addition to the bacterial consortium, a detailed study is needed for the use of bacterial granules in bioremediation of dye wastewaters.

8 Future Prospectives

Biodegradation of textile dyes using different microbes, like bacteria, fungi, and algae has become a promising approach for the treatment of different dyes from industrial wastewaters. To improve the degradation potential of microorganisms, they can be exposed gradually to higher concentration of dyes which will ultimately make them acclimatize and evolve. It has been observed that the microbial communities adapting to higher concentrations of dyes showed better decolorization ability. In this process, the up regulated genes and proteins can be explored further for designing the microbes with enhanced degradation capabilities. Development of aerobic granules is a promising technique for the treatment of wastewater with high strength of textile dye. This granular system may be helpful in many conditions and can be operated for the treatment of larger organic loads. The granular system has good settling capacity which can treat the effluents better than any conventional systems. The formation of these granules and mechanisms of degradation of high organic loads and their application for different industrial effluent needs to be explored in depth for their application at a large scale for removal of dyes from wastewater.

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Application of Extremophilic Microorganisms in Decolorization and Biodegradation of Textile Wastewater

M.A. Amoozegar, M. Mehrshad and H. Akhoondi

1 Introduction

The release of colored wastewater from textile industries in the ecosystem has been a great environmental as well as public health concern over the decades. Textile industry is a promising market due to the customer's increasing demand for new products. To fulfill these demands, textile industries are using selective dyestuffs among 100,000 different commercially available dyes (Husain 2006). Over 20–40 % of the dyes used in textile industries released to the environment via effluent discharge (Song et al. 2008) which causes serious environmental problems. However, growing importance of green practices encourages the adaption of microbe-based wastewater treatment technologies for textile effluents (Christie 2007). Several physical, chemical and physicochemical methods have been used for textile wastewater treatment, but each of them has their own advantages and disadvantages. Physico-chemical methods of wastewater treatment are not only costly methods, but also, some cases, very difficult to apply. Biological degradation and decolorization using microorganisms, on the contrary, provides inexpensive, effective, and specific, less energy intensive and eco-friendly methods for textile wastewater treatment (Robinson et al. 2001).

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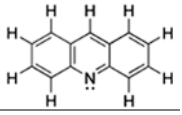
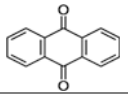
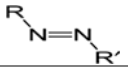
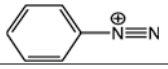
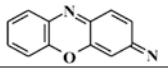
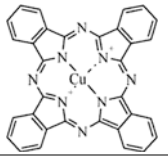
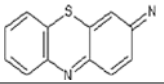
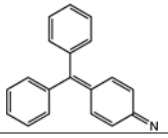
2 Textile Dyes

Textile industry is a growing sector which, in turn, uses of textile dyes in a large quantity (Dos Santos et al. 2007). Around 100,000 commercially available dyes can be classified according to their chromophoric groups, reactive groups, or even their applications (Husain 2006; Dos Santos et al. 2007). Considering their chromophoric groups, they fall into nine major classes as reflected in Table 1 (Rodríguez Couto 2009).

Azo dyes constitute more than half of commercial dyes available in the market. Any synthetic organic dyes, that contain nitrogen in azo group (–N=N–) as part of their molecular structures, fall into this class of dyes. Now-a-days more than 2000 different azoic dyes are being used in different industries and most of them are also

Table 1 Chromophoric groups of textile dyes

Chromophoric group	Structure
Acridine	
Anthraquinone	
Azo	
Diazonium	
Nitro	
Oxazine	
Phthalocyanine	
Thiazine	
Triarylmethane	

used in textile industries (Amoozegar et al. 2011). Textile process consists of several steps. Dying and scouring steps are the main sources of color in wastewaters and effluents (Dos Santos et al. 2007).

3 Textile Wastewater Effluents Properties

Industrial effluents treatment approaches should be designed in a way to meet the goals of protecting the assimilative capacity of surface waters; saving different kinds of life; preserving or restoring the aesthetic and recreational value of surface waters and protecting human beings from adverse effects of deteriorating water quality conditions. Selection among different wastewater treatment facilities is based on the knowledge of different factors, like physical, chemical and biological characteristics of wastewater, the quality of water reservoirs (rivers, ponds, streams, etc.) must be maintained in which the wastewater is to be released or the quality that the wastewater should meet environmental standards after treatment for reuse for various purposes. The main physical characteristics of industrial wastewaters are their solid content, color, odour and temperature. However, chemical characteristics of wastewaters are divided into two main classes: organic and inorganic characteristics. Inorganic chemicals, that enable us to choose the proper method for the wastewater treatment, are varying according to the wastewater origin. In organic factors include free ammonia, organic nitrogen, nitrites, nitrates, organic and inorganic phosphorous, chloride, sulphate, heavy metals, hydrogen sulphide, oxygen, methane and carbon dioxide. In order to assess the organic content of wastewaters, biological oxygen demand (BOD), chemical oxygen demand (COD), total organic compound (TOC) and volatile organic compounds (VOC) need to be determined carefully, as they have a great impact on choosing proper biological treatment technology. Typical characteristics of textile industry wastewater are presented in Table 2 (Al-Kdasi et al. 2004).

The textile industries wastewaters are a complex mixture of salts, acids, heavy metals, organochlorine-based pesticides, pigments, dyes etc. Dyes and dyestuff are of primary importance in textile manufacturing. In discriminate release of colored

Table 2 Composite textile industry wastewater characteristics

Parameters	Values
pH	7.0–9.0
Biochemical oxygen demand (mg l^{-1})	80–6,000
Chemical oxygen demand (mg l^{-1})	150–12,000
Total suspended solids (mg l^{-1})	15–8,000
Total dissolved solids (mg l^{-1})	2,900–3,100
Chloride (mg l^{-1})	1,000–1,600
Total kjeldahl nitrogen (mg l^{-1})	70–80

textile effluents to the environment HAS undesired impact on neighboring receptor water bodies because of presence of toxic reactive dyes and dark coloration. Due to environmental and health effects of dyes released in textile industry wastewater made the textile wastewater decolorization a subject to scientific scrutiny (Christie 2007).

Textile dyeing industries are facing problems to meet the green practices standards for safe discharge of wastewater due to complex nature and hard-to-treat by conventional methods. Therefore, in recent years, biological decolorization, using microorganisms capable of decolorizing and detoxifying the synthetic dyes, has been considered as a promising and environmentally benign method (McMullan et al. 2001).

4 Microbial Degradation of Textile Dyes

Amongst different decolorization methods for textile wastewaters; the biological methods seem to be most applicable. Bacteria, fungi and yeasts could be used based on their ability to decolorize dyes in the wastewater through aerobic, anaerobic or anaerobic/ aerobic systems. Among different techniques for wastewater decolorization, the live or dead microbial biomass adsorption has a great significance in biorecovery of dyes after decolorization of the effluents (McMullan et al. 2001). Some of microorganisms that are able to decolorize textile dyes, are of different taxonomic groups and their efficiency in dye removal has been summarized in Table 3. Some microbes show a very high efficiency in waste water decolorization which is attributed to their growth rate and versatile metabolism. However, harsh condition of wastewater effluents poses a limiting factor for mesophilic microorganisms to remediate the wastewaters properly. But the ability of extremophilic microorganism to survive in such harsh condition has some advantages over other mesophilic microorganisms for the bioremediation of colored wastewaters.

5 Extremophilic Microorganisms

Extremophiles are organisms that are adapted to grow optimally at or near to the extreme ranges of environmental variables. Most of them thrive under conditions that are clearly hostile from a human perspective. Extremophiles can fall in different categories based on single environmental extreme they survive. Different categories of extremophiles include acidophile, alkaliphile, endolith, halophile, hyperthermophile, hypolith, metallotolerant, oligotroph, piezophile, psychrophile, radioresistant, thermophile, toxitolerant and xerophile (Horikoshi 2011). Besides, there are many extremophiles which fall in two or more of above categories. They represent the polyextremophiles which refer to microorganisms adapted to two or more different environmental extremes (Rothschild and Mancinelli 2001).

Table 3 Different microbes used in microbial decolorization of textile dyes

Microorganisms	Dye (concentration)	Efficiency (time)	Medium NaCl content	Reference
Brevibacillus panacihumi strain ZB1,	Acid orange 7	98 % (2 h)		Bayet al. (2014)
Lysinibacillus fusiformis strain ZB2				
Enterococcus faecalis strain ZL				
Pseudomonas putida	Navy-Blue HER (100 mg l^{-1})	84.78 % (5 h)		Preethi et al. (2014)
Bacillus subtilis				
Escherichia coli				
Enterobacter sp. SXCR	Congo red (200 mg l^{-1})	100 % (93 h)		Prasad and Aikat (2014)
Saccharomyces cerevisiae	Congo Red			Kumar et al. (2013)
Lactobacillus sporogenes				
Deinococcus radiodurans R1	Malachite green (200 mg l^{-1})	97.20 %		Lv et al. (2013)
Micrococcus sp. strain BD15	Malachite Green (100 mg l^{-1})			Du et al. (2013)
Shewanella oneidensis MR-1	Mixture containing methyl orange (10 mM) and naphthol green B (10 mM)	>95 % (25 h)		Cao et al. (2013)
Shewanella aquimarina	Acid red 27 (200 μM)	100 % (5 h)	50 g l^{-1}	Meng et al. (2012)
Pleurotus ostreatus	Crystal violet (20 mg l^{-1})	92 % (10 days)		Kunjadia et al. (2012)
Staphylococcus sp. NIU-K1	Reactive black 5 (200 mg l^{-1})	>50 % (7 days)	60 g l^{-1}	Chen et al. (2011)
	Reactive blue 160 (200 mg l^{-1})			
	Reactive red 198 (200 mg l^{-1})			

(continued)

Table 3 (continued)

Microorganisms	Dye (concentration)	Efficiency (time)	Medium NaCl content	Reference
Exiguobacterium sp. NIU-K2	Reactive black 5 (200 mg l^{-1})	>50 % (7 days)	60 g l^{-1}	Chen et al. (2011)
	Reactive blue 160 (200 mg l^{-1})			
	Reactive red 198 (200 mg l^{-1})			
Exiguobacterium sp. NIU-K4	Reactive black 5 (200 mg l^{-1})	>50 % (7 days)	60 g l^{-1}	Chen et al. (2011)
	Reactive blue 160 (200 mg l^{-1})			
	Reactive red 198 (200 mg l^{-1})			
Klebsiella sp. P11	Reactive black 5 (200 mg l^{-1})	>50 % (7 days)	60 g l^{-1}	Chen et al. (2011)
	Reactive blue 160 (200 mg l^{-1})			
Comamonas sp. UVS	Reactive Blue HERD (50 mg l^{-1})	98 % (6 h)		Jadhav et al. (2011)
Halomonas sp. IP8	Cibacron Black (50 mg l^{-1})	60 % (8 h)	50 g l^{-1}	Pourbabee et al. (2011)
Bacillus cereus	Reactive red195 (200 mg l^{-1})	96 % (20 h)		Modi et al. (2010)
Pseudomonas sp.	Reactive Blue 13 (200 mg l^{-1})	83.2 % (70 h)		Lin et al. (2010)
Pseudomonas aeruginosa	Remazol Orange (200 mg l^{-1})	94 % (24 h)		Sarayu and Sandhya (2010)
Micrococcus glutamicus NCIM 2168	Reactive Green 19 (50 mg l^{-1})	100 % (42 h)		Saratale et al. (2009)
Enterobacter EC3	Reactive Black 5 (1,000 mg l^{-1})	92.56 % (36 h)		Wang et al. (2009b)
Mutant *Bacillus* sp. ACT2	Congo Red (3,000 mg l^{-1})	12–30 % (37–48 h)		Gopinath et al. (2009)
Lactobacillus acidophilus	Water and oil soluble azo dyes (6 mg l^{-1})	86-100 % (36 h)		Chen et al. (2009a, b)
Lactobacillus fermentum				

(continued)

Table 3 (continued)

Microorganisms	Dye (concentration)	Efficiency (time)	Medium NaCl content	Reference
Geobacillus stearothermophilus UCP 986	Orange II (0.050 mM)	96–98 % (24 h)		Evangelista-Barreto et al. (2009)
Aeromonas hydrophila	Reactive Red 198 (300 mg l^{-1})	60.20 %		Hsueh et al. (2009)
	Reactive Black 5 (300 mg l^{-1})	80.90 %		
	Reactive red 141 (300 mg l^{-1})	66.50 %		
	Reactive blue 171 (300 mg l^{-1})	36.00 %		
	Reactive Yellow 84 (300 mg l^{-1})	33.70 %		
Aeromonas hydrophila	Reactive Red 141 (3,800 mg l^{-1})	100 % (48 h)		Chen et al. (2009a)
Mutant *Escherichia coli* JM109 (pGEX-AZR)	Direct Blue 71 (150 mg l^{-1})	100 % (12 h)		Jin et al. (2009)
Bacillus sp. VUS	Navy blue 2GL (50 mg l^{-1})	94 % (18 h)		Dawkar et al. (2009)
Citrobacter sp. CK3	Reactive red 180 (200 mg l^{-1})	96 % (36 h)		Wang et al. (2009a)
Acinetobacter calcoaceticus NCIM-2890	Direct brown MR (50 mg l^{-1})	91.3 % (48 h)		Ghodake et al. (2009)
Bacillus sp.	C.I. Reactive orange 16 (100 mg l^{-1})	88 % (24 h)		Telke et al. (2009)
Enterococcus gallinarum	Direct black 38 (100 mg l^{-1})	100 % (20 days)		Bafana et al. (2009)
Pseudomonas sp. SU-EBT	Congo red (1,000 mg l^{-1})	97 % (12 h)		Telke et al. (2009)
Brevibacillus laterosporus MTCC 2298	Golden yellow HER (50 mg l^{-1})	87 % (48 h)		Gomare et al. (2009)
Bacillus sp.	Congo red (100-300 mg l^{-1})	100 % (24-27 h)		Kannappan et al. (2009)
Sphingomonas paucimobilis	Malachite green	100 % (24 h)		Ayed et al. (2009)

(continued)

Table 3 (continued)

Microorganisms	Dye (concentration)	Efficiency (time)	Medium NaCl content	Reference
Pseudomonas sp. SUK1	Reactive Red 2 (100 mg l^{-1})	95 % (18 h)		Kalyani et al. (2008)
Vibrio harveyi strain TEMS1	Acid black 210 (100 mg l^{-1})	93.9 % (24 h)	5 g l^{-1}	Ozdemir et al. (2008)
	Acid black 24 (100 mg l^{-1})	39.4 % (24 h)		
	Acid blue 7 (100 mg l^{-1})	4 % (24 h)		
	Acid green 20 (100 mg l^{-1})	16.3 % (24 h)		
	Acid yellow 36 (100 mg l^{-1})	1.5 % (24 h)		
Rhizobium radiobacter MTCC 8161	Reactive red 141 (50 mg l^{-1})	90 % (48 h)		Telke et al. (2008)
Comamonas sp. UVS	Direct red 5B (1,100 mg l^{-1})	100 % (13 h)		Jadhav et al. (2008)
Exiguobacterium sp. RD3	Navy blue HE2R (50 mg l^{-1})	91 % (48 h)		Dhanve et al. (2008)
Shewanella putrefaciens strain AS96	Reactive blazck-5 (100 mg l^{-1})	100 % (6 h)	40 g l^{-1}	Khalid et al. (2008b)
	Direct red-81 (100 mg l^{-1})	100 % (8 h)		
	Acid red-88 (100 mg l^{-1})	100 % (8 h)		
	Disperse orange-3 (100 mg l^{-1})	100 % (8 h)		
Pseudomonas sp. SUK1	Red BLI (50 mg l^{-1})	99.28 %		Kalyani et al. (2009)
	Reactive Navy blue RX (50 mg l^{-1})	85.33 %		
	Reactive Red M5B (50 mg l^{-1})	92.33 %		
	Reactive red 6BI (50 mg l^{-1})	97.30 %		
	Reactive red HE18 (50 mg l^{-1})	93.49 %		
	Reactive red HE3B (50 mg l^{-1})	93.47 %		

(continued)

Table 3 (continued)

Microorganisms	Dye (concentration)	Efficiency (time)	Medium NaCl content	Reference
	Reactive orange HE2R (50 mg l^{-1})	99.29 %		
	Reactive orange M2R (50 mg l^{-1})	90.50 %		
Comamonas sp. UVS	Direct red 5B	100 % (13 h)		Umesh et al. (2008)
Coriolus versicolor f. *antarcticus*	Malachite green			Diorio et al. (2008)
Bacillus fusiformis KMK5	Disperse blue 79 (1,500 mg l^{-1})	100 % (48 h)		Kolekar et al. (2000)
	Acid orange 10 (1,500 mg l^{-1})			
Trametes pubescens	Reactive black 5			Enayatzamir et al. (2008)
Schizophyllum sp.	Congo red			Li and Jia (2008)
Phanerochaete chrysosporium	K-2BP			Gao et al. (2008)
Trametes pubescens	Reactive black 5			Rodríguez Couto et al. (2008)
Aspergillus fumigatus	Reactive blue 19			Wang and Hu (2008)
Clostridium bifermentans SL186	Reactive red 3B-A (100 mg l^{-1})	>90 % (36 h)		Joe et al. (2008)
	Reactive black 5 (100 mg l^{-1})			
	Reactive yellow 3G-P (100 mg l^{-1})			
Halomonas sp. GTW	Reactive brilliant red K-2BP (100 mg l^{-1})	100 % (24 h)	150 g l^{-1}	Guo et al. (2008a)
	Acid red G (50 mg l^{-1})	100 % (24 h)		
	Acid Red B (50 mg l^{-1})	100 % (24 h)		
	Acid scarlet GR (50 mg l^{-1})	90 % (24 h)		
	Acid black 10B (50 mg l^{-1})	90 % (24 h)		
	Reactive brilliant red X-3B (50 mg l^{-1})	60 % (24 h)		

(continued)

Table 3 (continued)

Microorganisms	Dye (concentration)	Efficiency (time)	Medium NaCl content	Reference
Shewanella decolorationis strain S12	Fast acid red GR (150 μM)	100 % (10 h)	5 g l^{-1}	Xu et al. (2007)
Gracilibacillus sp. GTY	Acid red B (100 mg l^{-1})	100 % (96 h)	150 g l^{-1}	Salah Uddin et al. (2007)
Halomonas sp. D2	Remazol black B (50 mg l^{-1})	72 % (96 h)	50 g l^{-1}	Asad et al. (2007)
	Remozal black N (50 mg l^{-1})	82 % (96 h)		
	Sulphonyl green BLE (50 mg l^{-1})	94 % (96 h)		
	Sulphonyl scarlet BNLE (50 mg l^{-1})	72 % (96 h)		
	Sulphonyl blue TLE (50 mg l^{-1})	56 % (96 h)		
	Maxilon blue (50 mg l^{-1})	37 % (96 h)		
	Entrazol blue IBC (50 mg l^{-1})	21 % (96 h)		
	Mixyure of above seven dyes (50 mg l^{-1})	100 % (120 h)		
Halomonas sp. A3	Remazol black B (50 mg l^{-1})	56 % (96 h)	50 g l^{-1}	Asad et al. (2007)
	Remozal Black N (50 mg l^{-1})	87 % (96 h)		
	Sulphonyl green BLE (50 mg l^{-1})	97 % (96 h)		
	Sulphonyl Scarlet BNLE (50 mg l^{-1})	60 % (96 h)		
	Sulphonyl blue TLE (50 mg l^{-1})	85 % (96 h)		
	Maxilon Blue (50 mg l^{-1})	46 % (96 h)		
	Entrazol blue IBC (50 mg l^{-1})	41 % (96 h)		
	Mixyure of above seven dyes (50 mg l^{-1})	100 % (120 h)		
Shewanella decolorationis S12	Acid red GR (150 mM)	100 % (68 h)		Xu et al. (2007)

(continued)

Table 3 (continued)

Microorganisms	Dye (concentration)	Efficiency (time)	Medium NaCl content	Reference
Halomonas sp. Gb	Remazol black B (50 mg l^{-1})	64 % (96 h)	50 g l^{-1}	Asad et al. (2007)
	Remozal black N (50 mg l^{-1})	82 % (96 h)		
	Sulphonyl green BLE (50 mg l^{-1})	95 % (96 h)		
	Sulphonyl scarlet BNLE (50 mg l^{-1})	74 % (96 h)		
	Sulphonyl blue TLE (50 mg l^{-1})	56 % (96 h)		
	Maxilon blue (50 mg l^{-1})	55 % (96 h)		
	Entrazol blue IBC (50 mg l^{-1})	32 % (96 h)		
	Mixyure of above seven dyes (50 mg l^{-1})	100 % (120 h)		
Fomes sclerodermeus	Malachite green			Papinutti et al. (2006)
Funa liatrogii	Acid black 52			Park et al. (2006)
Trametes hirsuta	Bromophenol blue			Rodríguez Couto et al. (2006)
	Methyl orange			
	Poly R-478			
Irpex lacteus	Reactive orange 16			Tavčar et al. (2006)
Rhodopseudomonas palustris AS1.2352	Reactive brilliant red	90 % (24 h)		Liu et al. (2006)
	X-3B			
Dichomitus squalens	Remazol brilliant blue R			Šušla et al. (2007)
	Reactive orange16			
	Copper(II) phthalocyanine			
Trametes hirsuta	Phenol red			Domínguez et al. (2005)
Pleurotus sajor-caju	Real textile effluen			Kamida et al. (2005)

(continued)

Table 3 (continued)

Microorganisms	Dye (concentration)	Efficiency (time)	Medium NaCl content	Reference
Phanerochaete chrysosporium	Direct black 38			Pazarlioglu et al. (2005)
	Direct brown 2			
	Direct red 23			
	Direct blue 15			
	Direct orange 26			
	Direct green 6			
	Tartrazine			
	Chrysophenin			
	Congo red			
Phanerochaete chrysosporium	Methyl violet			Radha et al. (2005)
	Acid orange			
	Acid red 114			
	Vat magenta			
	Methylene blue			
	Acid green			
Funa liatrogii	Reactive black 5			Mazmanci and Ünyayar (2005)
Pseudomonas aeruginosa NBAR12	Reactive blue 172	83 % (42 h)		Bhatt et al. (2005)
	(500 mg l^{-1})			
Trametes hirsuta	Lissamine green B			Rodríguez Couto and Sanromán (2005)
Phanerochaete sordida	Basic bue 22			Ge et al. (2004)
Bjerkandera adusta	Reactive black 5			Mohorčič et al. (2004)

(continued)

Table 3 (continued)

Microorganisms	Dye (concentration)	Efficiency (time)	Medium NaCl content	Reference
Trametes versicolor	Carpet dye effluent			Ramsay and Goode (2004)
Trametes hirsuta	Indigo carmine			Rodríguez Couto et al. (2004a,b)
	Lanaset marine			
Trametes hirsuta	Sella solid blue			Rodríguez Couto and Sanromán (2004)
Pleurotus pulmonarius	Remazol brilliant blue			Tychanowicz et al. (2004)
	Ethyl violet			
	Methyl violet			
	Methyl green			
	Brilliant cresyl blue			
	Methylene blue			
	Poly R-478			
	Congo red			
	Trypan blue			
	Amido black			
Aeromonas hydrophila	Red RBN (3,000 mg l^{-1})	90 % (8 h)		Chen et al. (2003)
Irpex lacteus	Remazol			Kasinath et al. (2003)
	Brilliant blue R			
Trametes versicolor	Acid Fuchsine			Rodríguez Couto et al. (2003)
	Indigo Carmine			
	Congo red			
Citrobacter sp.	Azo and triphenylmethane dyes (5 mM)	100 % (1 h)		An et al. (2002)
Trametes versicolor	Amaranth			Shin et al. (2002)
Paenibacillus azoreducens	Remazol black B (100 mg l^{-1})			Meehan et al. (2001)

(continued)

Table 3 (continued)

Microorganisms	Dye (concentration)	Efficiency (time)	Medium NaCl content	Reference
Phanerochaete chrysosporium	Poly R-478	98 % (24 h)		Rodriguez Couto et al. (2000)
Coriolus versicolor	Everzol			Kapdan et al. (2000)
	turquoise blue G			
Pseudomonas luteola	Reactive red 22			Chang and Lin (2000)
Desulfovibrio desulfuricans	Reactive orange 96	95 %		Yoo et al. (2000)
	Reactive red 120			
Sphingomonas sp. BN6	Acid azo dyes			Russ et al. (2000)
	Direct azo dyes			
	Amaranth			
Proteus mirabilis	RED RBN (1,000 mg l^{-1})	95 % (20 h)		Chen et al. (1999)
Trametes versicolor	Poly R-478			Leiding et al. (1999)
Bacteroides fragilis	Amaranth, orange II (100 mg l^{-1})	95 %		Bragger et al. (1997)
	Tartrazine (100 mg l^{-1})			
K1ebsi1e11a pneumonia R5-13	Methyl red (100 mg l^{-1})	100 % (168 h)		Wong and Yuen (1996)
Pycnoporus cinnabarinus	Remozal brilliant			Schliephake and Lonergan (1996)
	Blue R			
Pseudomonas cepacia 13NA	Acid orange 12	90 % (68 h)		Ogawa et al. (1986)
	Acid orange 20			
	Acid ted 88			
Pseudomonas sp.	Orange I (1,000 mg l^{-1})	90 % (35 h)		Kulla et al. (1983)
	Orange Il (1,000 mg l^{-1})			
Aeromonas hydrophila var 24 B	Various azo dyes (10-100 mg l^{-1})	50-90 % (24 h)		Idaka et al. (1978)

The exploration of extremobiosphere targets at discovery of extremophile microorganisms with new metabolisms, natural products, biocatalysts and other services (Schiraldi and De Rosa 2002).

Extremophilic microorganisms have many applications in biotechnology, medicine and industry. Extremozymes are one of the most important products of extremophiles not only because of their industrial application, but also because they can also be used as a model system for the study of stabilization and enzyme activation mechanisms of protein structure-functional properties (Demirjian et al. 2001). Enzymes of thermophilic, hyperthermophilic, alkaliphilic and psycrophilic groups of extremophilic microorganisms are the most promising for industrial applications (Van Den Burg 2003). Highly thermostable hydrolases, like cellulases, amylases, pectinases, chitinases, xylanases, lipases, proteases, pullulanases, glucose isomerases, alcohol dehydrogenases, and esterases with broad industrial application can be extracted from thermophilic and hyperthermophilic microorganisms. Some other kinds of thermostable enzymes, like DNA polymerase, DNA ligase, restriction enzymes and phosphatase with application in molecular biology and medicine, are also produced by extremophilic microorganisms (Gomes and Steiner 2004; Egorova and Antranikian 2005). Psychrophilic microorganisms with hydrolases, like B-glucanases, pectinases, cellulases, and proteases, have some potential applications in the waste treatment and food industry, while cold adapted enzymes are of emerging interest in the detergent production industries (Cavicchioli et al. 2011). Alkaliphilic microorganisms are source of enzymes which are stable at high pH values. Some examples of these enzymes with application in industrial sector are elastase and keratinase in cosmetic industries and some other hydrolases, like cellulases, proteinases, amylases, lipases with application in detergent production industries. Some of extremophilic microbial enzymes have the potential to be used in the biosensor systems (D'Auria et al. 2002).

Along with these enzymes, other biologically active substances and biopolymers of extremophiles have also put their mark on industry and medicine, osmoprotectant compounds, like ectoin and betain, bacteriorhodopsin (Oesterhelt and Stoeckenius, 1973; Trivedi et al. 2011), β-carotene (León et al. 2003; Lamers et al. 2008), halocins and microhalocins (Haseltine et al. 2001; O'connor and Shand 2002) and long-chained poly unsaturated fatty acids are some examples of biologically active substances of extremophiles with biotechnological applications. Extremophiles are also source of useful biopolymers (Barbara et al. 2012) like bioplastics (Lu et al. 2009) and exopolysaccharides (Nicolaus et al. 2010). Gas vesicle and liposomes of some halophilic bacteria can be used for vaccine development (Stuart et al. 2001, 2004).

One of the most interesting applications of extremophilic microorganisms is their potential in bioremediation. Bioremediation is one of the most effective and successful cleaning techniques for removal of toxicants from polluted environments (Kumar et al. 2011). There are some strains of psychrophilic (Aislabie et al. 2006) and halophilic microorganisms (Nicholson and Fathepure 2004, 2005; Liebgott et al. 2007; Feng et al. 2012) which have been reported to degrade hydrocarbon compounds. These strains have the potential to be used for oil spill or oilfield

remediation. Heavy metals are one of the most important environmental concerns, as their accumulation through food chain can cause serious health problems. Some halophilic microorganisms have been reported with the ability to remediate heavy metal pollution through absorption. Hence, they can be used as biological agents for removal of heavy metals from highly saline industrial wastewaters (Popescu and Dumitru 2009; Francis et al. 2000; Amoozegar et al. 2012). Exploring extremophilic microbial potential for bioremediation purposes will result in using organisms that have a high tolerance to the environmental harsh conditions of salinity and high temperature for in situ and ex situ remediation in bioreactors (Kumar et al. 2011).

6 Extremophilic Microorganisms and Textile Dyes

In discriminate release of colored wastewater into the environment has become today a serious ecological obstacle. Therefore, green practices are tried to assort a proper decolorization or degradation approaches for the colored industrial effluents before releasing them to the environment. Extremophilic microorganisms are one of the most attractive biological tools for bioremediation in the harsh condition of most effluents. Factors, like pH, temperature, salinity, and dye concentration have a great effect on dye removal by microorganisms.

In most cases, sodium concentrations above 3,000 ppm moderately inhibit most of microbial activities except for halotolerant and halophilic microbes which can tolerate or may require salt to be active (Anjaneya et al. 2011). Halophilic microorganisms can be found in hypersaline environment which are widely distributed around the world. These microorganisms are a group of extremophiles which not only cope with salinity as an environmental extreme (Oren 2011), but also subjected to other kinds of extreme conditions, like high pH values, high or low temperature, low oxygen availability, pressure, heavy metals and/ or other toxic compounds (Oren 2002). Based on optimal growth with respect to the NaCl concentration, halophilic microorganisms fall into two physiological groups which include extreme halophiles (optimal growth at 2.5–5.2 M NaCl) and moderate halophiles (optimal growth at 0.5–2.5 M NaCl). Besides, there are some non-halophilic microorganisms with optimal growth in medium with less than 0.2 M NaCl concentration, but also they are able to tolerate high concentration of NaCl and hence defined as halotolerant microbes (Kushsner and Kamekura 1988).

In textile dyeing process, different salts are used for different purposes which include separating organic contaminants, inducing dyestuff precipitation, and mixing with concentrated dyes to standardize them. Addition of sodium hydroxide into dye bath to increase the pH could be another reason for elevated Sodium level (Khalid et al. 2008a). High salt concentration could decrease the decolorization process because of inability of microorganism to be active in this condition. Therefore, halophilic and halotolerant microorganisms can be only useful in this respect.

Fig. 1 Decolorization of azo dye, remazol black B by *Halomonas* sp. D2. The *right tube* contains decolorization medium without inoculation and the *left tube* is inoculated with the strain and it shows decolorization after 96 h incubation

Exiguobacterium acetylicum, *Exiguobacterium indicum* and *Staphylococcus gallinarum* are able to decolorate Reactive Black 5 dye in medium containing 60000 ppm NaCl (Chen et al. 2011). Halotolerant *Exiguobacterium* sp. has the ability to efficiently decolorize azo dye X-3B at 15 % (w/v) NaCl (Tan et al. 2009). Three halophilic and halotolerant strains of the genus *Halomonas* have been reported with the high ability of azo dye decolorization (Fig. 1) in a wide range of NaCl concentration (up to 20 % w/v), temperature (25–40 °C) and pH (5–11) after 5 days of incubation (Asad et al. 2007).

Halomonas sp. strain GTW, which was isolated from the coastal sediments, is able to grow well and completely decolorize K-2BP (98 %) at 30 °C (Guo et al. 2008a). Azo dye decolorization has been also reported with *Shewanella aquimarina*, which is able to grow at up to 7 % (w/v) NaCl (Meng et al. 2012). Further, research also showed that *Shewanella putrefaciens* strain AS96 could be effective for treatment of colored industrial wastewater containing high salt concentration up to 60 (g l^{-1}) NaCl (Khalid et al. 2008b). *Psychrobacter alimentarius* strain KS23 and *Staphylococcus equorum* strain KS26 which were isolated from seawater sediment, were able to decolorize three reactive dyes including Reactive Black 5, Reactive Golden Ovifix, and Reactive Blue BRS in medium with range of 0–100 g l^{-1} NaCl concentration (Khalid et al. 2012). A halophilic strain was isolated from a solar sea-saltern in Turkey and found to be resistant against Lanaset Navy R and Lanaset Brown B dyes. According to 16S rRNA gene sequence analysis, the strain C-22 belongs to the genus *Halobacillus* which was the first report for its ability of this genus in azo-metal complex dyes decolorization (Demirci et al. 2011). A novel halotolerant bacterium *Gracilibacillus* sp. GTY was isolated, showing the ability of dye decolorization by growing and resting cells, as well as by extracted azo reductase. This strain was able to grow in the media with 15 % (w/v) of NaCl. Decolorization efficiency of the strain grown in very low, or high concentrations did not suggest that salt concentrations controlled the

production of azo reductase (Salah Uddin et al. 2007). Decolorization of Acid Black 210 by a *Vibrio harveyi* TEMS1, isolated from coastal seawater of Turkey, has been also studied. Decolorization studies were performed in medium with 5 g l^{-1} NaCl concentration (Ozdemir et al. 2008). *Shewanella algae* and *Shewanella marisflavi*, isolated from marine environments, demonstrated better azo dye decolorization ability as compared to their strains isolated from non-saline sources. *S. algae* and *S. marisflavi* are able to decolorize amaranth dye at up to 100 (g l^{-1}) NaCl or Na_2SO_4 (Liu et al. 2013). The moderately halotolerant bacterial strain *Bacillus firmus* effectively decolorized Polar red B (an azo dye) in synthetic saline wastewater medium. Decolorization occurred in a wide range of sodium chloride (1–6 %, w/v), dye (5–100 mg l^{-1}) and at pH range of 6–10 after 24 h of incubation. Cell immobilization studies of this strain clearly indicated that color removal was significantly higher in immobilized cell systems especially at salt concentrations higher than 4 % (Ogugbue et al. 2011).

Thermophilic microorganisms are a group of extremophiles which are able to thrive and grow at high temperatures from 45 to 122 °C. Many of thermophiles belong to the domain *Archaea* (Brock 1967). Thermophilic microbes are among well studied extremophiles, as their enzymes are well suited for industrial processes (Prieur 2007). Based on advantages of these organisms, natural and artificial hot environments have been widely screened for novel thermophilic microorganisms and bioactive compounds (Torkamani et al. 2008; Kublanov et al. 2009). The biggest disadvantage of such microorganisms for biotechnological application is higher equipment corrosion and liquid evaporation which haven't been properly tackled for large scale operations. Eight thermophilic consortia were separated from Spain's northwest hot springs with the ability of Reactive Black 5 dye decolorization at 65 °C. From these consortia, 3 bacterial strains were isolated which showed closest similarity to *Anoxybacillus pushchinoensis*, *Anoxybacillus kamchatkensis* and *Anoxybacillus flavithermus* (Deive et al. 2010; Sanromán et al. 2010). *Anoxybacillus rupiensis* is a thermophilic bacterium which was isolated from hot springs of Maharashtra state in India. When reddish-black effluents of dyeing unit of a textile factory in Aurangabad, Maharashtra with the pH of 10.5 were subjected to this bacterium for decolorization, the results showed 75 % decolorization through degradation at 60 °C in eight days (Gursahani and Gupta 2011). Batch assays of mesophilic (30 °C) and thermophilic (55 °C) anaerobic consortia were studied for decolorization of Reactive Red 2 and Reactive Orange 14 azoic dyes. The contribution of fermentative and methanogenic microorganisms in both temperatures was also evaluated. Results revealed that the application of thermophilic anaerobic treatment was an interesting option for the reductive decolorization of azo dyes compared to mesophilic conditions (Dos Santos et al. 2005). Two facultative anaerobic bacteria consortia and a bacterial isolate DTB showed the ability of decolorization of textile colored discharge effluents. Both cultures were able to grow and decolorize the effluents at elevated temperatures up to 60 °C. These isolated bacteria can be used for textile colored wastewater treatment which is normally discharged at elevated temperatures (Banat et al. 1997).

Alkaline-adapted microorganisms can be divided into two main groups which include; alkaliphiles and alkalitolerants. The term alkaliphiles is restricted to microorganisms that require alkaline media for growth and their optimum growth rate could be observed in at least two pH units above neutrality. Alkalitolerants are able to grow at pH values more than 9 or 10, but their optimum growth rates occur around neutrality or less (Grant and Tindall 1986; Jones et al. 1994). One of the most important characteristic of textile wastewater effluents is their alkalinity. Using alkaliphiles is inevitable for bioremediation process, because they are adapted to sustain in the harsh conditions of dyeing process. An obligate alkaliphilic bacterium *Bacillus cohnii* MTCC 3616 was used for textile azo dye Direct Red-22 aerobic decolorization, showing 95 % efficiency for decolorization at 37 °C and pH 9 in 4 h incubation under static conditions. The decolorization occurred in a broad pH range (7–11), temperature (10–45 °C) and salinity (1–7 %) (Prasad and Rao 2013). Alkaliphilic bacterial strain, *Bacillus badius*, isolated from a lake in India, showed high potential towards the degradation of azo dyes up to 100 mg l^{-1} in 24 h under aerobic condition. Azoreductase enzyme, which is able to cleave azo and nitro groups of various compounds, has also been purified from this strain (Misal et al. 2011). *Clostridium bifermentans* strain SL186 was isolated from a contaminated site and investigated for Reactive Red 3B-A, Reactive black 5 and Reactive Yellow 3G-P dyes decolorization. The bacterium retained decolorizing activity over a wide range of pH values (6–12) with optimum activity at pH 10 (Joe et al. 2008).

7 Polyextremophilic Microorganisms and Textile Dyes

Extremophilic microorganisms are able to live under different types of stressful conditions which provide them an opportunity to extend habitable space on earth which can support essential biological processes like cell growth and main metabolism. It is important to note that there are some kinds of extremophiles that are adopted to grow optimally under multiple stress factors, known as polyextremophiles. The term polyextremophiles was first coined by Rothschild and Mancinelli (2001) to describe this group of micro-organisms. In comparison to other types of microorganisms that will die or become dormant in harsh conditions, extremophiles and polyextremophiles are able to grow with active metabolism in the environmental harsh conditions, as they have application in such environments. Different types of polyextremophiles are adopted to different combinations of environmental extremes i.e. high temperature and low pH, high temperature and high pH, high temperature and high pressure, low temperature and low pressure and high salt concentration and high pH. The chemolithotrophic archaum *Sulfolobus acidocaldarius* can easily flourish at 75 °C at pH 2–3, thus showing adaptation to grow in high temperature and low pH (Reysenbach et al. 2006). The archaum *Thermococcus alcaliphilus*, which is able to grow at 90 °C and at pH 10.5, was first isolated from shallow marine hydrothermal springs (Keller et al., 1995). *Thermococcus barophilus*, which flourishes at 100 °C and needs 15–17.5 MPa at the

highest temperature, is an example of polyextremophiles at high temperature and high pressure extremes (Marteinsson et al., 1999). Most of deep sea bacteria are adapted to low temperature (2–4 °C) and high pressure (50–110 MPa) as polyextremophiles. Soda lakes are the source of haloalkaliphilic microorganisms like *Natronobacterium gregoryi* which can thrive in high pH and high salt concentrations (Tindall et al. 1984). These are some examples of poly environmental extremes which were explored for polyextremophilic life. Culture independent methods revealed microbial life in the environmental extremes wherein we don't have expectation of life (Antunes et al. 2011; Stock et al. 2012). This helps us to understand the true shape of habitable space on earth.

We are indirectly benefitted by extremophilic and polyextremophilic microorganisms which are used in biotechnology and bioremediation (Rothschild and Manicelli 2001). Many of industrial wastes have harsh conditions which make extremophiles and polyextremophiles a good choice for their treatment before releasing them into the environment. Understanding the physical, geochemical and biological limits of life is an emerging biotechnological interest in view of applications of extremophiles, polyextremophiles and their biomolecules in industrial processes and waste treatments (Podar and Reysenbach 2006; Taylor et al. 2012).

Textile colored effluent is one of the complex industrial effluents wherein microorganisms, which are used for their decolorization, are subjected to a harsh condition due to the high salinity, alkaline pH and high temperature of the effluents (McMullan et al. 2001; Kandelbauer and Guebitz 2005). Extremophilic microorganisms, which are naturally adapted to this harsh condition, are a perfect choice for treatment of the wastewaters. As these effluents have a combination of environmental extremes, polyextremophilic microorganisms attracted the attention of scientists and became the subject of scientific scrutiny for finding new highly capable microorganisms for textile colored wastewater treatment.

There are some examples of polyextremophilic microorganisms which have been able to decolorize textile wastewaters. Four fungal strains, isolated from environmental samples, were assayed for their ability for Brown GR dye decolorization. These strains belonged to the genus *Aspergillus* which showed the highest decolorization efficiency at pH 4 and 2 % (w/v) NaCl concentration (Singh et al. 2013).

A moderately halophilic and alkalitolerant bacterium was isolated from the salty effluents of textile industries in central Iran with remarkable azo dyes decolorizing ability over wide ranges of pH (7–11) and temperature (25–45°C), in presence of NaCl and Na_2SO_4 (0.5–1.5 M) under both anaerobic and aerobic conditions (Fig. 2). According to 16S rDNA sequence similarity analysis, this strain belonged to the genus *Halomonas* with the highest similarity to *Halomonas axialensis* (Pourbabaee et al. 2011).

Bacillus sp. strain SF was isolated from wastewater drain of textile finishing company and showed growth at pH 9.3–10 and 60–65 °C temperature. This alkali-thermophilic microorganism has the ability of azo dye decolorization. An

Fig. 2 Microbial decolorization of textile dyes by *Halomonas* sp. strain IP8. From left to right tube *T1* contains decolorization medium with remazol black B without inoculums and *T2* tube is the same medium after decolorization with *Halomonas* sp. strain IP8; tube *T3* contains decolorization medium with remazol black GF without inoculums and *T4* tube is the same medium after decolorization with *Halomonas* sp. strain IP8 and *T5* tube contains Cibacron Red 6B and *T6* tube shows decolorization of dye by *Halomonas* sp. strain IP8

NADH-dependent azoreductase was found to be responsible for the decolorization of azo dyes, showing optimum range of 8 to 9, and the temperature 80 °C for maximum activity (Paar et al. 2001; Maier et al. 2004).

8 Conclusion

Proper decolorization of colored wastewater effluents of textile industries is a major environmental concern. Amongst different chemical, physical and biological treatment methods, the biotechnological approaches based on microorganisms, are the most effective and environmental friendly methods. Different strains of microorganisms have shown the ability of textile dye decolorization. One of the most important factors, which have a great impact on the setting of a proper bioremediation plant for textile wastewater, is the effluent characteristics, high salinity, temperature and alkalinity. Extremophilic microorganisms and their bioactive molecules have been found to have a great potential for treatment of textile wastewaters. Most of researches concerning extremophilic microorganisms for bioremediation of textile waste waters have focused on their ability in decolorization, but their final products haven't been examined properly. Finding the microbial enzymes and genes responsible for decolorization in extremophilic microorganisms and using them for bioremediation is a future perspective.

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The Biodegradation of Azo Dyes by Actinobacteria

Azeem Khalid and Shahid Mahmood

1 Introduction

Synthetic dyes have wide application in the textile, leather, pharmaceutical, cosmetic, paper and food industry. According to an estimate, global production of synthetic dyes is more than 700,000 tonnes and textile sector consumes about 60 % of the total production of dyes (Robinson et al. 2001; Shinde and Thorat 2013). Since dyeing process is not very efficient, production of highly colored wastewater is enormous. The amount lost in wastewater is a function of the class of dyes and in general, their loss through discharge in the wastewater can be 2 % of the initial concentration of basic dyes to as high as 50 % of a reactive dye (Tan et al. 2000; Boer et al. 2004).

Azo dyes are aromatic compounds containing one or more azo (−N=N−) chromophores. Such dyes are considered to be electron-deficient compounds, as they possess the azo (−N=N−) and sulfonic acid (SO_3^-) electron withdrawing groups, resulting in a deficit of electrons in the molecule which renders the compound more sensitive to oxidative catabolism by bacteria. Hence, azo dyes tend to persist under aerobic environmental conditions (Rieger et al. 2002). Because of the persistence nature, azo dyes have negative impact on the environment in terms of total organic carbon (TOC), chemical oxygen demand (COD) and biological oxygen demand (BOD) (Saratale et al. 2009). Many synthetic dyes and their metabolic intermediate products are found to be toxic, mutagenic and carcinogenic (Dafale et al. 2010; Poljsak et al. 2010; Sellamuthu et al. 2011; Yang et al. 2013). The treatment of dye-contaminated wastewater in an environmentally safe manner is essentially required prior to its disposal. Various physical, chemical and biological strategies can be used for the treatment of azo dyes (Qin et al. 2007;

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Onat et al. 2010). However, physico-chemical methods generally produce a huge amount of additional residues that make these methods non eco-friendly (Sharma et al. 2013). Complete mineralization of dyes with microorganisms is one of the most viable options for remediation of industrial effluents containing azo dyes. The versatility of microorganisms and their various metabolic pathways can be used to target the major chemical classes of dyes (Mohanty et al. 2006; Sadettin and Donmez 2007; Liu et al. 2011; Saratale et al. 2011; Khalid et al. 2012; Prasad and Aikat 2014). Although a variety of culturable bacteria, fungi and algae, capable of degrading dyes through the oxidoreductase enzymes, have been intensively studied (Table 1), but a group of microorganisms, such as actinomycetes, has not previously been focused much for their biodegradability potential.

Actinomycetes belong to Enterobacteriaceae family and now, they are known for their degradation capabilities. Actinobacteria are considered a very attractive option for the production of many secondary bioactive compounds which are metabolites (Nathan et al. 2004; Jensen et al. 2005; Suthindhiran and Kannabiran 2009). Actinobacteria are a group of filamentous Gram positive bacteria which are known as prokaryotes with high GC contents (Stackebrandt et al. 1997; Deepika and Kannabiran 2009; Deepika et al. 2009). They are widely distributed in terrestrial and aquatic ecosystems (Chronakova et al. 2009), *Streptomyces* spp. in soil consist of 50 % of the total microbial population and play a crucial role in the recycling of refractory biomaterials by decomposing complex mixtures of polymeric materials from dead plants, animals and fungi. Considering their diverse habitat and role in the environment, these microorganisms can be studied for their potential for biodegradation and bioremediation of contaminated soil and aquatic environment.

Very recently, *Streptomyces* species have been exploited for the degradation of synthetic dyes (Lu et al. 2013; Gousterova et al. 2014). Researchers have developed a great interest in actinomycetes because of their high potential in the treatment of industrial waste (Gousterova et al. 2011). Actinobacteria are particularly suitable for the wastewater treatment, because of their different metabolic pathways working in a wide range of environmental conditions and survival under extreme conditions. Microorganisms, isolated from extreme environments, were found to be producers of enzymes and metabolites with promising properties (Lee et al. 2012; Mahajan and Balachandran 2012). In this chapter, the role of actinomycetes in the degradation of azo dyes is discussed thoroughly.

2 Environmental Concerns of Azo Dyes

Azo dyes and their intermediates are toxic, mutagenic and carcinogenic (Lu et al. 2010; Modi et al. 2010) and affect organisms in both aquatic and terrestrial systems (Puvaneswari et al. 2006). Among azo dyes, those produced from aromatic amines, such as benzidine and 4-biphenylamine, 4-aminobiphenyl, monoacetylbenzidine and acetylaminobiphenyl, pose a serious threat to the environment and human health (Manning et al. 1985; Cerniglia et al. 1986; Choudhary 1996; Chung 2000).

Table 1 Biodegradation of structurally different synthetic dyes by various microorganisms

Culture species	Dyes	Type of microorganisms	Comments	References
Pseudomonas aerogenosa	Direct orange, disperse brown, reactive green	Bacteria	About 94 % color removal of the dyes was observed after 7 days incubation under aerobic conditions	Ahmed (2014)
Bjerkandera adusta Dec1	Amaranth	Fungi	After 10 days, 98 % decolorization was achieved under aerobic conditions.	Gomi et al. (2001)
Enteromorpha sp.	Basic red 46 (BR46)	Algae	The maximum decolorization efficiency (83.45 %) was achieved with 4 g initial biomass of algae, after 5 h at temperature 25 °C	Khataee et al. (2013)
Aspergillus niger *Mucor racemosus*	Red HE7B, reactive red 141, yellow FN2R, reactive yellow 206	Fungi	Up to 94 % dyes degradation was achieved by fungi after 5 day incubation under static conditions	Balaji et al. (2012)
Aeromonas hydrophila	Crystal violet, Fuchsin green, brilliant green, malachite green	Bacteria	About 90 % color of dye was removed in 10 h under anaerobic conditions	Ren et al. (2006)
Trametes villosa	Cibacron blue 19, cibacron blue 20	Fungi	Both the dyes were degraded in 10 days of incubation under shaking conditions.	Machado et al. (2006)
Bacillus subtillis	Orange	Bacteria	More than 94 % decolorization was achieved in 23.7 h under static conditions.	Sha et al. (2014)
Pseudomonas fluorescen	Reactive orange-M2R, reactive blue-M58, reactive yellow-M4G, reactive black-B	Bacteria	The dye decolorization for the mentioned dyes was 59, 90, 77 and 79 % respectively within 16 days under shaking conditions.	Sriram et al. (2013)
Phanerochaete chrysosporium	aminobenzoic or aminosulphonic acids (diazo components)	Fungi	The decolorization was 80–89 % after 28 days under shaking conditions.	Martins et al. (2001)

(continued)

Table 1 (continued)

Culture species	Dyes	Type of microorganisms	Comments	References
Marasmius cladophyllus	Remazol brilliant blue R	Fungi	About 97 % of the dye was degraded in 16 days under static conditions.	Ngieng et al. (2013)
Entrobacter spp.	Congo red	Bacteria	Complete decolorization of the dye (200 mg L^{-1}) was observed within 93 h under static conditions	Prasad and Aikat (2014)
phanerochaete chrysosporium	Amido black B, procion blue-2G	Fungi	The dye decolorization was 20–100 % depending on the concentration of dye in 25 days under static conditions	Kumar et al. (2013)
Lyngbya sp. BDU 9001	Textile dye effluent	Cyanobacterium	The decolorization efficiency was 73 % at the 15th day of incubation	Henciya et al. (2013)
Bacillus cereus	Reactive red 195	Bacteria	97 % dye was degraded after 72 h under anaerobic conditions.	Modi et al. (2010)
Bacillus sp. VUS	Orange T4LL	Bacteria	Complete color removal of the dye was achieved within 24 h under static anoxic conditions.	Dawkar et al. (2010)
Enterococcus faecalis	Acid red 27, reactive red 2	Bacteria	Decolorization was between 95–100 % within 12 h under anaerobic conditions	Handayani et al. (2007)

A small amount of dye in the water (<1 mg l^{-1} for some dyes) can cause a very visible color change (Banat et al. 1996), which affects not only the aesthetic aspects of the water, but also raises environmental concerns (Lu et al. 2010; Modi et al. 2010). Previously, several researchers have reported the toxicity of synthetic dyes on ecosystems (Fraga et al. 2009; Osugi et al. 2009; Dafale et al. 2010). The effluents containing dyes affect the photosynthetic activity of aquatic plants and algae by changing the light and gas penetration into water bodies. Hence, wastewater is also considered very toxic to aquatic organisms, resulting in the disruption of the ecological balance (Modi et al. 2010). Also, azo dyes significantly increase the chemical and biological oxygen demand in water bodies, which also affects aquatic life indirectly (Sun et al. 2009).

Some dyes are reported to cause irritation allergy, dermatitis, eye irritation and respiratory tract problems in human beings (Keharia and Madamwar 2003; Kousha et al. 2012). Induction of bladder cancer in humans, splenic sarcomas, hepatocarcinomas and nuclear anomalies in some experimental animals were commonly observed (Rafii et al. 1997; Puvaneswari et al. 2006). Intestinal cancer in 1970's was of common occurrence in highly industrialized locations and linked to use of azo dyes (Wolff and Oehme 1974; Chung et al. 1978). Furthermore, the mutagenic activity of disperse azo dyes was also observed in *Salmonella* species (Ferraz et al. 2010). These studies suggest that the azo compounds in the water and the soil environment can affect the activity and composition of the microbial communities which are sensitive to these toxins. Similarly, Chen (2006) found that aromatic amines, which are common metabolic products of reductive cleavage of azo dyes, were found highly toxic to bacterium *Pseudomonas luteola*.

Recent studies have indicated that azo dyes also cause phytotoxicity. Ayed et al. (2011) reported a reduction of 55 and 30 % in the germination of *Triticum aestivum* and *Sorghum bicolor* respectively with a Methyl Red azo dye. However, degradation products of the dye were found to be non-toxic to the germination of the both plant species. On the contrary, Chaube et al. (2010) observed that even the degradation products of Direct Violet 51 and Tartrazine were toxic and a significant decrease in the germination and growth of *Triticum aestivum* and *Phaseolus mungo* was observed. Similarly, the application of dye-contaminated water inhibited the growth of peas and wheat plants in laboratory conditions, although the same water, after biological treatment, improved plant growth (Khalid et al. 2013; Saba et al. 2013).

3 Biodegradation of Azo Dyes by Actinobacteria

Actinomycetes were used mainly for the production of bioactive compounds, such as antibiotics and neglected earlier in relation to their biodegradation capacities. Now-a-days, they are becoming potential degraders of organic compounds, including azo dyes (Table 2). These bacteria have gained attention all over the world because of their versatile nature and enzyme system which is required for the

Table 2 Biodegradation of synthetic dyes by Actinobacteria via oxidation reduction processes

Actinomycetes sp.	Dyes	Conditions	Comments	References
Streptomyces krainskii SUK-5	Reactive blue-59	Shaking	Complete (100 %) degradation of the dye was observed within 24 h at pH 8 and temperature 30 °C	Mane et al. (2008)
Streptomyces sp. SS07	Xylidine ponceau-2R, direct black-38, direct brown-1	Reduced	Complete reduction of the dyes was achieved in 24 h at pH 9.2 and 37 °C	Bhaskar et al. (2003)
Streptomyces sp.	Reactive blue 160	Shaking	Actinomycetes had the potential to decolorize more than 98 % of dye in the presence of lactose and urea within 48 h.	Khobragade and Deshmukh (2013)
Streptomyces sp. C1	Indigo carmine diamond black PV	Oxidized	The 83.7 % decolorization was observed at pH of 8.0 and a temperature of 40 °C in 2 h with syringaldehyde as mediator	Lu et al. (2013)
Georgenia sp. CC-NMPT-T3	Reactive orange 16	Static anoxic	Decolorization of the dye was 94.2 % in 8 h at pH 6–8 and temperature 28-45 °C	Sahasrabudhe and Pathade (2013)
Actinomycetes sp. consortium	Reactive yellow	Static	The 90 % of dye was removed in 1 h	Bagewadi et al. (2011)
Streptomyces globosus	Acid fast red	Static/ shaking	Acid fast red was decolorized up to 82 % under static condition while 70 % dye removal was observed under shaking conditions.	El-Sersy et al. (2011)
Streptomyces psammoticus	Acid orange, methyl orange, and bismarck brown	Oxidized	The selected dyes were decolorized at the rates of 86, 71 and 75 %, respectively in the presence of 1-hydroxybenzotriazole as mediator.	Niladevi et al. (2008)
Streptomyces coelicolor	Acid blue, direct sky blue 6b, reactive black 5	Shaking	Decolorization was 94 % for acid blue 74, 91 % for direct sky blue 6b and 65 % for reactive black 5 in the presence of acetosyringone in 10 min.	Dube et al. (2008b)
Streptomyces cyaneus CECT 3335	Methyl orange and orange II,	Oxidized	Color removal was 90 % in the presence of acetosyringone (0.1 mM) redox mediator.	Moya et al. (2010)

degradation of dye chemicals. Usually, the dye degradation occurs by the activity of lignin peroxidase, laccase, NADH-DCIP reductase or azoreductase enzymes (Sahasrabudhe and Pathade 2013).

Actinomycetes show a variable potential to degrade various synthetic dyes. Chengalroyen (2011) examined the decolorization behaviour of *Streptomyces* species against two structurally different dyes (Congo red and Orange II). *Streptomyces* species were able to decolorize structurally complex dye Congo red with two azo bonds and poly aromatic and sulfonated groups, while structurally simpler dye Orange II (with a single azo bond and sulfonated group) was not decomposed. Biodegradation of triphenyl methane dyes by two actinomycetes, such as *Nocardia corallina* and *Nocardia globerula*, was reported by Yatome et al. (1991).The Crystal Violet dye was completely decolorized within 24 h. *Nocardia coralline* was also able to decolorize four triphenyl methane dyes, such as Methyl Violet, Ethyl Violet, Basic Fuchsin and Victoria Blue, but maximum decolorization was observed in Crystal Violet dye. They suggest that the decolorization activity of actinomycetes is intracellular, as there was no activity in the culture filtrate. Similarly, the decolorization activity was not observed in washed cells of *N. corallina*, when the cells were incubated in a buffer, but the activity was recovered when the cells were incubated in LB medium. Bhaskara et al. (2003) reported that extracellular fluid protein (ECFP) of *Streptomyces* species could be used for the reduction of azo dyes soluble in water.

Sahasrabudhe and Pathade (2013), in a study, indicated that *Georgenia* sp. CC-NMPT-T3 could degrade the individual as well as mixture of five different dyes under static anoxic conditions (Sahasrabudhe and Pathade 2013). This suggests its potential to be used as inoculum in the bioreactor for the treatment of textile wastewater containing a variety of synthetic dyes. Under shaking condition, *Streptomyces krainskii* strain SUK -5 completely degraded textile dye Reactive blue-59 within 24 h (Mane et al. 2008). During the process of degradation of Reactive blue-59, the involvement of lignin peroxidase, and NADH-DCIP reductase and MR reductase enzymes was confirmed.

Consortium of different actinomycetes can also be used for the degradation of azo dyes. Bagewadi et al. (2011) developed a consortium having five actinomycetes strains. About 97.44 % of degradation of reactive yellow dye (5 mg 100 ml^{-1}) was observed by this consortium in 15 days. They concluded that degradation of the dye depends on the concentration of dye, as well as on the growth of actinomycetes. El-Sersy et al. (2011) studied the potential of five actinomycetes (*Streptomyces globosus, Streptomyces alanosinicus, Streptomyces ruber, Streptomyces gancidicus,* and *Nocardiopsis aegyptia*) for dye decolorization. *Streptomyces globosus* had the maximum potential for the degradation of acid fast red dye under static (81.6 %) and shaking (70.2 %) conditions. The authors suggested biosorption as dominant mechanism for removal of dye from the solution. A 1.14-fold more biosorption was observed with an increase in the size of the inoculum and a decrease in the concentration of starch. Studies conducted in 1990's, also revealed that the *Streptomyces* species can be used for the degradation of azo dyes (Pasti et al. 1991; Paszczynski et al. 1991; Burke and Crawford 1998).

4 Enzymes Involved in the Biodegradation of Azo Dyes by Actinobacteria

Possible mechanisms of microbial removal of azo dyes include biosorption, bioaccumulation, reduction and oxidation. However, bioremediation by biosorption and bioaccumulation are slow and dyes are often not completely mineralized (Gadd 2009; Wang and Chen 2009). In this scenario, role of actinomycetes in the degradation of azo dyes or toxic by-products through oxidation-reduction processes is very important.

4.1 Reductive Degradation of Azo Dyes

Azo dyes are generally decomposed by microorganisms in a two-step process (Dawkar et al. 2010; Khalid et al. 2010; Liu et al. 2011). In the first step, microbes breakdown the azo bond (−N=N) of azo dyes with the help of azoreductase enzyme in low oxygen condition. This process is also called decolorization, resulting in the formation of colorless aromatic amines, which are mineralized in the oxidation process. So far, a little is known about the azo dyes degradation pathways of actinomycetes. Azoreductase enzyme has been reported in a few species of actinomycetes. Usually, the azoreductase enzyme is not easily identified in actinomycetes, due to its low concentration. However, the surfactant can be used to improve the production of the enzyme in the microorganisms (Reese and Maguire 1969). For this purpose, Chengalroyen (2011) used two surfactants, Tween-80 and Tween-20, to improve the production of azoreductase in *Streptomyces*. A closer monitoring of different enzymes in the same host showed that Tween-80 might result in the selective inhibition or enhancement of certain enzymes, suggesting that a complex interaction is involved. The identification of decolorizing strains in the presence of Tween-20 suggests the release of superior stabilized-protein into culture media (Chou et al. 2005). Chengalroyen (2011) also identified azoreductase in *Streptomyces coelicolor* by genome sequencing. This study provides an evidence that the azoreductase is also widespread in Actinobacteria.

4.2 Oxidative Degradation of Azo Dyes

In the case of actinomycetes, oxidation process is the most dominant mechanism for the degradation of azo dyes (Lu et al. 2013; Priyaragini et al. 2013). Usually, peroxidase or lignin-degrading enzymes are involved in the degradation process; either a single enzyme is being involved in the process or a group of enzymes act synergistically. Dye degrading peroxidases are reported to degrade hydroxyl free anthraquinone dyes (Sugano et al. 2006; Marchis et al. 2011). A combination of

lignin peroxidases and veratryl alcohol was found to enhance the decolorization of azo and anthraquinone dyes (Joshi et al. 2010). The role of lignin-degrading enzymes in the treatment of contaminated effluent is very critical, as it demands a thorough understanding of lignin degrading organisms and their enzyme systems. The laccases produced by *Streptomyces* were also found to be very effective for the decolorization of textile dyes (Dube et al. 2008a; Molina-Guijarro et al. 2009; Lu et al. 2013). Gottlieb et al. (2003) demonstrated the usefulness of a laccase enzyme produced by *Streptomyces cyaneus* CECT 3335 for the decolorization of azo dyes.

Often laccases are coupled to the reduction of oxygen to water along with oxidation of various substrates (Kurniawati and Nicell 2007, 2009; Morozova et al. 2007).The role of redox mediators is very important in a system for oxidative degradation, since it mediates the movement of electrons in the system and increases the degradation rate indirectly (Gonzalez-Gutierrez and Escamilla-Silva 2009). Lu et al. (2013) demonstrated the degradation of indigo carmine and diamond black PV with syringaldehyde as mediator. In this study, eight strains with laccase activity were isolated from composting samples in different phases, among which a novel strain was identified as *Streptomyces* sp. C1. The purified laccase-like multi copper oxidases (LMCO) of strain C1 showed a single protein band on SDS-PAGE gel with a molecular mass of about 38 kDa. The novel laccase showed alkaline resistance and moderate thermostability. The enzyme activity was activated by some metal ions, such as Cu^{2+}, Co^{2+} and Fe^{3+}at the concentration of 1 mM, while it was strongly inhibited in the presence of Hg^{2+}.

Some studies showed that two-domain small laccase isolated from *Streptomyces* species could tolerate high temperature and alkaline conditions (Steger et al. 2007; Dube et al. 2008a; Molina-Guijarro et al. 2009) and also caused degradation of pigment and phenolic compounds along with antibiotic formation (Nakamura et al. 2003; Molina-Guijarro et al. 2009). The ability of a laccase (EC 1.10.3.2), produced by *Streptomyces cyaneus* CECT 3335, to decolorize and detoxify azo dyes was also assessed by Moya et al. (2010). Acetosyringone was used as the redox mediator in this study. A significant decrease in the toxicity of the dyes, New Coccine and Chromotrope 2R, was observed after the decolorization process. Laccases had a large catalytic versatility due to their low substrate specificity against phenolic compounds and aromatic amines. In the presence of redox mediators, these enzymes can extend their oxidative action even to non-phenolic compounds (Bourbonnais and Paice 1990; Call and Mücke 1997). A large number of phenolic compounds were produced by the selected *Streptomyces* strains, when grown under solid state fermentation conditions on wheat straw (Hernandez-Coronado et al. 1998). These compounds can act as natural mediators. Usually these compounds are produced by microorganisms during their growth or lignin depolymerisation (Camarero et al. 2005), whereas Maneet al. (2008) isolated lignin peroxidase from *Streptomyces krainskii* SUK-5. In the presence of this enzyme, reactive blue-59 dye was completely degraded in less than 24 h. Recently, titanium oxide nano-particles (TiO2 NPs) were synthesized from the marine Actinobacteria (Priyaragini et al. 2013). Actinobacteria crude extract and NP synthesized TiO2 were found very effective for the degradation of azo dyes, such as acid red 79 and acid red 80.

Immobilized nano-particles and bacterial cells have shown promising results in the dye degradation (Lachheb et al. 2002; Daneshvar et al. 2005).

From these studies, it may be concluded that oxidative enzymes, produced by actinomycetes in the presence of some redox mediators, may have a practical application in the degradation of azo dyes.

5 Factor Affecting Biodegradation of Azo Dyes

The effectiveness of any biological treatment depends on the environmental conditions. The thermal stability and functioning of the enzyme in the Actinobacteria strain is very important for practical application. The isolation of *Streptomyces* spp. from unusual environment may produce a variety of bioactive compounds (Chronakova et al. 2010; Gousterova et al. 2014).Therefore, it is urgent to isolate and identify strains that can survive and maintain their activities under varying environmental conditions.

Thermostable laccases have been isolated from various strains of actinomycetes (Endo et al. 2003; Suzuki et al. 2003; Lu et al. 2013). Thermostable laccases have been also reported in Actinobacteria, such as *Streptomyces lavendulae* REN-7, *Streptomyces griseus* and *Streptomyces* sp. C1, which retained their original activity even at 70 °C (Endo et al. 2003; Suzuki et al. 2003; Lu et al. 2013). Recently, Sahasrabudhe and Pathade (2013) reported degradation of reactive orange dye using *Georgenia* sp. CC-NMPT-T3 at 28–45 °C. The actinobacteria could decolorize 94.2 % reactive orange dye (50 mg l^{-1}) within 8 h. In general, most laccases have an optimum enzymatic reaction at an acidic pH and the optimum temperature about 30 °C (Baldrian 2006). Lu et al. (2013) reported optimum activity of laccase enzyme isolated from *Streptomyces* sp. C1 at pH 8.0. Moreover, this enzyme was capable of retaining 70 and 50 % of its initial activity after 3 h incubation at pH 11.0 and 12.0, respectively. This demonstrated that the enzyme showed a moderate resistance to alkaline conditions. Mane et al. (2008) reported pH 8.0 as the best suitable condition for *Streptomyces krainskii* strain SUK-5 for optimal decolorization of reactive blue-59 dye. Similarly, *Georgenia* sp. CC-NMPT-T3 was able to degrade the azo dye reactive orange at pH 6–8 in static anoxic conditions (Sahasrabudhe and Pathade 2013).

Previously, azo reductase activities have been reported in static conditions in several species of Actinobacteria, such as *Streptomyces coelicolor, Nocardia corallina* and *Nocardia globerula* (Yatome et al. 1991; Bhaskara et al. 2003; Chou et al. 2005; Chengalroyen 2011). However, agitation (aerobic) is more effective in case of laccase enzyme and lignin-degrading enzymes. Actinobacteria *Georgenia* sp. CC-NMPT-T3, *Streptomyces cyaneus* CECT 3335, *Streptomyces krainskii* SUK-5 and *Streptomyces* sp. C1 have been reported to perform their activities best under aerobic conditions (Mane et al. 2008; Lu et al. 2013; Sahasrabudhe and Pathade 2013).

The presence of metals in the medium may also affect the performance of the microbial enzyme system. For example, the presence of Cu^{2+} in the medium increased the activity of laccase of *Streptomyces* sp. (Lu et al. 2013). Enhanced activity can be due to filling of the type-2 copper binding sites with copper ions (Bao et al. 1993). Similarly, the presence of some metalions Co^{2+}, Ba^{2+}, Mn^{2+} and Fe^{3+} at low concentrations also stimulate the activity of the enzyme from *Streptomyces* species (Nagai et al. 2002; Baldrian and Gabriel 2006). However, high concentration of these ions decreases the performance of the microbial enzyme systems. This decrease can be attributed to interference of these metals with proteins or enzymes, which ultimately form complexes with molecules of protein which may render it inactive or even completely inactivate the enzyme (Mills and Colwell 1977; Jadhav et al. 2012).

Carbon source is required by the microorganism as a source of energy and the electron donor for the removal of color of azo dyes (Moosvi et al. 2007; Perumal et al. 2007; Yemendzhiev et al. 2009). Among the various carbon sources, such as glucose, lactose, maltose, xylose, fructose, galactose, mannitol and sucrose, Khobragade and Deshmukh (2013) found lactose as a carbon source for the strongest decolorization of Reactive Blue 160. They also tested the efficacy of different nitrogen sources such as urea, peptone, yeast extract, ammonium nitrate, ammonium chloride and potassium nitrate, for color decolorization. Among all, urea was found to be the best source of nitrogen for the decolorization of dye. Sahasrabudhe and Pathade (2013) found yeast extract and sucrose as the best carbon sources for *Georgenia* sp. CC-NMPT-T3 to degrade Reactive orange 16 dye. However, contrary to the findings of Khobragade and Deshmukh (2013), they observed a very low degradation of the dye in the medium containing urea as a nitrogen source.

6 Conclusions

Actinobacteria are new options for the treatment of azo dye wastewater for safe disposal into the environment. These bacteria are widely distributed in the ecosystem and can operate in a broad range of environmental conditions. In particular, under extreme environmental conditions, they can have a better survival due to the presence of different adaptation mechanisms. These bacteria typically have polyphosphate reserves, which not only act as reserves of phosphorus, but also space for metal chelation, which can reduce the toxicity of the metal ions present in the industrial wastewater. Presence of superoxide dismutase genes in Actinobacteria may also play an important role in their resistance against environmental stresses. This can result in the production of biosurfactant comprising haemolytic, drop collapsing and activity of lipase production. Biosurfactant increases the surface area of hydrophobic substances, which enhance the bioavailability of these substances and modify the properties of the surface of the bacterial cell. Spore formation is another very important feature in actinomycetes which can be exploited in a stressed environment for its long-term application in the treatment system.

Actinomycetes degrade azo dyes by enzyme involved in reduction and oxidation process. However, further work is still needed for the isolation and selection of strains carrying the effective enzyme systems for bioremediation of wastewater containing dyes. Besides, there is an urgent need to study the source of low carbon like potatoes peel or other organic waste for the production of low-cost actinomycetes biomass. The adaptability and long-term survival of degrading Actinobacteria in the bioreactor system should also be taken into account to develop an effective treatment strategy that can be used across the field. Maintaining high levels of specific microorganisms in the treatment system is a great challenge. Advanced molecular techniques should be applied to monitor the microbial community structure, the persistence of the inoculum added, and their interactions with indigenous populations.

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Degradation of Azo Dyes by White-Rot Fungi

Susana Rodríguez-Couto

1 Introduction

The first synthetic dye, mauveine, was discovered in 1856 by the English chemist W.H. Perkin. Since then, natural dyes have been progressively replaced by synthetic dyes (Cardon 2003). Thus, around 800,000 tons of synthetic dyes are produced annually worldwide, 40 % of which are produced in Europe (Hessel et al. 2007). Synthetic dyes are xenobiotic compounds, i.e. extraneous to the biosphere. Consequently, they are resistant to biodegradation by the indigenous micro-organisms and, therefore, they persist in the environment for a long time, causing serious detrimental effects on ecosystems.

Synthetic dyes are used extensively in many industrial sectors, such as printing, pharmaceutical, food, cosmetic and textile industries (McMullan et al. 2001). These industries generate large quantities of coloured wastewater containing about 2–60 % of the dyes used, depending on the type of dyes (Hessel et al. 2007). Among synthetic dyes, since azo dyes are the most widely used, they are mostly found in industrial effluents. Azo dyes are characterised by the presence of one or more azo groups (–N=N–) (monoazo, diazo, triazo, polyazo) linked to phenyl and naphthyl radicals, which usually have some combinations of functional groups including amino ($–NH_2$), chlorine (–Cl), hydroxyl (–OH), methyl ($–CH_3$), nitro ($–NO_2$), sulphonic acid and sodium salts ($–SO_3Na$) (Fig. 1). They are designed to have high photolytic stability and resistance towards the main oxidising agents (Reife and Othmer 1993).

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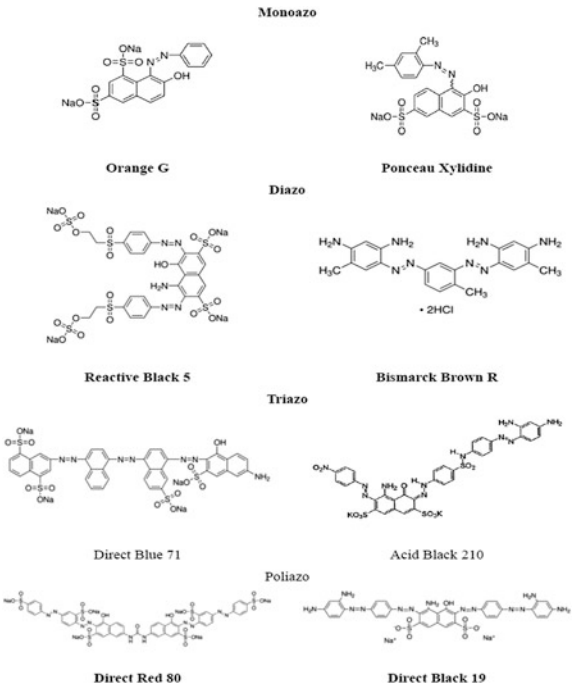

Fig. 1 Chemical structures of different monoazo, diazo, triazo and poliazo dyes

Colour is the first pollutant to be recognised in the wastewater and even the presence of a concentration as low as 1 mg l^{-1} is highly visible. An average concentration of 300 mg l^{-1} of dyes has been reported in effluents from textile-manufacturing processes (Tony et al. 2009). A river polluted by the discharge of dye-containing wastewater can be seen in Fig. 2.

In addition, dyes have diverse negative effects on the environment, such as inhibition of aquatic photosynthesis, depletion of dissolved oxygen and toxicity to flora, fauna and humans. Furthermore, the reduction of azo bonds forms amines which are highly toxic and carcinogenic in nature (Puvaneswari et al. 2006). Therefore, the removal of azo dyes from wastewater before being discharged into the environment is a matter of serious concern. Hence, environmental regulations in most of the countries make their removal mandatory before discharging wastewater into water bodies. However, the current existing chemical and physical methods to remove synthetic dyes from effluents, e.g. adsorption, flotation, Fenton oxidation, reduction ($Na_2S_2O_4$), ion exchange, chlorination/ozonation and incineration, are rather costly, time-consuming, mostly ineffective and sometimes generate hazardous sub-products (Grassi et al. 2011). Although azo dyes are not degraded by bacteria under aerobic conditions (Hu 1998), Kulla (1981) has reported the ability of *Pseudomonas* strains to degrade certain azo dyes under aerobic conditions. As dyes were not mineralised, novel strategies are needed to remove azo dyes from wastewater. In this regard, white-rot fungi have been a subject of intensive research in the last few years, as they have known to be the most efficient micro-organisms in

Fig. 2 Polluted river by dye-containing effluents (*Source* www.blogairdye.com)

degrading synthetic dyes so far (Kaushik and Malik 2009). This ability is related to the secretion of extracellular non-specific ligninolytic enzymes, which are well known to be involved in the degradation of the recalcitrant polymer lignin in nature (Fig. 3).

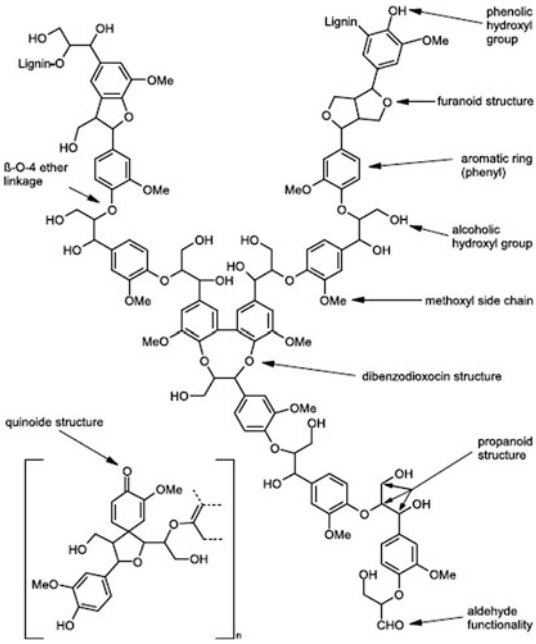

Fig. 3 Structural model of lignin by (Brunow 2001; Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission)

Due to the similarity between the chemical structure of lignin and those of several recalcitrant pollutants including synthetic dyes, the use of white-rot fungi and/or their enzymes for the degradation of recalcitrant dyes has been widely used (Paszczynski et al. 1991; Zhou and Zimmerman 1993).

Decolouration of azo dyes by the well known ligninolytic fungus *Phanerochaete chrysosporium* was first described in 1990 (Cripps et al. 1990). Since then, *P. chrysosporium* has been subject of an intensive research for bioremediation of recalcitrant compounds. Besides, it was found to be capable of decolourising different types of azo dyes (Knapp et al. 1995; Moreira et al. 2000; Chagas and Durrant 2001). The promising results obtained by *P. chrysosporium* have accelerated the search for other species of white-rot fungi with azo dye-degrading ability. Thus, other white-rot fungi, such as *Pleurotus ostreatus*, *Trametes versicolor, Bjerkandera adusta* and *Thelephora* sp., were also found to be able of decolourising different azo dyes (Heinfling et al. 1997; Cao 2000; Selvam et al. 2003).

2 White-Rot Fungi

White-rot fungi are the only micro-organisms able to degrade the whole wood components i.e. cellulose, hemicellulose and lignin (Fig. 4). Their name derives from the white appearance of the wood due to lignin removal when attacked by these fungi (Pointing 2001).

Although some white-rot fungi are also Ascomycetes, but most of them are Basidiomycetes taxonomically (Eaton and Hale 1993). White-rot caused by fungi can be divided into simultaneous and selective lignin degradation types. Simultaneously, white-rot fungi degrade all wood cell wall polymers progressively, but preferably they metabolise lignin and hemicellulose. The main characteristics of white-rot fungi are presented in Table 1 (Martínez et al. 2005).

In general, white-rot fungi are unable to use lignin as sole carbon source, but they degrade it in order to gain access to polysaccharides (Sinsabaug and Liptak 1997; Yeo et al. 2007). The ability of white-rot fungi to degrade lignin is due to the production of one or more extracellular non-specific lignin-modifying enzymes (LMEs), which generate free radicals that randomly attack the lignin molecule, breaking covalent bonds and releasing a range of mainly phenolic compounds. Since radicals are highly reactive but short-lived molecules, they can reach only closer lignocellulose material, forming erosion zones around the hyphae (Mester and Field 1998; Carlile et al. 2001). There are two main types of LMEs: peroxidases [lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP)] and phenoloxidases (laccases). Some fungi are able to produce both types of enzymes, whereas others produce only one of them. In addition, white-rot fungi secrete low-molecular weight mediators which increase the range of compounds that they can degrade (Pointing 2001). A detailed description of LMEs and mediators are included in Sect. 4.

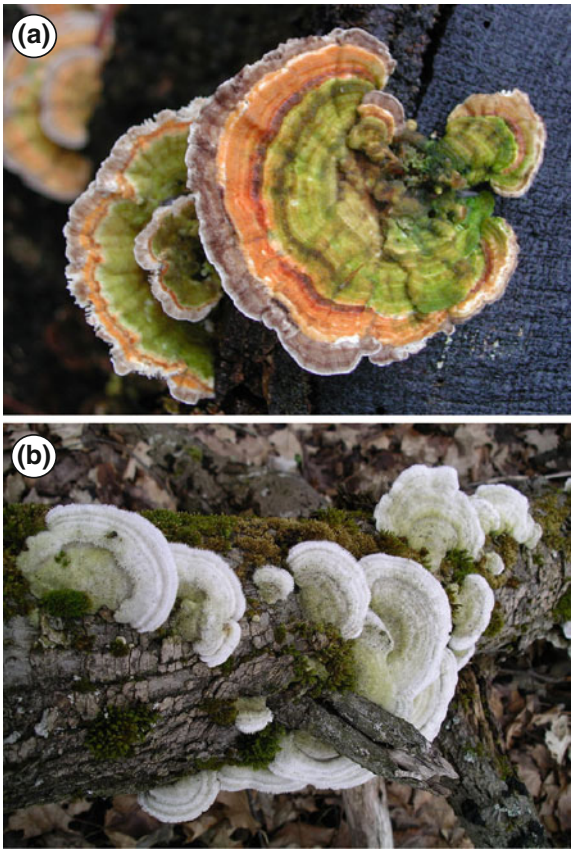

Fig. 4 White-rot fungi as they grow in nature. **a** *Trametes pubescens,* **b** *Trametes versicolor*

3 Factors Affecting the Fungal Degradation of Azo Dyes

Micro-organisms are sensitive to the presence of chemical substances, such as dyes, high salinity, variations in pH and high content of organic compounds (Dua et al. 2002; Ang et al. 2005; Megharaj et al. 2011). The biodecolouration process depends on the following factors: the azo dye structure, carbon and nitrogen sources, salinity, pH, temperature, dye concentration and the presence or absence of oxygen.

3.1 Effect of the Azo Dye Structure

It was observed that dye structure highly affects its degradation (Pasti-Grigsby et al. 1992; Hsueh et al. 2009; Kuberan et al. 2011; Rajee and Patterson 2011; Sawhney and Kumar 2011). Thus, it was found that azo dyes containing electron-withdrawing groups such as SO_3^- were degraded easier than those with electron-donating groups,

Table 1 Anatomical and chemical features of different types of wood decaying by white-rot-fungi (Martínez et al. 2005)

	Simultaneous rot	Selective delignification
Decay aspect and consistency	Bleached appearance, lighter in colour than sound wood, moist, soft, spongy, strength loss after advanced decay	
Host (wood-type)	Hardwood, rarely softwood	Hardwood and softwood
Cell-wall constituents degraded	Cellulose, lignin and hemicelluloses	Initial attack selective for hemicelluloses and lignin, later cellulose also
	Brittle fracture	Fibrous feature
Anatomical features	Cell wall attacked progressively from lumen. Erosion furrows associated with hyphae	Lignin degradation in middle lamella and secondary wall. Middle lamella dissolved by diffusion mechanism (not in contact with hyphae), radial cavities in cell wall
Fungi	Basidiomycetes (e.g. *T. versicolor, Irpex lacteus, P. chrysosporium* and *Heterobasidium annosum*) and some Ascomycetes (e.g. *Xylaria hypoxylon*)	Basidiomycetes (e.g. *Ganoderma austral, Phlebiatremellosa, C. subvermispora, Pleurotus* spp. and *Phellinus pini*)

such as –NH-triazine. In addition, it was observed that if the electro-withdrawing groups were in the *para* or *ortho* positions to the azo bond, degradation was faster than, if they were in the *meta* position. This is due to the fact that they make azo dyes highly electrophilic (Pricelius et al. 2007; Tauber et al. 2008; Hsueh et al. 2009) and with less steric hindrance near the azo bond (Chen et al. 2011).

3.2 Influence of Carbon and Nitrogen Sources

Carbon and nitrogen sources considerably affect azo dye decolouration by micro-organisms. Carbon sources function as sources of carbon and energy for growth and survival of the fungus and as electron donors to break the azo bond (Yang et al. 2009; Yemendzhiev et al. 2009). Carbon sources are accepted differently by different micro-organisms and have an important effect on the extent of decolouration. Thus, in several cases, the microbial decolouration of azo dyes is increased in the presence of glucose, whereas in other cases, the presence of several carbon sources is required and in others, the presence of a carbon source inhibits azo dye decolouration (Solis et al. 2012). Besides the type of carbon source, its concentration is also an important parameter affecting dye decolouration, as high carbon concentrations can diminish decolouration (Solis et al. 2012).

Nitrogen sources are also important for microbial decolouration. The metabolism of organic nitrogen sources is considered essential for the regeneration of NADH (Saratale et al. 2009). However, several fungal species suppress their ligninolytic activity in the presence of high concentrations of nitrogen (25–60 mM) (Kaushik and Malik 2009).

3.3 pH

Kaushik and Malik (2009) stated that the optimal decolouration pH for most fungi was in the acidic range as fungal ligninolytic enzymes have maximal activity at acid pH. In addition, fungi grow normally at a pH value ranging from 4 to 5 (Fu and Viraraghavan 2001). However, low pH values are not always suitable for the treatment of dye-containing effluents. Hence, fungal strains capable of dye decolouration at wide pH ranges are more suitable for industrial applications.

3.4 Temperature

Different micro-organisms have different optimum growth temperatures. Thus, most fungi grow at 25–35 °C (Fu and Viraraghavan 2001). Also, several studies have indicated that micro-organisms could degrade synthetic dyes best in the temperature range of 25–37 °C (Ali 2010).

3.5 Effect of Initial Dye Concentration on Microbial Decolouration

The microbial decolouration of dyes decreases with increasing dye concentration. The results of some studies showed that with increasing initial dye concentration, the decolouration decreased considerably. This could be attributed to the toxicity of the dyes to the growing microbial cells at higher dye concentrations (Ali 2010).

3.6 Salts

Wastewater from textile processing and dyestuff manufacture industries contains substantial amounts of salts in addition to azo dyes (Khalid et al. 2008). Thus, microbial species capable of tolerating high salt concentrations will be suitable for treating such wastewater. However, there are very few papers which report the

ability of white-rot fungi to degrade coloured effluents in alkaline-saline conditions (Ottoni et al. 2013). Recently, Ottoni et al. (2014) reported that the white-rot fungus *T. versicolor* was able to completely decolourise the diazo dye Reactive Black 5 (0.1 g l^{-1}) in a liquid medium containing 15 g l^{-1} NaCl at pH 9.5 in batch culture. Also, they found that *T. versicolor*, immobilised on either polyurethane foam or nylon sponge, was able to operate in continuous mode for 40 days in a fixed-bed bioreactor with decolouration range of 85–100 %.

3.7 Aeration and Shaking

White-rot fungi are aerobic in nature; hence they need oxygen for growth and maintenance. Also, ligninolytic enzymes need oxygen to bring about their catalytic action. This oxygen requirement depends on the fungus and its ligninolytic system. Shaking favours oxygen transfer, but it affects fungal morphology and can diminish the rate of the enzyme synthesis (Žnidaršič and Pavko 2001).

4 Involvement of Oxidoreductive Enzymes in the Degradation Process

The ability of white-rot fungi to degrade lignin is due to the secretion of several extracellular non-specific LMEs (Table 2). LMEs production by white-rot fungi occurs usually at the onset of secondary metabolism (Hatakka 1994; Camarero et al. 1999). The main LMEs are two glycosylated hemo-containing peroxidases, lignin

Table 2 Ligninolytic enzymes and their main reactions

Enzyme and abbreviation	Cofactor	Substrate, mediator	Reaction
Lignin peroxidase (LiP)	H_2O_2	Veratryl alcohol	Aromatic ring oxidised to cation radical
Manganese-dependent peroxidase (MnP)	H_2O_2	Mn, organic acids as chelators, thiols, unsaturated fatty acids	Mn(II) oxidised to Mn(III); chelated Mn(III) oxidises phenolic compounds to phenoxyl radicals; other reactions in the presence of additional compounds
Laccase	O_2	Phenols, mediators, e.g. hydroxybenzotriazole or ABTS	Phenols are oxidised to phenoxyl radicals; other reactions in the presence of mediators

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peroxidase (LiP, E.C. 1.11.1.14) and manganese-dependent peroxidase (MnP, E.C. 1.11.1.13) (Orth and Tien 1995), and a copper-containing phenol oxidase, laccase (E.C. 1.10.3.2) (Thurston 1994). Some authors have also reported Mn-independent peroxidase (MiP) and versatile peroxidase (VP) activities in some white-rot fungi (Heinflig et al. 1998; Camarero et al. 1999; Ruiz-Dueñas et al. 2001). In addition, they also produce several accessory enzymes, such as glyoxal oxidase (E.C. 1.2.3.5), superoxide dismutase (E.C. 1.15.1.1), glucose oxidase (E.C. 1.1.3.4), aryl alcohol oxidase (E.C. 1.1.3.7) and cellobiose dehydrogenase (E.C. 1.1.99.18), which either produce H_2O_2 required by peroxidases or serve to link lignocellulose degradation pathways (Leonowicz et al. 2001). Moreover, apart from the above-mentioned extracellular enzymes, intracellular enzymes such as cytochrome P450 monooxygenases might also be involved in the decolouration process (Low et al. 2009).

Lignin peroxidase (LiP, E.C. 1.11.1.14) is an extracellular glycosylated heme-containing peroxidase, which in the presence of endogenously generated hydrogen peroxide, catalyses the oxidation of non-phenolic aromatic structures in lignin generating aryl cation radicals. The molecular mass of LiPs from different white-rot fungi varies from 37 to 50 kDa (Hirai et al. 2005; Asgher et al. 2006).

pH and temperature activity profiles of LiPs from different sources vary significantly and optimum activities have been found between pH 2–5 and 35–55 °C, respectively (Yang et al. 2004; Asgher et al. 2007). The natural fungal secondary metabolites, such as veratryl alcohol (VA) and 2-chloro-1,4-dimethoxybenzene, act as redox mediators to stimulate the LiP catalysed oxidation of a wide range of recalcitrant substrates (Teunissen and Field 1998; Christian et al. 2005). Its catalytic cycle has been shown in Fig. 5. LiP is oxidised by H_2O_2 to form a two-electron oxidised intermediate (compound I) which, in turn, oxidises substrates by removing one electron and producing a more reduced enzyme intermediate (compound II) and a substrate radical. This intermediate can then oxidise substrates by one electron, returning the enzyme to its initial state. However, compound II has a very high

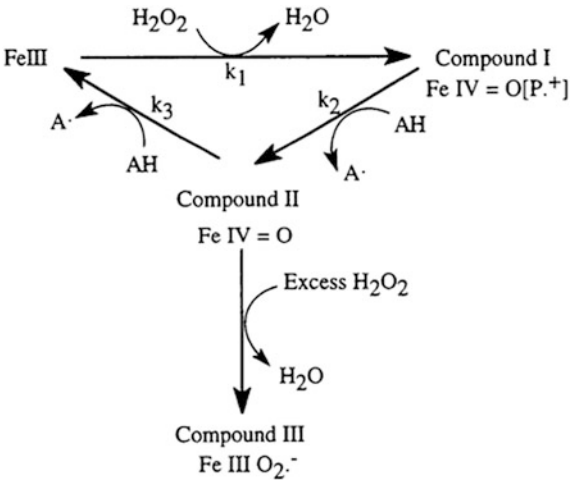

Fig. 5 The catalytic cycle of lignin peroxidase (Wariishi and Gold 1989; reprinted with permission from Elsevier Ltd., UK.)

reactivity with H_2O_2, and in the presence of a poor substrate and excess H_2O_2, it is instead converted to an inactive form of the enzyme (compound III) (Gold et al. 1989; Wariishi and Gold 1989). Compounds, such as veratryl alcohol and tryptophan, exert a protective effect against the enzyme inactivation by excess of H_2O_2 (Collins et al. 1997) and when present, they convert compound II into the resting enzyme, completing the catalytic cycle.

Manganese-dependent peroxidase (MnP, E.C. 1.11.1.13) is an extracellular glycosylated heme-containing peroxidase that catalyses H_2O_2-dependent oxidation of Mn^{2+} to highly reactive Mn^{3+}. It is often produced in multiple isoforms with molecular masses between 32 and 62.5 kDa, optimum pH of 4–7 and optimum temperature of 40–60 °C (Ürek and Pazarlioglu 2004; Baborová et al. 2006). MnP oxidises the phenolic units of lignin producing free radicals. The catalytic cycle of MnP (Fig. 6) is similar to that of the LiP. It includes the native ferric enzyme as well as the reactive intermediates (compound I and compound II) (Hofrichter 2002). In contrast to other peroxidases, MnP uses Mn^{2+} as the preferred substrate. The cycle starts by binding of H_2O_2 to the native ferric enzyme and the formation of an iron peroxide complex (Hofrichter 2002). The subsequent cleavage of the peroxide oxygen-oxygen bond requires a two-electron transfer from the heme, resulting in the formation of MnP-compound I. Afterwards, one molecule of water is released. A subsequent reduction proceeds through MnP-compound II. A mono-chelated Mn^{2+} ion acts as the one-electron donor for this enzyme intermediate and is oxidised to Mn^{3+}, leading to the generation of the native enzyme and the release of a second molecule of water (Hofrichter 2002).

Laccase (E.C. 1.10.3.2; benzendiol-oxygen oxidoreductase) belongs to a group of polyphenol oxidases containing copper atoms in the catalytic centre, usually called multi-copper oxidases. Fungal laccases are produced mainly extracellularly in multiple isoforms with a typical molecular mass of 60–80 kDa and an acidic p*I* value of 3–6 (Thurston 1994; Baldrian 2006). In addition to lignin degradation, fungal laccases are also involved in the formation of fruiting bodies, synthesis of melanin and other pigments, sporulation, conidiation and plant pathogenesis (Alexandre and Zhulin 2000; Mayer and Staples 2002).

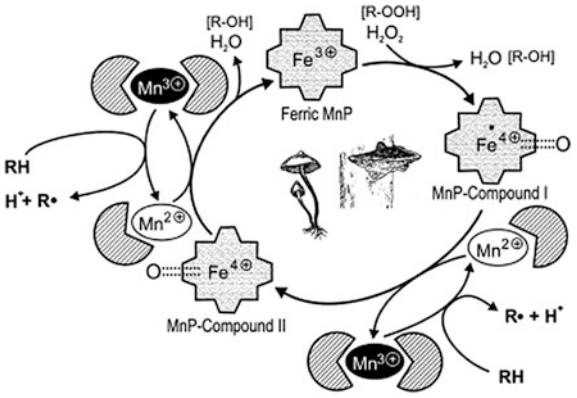

Fig. 6 The catalytic cycle of manganese-dependent peroxidase (MnP) (Hofrichter 2002; reprinted with permission from Elsevier Ltd., UK.)

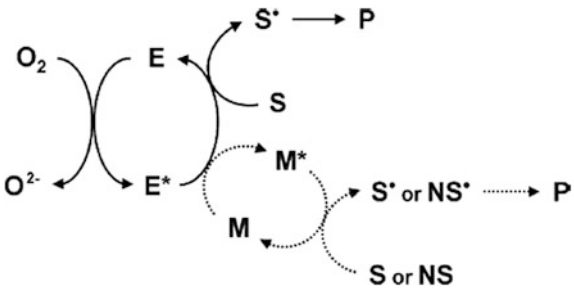

Fig. 7 Catalytic cycle of laccase enzyme (Kurniawati and Nicell 2007; reprinted with permission from Elsevier Ltd., UK.)

Laccases oxidise different compounds, such as phenols, polyphenols and aromatic amines with the concomitant reduction of molecular oxygen to water. Their broad substrate spectrum can be additionally extended by the presence of the so-called redox mediators (Bourbonnais et al. 1995; Johannes and Majcherczyk 2000). The basis of the laccase mediator system (LMS) is the use of low-molecular weight compounds that are oxidised by laccase to organic radicals or positively charged intermediates, which, in turn, act as redox mediators (Morozova et al. 2007). These radicals or charged intermediates are capable of oxidising compounds that are not substrates of laccases because of either their large size or their particular high redox potential.

The catalytic cycle of laccase has been shown in Fig. 7. In this cycle, the copper centre at the catalytic site of laccase donates electrons to oxygen, leaving the laccase enzyme in an oxidised state. In typical interactions of laccase with a substrate, the catalytic site of laccase abstracts electrons from the substrate and releases an oxidised product. When a mediator is present, the mediator is oxidised by laccase and further oxidises another compound that is either a substrate or a non-substrate of laccase, resulting in the formation of oxidised product(s) and the mediator regeneration (Banci et al. 1999). Examples of laccase mediators are 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate (ABTS), 1-hydroxybenzotriazole (HBT), and 3-hydroxyanthranilate (3-HAA), of which the last one is found to occur naturally in the cultures of *Pycnoporus cinnabarinus* (Eggert et al. 1996; Morozova et al. 2007). Also some phenolic lignin precursors and degradation products might act as laccase mediators in nature (Camarero et al. 2005).

5 Microbial Toxicity of Dyes and Their Degradation Products

The acute toxicity of azo dyes, according to the criteria of the European Union for the classification of dangerous substances, is low and the values of 50 % lethal dose (LD_{50}) are 250–2,000 mg kg^{-1} body weight (Clarke and Anliker 1980). However,

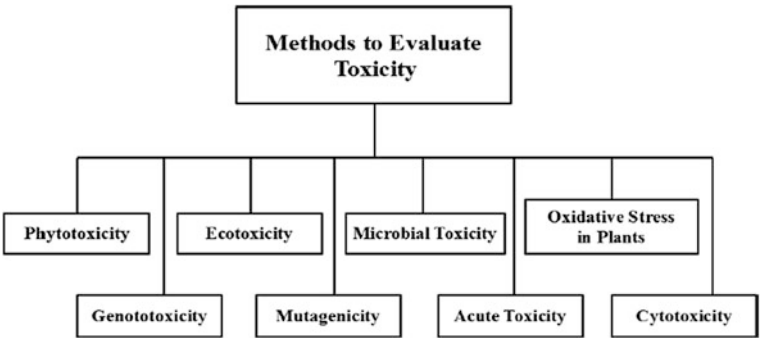

Fig. 8 Methods to evaluate the toxicity of azo dyes and their degradation products present in treated effluents (Solis et al. 2012)

occupational sensitivity to azo dyes has been shown in textile industries since 1930 (Foussereau et al. 1982). In addition, some azo dyes have been linked to bladder cancer in humans, to splenic sarcoma, hepatocarcinomas and nuclear anomalies in experimental animals and to cause chromosomal aberration in mammalian cells (Mendevedev et al. 1988; Percy et al. 1989).

It is very important for any bioremediation technology to assess the toxicity of the pollutants and metabolites formed after dye degradation in order to study the feasibility of the method (Jadhav et al. 2011). Toxicity of dyes and their metabolites is evaluated by using various methodologies as indicated in Fig. 8.

On the other hand, some studies have reported the metabolic pathway of azo dye degradation by white-rot fungi (Martins et al. 2003; Zhao et al. 2006; Zhao and Hardin 2007; Lu et al. 2008). These studies are very interesting, since they could predict the expected by-products from azo dye biodegradation based on their structure which would allow selecting the best strategy for the treatment of these recalcitrant dyes.

6 Future Prospectives

Treatment of dye-containing effluents is a challenging task. Despite the research efforts performed in the last decades, there is no single and economically attractive method to decolourise and detoxify wastewater polluted with azo dyes. Therefore, the need of an effective and environmentally friendly treatment at affordable cost is today of utmost importance.

To apply biodegradation for the treatment of dye-containing wastewater, fungal strains, capable of growing in a wide range of pH and temperature conditions and capable of resisting the toxicity of the dyes even at higher concentrations, should be chosen. Further, toxicity studies must be done to ensure the safety of the decolourised wastewater. In this regard, a few studies have been conducted on testing the

toxicity of the treated effluent/dye solution (Ambrosio and Campos-Takaki 2004; Romero et al. 2006; Eichlerova et al. 2007; Diorio et al. 2008). Based on the successful laboratory results, efforts should now be made to scale-up and apply fungal decolouration techniques in real industrial effluents. In addition, there is a hope that recombinant strains with higher biodegradation capacities will be applied in the future for the removal of dyes in wastewater.

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Degradation of Anthroquinone Dyes Stimulated by Fungi

S.N. Singh, Shweta Mishra and Nitanshi Jauhari

1 Introduction

Synthetic dyes are widely used in textile dyeing, paper printing, color photography, pharmaceuticals, food, leather industries, and cosmetics (Couto 2009). Annual production of synthetic dyes has already exceeded over 7×10^5 metric tons (Chen et al. 2003). Different industries, such as paper and pulp mills, textiles anddyestuff industries, distilleries and tanneries, discharge an enormous amount of highly colored waste water (Raghukumar 2000). Among the industries, the textile industry generates maximum liquid effluent, as a large quantity of water is used in thedyeing processes (Kalyani et al. 2009). As all the dyes do not bind to fabric, their loss in waste water varies from 2 % of basic dyes to 50 % for reactive dyes, thus causing severe contamination of both surface and ground waters through the discharge of textile industries (O'Neill et al. 1999). According to an estimate, 280,000 tons of textile dyes are globally discharged in the textile effluent every year (Jin et al. 2007). Presence of color in the dye effluents is itself a clear indication of water being polluted and their discharge to water bodies hampers water quality and causes toxicity to aquatic animals, plants and microbes. Thus, a disposal of untreated dyeing effluent without treatment is a serious offence which causes both environmental and health hazards (Shedbalkar et al. 2008). Besides, colored effluents from the industries hamper the light penetration of water bodies and also enhance BOD level affecting the aquatic flora which serves as a food source for aquatic animals (Annuar et al. 2009).

In dyes, twelve different chromophores have been reported and among them, azo and anthraquinone are the major ones. Azo dyes contain N=N double bonds and account for about 70 % of all textiledyestuffs produced. It is the most common

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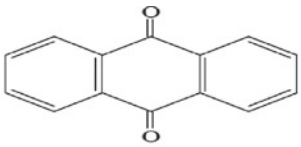
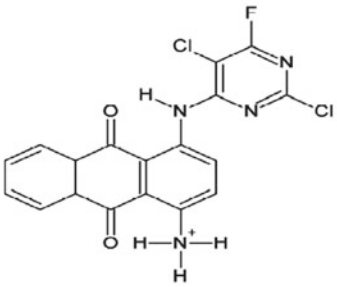
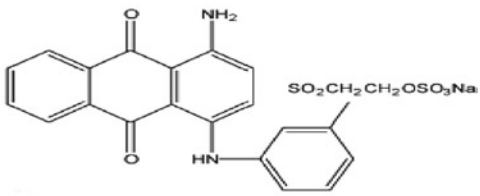


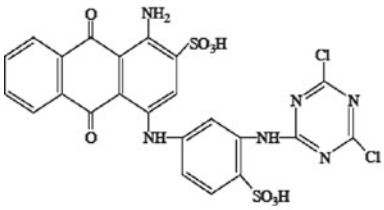
Anthraquinone



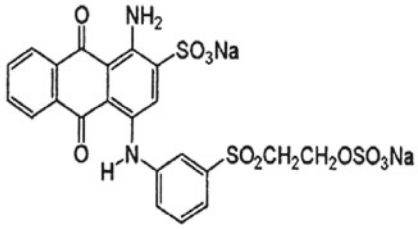
Drimarene Blue K_2RL



Remazol Brilliant Blue R (RBBR)



Reactive Blue 4 (RB4)



Reactive Blue 19 (RB19)

Fig. 1 Chemical structure of anthraquinone and its derivatives

chromophore of reactive dyes. However, anthraquinone dyes are derived from anthraquinone having a quinoid ring as the chromophore. It may also contain hydroxyl groups or amino groups in their general structure acting as a chromophore (Fig. 1). Anthraquinone dyes are not only resistant to microbial degradation but also toxic, carcinogenic and mutagenic in nature (Itoh et al. 1996). During decolorization, anthraquinone dyes get converted into a very harmful compound, i.e., benzidine.

Since synthetic dyes are generally very recalcitrant in nature, they can't be removed by conventional wastewater treatments, such as adsorption, photooxidation, coagulation, flocculation, photo and chemical degradation. The chemical methods produce toxic compounds by the cleavage of chromophoric groups present in the dye which is a major disadvantage associated with this method (Robinson et al. 2001). However, the biological treatment is often economical and eco-friendly

as compared to physical and chemical processes and its degradation may lead to complete mineralization at a very low cost (Pandey et al. 2007). Several potential microorganisms have been identified among fungi, bacteria, yeasts and algae, for their high ability to decolorize many anthraquinone dyes (Pandey et al. 2007).

In the bioremediation methods, microbial decolorization/degradation as well as dye adsorption by living or dead microbial biomass are commonly recommended for the treatment of industrial dye effluents (Fu and Viraraghavan 2001; McMullan et al. 2001).

In biological treatments, white rot fungi (WRF) have sustained our increasing interest, because several studies have reported their high ability for the decolorization and toxicity reduction of dyes (Fu and Viraraghavan 2001). White rot fungi secrete severalextracellular enzymes which facilitate the remarkable biotransformation or biodegradation of a broad variety of synthetic dyes (Wesenberg et al. 2003). In the enzyme-based methods, the energy requirement is very low and also poses a minimal adverse impact on the ecosystems. Among extracellular enzymes, laccases have aroused immense interest, because of their remarkable ability to transform a wide variety of hazardous chemicals (Majeau et al. 2010). They also act as useful biocatalysts for wider biotechnological applications, due to their non-specific oxidation capacity and ability to use available molecular oxygen as an electron acceptor (Baldrian 2006). Dead fungal biomass is also considered as a suitable alternative for bioremediation, as its wastes don't have toxic effect. In addition, it does not require any nutrients, nor does it release toxins or propagules in the environment (Tigini et al. 2011).

2 Degradation of Anthraquinone Dyes by Fungi

Knapp et al. (1995) have reported that wood rotting basidiomycetes fungi (WRF) are highly capable of degrading synthetic dyes. While working on the biodegradation of 14 structurally different synthetic dyes by wood-rotting basidiomycetes fungi, it was found that the decomposition rate highly depends on both the chemical structure of the dye and the characteristics of the fungi. Therefore, white-rot fungi have been widely used for the decoloration of different dyes. *Trametes versicolor* has ability to decolorize anthraquinone, azo and indigo-based dyes (Wang and Yu 1998). A rapid decoloration of Remazol Brilliant Blue (RBBR) by *Pycnoporus cinnabarinus* in packed-bed bioreactor was reported by Schliephaje and Lonergan (1996). Abadulla et al. (2000) also observed decoloration of different synthetic dyes, such as anthraquinone, triarylmethane, indigoid, by a fungus *Trametes hirsute*. Verma et al. (2012) reported a rapid two- step process for the bioremediation of 1,000 ppm anthraquinone Reactive Blue 4 (RB4) dye by laccase enzyme produced by marine-borne basidiomycetous fungus *Cerrena unicolor*, which caused 61 % of color removal and two-fold decrease in COD after 12 h of incubation. A change in aromatic structure of dye with concomitant formation of many low molecular weight phenolic compounds was also observed during degradation of

RB4. Many metabolites were reported duringenzymatic degradation of this dye, such as 2-formylbenzoic acid, 1,2,4,5-tetrahydroxy-3-benzoic acid, 2,3,4-trihydroxybenzenesulfonic acid and 1,2,3,4-pentahydroxybenzene. In the second step, when the enzyme -transformed solution was subjected to sorption on the powdered fungal biomass, the reduction was further enhanced to 93 % within 10 min of incubation.

Zhang et al. (2007) also recorded 90 % decolorization of 80 ppm of anthraquinone dye, Remazol Brilliant Blue (RBBR), by a non-ligninolytic fungus, *Myrothecium* sp. IMER1 after 7 days of cultivation. The production of bilirubin oxidase (BOX) was also found during adsorption of dye by cells at the initial stage of decolorization which fainted the color initially and then led to complete disappearance. This observation clearly indicated that both the strain and the extracellular enzyme BOX had promising applications in the decolorization of dye effluent.

Working on this aspect, Yang et al. (2009) also identified a white rot fungus *Trametes* sp. strain SQ01, from decayed wood in a temperate forest, for its ability to degrade azo, triphenylmethane and anthraquinone dyes. It was found that except acid blue, about 97–99 % of azo dyes and RRBR (anthraquinone dye) were degraded by *Trametes* sp. SQ01 after 7 days of incubation. Besides, 30–70 % of triphenymethane dyes was also removed during the same period, while bromophenol blue was completely degraded.

Levin et al. (2012) investigated the role of grape stalks as an agroindustrial waste, for growth, enzyme production and decolorization of different dyes, such as indigo carmine, malachite green, azure B, remazol brilliant blue, crystal violet and xylidine by three white rot fungi: *Trametes trogii*, *Stereum hirsutum* and *Coriolus antarcticus*. The laccase (33.0 U g^{-1}) and Mn-Peroxidase (1.6 U g^{-1} dry wt.) activities were found highest in *C. antarcticus,* while endoglucanase (10.4 U g^{-1} dry wt.) and endoxylanase (14.6 U g^{-1} dry wt.) activities were maximum in *S. hirsutum and T. trogii* respectively. It was also noted that in the presence of grape stalk, *C. antarcticus* was found very effective in bioremediation of textile possessing effluents, attaining percent decolorization of 93, 86, 82, 82, 77 and 58 % for indigo carmine, malachite green, azure B, remazol brilliant blue R, crystal violet and xylidine, respectively, in 5 h incubation.

Hsu et al. (2012) identified a new fungal strain, *Lentinus* sp., that produces an extracellular enzyme laccase Lentinus lcc3 with an activity of approximately 58,300 U l^{-1}, This enzyme has the ability to reverse the toxicity of anthraquinone and azo dyes on rice seed germination and also decolorized industrial textile effluent. Initially, at 1 U ml^{-1} of lcc3, the decolorization efficiencywas found to be 97, 29, 48, 22, 22 and 4 % for Acid Blue 80, RBBR, Acid Red 37, Acid Black 1, Direct Blue 71, andDirect Black 19, respectively. The decolorization efficiencywas further improved to 88 and 61 % for RBBR and Acid Red 37, respectively, when the concentration of laccase was increased to 20 U ml^{-1}. This indicates that this enzyme is very useful for bioremediation of textile dyes in waste water.

According to Casieri et al. (2008), two fungal species *Trametes pubescens* Mut 2295 and *Pleurotus ostrea*tus Mut 2976, obtained from Mycotheca Universitatis Taurinensis (MUT) Collection, were highly capable of decolorizing three industrial

dyes, anthraquinone dye (RBBR and B49) and azo dye R243, in a concentration range between 200–2,000 ppm. It was also noted that both the species decolorized anthraquinone dye RBBR and B49 more than azo dye R243 during 5 sequential cycles. Interestingly, laccase induction was found invariably higher during decolorization of all dyes than the control without dyes.

Gurav et al. (2011) have also reported microbial decolorization of a very recalcitrant insoluble Vat Red 10 dye, an anthraquinone oxazole dye, by *Pseudomonas desmolyticum* NCIM2112 and *Galactomyces geotrichum* MTCC 1360 at pH 9 and 25 °C. It was observed that both microbes *P. desmolyticum* and *G. geotrichum* could decolorize this dye by 55.5 and 45 %, respectively, after 23 days of incubation. During degradation of Vat Red 10 dye, a metabolite 2,6-diisopropyl Naphthalene (2,6-DIPN) was produced as an end product, which was found non-toxic in nature and also served as a plant growth factor.

Murugesan et al. (2006) have observed 90 % decolorization of 50 ppm anthraquinone dye by a laccase enzyme produced by fungus *Ganoderma lucidum*in the absence of redox mediator HBT, after 20 h of incubation. It was noted that in the presence of redox mediator HBT, the decolorization of RBBR was attained to 92 % by laccase enzyme within 2 h of incubation. Subsequently, Hadibarata et al. (2011) also investigated the degradation ability of a white rot fungus *Polypor*us sp. S133 for the decolorization of 200 ppm anthraquinone dye RBBR, mediated by laccase, induced by the presence of dye. About 26 and 60 % decolorization of this dye were obtained after 24 and 48 h of incubation period, respectively and complete decolorization was obtained after 72 h of incubation. It was also found that laccase with redox mediator increased the decolorization of anthraquinone dye by 20 %.

Similarly, Forootanfar et al. (2012) also studied the decolorization of six synthetic dyes using three different fungal laccases, produced by three different species of fungi i.e. *Aspergillus oryzae*, *Trametes versicolor* and *Paraconiothyrium variabile*. Among these fungi, laccase produced by *P. variabile* was found more capable in facilitating the decolorization of all six synthetic dyes than other two fungal strains, *A. oryzae* and *T. versicolor*, using hydroxybenzotriazole (HBT; 5 mM) as a laccase mediator. *P. variabile* decolorized bromophenol blue, commassie brilliant blue, panseu-S, rimazol brilliant blue R (RBBR), congo red and methylene blue by 100, 91, 56, 47, 18.5 and 21.3 %, respectively, after 3 h of incubation period. An increase in HBT concentration from 0.1 to 5 mM further augmented the decolorization of dyes. It was also found that in the absence of HBT, laccase from *A.oryzae* was able to degrade 53 % of methylene blue and 26 % of RBBR after 30 min incubation, while 93 % of RBBR was decolorized by *T. versicolor* after 3 h of incubation.

Siddiqui et al. (2010) reported decolorization of increasing concentration (10–200 ppm) of a reactive anthraquinone dye Dimarene Blue K2RL by a immobilized fungus *Aspergillus niger* SA1. About 75 % of decolorization was achieved in 24 h incubation with 10 ppm concentration of the dye. It was also observed that the decolorization of dye was gradually reduced with increasing concentration of dye i.e. 68, 40, 11, 3 and 2 % decolorization at 25, 50, 100, 200 and 300 ppm, respectively.

Andleeb et al. (2012) investigated the degradation and decolorization of anthraquinone dye Dimarene Blue K2RL by a fungus *Aspergillus flavus* SA2 in a lab scale immobilized fluidized bed bioreactor (FBR) system. It was observed that the fungus decolorized dye having higher concentration (up to 500 ppm), to a greater extent with a lower retention time (72 h) than that previously reported by Sharma et al. (2004a, b, c). About 71.3 % of the (50 ppm) Dimarene Blue K2RL dye was removed by *A. flavus* in the bioreactor system (FBR) after 24 h of incubation period. Different metabolites, formed during decolorization and degradation of this dye were identified as phthalic acid, benzoic acid, 1,4-dihydroxyanthraquinone, 2,3-dihydro-9,10-dihydroxy-1,4-anthracenedione, and catechol.

Ngieng et al. (2013) have also examined the decolorization of various synthetic dyes i.e., Congo red, Orange G, and Methyl red and Remazol brilliant blue R (RBBR) by twentyendophytic fungi isolated from *Melastoma malabathricum* (Senduduk). It was observed that out of twenty, only one strain MS8 was able to decolorize all the four dyes with 200 ppm concentration. This fungus completely decolorized the RBBR and Orange G dye in the agar medium within 8 days. When the decolorization was analyzed quantitatively in aqueous minimal medium, the percent decolorization for RBBR, Orange G, Congo red and Methyl red was found to be 97, 33, 48 and 56 %, respectively, within 16 days of incubation.

More recently, Korniollowicz-Kowalska and Rybczynska (2014) reported decolorization of two anthraquinone dyes, Ac and Poly R-478 by anamorphic fungus*Bjerkandera adusta* CCBAS 930. It was found that about 72 % of the color, which was generally caused by 0.01 % Ac, was removed by *B. adusta* CCBAS 930 after 4 days of incubation. This corresponded to 76.82 % reduction of the concentration of dye. Similarly, 70 % decolorization of 0.01 % Poly R-478 was observed, corresponding to a reduction of 77.28 % in the dye concentration by this fungus after 14 days of incubation.

3 Degradative Enzymes for Synthetic Dyes

The fungi are able to metabolize a wide range of carbon and nitrogen sources for their survival, mediated byextracellular enzymes, such as lignin peroxidase, manganese peroxidase and laccase (Saratale et al. 2007). Since these enzymes degrade many complex organic pollutants, they are found to be the most appropriate in the treatment of colored and metallic effluents (Ezeronye and Okerentugba 1999).

3.1 Lignin Peroxidase (LiP): (EC: 1.11.1.14)

Lignin peroxidase (LiP) enzyme is a heme containing protein and was first time detected in cultures of *Phanerochaete chrysosporium*. It is a monomeric N- and O-glycosylated protein and found in several iso-forms. (Tien and Kirk 1983;

Wesenberg et al. 2003). This enzyme is basic in nature having an isoelectric point between 3 and 5 depending on the isoform (Leonowicz et al. 2001). It essentially requires hydrogen peroxide (H_2O_2) to catalyze a reaction that occurs through a cycle. It has high redox potential, which facilitates the LiP enzyme to directly oxidize non-phenolic lignin units (Sarkar et al. 1997). LiP abstracts single electron from the aromatic rings of aromatic compound, leading to the formation of a cation radical and subsequent cleavage reactions (Zheng and Obbard 2002). A characteristic of LiP, which is also shared by non-ligninolytic peroxidases, is its relative unspecificity for substrates, such as phenolic compounds and dyes (Martinez 2002). During oxidation of aromatic ring of non-phenolic compounds, aromatic cations are formed by LiP and phenoxy radicals by the oxidation of phenolic substrates by peroxidases (Kersten et al. 1985; Martinez 2002).

The native enzyme having heme group (ferric form) at the active site is oxidized by H_2O_2 with two electrons to compound I as reflected in Fig. 2. One electron is abstracted from ferric [Fe(III)] iron to form ferryl [Fe(IV)], while the second electron is removed from the porphyrin ring to form a porphyrin cation radical

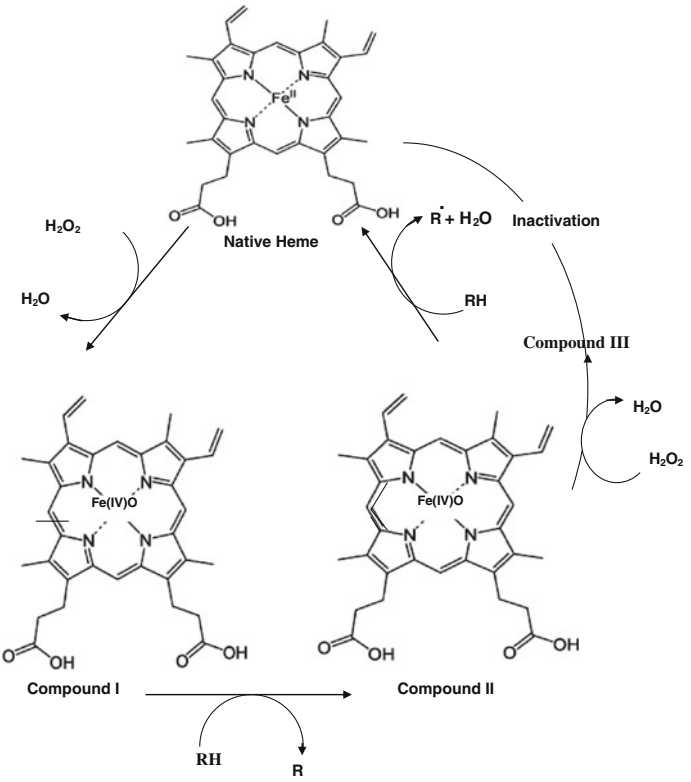


Fig. 2 Catalytic cycle of heme-containing peroxidases (modified after Torres et al. 2003)

(Aust 1995). During this reaction, H_2O_2 is reduced to water. Subsequently, compound I oxidizes substrates by one electron and gets reduced to compound II. In this step, the porphyrin ring gains the electron. Thus, compound I has higher ability to oxidize substrates with higher redox potential than compound II (Mester and Tien 2000). After this, compounds II reacts with reducing substrates by gaining one electron and returns to resting enzyme. There is also possibility that RH of compound I can react with compounds II to form radical $R^{\bullet}$., and compound II can also react with H_2O_2 leading to formation of compound III (Cai and Tien 1992), which causes the inactivation of peroxidase (Torres et al. 2003). The inactivation of LiP can be avoided by veratryl alcohol which completes the catalytic cycle of LiP by reducing compound II to resting enzyme, as illustrated below:

Catalytic cycle of lignin-peroxidase (Adapted from Banci 1997)

1. $Enz - heme[\mathrm{Fe(III)}]_{(PX)} + H_2O_2 \rightarrow (Enz - heme^{\bullet+})[\mathrm{O = Fe(IV)}]_{(Compound\ I)} + H_2O$
2. $(Enz - heme^{\bullet+})[\mathrm{O = Fe(IV)}]_{(Compound\ I)} + RH \rightarrow Enz - heme[\mathrm{O = Fe(IV)}]_{(Compound\ II)} + H^{+}R^{\bullet}$
3. $Enz - heme[\mathrm{O = Fe(IV)}]_{(Compound\ II)} + \mathrm{RH} \rightarrow Enz - heme[\mathrm{Fe(III)}]_{(PX)} + H^{+}R^{\bullet}$

where PX = native or resting enzyme, Enz = enzyme

3.2 Manganese Peroxidases (MnP): (EC: 1.11.1.13)

MnP is also a heme-containing peroxidase having heme (ferric protoporphyrin) as a prosthetic group and glycoprotein in nature (Zapanta and Tien 1997). This is the most common ligninolytic peroxidase, produced by almost all white rot basidiomycetes (Glenn and Gold 1985; Hofrichter 2002; Wesenberg et al. 2003). Its mechanistic properties are similar to LiP and it forms the oxidized intermediates, compound I and compound II (Cai and Tien 1993; Zapanta and Tien 1997). H_2O_2 is essentially required by MnP for the oxidation of lignin and lignin-related compounds (Mester and Tien 2000). MnP is dependent on Mn (II) as a substrate for the formation of compound II (Wariishi et al. 1988). Mn (II) is also a preferred substrate for compound I. During degradation of lignin and other substrates, MnP oxidizes Mn (II) to Mn (III) and subsequently, Mn (III) oxidizes a variety of compounds (Glenn et al. 1986; Mester and Tien 2000). It was also observed that the chelation of Mn (II) and Mn (III) by organic acids, such as oxalate, was essential for MnP activity (Zapanta and Tien 1997). Oxalate is an organic acid chelator produced by white rot fungi at the same time when MnP is synthesized in the liquid cultures of *Phanerochaete chrysosporium* (Wariishi et al. 1992; Kuan and Tien 1993; Zapanta and Tien 1997).

3.3 Laccase (Lac): (EC 1.10.3.2)

Laccase belongs to a group of polyphenol oxidases in which the catalytic center is found to be occupied by Cu atoms (Fernaud Hernández et al. 2006) This enzyme was first discovered in the exudates of Japanese lacquer trees and then found in a wide range of plants and fungi (Giardina et al. 2010). Fungal laccases are found to contain 4 Cu ions in three types of sites: type 1 (blue Cu) is characterized by a strong absorption at ~610 nm; type 2 is weakly absorbing and acts as a one-electron acceptor; and type 3 contains a pair of Cu ions that absorb at 330 nm and function as electron acceptors (Baldrian 2006). Laccases have the ability to surprisingly oxidize a wide range of organic and inorganic compounds including diphenols, polyphenols,diamines, and aromatic amines by reducing molecular oxygen to water (Kiiskinen et al. 2002).

The function of fungal laccases is that four copper atoms are organized into 2 clusters; where type 1 copper is located near the active site and the 2 and type 3 are located at the core of the enzyme forming a triangle and trinuclear cluster. The type 1 copper receives electrons extracted from the substrate and transfers it to type 3 copper, which subsequently relays the electrons to a type 2 copper where oxygen gets reduced to water (Fig. 3). For the reduction of oxygen, it is necessary that it

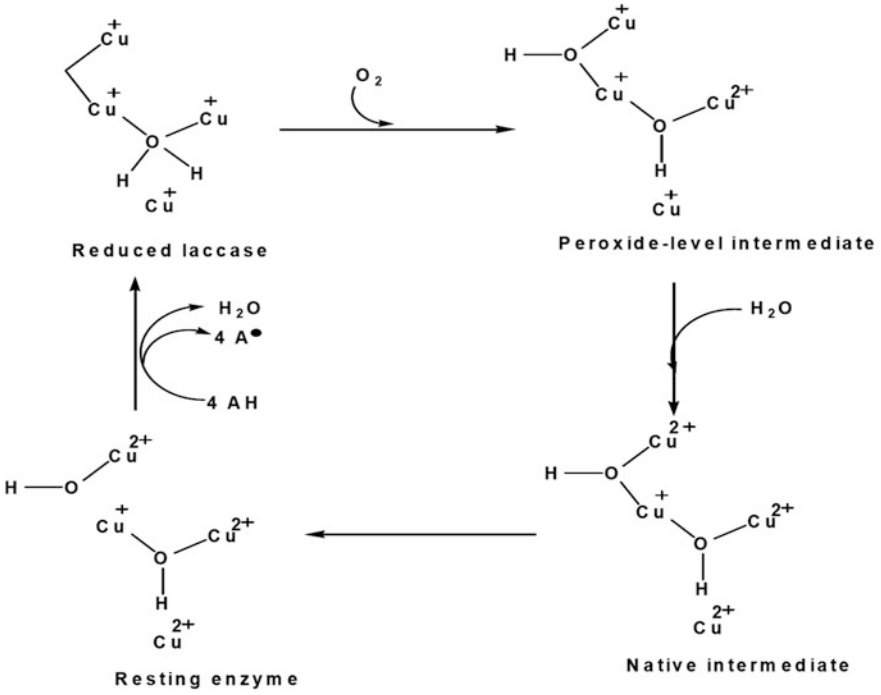


Fig. 3 Catalytic cycle of laccases (Wesenberg et al. 2003)

should reach the trinuclear cluster through a channel. However, sodium azide is a known non-competitive inhibitor of laccase (Mishra and Kumar 2009).

It has a broad substrate range, and does not require H_2O_2 for oxidation reactions in contrast to other oxidases and peroxidases. Therefore, laccases are very useful in biotechnological applications, such as paper pulp bleaching, synthetic dye decolorization, bioremediation, biosensing, chemical synthesis, and immunoassay (Champagne and Ramsay 2005; Couto and Herrera 2006). The decolorization activity of laccases generally depends on the source of the enzyme and also on the chemical structure of the dye (Abadulla et al. 2000; Rodriguez-Couto 2007; Michniewicz et al. 2008). Therefore, laccase enzymes obtained from different strains and also from different fermentation processes may have differentdye decolorization potential.

Anthraquinone dyes with fused aromatic rings, such as Acid Blue 80 and RBBR, are generally used as starting materials for polymeric dyes and hence, represent an important class of toxic and recalcitrant organopollutants. Laccases are considered remarkable "green" catalysts in removing highly toxic phenolic pollutants from the industrial wastewater. According to Hsu et al. (2012), *Lentinus* sp. lcc3 possesses a novel decolorization efficiency (1 h reaction at room temperature) for Acid Blue 80 (97 % at 1 U ml^{-1} lcc3) and RBBR (88 % at 20 U ml^{-1}). Similarly BenYounes et al. (2007) have observed 91 % decolorization of RBBR by laccase produced from *Perenniporia tephropora* after 48 h of the treatment.

Erkurt et al. (2007) reported that *F. trogii*was found to be an efficient fungal strain that produce laccase for decolorization of dyes. It was found that about 90 mg l^{-1} of RBBR was decolorized by *F. trogii* after 48 h of incubation, which was mediated by the laccase enzyme produced by the fungal strain. Forootanfar et al. (2012) isolated three laccases from three fungal strains and observed their effect on anthraquinone RBBR dye decolorization both in the presence and absence of redox mediator HBT (N-hydroxy benzotriazole). It was observed that in all the cases, the percent of decolorization increased with an increase in HBT concentration. The purified laccase of *T. versicolor* showed the highest (80.5 %) decolorization after 30 min incubation in absence of HBT. In case of laccase from *A. oryzae* and in absence of HBT, decolorization was found to be only 28.3 % after 30 min. However, *P. variabile* caused only 16.6 % decolorization at the same time in presence of HBT (5 mM). Similarly, it was also reported that laccase enzymes produced by both *Pycnoporus cinnabarinus* and genetically modified *Aspergillus*, together with a redox mediator and a nonionic surfactant were able to degrade an azo and an anthraquinone dye, respectively (Schliephake et al. 2000; Soares et al. 2001).

Murugesan et al. (2006) studied the role of crude laccase enzyme both in the presence and absence of redox mediator (HBT) isolated from a white rot fungus *Ganoderma lucidum* KMK2 for the decolorization of different dyes, such as Remazol Black-5 and anthraquinone dye Remazol Brilliant Blue R (RBBR). It was found that crude enzyme showed maximum decolorization activity to anthraquinone dye Remazol Brilliant Blue R (RBBR) without redox mediator, whereas diazo dye Remazol Black-5 (RB-5) required a redox mediator. Besides, a concentration of

1 mM HBT was found to be suitable to decolorize RB-5 (50 mg l^{-1}) by 62 and 77.4 % within 1 and 2 h, respectively by the crude laccase (25 Um l^{-1}) where RBBR (50 mg l^{-1}) was decolorized by 90 % within 20 h. However, laccase was found to be more efficient in presence of HBT, showing 92 % decolorization within 2 h.

Hadibarata et al. (2011) have also reported decolorization of anthraquinone RBBR dye by laccase enzyme extracted from white rot fungus *Polyporus* sp., both in the presence and absence of a redox mediator HBT. It was observed that laccase individually could also decolorize dye but in the presence of HBT, the decolorization was increased by 20 %.

Several workers have reported use of HBT as a laccase mediator and the reason for selection is that the dyes with NH_2 and OH groups in their structure create steric hinderance and reduce the accessibility of laccases. Thus, these dyes are more degradable by laccase attack (Soares et al. 2001; Camarero et al. 2005; Rodriguez-Couto 2007). The affinity of the oxidized mediator for the substrate reaction is an important factor for the efficiency of a laccase-mediator system.

3.4 Dye-Decolorizing Peroxidase (DyP)

Dye decolorizing peroxidase, a heme-containing enzyme, is broadly present in plants, microorganisms and animals (Duarte-Vazquez et al. 2003). The metabolism of aromatic dyes is mediated either by precipitation or by opening the aromatic ring structure. So, this group of enzyme has opened new prospects for the development of biotechnological processes aimed at the degradation of xenobiotic compounds (Field et al. 1993), effluent decolorization (Banat et al. 1996) and bio bleaching of Kraft pulp (Moreira et al. 1997). DyP, isolated from fungus *Thanatephorus cucumeris*, is a glycoprotein novel extracellular peroxidase and contains one heme as a co-factor. This enzyme has molecular mass of 58 kDa and essentially requires H_2O_2 for all enzyme reactions. This clearly indicates that it works as a peroxidase. It functions well under lower pH (3-3.2) conditions and shows no homology to other peroxidases. Besides, it has broad substrate specificity, as DyP degrades not only the typical peroxidase substrates, but also degrades hydroxyl-free anthraquinone, which is not a substrate of other peroxidases (Kim and Shoda 1999; Sugano et al. 2000, 2006). Most of the synthetic dyes are derived from anthraquinone compounds. Hence, DyP is a promising enzyme for the treatment of the dye-contaminated water, because it degrades synthetic dyes very efficiently and effectively (Kim and Shoda 1999; Sugano et al. 2000; Shakeri et al. 2007).

So far, degradation of anthraquinone by peroxidases is found to be difficult, as it contains no hydrogen atoms that can be withdrawn by peroxidase. In fact, no model related to in vitro degradation of anthraquinone dyes by peroxidase has been published. Two peroxidases, namely pseudoperoxidase and horseradish peroxidase have been found which directly or indirectly mediate the degradation of anthraquinone. Oxidative degradation of a hydroxyl anthraquinone compound is mediated by pseudoperoxidase activity offerrylmyoglobin (Cartoni et al. 2004), while

horseradish peroxidase indirectly degrades anthracycline by acting on hydroquinone (Reszka et al. 2005).

Sugano et al. (2009) have reported complete decolorization of Reactive Blue 5, which is one of the derivatives of anthraquinone, as shown in Fig. 3 by the concerted action of two peroxidases, one versatile peroxidase (TcVP1) and the otherdye-decolorizing peroxidase (DyP) from *Thanatephorus Cucumeris* (Fig. 4). The decolorization processproceeded in a sequence; initially, DyP decolorized Reactive blue 5 to light red-brown compounds, and then TcVP1 decolorized these colored intermediates to colorless DyP. This was the first description of the *in vitro* complete decolorization of an anthraquinone dye and the first report of using adual-enzyme system for such a purpose. This strongly supports the notion that DyP and

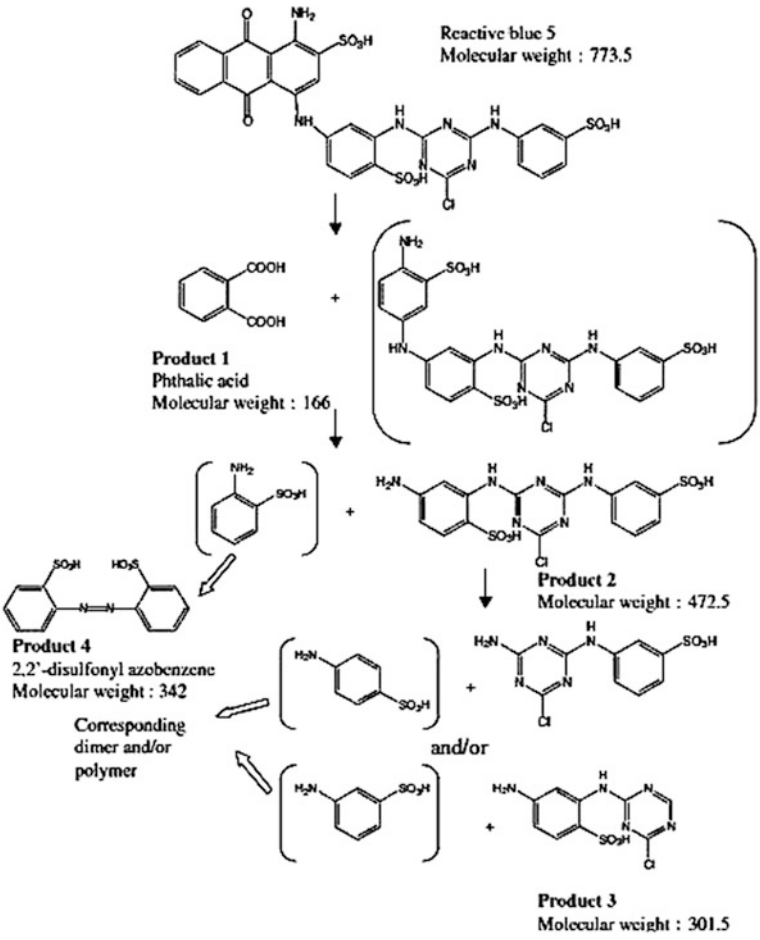


Fig. 4 Degradation pathway of RB5 treated with dye decolorizing peroxidase (DyP) (Sugano et al. 2009)

TcVP1 are good candidates for development of a novel strategy for the treatment of dye wastewater.

It is also reported that the putative oxidative cycle of fungal DyPs is largely equivalent to that found in other peroxidases. Yoshida et al. (2011) proposed a swinging mechanism of a distal aspartate residue during Compound I formation, as reflected in Fig. 5. In the first step, H_2O_2 enters the heme cavity of the enzyme in resting state, where it displaces a water molecule that occupies the sixth ferric iron coordination site of the protoporphyrin IX system. A distal basic amino acid residue mediates the rearrangement of a proton in H_2O_2. In peroxidases, the base residue is a histidine, whereas in DyPs, this key residue is substituted by an aspartate. The heme molecule is then oxidized to the radical-cationic oxoferryl species Compound I by two-fold single electron transfer, releasing a water molecule. Two electrons are successively drawn from substrate molecules, to from their oxidized counterparts. Concomitantly, the heme is stepwise reduced back to its initial oxidation state, leading to the resting phase of the enzyme in this process.

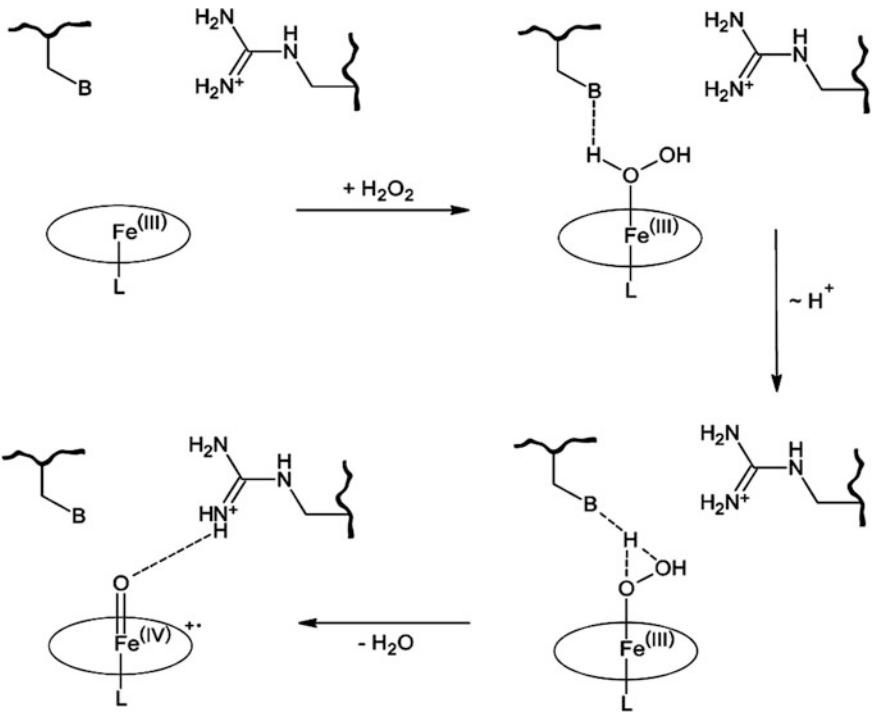


Fig. 5 Catalytic features of a DyP-type peroxidase

$$\text{Resting peroxidase} + H_2O_2 \rightarrow \text{Compound I} + H_2O$$
$$\text{Compound I} + AH_2 \rightarrow \text{Compound II} + AH^{\cdot}$$
$$\text{Compound II} + AH_2 \rightarrow \text{Resting peroxidase} + AH^{\cdot}$$
$$2AH^{\cdot} \rightarrow A_2H_2 \text{ or } AH_2 + A \text{ (non - enzymatic reaction)}$$

The above reactions summarize the general catalytic cycle of peroxidases. The term resting peroxidase indicates the peroxidase resting state and AH_2 and AH are substrate molecules and the corresponding radical species, respectively.

This is the key step in heme peroxidase catalysis. After proton rearrangement by the distal base catalytic residue, two electrons are transferred from heme to hydrogen peroxide, which turns enzyme active, called Compound I (Poulos and Kraut 1980). **B** indicates a basic residue, i.e., a histidine or aspartate/glutamate in peroxidases. **L** indicates the proximal heme ligand, usually a histidine residue (Strittmatter et al. 2013).

4 Mechanisms of Fungal Dye Degradation and Decolorization

In comparison to physical and chemical methods, biological treatment is often found to be economical and eco-friendly alternative. Biological methods, such as microbial decolorization and degradation, adsorption by living or dead microbial biomass, are commonly used for the treatment of industrial effluents (Fu and Viraraghavan 2001; McMullan et al. 2001).

In the decolorization and degradation of dyes by fungal strains, the chemicals are adsorbed on the microbial biomass. This process is known as biosorption which takes place on both living and dead biomass. Dead microbial biomass contains a natural polysaccharide chitin and its derivative chitosan in their cell walls which has a unique molecular structure with a high affinity for many classes of dyes. Hence, microbial biomass can be used as an efficient absorbent for synthetic dyes (Joshi et al. 2004).

During decolorization and degradation of dyes by fungal strains, at least two or three highly non-specific enzymes, like lignin (LiP), manganese peroxidase (MnP) and laccase (Lac), are induced which generate free radicals in a variety of reactions (Knapp et al. 2001; Pointing 2001). The degradation of dyes generally depends on their chemical structure which strongly influences their degradability by pure cultures and isolated enzymes.

The fungal action rarely leads to the mineralization of dyes, depending on their chemical structures. It was also observed that dyes containing substituted aromatic rings in their structure were mineralized faster than unsubstituted rings. Nitrogen-limited conditions also mediate faster mineralization of dyes. A few researchers have also reported that the dyes can be utilized as a carbon source by the fungal

strains. To be used as a carbon source, certain bonds in the dye molecule are cleaved, but the chromophore is not affected. This mechanism occurs generally in the case of consortium of microorganisms (Knapp et al. 2001; Singh 2006).

5 Factors Affecting Fungal Decolorization and Degradation of Dyes

There are various factors, such as media composition, pH value, agitation and aeration, temperature and initial dye concentration, which can impact the growth of fungal strains and also regulate decolorization and degradation of synthetic dyes.

5.1 Media Composition

The media composition plays a very important role in facilitating the fungal growth and decolorization process. The media usually contain carbon and nitrogen sources along with mineral nutrients and other additives (Singh 2006).

5.2 Carbon Source

There is an essential requirement of carbon source for the fungal growth, as it supplies the oxidants which mediate the decolorization of dyes. Generally glucose has been widely used by many workers as a carbon source in their studies for the fungal culture. Besides, sucrose, maltose, xylose and glycerol, starch and xylan were also found to be useful as a carbon source. But cellulose and its derivatives were not found effective as a source of carbon. For initial experiments, glucose at concentration of 5–10 g l^{-1} is a good choice. A wide difference in the nature of carbon substrates used for the effluent treatment of the dyes was observed. It was found that effluents from dyeing or chemical dye production usually do not contain usable carbon substrates, while others from distilling or paper pulping contain a range of carbohydrates which are useful substrates for certain white-rot fungi. So, the addition of carbon source largely depends on the organism and type of the dye to be treated for decolorization/degradation.

Swamy and Ramsay (1999b) observed that glucose was an essential carbon source and a minimum of 0.34 g glucose l^{-1} was required for the decolorization of dyes. In addition to being a potential carbon and energy source, glucose is also a substrate for pyranose oxidase which can generate H_2O_2 (Giffhorn 2000) for peroxidase activity and oxygen radicals.

Radha et al. (2005) have also investigated the role of glucose in the decolorization of dyes. It was noted that when the initial concentration of glucose was enhanced from 1 to 5.0 g l^{-1} in the basal medium, the decolorization of dyes was significantly enhanced. This clearly indicates that glucose enhances the decolorization of dyes and a concentration of 5.0 g l^{-1} glucose was found to be sufficient to achieve the maximum decolorization. It was also noted that as the glucose concentration was increased from the optimal level, it caused a decrease in dye decolorization rate due to change in the metabolic pathways. In such situation, glucose was more utilized for the growth of fungus than for decolorization of dyes.

5.3 Nitrogen Source

Nitrogen is a second essential component of media required for the growth and enzyme production in fungal species. Therefore, the demand of nitrogen varies widely among fungal species. For example, in *P. chrysosporium,* the ligninolytic enzymes are produced under nitrogen-limited conditions, while in *B. adusta,* more LiP and MnP are produced in nitrogen-sufficient media. However, white-rot fungi have the ability to use both inorganic and organic nitrogen sources. Ammonium salts in the form of inorganic nitrogen are generally used for the fungal growth and enzyme production, while organic nitrogen is not found suitable. Therefore, the presence of usable nitrogen source in the case of effluent treatment should be considered from the angle of dye decolorization.

Radha et al. (2005) studied the effect of nitrogen source on decolorization of dyes. In their study, the ammonium chloride was chosen as nitrogen source (0–0.2 g l^{-1}) Without nitrogen source, the decolorization of dyes was found to be 45 %, but when medium was supplemented with 0.05 g l^{-1} of ammonium chloride, the dye decolorization was attained to 96 %. However, when the amount of nitrogen source was increased from its optimum level, the decolorization processwas adversely affected. However, Swamy and Ramsay (1999a) have reported that production of manganese peroxidase (MnP) and laccase enzyme was enhanced in nitrogen-limited conditions duringdye decolorization.

5.4 Mineral Nutrients

In addition to carbon and nitrogen sources, certain mineral nutrients are also required by microbes, for example, white-rot fungi need iron, copper and manganese for the decolorization of dyes. These mineral nutrients can be either a part of effluent or must be added to the media. In addition, fungal lignin peroxidase requires a variety of other materials, like veratryl alcohol, tryptophan and aromatics (phenol and aniline) for its activity, as these serve a low molecular mass redox mediators and therefore, facilitate the decolorization process (Knapp et al. 2001; Singh 2006).

It was also found that wood and straw contain some components which induce the production of enzyme in whit rot fungi. According to Pavko and Novotny (2008), the production of ligninolytic enzyme in *Dichomitus squalens* could be substantially induced by the addition of beech wood and straw particles to the liquid media.

5.5 pH

Filamentous fungi together with white-rots generally grow optimally at acidic pH. However, during cultivation, a change in pH was observed which usually depended on the substrate used for the growth of fungi. When the carbohydrate containing media was used for the growth of fungi, it caused acidification of the medium. The decolorization of the dye can be carried out with a wholefermentation broth (mycelium and enzymes) or with isolated enzymes. Therefore, a distinction has to be made between the optimum pH for growth and for the enzyme production, the optimum pH for the action of isolated enzymes and the optimum pH for dye degradation. According to Knapp et al. (2001), the optimum pH is to be maintained in the range of 4–4.5 for the growth of fungi as well as action of enzyme systems which facilitate the decolorization of dyes.

Radha et al. (2005) also observed the effect of pH on the decolorization of dyes. The growth of fungus *P. chrysosporium* and decolorization of dyes are regulated by the pH of the medium. Maximum decolorization of most of the dyes was observed at a pH range of 4.0–5.0 and the level of decolorization was decreased at both extremes of pH (<4.0 and >5.0). Most of the dyes were found to decolorize at a pH range of 4.0–5.0. However, methylene blue, Acid green, Congo red and Vat magenta were mostly decolorized at around pH 5.0.

The effect of pH on the decolorization of anthraquinone dye RBBR was also studied by Yang et al. (2009) and it was found that optimal pH value for decolorization was 4.5 at which the decolorization yield reached to 85 % after incubation at 25 °C for 25 min.

5.6 Temperature

Temperature is also a very important factor, as it influences the growth and enzyme production, the enzymatic decolorization rate and the temperature of the waste stream. Most white-rot fungi are mesophiles and their optimum temperature is in the range of 27–30 °C for the culture. For enzymatic reactions, the optimal temperature is found usually higher, but the enzyme stability and degradation are adversely affected at temperatures beyond 65 °C. A variety of textile and dye effluents are produced at temperatures 50–60 °C, most of the researchers have suggested that the optimal temperature for the decolorization of dyes has to be selected on case to case basis (Knapp et al. 2001; Singh 2006).

Radha et al. (2005) studied the effect of temperature on fungal growth of *P. chrysosporium* and the decolorization process. Different temperatures were selected ranging from 20–45 °C for decolorization of dyes. It was observed that thedecolorizing activity of fungi was reduced at both higher and lower temperature, indicating the lack of peroxidase production which is generally required for the decolorization of dyes or the enzyme gets denatured at higher (>35 °C) or lower (<35 °C) temperature. Hence, for *P. chrysoporium*, a temperature of 35 °C was found most suitable for the fungal growth as well as decolorization process.

The effect of temperature on decolorization of RBBR was also studied by Yang et al. (2009) and it was observed that the variation in temperature had minimum effect on decolorization of RBBR below 70 °C and maximum decolorization was attained at 50–60 °C by a white-rot fungus, *Trametes* sp. strain SQ01 after 10 min of incubation.

5.7 Agitation and Aeration

Since most of the ligninolytic fungi are obligate aerobes, they essentially, require oxygen for the maintenance of their viability. There is a need for oxygen for multipurpose in fungi i.e., lignin degradation also requires oxygen, either for the mycelial generation of H_2O_2 for peroxidases or for the direct action of oxidases. Oxygen can also degrade lignin fragments directly. Hence, the demand for oxygen generally depends on the fungus and its ligninolytic system. The water solubility of oxygen is very low i.e. 8 mg l^{-1} at 20 °C. As the oxygen supply to the culture media is necessary for cultivation, its solubility can be enhanced by both aeration and agitation.

5.8 Initial Dye Concentration

For the removal of dyes, it is important to optimize the initial dye concentration. Dyes are usually toxic to microorganisms and its toxicity depends on the type of dye. Higher concentrations of dyes are invariably toxic. The range of initial dye concentration studied generally varies from 50–1,000 mg l^{-1}, and also depends on microorganism and type of dye (Singh 2006).

6 Conclusion

A lot of studies have been carried out on fungal decolorization of chemical dyes on a laboratory scale to find the potential fungal strains with effective enzymes. The management of textile industrial effluents is no doubt a complicated task, taking into consideration the complexity of the waste compounds present in the waste

water, in addition to dyes. Many options were explored for treatment of textile effluent water and reuse of water. A wide range of water pH, temperature, salt concentration and chemical dyes in use today add to the complications of biological treatments. Hence, color removal by microbes from effluents in an economical way still remains a formidable task. Although a number of systems employing various physico-chemical and biological processes have been successfully implemented, but regulatory agencies are stressing on only new, efficient, and improved decolorization technologies. In view of emerging demand for a technically feasible and economical treatment technology, a number of emerging technologies are being proposed and are presently at different stages of commercialization. New technologies with amalgamation of different methods are most likely to be available in the near future, which will be both efficient and economically viable. However, high cost involved in sophisticated technologies may have many limiting factors as well. Therefore, bioremediation of textile effluents is still an attractive solution being a low-cost, sustainable and publicly acceptable technology. Use of microbes and their isolated enzymes for textile dyes degradation is not very expensive option. That is why many microorganisms and enzymes have been isolated and explored for their ability and capacity to degrade dyes. Microbes have been also modified by the genetic engineering tools to obtain "super and faster degraders". In some cases, a combination of biological with physical process such as adsorption or filtration, or chemical, such as coagulation/oxidation-processes may be inevitable to achieve the desirable goal of textile effluent treatment. Several low cost and efficient sorbents including natural wastes are also very promising, due to their low cost and high availability.

7 Future Perspectives

In view of increasing application of synthetic dyes, there is an urgent need for the effective treatment process of colored effluents, prior to their discharge as waste water into waterways: Biodegradation of synthetic dyes using different microbes and isolated enzymes offers a promising approach by individually or in combination with conventional treatments available. The complexity of dyes degradation of structurally different dyes, undoubtedly demands focused research for microbial degradation. As of now, most of the investigations addressed dye degradation of azo dyes. Now, there is also a need to focus on microbial degradation of other classes of textile dyes, anthraquinone, indigoid, xanthene, arylmethane and phthalocyanine derivatives. The pathways for dye degradation are also still not fully explored. Intensive research in microbiology, molecular biology, chemistry and genetic fields is needed to explore the degradation pathway and to develop a new technology which will be highly effective for degradation of different dyes. New microbes and enzymes, with broader substrate specificity and higher activity, have to be isolated and studied for their ability and capacity as key agents in dye remediation. Through the process of genetic engineering, random or selective

modification of the microorganisms and enzymes would help us in tailoring microbes with higher catalytic power to target a range of toxic compounds. Optimization of the biological remediation process with respect to time, efficiency, stabilization and costs will promote to develop new bioremediation technology. Emerging effective technology is likely to be a combination of more than one treatment, either biological or chemical, for the complete mineralization and detoxification of the colored effluents from the textile industries. However, adaptation of new treatments has not to target on pollution reduction alone, but also on reuse of waste water and likely use of by-products for other applications, if possible.

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