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Improved Bacterial-Fungal Consortium as an Alternative Approach for Enhanced Decolourisation and Degradation of Azo Dyes: A Review

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ABSTRACT

Over the past five decades, the Indian textile industries have increasingly become a major user of azo dyes due to their wide range of applications. India is considered as one of the second largest producers of textiles in the world. Due to the rapid increase in the production rate of fabrics, the textile industry generates a huge amount of dyestuff and dyes in the environment. Continuous discharge of wastewater into the environment creates various types of pollution and also health impact on human beings. Azo dyes are toxic aromatic man-made compounds, which cannot be easily degraded by the indigenous microbes, and thus persist in ecosystems over a long period of time. In the current scenario, most wastewater treatment plants are obsolete and especially physico-chemical methods failed to achieve the satisfactory result because they are more expensive and produce a huge amount of sludge which again causes the secondary disposal problems. So, there is an urgent need to develop an alternative method to improve the degradation of azo dyes in an eco-friendly manner with low cost. In order to achieve the higher degradation efficiency in bioremediation technology, the present review mainly focuses on three important factors such as strain development for hyper-production of oxidoreductive enzymes by random mutagenesis, development of bacterial-fungal consortium and reusability of microbial cell cultures. Additionally, this review also compiles the effect of various physico-chemical conditions in decolourisation and degradation of azo dyes.

INTRODUCTION

Dyes are considered as complex organic substances, which give a wide range of colours to substrates when applied to them. They firmly get attached to the substrate by physical adsorption, chemical modification or by the covalent linkage (Kirk-Othmer 2004 & Bafana et al. 2011). Dyes consist of two important compounds, they are chromophore and auxochrome. The colour property of dyes depends on the chemical constituents of dyes and their absorption ability for the visible light. But, according to the Witt theory, the colour of the dye is due to the presence of chromophores, whereas the modern electronic theory states that the colour changes occur due to the excitation of the valance electrons (Murrell 1973).

Historical view about dyes: Natural dyes have been used by the mankind since ancient times or Neolithic period, which took place around 10,200 BCE. These dyes were made by using natural pigments obtained from the plant extracts, insects, sea animals and minerals (Kadolph 2008). The man lived in that age mixed the dyes with solvents and used them for the painting on caves, skin, jewellery and clothes. Nearly, 40,000 years ago during the New Stone Age period, the yellow and blue colours were considered as the most common one obtained from the plant sources. The types of natural dyes and their sources are presented in Table 1.

Rise of synthetic dyes and the current situation of dying industries in India: The first artificial dye "mauve" was accidentally identified by W. H. Perkin in 1856, which gives a permanent colour when applied to the fabrics. Further, these dyes are highly stable and do not undergo fading even by the exposure to radiation, water, various chemicals and microbial degradation (Rai et al. 2005). In the recent past, original dyes obtained from natural sources have been completely modified by the artificial dyes as the former are highly expensive, less applicability and tend to fade quickly. Over the past five decades, 10,000 artificial dyes have been synthesized worldwide and used for the different applications (Robinson et al. 2001). Currently, the European countries have established many dyeing industries and are able to produce 40% of dyestuff, annually. These dying industries discharge huge amount of effluents into water bodies. Moreover, the water discharged from the textile industries is quite resistant to microbial attack due to unfavourable pH, organic and inorganic matters, and it also contains a

Table 1: Different sources of natural dyes.

S.No	Natural Dyes	Color	Sources
1	Indigo	Deep blue	Obtained from the Dyer's Woad herb (leaves), *Isatis tinctoria*, *Indigofera tinctoria*
2	Tyrian Purple	Purple	Glands of snails
3.	Alizarin	Red	Extracted from the madder plant
4.	Carthamin	Yellow	Extract obtained from the flowers of *Carthamus tinctorius* and weld (leaves) and oak tree (bark)

Table 2: Society of Dyers and Colorists (SDC) classify the azo dyes in colour index (CI) based on the structural complexity.

S.No.	Azo dyes based on chemical structure	Color Index (CI)
1.	Monoazo	11000-19999
2.	Bis-azo	20000-29999
3.	Tri-azo	30000-34999
4.	Polyazo	35000-36999
5.	Azoic	37000-39999

mixture of different types of dyes (Banat et al. 1996). Worldwide, it was reported that 2,80,000 tons of treated or untreated effluents were discharged onto the land or in water every year (Jin et al. 2007). In India, the dye industry grew rapidly for the past 50 years due to the high demand for fabrics. Nevertheless, Gujarat is considered the first leading producer of dyestuff, whereas Mumbai and Tiruppur are the second most producers. Over the past 50 years, the number of bleaching and dying units in Tiruppur have considerably increased and currently, the town has established 728 dying units out of which 430 units are in operation (Fig. 1). It is estimated that 80,000 tons of dyestuff and pigments are produced every year in India (Marimuthu et al. 2013). The effluents discharged from the dying industries contain 2% of dyes, which have been released during the dying process. Till 1977, the untreated effluents from the textile industries have been discharged into Noyal River that caused a severe environmental pollution in and around Tiruppur region. To solve this problem, the Tamil Nadu Pollution Control Board (TNPCB) established several Common Effluent Treatment Plants (CETPs), which use physical, chemical and biological methods to eradicate the dye-containing wastewater released from the textile industries. However, these methods failed to achieve satisfactory results to remove completely the structurally different dyes from effluents and also produce the secondary disposal problems.

Classification of synthetic dyes: Generally, the dyes have been classified in two ways based on their structural complexity (nature of chromophores) and their mode of application on fabrics. Based on the nature of chromophores and chemical classification system, the dyes are classified into Acridine dyes, Anthraquinone dyes, Arylmethane dyes, Triarylmethane dyes, Azo dyes, Cyanine dyes, Diazonium dyes, Nitro dyes, Nitroso dyes, Phthalocyanine dyes, Quinone-imine dyes, Azin dyes, Xanthene dyes, Indophenol dyes, Oxazin dyes, Safranin dyes, Thiazin dyes, Thiazole dyes, Fluorene dyes and Rhodamine dyes. Similarly, based on their mode of applications these dyes have been classified as Reactive dyes, Acid dyes, Basic dyes, Direct dyes, Mordant dyes, Disperse dyes, Vat dyes, Sulphur dyes and Solvent dyes. Azo dyes are predominantly utilized as a dying agent in textile industries due to their wide range of applicability. Until 2001, there was no proper nomenclature system to classify the dyes, but presently the Generic Name and Colour Index have been compiled to each dye by the Society of Dyers and Colorists (SDC) and the American Association of Textile Chemists and Colorists (AATCC).

Azo dyes: Azo dyes are synthetic compounds and have been mostly used in different fields, especially in textile industries. As they are very stable, require very less amount for production and generate a wide variety of colours, which accounts for 60-70% of total synthetic dyes (Yingying et al. 2016). Azo dyes have a complex structure with high molecular weight compounds and contain one or more azo bond (-N=N-), which has the ability to absorb light in the visible spectrum of range 400-700 nm (Chang & Kuo 2000). The first azo dyes synthesis was started in the year 1861 by Mene (Aniline Yellow) and followed by Martius in 1863 (Bismarck Brown). The modern azo dyes are synthesized by two-step reactions in which during the first step the aniline is used as a derivative for the synthesis of an aromatic diazonium ion, and in the next step, diazonium salt is coupled with an aromatic compound (Fig. 2).

The American Association of Textile Chemists and Colorists (AATCC) classify the azo dyes based on the chemical constituent or by the colour. According to the colour index system, azo dyes are specifically assigned in the range of 11000 to 39999 based on the structural variation and chemical nature of the dyes (Table 2).

Persistent nature of azo dyes: Azo dyes are xenobiotic organic compounds, which cannot be easily degraded by the chemical, light or even by the microbial attack (Zeenat et al. 2014). These dyes are highly recalcitrant in nature due to

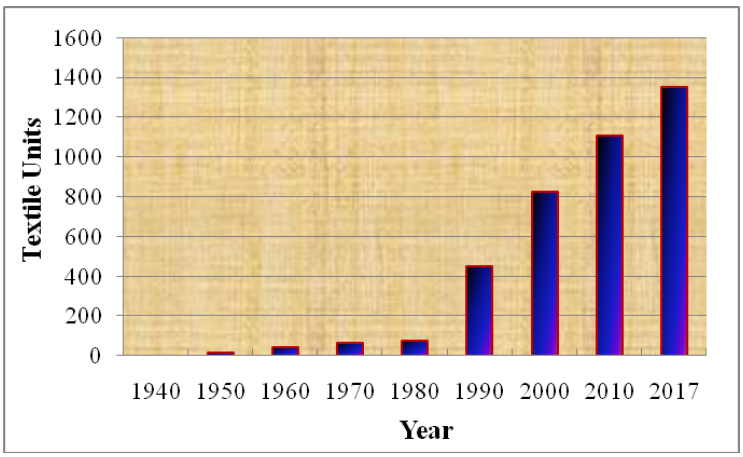

Fig. 1: Number of bleaching and dyeing units in Tirupur (1940-2017).

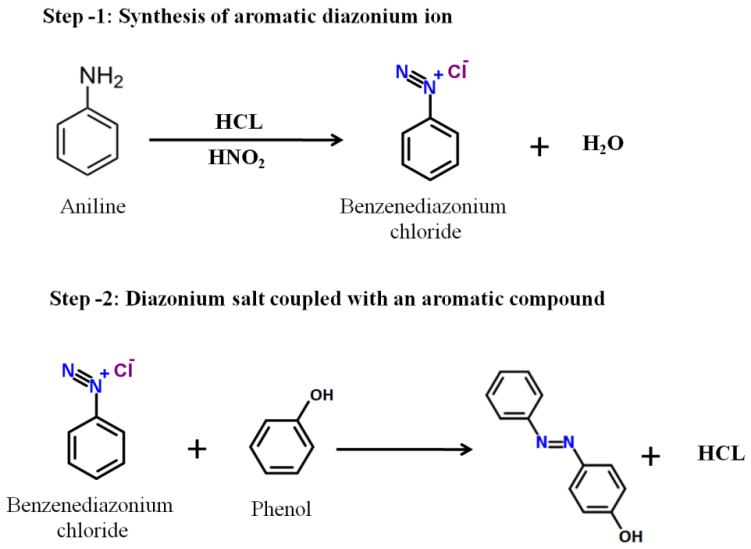

Fig. 2: Synthesis of modern azo dyes.

the presence of one or more azo bond (Anjaneya et al. 2013) and sulphonic (-SO_3^-) electron-withdrawing group (EWG). Sulphonated azo dyes readily obtain electrons from the nearest orbit and generate electron deficiency in the molecules; thereby it provides more stable azo compounds which are found to be more resistant to the oxidative catabolism of microbes (Akpanand & Hameed 2013). Further, the azo dyes containing an EWG in an ortho position are more stable than the azo dyes that have an EWG at the para position. This effect is called a "meta effect" and it could be due to the steric hindrance of the substitute molecules near to the azo bond. Indeed, the azo dyes combined with a different substituent and additionally with one or more sulphonic compounds make them highly persistent to the aerobic environmental conditions.

Impact of azo dyes: Among all the industrial sectors, textile industries are considered as one of the leading polluters of environment due to the release of a large amount of treated and untreated dyes in wastewater. Unfortunately, it is estimated that 2,00,000 tons of dyestuff are released into the environment every year. Azo dyes are dispersed in untreated or improperly treated effluents and their toxic products generated during the partial degradation are discharged into the water bodies that causes unpleasant odour which leads to abnormal changes in the quality of water. Further, it generates mutation of aquatic plants and small animals, and causes acute toxicity to them (Vandevivere et al. 1998). The products obtained after the anaerobic degradation of azo dyes using microorganisms lead to the formation of aromatic amines, which is resulted to have a toxic effect on

Table 3: Physico-chemical treatment methods for azo dye.

Physical/chemical treatment methods	Material used	Advantages	Disadvantages
Coagulation-flocculation	Mg salt, Ferric chloride, and chitosan	Simple and economically feasibleProcess	A large amount of Sludge generation
Electrocoagulation	Metal ions (Fe or Al)	-	The high cost of electricity, inefficiency technique
Adsorption	Activated carbon, chitosan, fruit peels, wood chips and alginate	Effectively remove the dyes from the effluent	High cost, requires long retention times
Ion exchange	Calyx arene-based polymer	Routinely used without changing the adsorbent	Dye removal occurs only for specific dyes
Irradiation	Gamma and electron beam radiation	Much effective only for small-scale industry	A huge amount of dissolved O_2 is required
Membrane filtration	Ultrafiltration and	Removes all type of dyes nanofiltration	Concentrated sludge production
Ozonation	O_3	High oxidationpotential, applied in gaseous state: no alteration of volume	Short half-life (20 min)
Redox-active metals	Fenton reagent (H_2O_2-Fe(II))	More efficiently remove the color from both soluble and insoluble dyes	The problem in the disposal of sludge
Photochemical treatment	UV rays	Sludge formation was not	Toxic metabolite production, found inefficient in color removal
Electrochemical destruction	Lead dioxide, and boron-dopeddiamond	Breakdown compounds are non-hazardous and no sludge formation	High cost of electricity and very poor color removal

aquatic ecosystems and even have carcinogenic and mutagenic effects on the organisms (Pinheiro et al. 2004). Besides, an azo dye such as metanil yellow, is found to have hepatotoxicity effect in albino rats (Singh et al. 1988). Nevertheless, in most literature survey it is reported that the continuous exposure of azo dyes on human beings lead to the causes of bladder cancer and also severe damage to the vital organs (Iscen et al. 2007). In this review, the primary investigation was carried out to characterize the azo dyes discharged from textile industries and their impact on the environment. In order to achieve high degradation efficiency in bioremediation technology, we focus on three important factors such as strain development through random mutagenesis, development of bacterial-fungal consortium and reusability of microbial cell cultures. Additionally, this review also compiles different strategies to develop optimal parameters for the effective bioremediation of azo dyes.

TREATMENT METHODS OF AZO DYE CONTAINING WASTEWATERS: PAST, PRESENT AND THE FUTURE

Treating azo dye-containing effluents these days has been found to be difficult because azo dyes are xenobiotic compounds having a high complex aromatic molecular structure with different substitutions. It is estimated that 2-50% of dyestuff was released into the environment as wastewater from the textile industries (McMullan et al. 2009). Several methods have been employed to eradicate the azo dyes from the industrial effluents and they can be broadly classified into three categories such as physical, chemical and biological methods.

Physical and chemical treatments: Over the past three decades, conventional physico-chemical methods have been widely employed to minimize the level of azo dyes present in the effluents. Physical methods such as coagulation-flocculation (Fang et al. 2013), electrocoagulation (Eyvaz et al. 2009), adsorption (Seo et al. 2010), ion exchange (Royer et al. 2010), irradiation (Hosono et al. 1993), ultrasound (Eren & Ince 2010) and membrane filtration (Uzal et al. 2010) were effectively used to remove the dyes from wastewaters. Similarly, the different kinds of chemical treatment methods were also in practice to remove the dyes using the techniques such as ozonation (Santana et al. 2009), sodium hypochlorite (Li et al. 2009), redox-active metals (Gomathi et al. 2009), photochemical treatment (Wang et al. 2010) and electrochemical destruction (Wang 2008 et al. 2008). Recently, among all the chemical methods, advanced oxidation processes (AOPs) are found to be effective methods to remove the dyes with the aid of hydroxyl radicals as strong powerful oxidizing agents. Merits and demerits of the physico-chemical techniques for the removal of dyes from the industrial effluents are presented in Table 3.

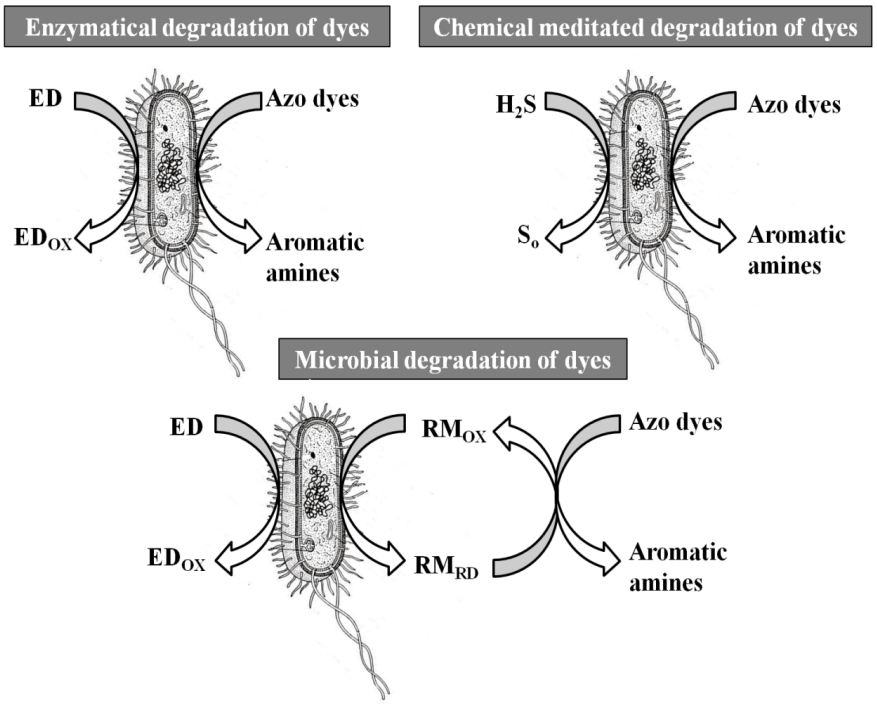

Fig. 3: General mechanism of azo dye degradation.

Biological treatment: "Bioremediation" is a process by which toxic nature of azo dyes is converted to non-toxic by the microbial processes. In the present scenario, using the microbes for treatment of dye polluted water is often considered as an eco-friendly technology when compared with the conventional methods (Liu et al. 2015). Nowadays, extensive research is carried out on biological methods due to their efficiency, low cost, less sludge generation and eco-friendly nature (Liang Tan et al. 2016). Complete detoxification of pollutants can be achieved in the microbial degradation process which results in the reduction of secondary pollution. In order to improve the treatment method feasibly effectively for azo dye polluted water, it is much important to find out new potent azo dye-degrading microbial strains and their mechanisms involved in dye degradation process. Several microorganisms have been intensively used in decolourisation of azo dyes, such as Gram-positive and Gram-negative bacteria (Moosvi et al. 2005), fungi (Miranda et al. 2013), yeast (Song et al. 2017) and algae (Mostafa et al. 2009).

***Bacteria and fungi*:** Bacteria are widely used in bioprocess technology to eradicate the dye-containing pollution due to their variability, adaptability, high activity and cost-effectiveness. Azo dye degradation initially begins with the breakage of the azo bond, aided by azoreductase produced from the bacteria in an anaerobic condition. Nevertheless, the resulting products formed during anaerobic degradation are aromatic amines, which are highly mutagenic and carcinogenic. These aromatic compounds are further converted into non-toxic molecules by the enzymatical action of hydroxylase and oxygenase produced by certain bacteria (Pandey et al. 2007). Mixed azo dyes present in the effluent could not be easily degraded by the single microbial strain. Further, azo dyes containing a sulphonic compound with strong anionic nature, are found to be more resistant to the bacterial attack and the intermediates formed during decolourisation can also inhibit the activity of bacteria. Considering the aforesaid drawback, the strain development is much required for bioremediation process to improve the activity of enzymes present in the bacteria. In contrast, several fungal biomasses have been reported in the recent literature to effectively degrade the azo dyes by secretion of extracellular oxidoreductive enzymes, including manganese peroxidase (MnP) and laccase (Tan et al. 2013). These days white rot fungi has received much attention in the field of bioremediation technology because it has strong adaptability and has the ability to degrade the phenolic compounds. Generally, the metabolites obtained after the bacterial degradation of azo dyes under anaerobic conditions are found to be toxic and can be converted to nontoxic only by the fungal system (Qu et al. 2012). Fungal cells have the ability to degrade the azo dyes by absorption or by the enzyme

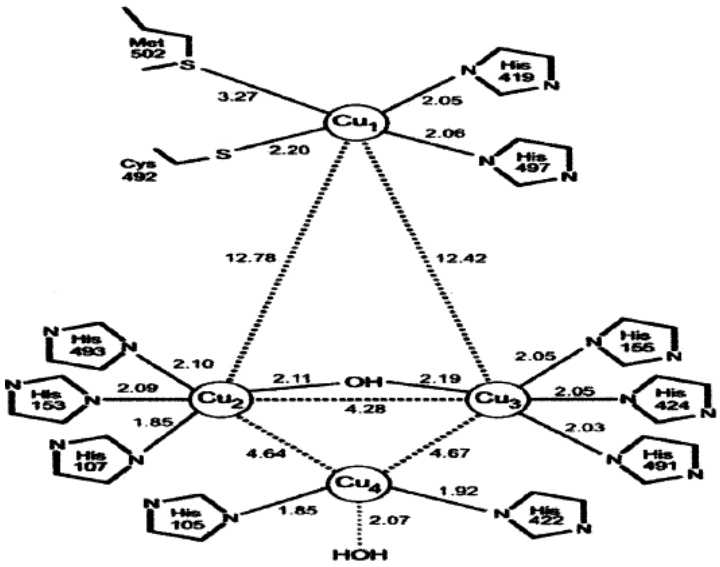

Fig. 4: Structure of laccase.

synthesized by them. Nevertheless, these enzymes could not degrade all types of azo dyes effectively. To establish effective bioprocess technology to remove azo dyes from wastewaters, it is necessary to discover the potent microorganisms which show high enzyme activity with a wide range of substrate specificity.

MICROBIAL OXIDOREDUCTIVE ENZYMES AND THEIR IMPACT ON AZO DYE DECOLOURISATION AND DEGRADATION

In the recent past, the enzymatical decolourisation or degradation of azo dye is found to be more efficient and an alternative method for treating azo dye containing wastewater. The oxidoreductive enzymes generate a high amount of reactive free radicals that help to cleave or remove or transform the phenolic compounds present in the textile effluents. The oxidation of phenolic compounds by oxidoreductive enzymes leads to the formation of oligomeric and polymeric products. These products formed are readily precipitated and settle down due to the increase in molecular weight, which further confirm the detoxification effect of dyes.

However, the initial step of azo dye reduction is breakage of the azo bond by the bacterial action either in aerobic or anaerobic conditions. This kind of reduction may be due to the different mechanisms involved in dye degradation by the oxidoreductive enzymes, redox mediator, sulfide or a combination of these compounds. Generally, this reaction could occur either intracellularly or extracellularly of microbial cells (Fig. 3). Among all the oxidoreductive enzymes, the laccases, manganese peroxidase (MnP) and azoreductase were seen to have a great potential for azo dye degradation (Ram et al. 2015).

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2): Laccase is multicopper enzyme containing proteins with four catalytic copper atoms. The copper atoms have been placed in two sites such as T1 site and T2/T3 sites. The T1 site contains one carbon atom in which substrate can bind and forms a bluish-green colour during oxidation state Cu^{2+}, whereas T2/T3 site contains a cluster of three copper atoms in which molecular oxygen readily binds to it (Jones et. al. 2015). The structure of laccase is presented in Fig. 4.

***Sources*:** Laccase was first developed by Yoshida in 1883 from the plant extracts obtained from *Rhus vernicifera*. It is found in higher plants, fungi, bacteria, archaea and insects. The laccase from white-rot fungi has great significance in the field of biotechnology due to its higher redox potential (up to +800 mV) when compared to bacterial and plant laccases. Thus, fungal laccases have paved much attention these days in the bioremediation process for the effective degradation of aromatic amines.

***Mode of action of laccase*:** Laccases have been greatly employed as oxidoreductive enzymes for the effective degradation of azo dyes because they have low specificity during the reduction of the substrate (Novotny et al. 2004). Further, these enzymes required NADH or redox mediator to enhance the degradation rate of azo dyes. Generally, the laccase-mediated degradation of azo dyes was performed in a step-wise manner. In the first step, the azo dyes degraded

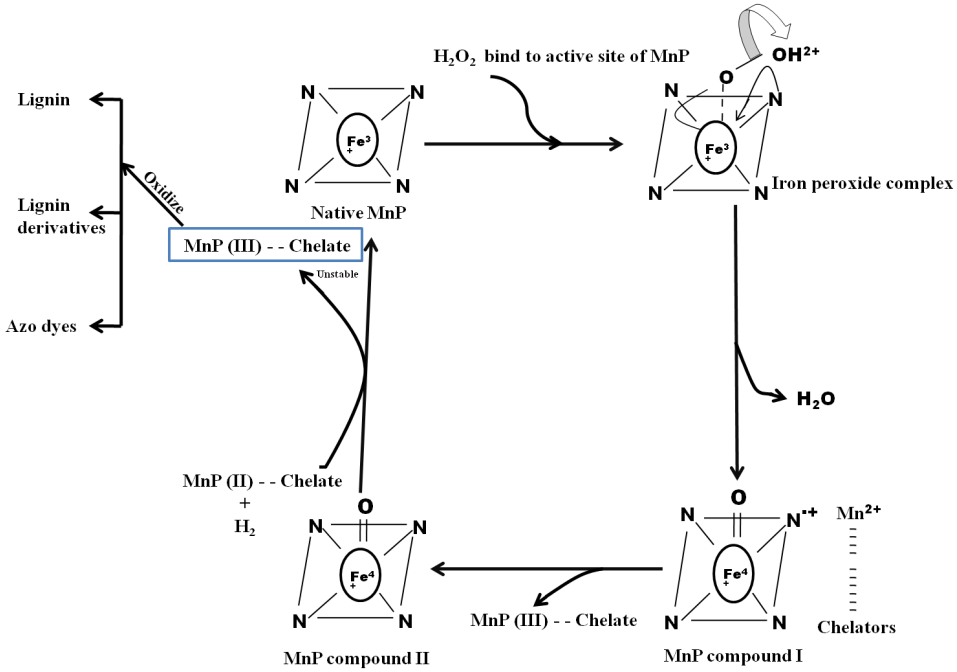

Fig. 5: Catalytic mechanism of MnP in azo dye degradation.

into phenolic compounds by the nonspecific free radical mechanism. In the second step, phenolic compounds are further oxidized to carbonium ion either by the nucleophilic attack or by the phenoxy radicals generated during the reaction. In addition, laccase is able to oxidize various non-phenolic compounds in the presence of redox mediators (Sharma et al. 2007). It is suggested that the laccases have the ability to remove hydrogen atoms from the hydroxyl group of phenolic compounds and aromatic amines (Ram et al. 2015).

***Production of laccase enzyme*:** In the present times, the researchers have focused on the high amount of laccase production by developing the new strains or by regulating the gene expression with the presence or absence of nutrient content (Palmieri et al. 2000). The presence of inducers such as redox mediator, aromatics and phenolic compounds bind to microbial cells, thereby enhancing the laccase production via stress response (De Souza et al. 2004). Many factors influence the level of laccase production such as types of cultivation method (solid-state or submerged), reactor or fermentor, the chemical composition of the nutrient and trace elements present in the cultivation medium. Nearly more than 100 laccases were isolated from the different sources of microorganisms. Nevertheless, the isolated enzymes are found to be similar in structural and functional properties but they are not thermostable and have very fewer activities towards the substrates. Therefore, most researchers have focused on to obtain novel laccases which have the higher stability to the various temperatures and also to possess high enzyme activity against the wide ranges of the substrate (Li-Qiong et al. 2011).

Azo dye degradation by laccases is a well-studied phenomenon; the reduction of organic dyes in the presence of laccase is considered as an effective method to remove the phenol containing aromatic compounds. Recently, several microorganisms have been employed for the degradation of azo dyes by using laccase as a catalysing agent (Table 4). Although the laccase has a wide range of substrate activity and low specificity, it takes much longer time for the process of dye degradation. To overcome this issue and to improve the bioprocess technology, the redox mediators such as veratryl alcohol (VA), 1-hydroxybenzotriazole (HBT), acetosyringone (AS) and 4-hydroxybenzoic acid (HA) have been extensively used by many researchers to improve the rate of degradation of dyes. The production of laccases in microorganisms is regulated by the nutrient types, amount of nutrients, strain, inducers and substrates (Palmieri et al. 2000). Nevertheless, the proper investigation is still required to improve the strain for effective degradation of azo dyes by using laccase as a catalysing agent.

Manganese Peroxidase (MnP; EC 1.11.1.13): MnP is a special kind of enzyme that belongs to the family of versa-

Table 4: Degradation of azo dyes using laccase as a catalyzing agent.

S.No	Microbial culture	Name of the dyes	% Decolorization	Time (h)	Factors influence laccase production	References
1	*Bacillus pumilus* W3	Reactive red 11	96	5	Methylsyringate (0.1%)	(Zheng-Bing et al. 2014)
		Reactive blue 171	95	5		
		Reactive blue 4	90	5		
		Reactive brilliant blue	91	5		
2	*Trichoderma*	Malachite green	100	16	1-hydroxybenzotriazole (HBT) (2 mM)	(Zabin et al. 2017)
		Methylene blue	90	18		
		Congo red	60	20		
3	*Marasmiellus palmivorus*	Reactive Blue 220	90	24	-	(Camila et al. 2017)
		Acid Green 28	75	24		
4	*Spirulina platensis* CFTRI	Remazol Brilliant Blue R	90	48	Syringaldehyde (0.1mM)	(Afreen et al. 2017)
5	*P. ostreatus* MTCC 142	Congo red	37	20	-	(Das et al. 2016)

Table 5: Taxonomic classification of *Pleurotus ostreatus.*

Domain	Eukarya
Super-group	Opisthokonta
Kingdom	Fungi
Phylum	Basidiomycota
Class	Agaricomycetes
Order	Agaricales
Family	Pleurotaceae
Genus	*Pleurotus*
Species	*ostreatus*

tile peroxidases (VPs) produced by several white rot fungi and plants (Moreira et al. 2005). This extracellular enzyme was first identified from *Phanerochaete chrysosporium* in 1983. MnP is a haeme-containing glycoprotein (MW 4 20-65 kDa) and initiate the catalytic reaction only in the presence of H_2O_2 and cofactors such as Mn^{2+} (Asgher et al. 2008). MnP catalytic reaction of azo dye degradation occurs in a step-wise manner in which H_2O_2 binds to the active site of the enzyme and then oxidized to form a Mn^{2+}. In the second step of the cyclic reaction, the Mn^{2+} is further oxidized to unstable Mn^{3+} which in turn chelated with the organic acid, especially with oxalate molecule. Mn^{3+} or chelated Mn^{3+} was able to oxidize wide range of substrates such as lignin, phenolic compounds and azo dyes due to its less specificity (Fig. 5). The remarkable potential of MnP gained more interest in modern biotechnology due to its wide range of applications in biopulping, biobleaching and bioremediation.

***White-rot fungi*:** White-rot fungi are Basidiomycetes capable of producing unique extracellular peroxidases LME (Lignin Modifying Enzymes) such as laccase, MnP and lignin peroxidases, which have great potential to decolourize/degrade/mineralize the wide range of toxic organic pollutants due to the broad spectrum of specificity in attacking the substrates (Cerniglia 1997). Recently, a vast number of white-rot fungi such as *Trametes versicolor*, *Phanerochaete chrysosporium*, *Irpex lacteus*, *Ceriporiopsis subvermispora*, *Dichomitus squalens*, *Ganoderma lucidum* and *Pleurotus ostreatus* have been employed to degrade the lignin and toxic azo dyes. In contrast, *Pleurotus ostreatus*, the oyster mushroom, is an easily available mushroom which predominantly releases LME during the degradation process of azo dye. The researchers have paved more attention to this fungus and their enzyme system due to its potential applications in bioremediation technology (Marco-Urrea et al. 2009). The taxonomical classifications of *P. ostreatus* have been summarized in Table 5. However, not much attention was given to the characterization of the extracellular enzymes (MnP) produced by the *P. ostreatus* and their effectiveness in the azo dye degradation.

Azoreductase (also known as Azobenzene reductase EC 1.7.1.6): Azoreductase is a key enzyme produced by azo dye degrading microorganisms, especially bacteria and fungi. It has the ability to decolourize the azo dyes by the reductive cleavage of an azo bond (HN=NH) in anaerobic conditions with the release of toxic aromatic compound (Pandey et al. 2007). This toxic aromatic compound is further oxidized to a non-toxic molecule in an aerobic condition by the other microorganisms (Stolz 2001). Azoreductase increases the catalytic reaction only in the presence of reducing molecules such as NADH, NADPH and $FADH_2$. The reduction reaction occurs in the cell membrane of bacteria by gaining electrons from the reducing molecules. Over the past few decades, the intensive research has been carried out by most of the researchers to demonstrate theoretically

Table 6: Catalytic activity of bacterial azoreductase on azo dyes decolorization.

S.No	Bacteria	Azo dyes and its concentration (mg/L-1)	% Decolorization	Time (h)	References
1	*Pseudomonas* species	Remazol black (100)	75	24	(Tuttolomondo et al. 2014)
		Methyl orange (100)	79	24	
		Benzyl orange (100)	83	24	
2	Bacterial consortium - (*Microbacterium* sp., *L. albus, Klebsiella* sp. and *S. arlettae*)	Disperse Red 1 (100)	80	60	(Franciscona et al. 2015)
3	*Bacillus* sp. strain CH12	Reactive Red 239 (100)	95	96	(Guadie et al. 2017)
4	*Comamonas* sp. UVS	Direct Red 5B (50)	78	6	(Jadhav et al. 2008)
5	*P. aeruginosa*	Reactive Blue (500)	48	80	(Bhatt et al. 2005)
6	Mutant *Bacillus* sp. ACT2	Congo Red (1000)	30	24	(Gopinath et al. 2009)
7	*Proteus* sp.	Congo Red (200)	67	48	(Perumal et al. 2012)
8	*Micrococcus glutamicus* NCIM 2168	Reactive Green 19 (50)	100	42	(Saratale et al. 2009)
9	Microbial consortium (white-rot fungus 8-4 and Pseudomonas 1-10)	Direct Fast Scarlet 4BS (50)	99	24	(Fang et al. 2004)
10	Bacterial consortium - (*A. faecalis, Sphingomonas* sp. EBD, B. subtilis, *B. thuringiensis* & *E. cancerogenus*)	Direct Blue-15 (50)	92	24	(Kumar et al. 2009)
11	Bacterial consortium - (*Citrobacter freundii* (2 strains), *Moraxella osloensis, P. aeruginosa* and *P. aeruginosa* BL22	Mordant Black 17 (100)	95	16	(Karunya et al. 2014)

about the structural diversity of catalytic protein performing azoreductase activity isolated from several microorganisms. Azoreductases obtained from different groups of bacteria and their effects on azo dye decolourisation are summarized in Table 6. Generally, azoreductase has been classified in two groups such as flavin dependant azoreductases (Chen et al. 2005) or flavin independent azoreductases (Blumel & Stolz 2003) based on the structure of amino acids and their functions. Further, flavin dependant azoreductases are subdivided into three groups based on the utilization of coenzymes for reduction of azo dyes such as NADH dependant or NADPH dependent or both. Although, the intracellular azoreductase expressed from the bacterial sources is not able to degrade the most of the azo dyes effectively. This problem is due to the structural complexity of the dyes and also the less specificity of the enzymes. So, there is an urgent need to search for new microorganisms and their enzymes which have broad substrate specificity. Further, the problem can be solved by using low molecular weight redox mediator which can act as an electron shuttle between the dyes and coenzymes. The azoreductase activity will be arrested if the reaction was performed under aerobic condition due to the presence of a redox mediator. Therefore, it is necessary to identify the oxygen-insensitive azoreductase from the different bacterial sources and make use of it for efficient degradation of azo dyes in wastewater treatment. Additionally, there is only a little information available on the enzyme kinetics and the characterization of these enzymes.

STRAIN DEVELOPMENT FOR IMPROVED DECOLOURISATION/DEGRADATION OF AZO DYES

Wastewater discharged from the textile industries containing different groups of azo dyes make them more resistant to microbial attack. Azo dye possesses a complex organic structure with one or more azo bond and hence, they are highly persistent in the environment for many years. A wild microbial strain cannot degrade the mixed azo dyes present in textile effluents. Therefore, it is much important to develop new strains for the effectiveness of the bioprocess technology. Nowadays, genetically modified microorganisms have been employed to eradicate the pollution in an excellent manner. Even though, microbes are considered as tiny organisms they contain a set of genes that is responsible for the production of different sort of catabolic or regulatory enzymes involved in the degradation process. Every

microbe is unique in characteristics with different metabolic regulations and produces various end products. This may be due to the alteration of the gene occurred during spontaneous mutation. Mutation is a permanent change and occurs in the base pair of DNA sequence.

Mutation is broadly classified into two types as spontaneous or induced mutation. The spontaneous mutation occurs naturally in the microbes whereas induced mutation occurs by the physical or chemical mutagens. Exposure of mutagen to the microbial cell will alter the base pair of DNA thereby it may change the pattern of amino acid sequence during protein formation. These variations of the gene may have a good or bad influence on the characteristics of microbes (Schofield & Hsieh 2003). In order to improve the productivity of enzymes from microbes with wide substrate specificity, many researchers have shown more interest in the random mutagenesis approaches. Strain development by exposing the DNA to the physical and chemical mutagenic agents offer a better opportunity to the biotechnologist for the hyperproduction of the enzyme with low cost (Lotfy et al. 2007). In random mutagenesis, mutations are induced to microbial cells by exposing them to one or more mutagenic agents such as UV irradiation, ethyl methyl sulphonate (EMS) and ethidium bromide (EtBr) for a certain period of time. The time of exposure, amount of mutagen and the distance between the microbial cells and radiation are important factors to be considered during the strain development.

In the recent past, a large number of studies have been carried out to improve enzyme production through random mutagenesis (Meleigy et al. 2008). Screened over-production of gibberellic acid mutant strain *Fusarium moniliforme* mutated by gamma rays (Taloria et al. 2012) described the increased alcohol dehydrogenase activity in mutated *S. cerevisiae* during ethanol production with UV mutation. Gopinath et al. (2009) showed the hyper-production of azoreductase by mutant *Bacillus* species which help to improve the degradation rate of Congo red dye. Tannler et al. (2008) developed high yield riboflavin producing bacterial mutant strain *B. subtilis* through the chemical mutagen method. Hyper-production of the oxidoreductive enzymes by mutant species and their potential of biodegradation of azo dyes has not been given much attention.

BACTERIAL-FUNGAL CONSORTIUM FOR DECOLOURISATION/DEGRADATION OF AZO DYES

Taking into account of the most significant environmental problem, the disposal of wastewater from the dyeing industries that are causing serious problems to the living organisms and livelihood, degradation of azo dyes is the topic of primary concern. As various dyes are wayward to biodegradation, researches are still progressing on developing new biological treatments (Borchert & Judy 2001). Over the past decades, a huge amount of microorganisms including bacteria, fungi, yeasts and algae have been screened out and utilized for the dye decolourisation in an eco-friendly manner. The mixed microbial consortia were suspected to have supremacy over pure isolates in treating azo dye containing the wastewater (Pandey et al. 2007). The azo dyes were supposed to have completely mineralized by co-metabolism of microorganisms (Kapdan & Ozturk 2005). Treating wastewater based on microbial consortium at the contaminated sites is quite efficient as microorganisms habituate to their environment (Thakur 2004). The bacterial-fungal consortium has proved to be effective, eco-friendly, and has the efficiency to degrade the dyes in a short span of time. The strenuous metabolism of the consortium degrades the dye molecule at various positions or carries out intermediate degradation for further mineralization. Further, the synergistic action of bacterial-fungal consortia may lead to faster and complete degradation or detoxification of the azo dyes (Khelifi et al. 2009). Additionally, this consortium releases a huge amount of extracellular oxidoreductive enzymes when compared to monoculture which paves the way for transforming the toxic pollutant into non-toxic metabolites (Kaushika & Malik 2009). The synergistic effect of the microbial consortium on azo dye degradation is given in Table 7. However, only a few reports suggested the bacterial-fungal consortium potential for efficient degradation of azo dyes, but the antagonistic activity among the consortium, positive and negative interaction, optimization and their enzyme system were not clearly elucidated.

OPTIMIZATION PARAMETERS FOR ACCELERATED DEGRADATION OF AZO DYES

The effluents that especially come from the textile industries consist of a different composition of organic pollutants. This effluent when mixed with the environment, undergoes various physico-chemical changes that makes it more resistant to microbial attack. Effective biological treatment methods totally depend upon the optimization parameters such as pH, temperature, the complexity of dye structure, the concentration of dye, treatment methods (aerobic or anaerobic), nutrient sources (carbon, nitrogen), inducers and presence or absence of redox mediators. Thus, to improve the biological treatment methods more effective and rapid in decolourisation, it is essential to find out the appropriate factors which influence the azo dye decolourisation.

Effect of physico-chemical factors on azo dye degradation:

Table 7: Synergistic effect of the microbial consortium on azo dye degradation.

S.No	Consortium	Species	Dye	% Degradation	References
1	Fungal	Trametes sp. SQ01 Chaetomium sp. R01	Acid Violet17 Acid Blue15 Crystal Violet Malachite Green Brilliant Green	86 85 82 82 85	(Sharma et al. 2004)
2	Fungal	*Aspergillus lentulus* *Aspergillus tereus* *Rhizopus oryzae*	Acid Blue Pigment Orange	98 100	(Mishra et al. 2014)
3	Bacterial	*Bacillus flexus* NBN2 *Bacillus cereus* AGP03 *Bacillus cytotoxicus* NVH *Bacillus* sp. L10	Direct Blue 151 Direct Red 31	98 95	(Lalnunhlimi et al. 2016)
4	Fungal- Bacterial	*Aspergillus ochraceus* NCIM-1146i *Pseudomonas* sp. SUK1	Rubine GFL	95	(Lade et al. 2012)
5	Bacterial	*Providencia* sp. SDS (PS) *Pseudomonas aeuroginosa* strain BCH	Red HE3B	100	(Phugare et al. 2011)
6	Fungal- Bacterial	White-rot fungus 8-4 *Pseudomonas aeuroginosa* 1-10	Direct Fast Scarlet 4BS	99	(Fang et al. 2004)
7	Bacterial	*Citrobacter freundii* (2 strains), *Moraxella osloensis, Pseudomonas aeruginosa* and *Pseudomonas aeruginosa* BL22	Mordant Black 17	95	(Karunya et al. 2014)
8	Bacterial	*Proteus vulgaris* *Miccrococcus glutamicus*	Green HE4BD	100	(Saratale et al. 2010)
9	Bacterial	*Bacillus vallismortis* *Bacillus megaterium*	Congo Red Brodeaux Ranocid fast Blue Blue BCC	96 89 81 82	(Tony et al. 2009)
10	Bacterial	*Bacillus cereus* (BN-7) *Pseudomonas putida* (BN-4) *Pseudomonas fluorescence* (BN-5) *Stenotrophomonas acidaminiphila* (BN-3)	Acid Red 88 Acid Red 119 Acid Red 97 Acid Blue 113 Reactive Red 120	78 99 94 99 82	(Khehra et al. 2005)
11	Fungal- Bacterial	*Pseudomonas* sp. SUK1 *A. ochraceus* NCIM-1146	Reactive Navy Blue HE2R	80	(Kadam et al. 2011)

***pH and temperature*:** The variation in pH of the medium has a major effect on azo dye decolourisation/degradation. The degradation efficiency of azo dye mostly depends upon the pH of the medium, tolerance ability of the azo dye degraders and pH stability of the oxidoreductive enzymes. In most of the literature survey, it was reported that the azo dye degradation occurs in the wide range of pH 5.0 to 10 (Kilic et al. 2007). Maximum degradation was achieved only at optimal pH, while the rate of degradation tends to decrease quickly when it reaches strong acid or in alkaline condition. Chang et al. (2001) reported that pH of the medium promotes the transfer of dye molecules across the cell membrane. Hence, pH directly influences or affects the rate of degradation of dyes. During the microbial degradation process, the reduction of azo bond occurs with the formation of aromatic amines. These aromatic amines increase the pH of the medium and it may be limiting to the growth of microbial biomass. Similarly, the temperature also has a profound impact on azo dye degradation. Microbes always adapt to integrate with the environmental temperature and its variation. But the microbial enzymes are very sensitive to the temperature because they decline from the original activity other than the optimal temperature. Microbes either combine with an endergonic or exergonic reaction during the time of bioremediation process which results in rapid changes in the temperature. This kind of temperature variation can also lead to the loss of cell viability or reduction in activation energy or a decline in growth rate and enzyme activity (Chang & Kuo 2000). However, certain microbes release thermostable oxidoreductive enzymes,

which can adapt to resist up to a temperature of 60°C over a short period of time (Pearce et al. 2003). Therefore, selection of microbial strain or development of that strain, which has the ability to tolerate a wide range of pH and temperature, is considered as prime factors for the effective degradation of azo dyes. Immobilization of cells offers a great protection for microorganisms against the environmental stress such as temperature and pH by the supporting matrix formed during the process.

***Dye structure and concentration of dye*:** Azo dyes are of a diverse group of synthetic dyes with one or more azo bond with different functional groups attached to them. This complexity of structure affects the efficiency of microbial decolourisation. It was reported that the decolourisation efficiency is much faster in monoazo dyes with low molecular weight compared to the diazo or triazo dyes (Hsueh et al. 2009). The dyes with hydroxyl or amino groups are easily degradable compared to those with methyl, methoxy, sulpho or nitro groups. Azo bond containing electron-dense region and a sulphonated group of reactive dye is normally considered to be high recalcitrant in nature which cannot be easily degraded by pure culture (Lorenco et al. 2000). Increasing the concentration of dye gradually decrease the dye degradation rate. This may be due to the toxic effect of the dye or improper biomass and dye ratio or inability of the strain or less amount of oxidoreductive enzymes present in the medium (Tony et al. 2009). Therefore, the development of microbial consortium may be an alternative approach to mineralize the azo dye completely.

***Nutrient source and conditions of treatment method (oxygenic or anoxygenic)*:** Bioreduction of azo dyes can be accomplished only with the supplement of carbon and nitrogen source in degradation medium. Degradation efficiency of azo dyes by the single microbe or consortium can be improved by the addition of one or more organic substances such as glucose, lactose, maltose and sucrose; and also by the addition of nitrogen sources such as sodium nitrate, peptone, beef extract and yeast extract. Pandey et al. (2007) demonstrated that the reductive cleavage of the azo bond by microbes occurs due to the energy obtained from the carbon source. Chang & Kuo (2000) observed that nitrogen source is responsible for the regeneration of NADH which acts as an electron donor for the reduction of dyes. Nitrogen source such as sodium nitrate influences the enzyme system involved in dye degradation (Jadhav et al. 2010). The microbial decolourisation of dyes occurs strictly under anaerobic or facultative anaerobic or aerobic conditions by the various groups of microorganisms. Complete degradation of azo dyes is possible only with the help of aerobic and anaerobic bacteria. Bioremediation of azo dyes occurs in two-step reactions. In the first step of the reaction, the reduction of azo dyes occurs in an anoxygenic condition by the action of azoreductase produced by the anaerobic bacteria with the release of toxic amines. In the second step, these amines are further converted into the non-toxic compounds with the help of oxidative enzyme produced by aerobic bacteria (Van der Zeea & Villaverde 2005). The earlier study suggested that the continuous aeration and agitation in the medium increases the oxygen concentration, thereby affecting the azoreductase activity. This is because the microbial cell utilizes the electron obtained from the oxidation process for the reduction of oxygen rather than the azo dyes. Keeping this in view, the researcher is in search of oxygen sensitive azoreductase from different groups of bacteria to make this process economically feasible by conducting the reaction in one-step mechanism under aerobic condition.

Inducers and redox mediators: Wastewater discharge from the textile industries contains a low amount of substrate, which cannot be readily utilized by the microbes and thus affecting the rate of degradation of azo dyes. This problem can be solved by using the inducer as an external substrate (electron donor) which enhances the degradation efficiency of microbes. Addition of inducer such as glycerol, tween 80 and copper sulphate apparently induces the oxidoreductive enzymes which favour the degradation of azo dyes. Dawkar et al. (2009) observed that the complete degradation of Navy blue 2GL occurs within 18 hrs of incubation by the addition of $CaCl_2$ due to overexpression of oxidoreductive enzymes. Similarly, Telke et al. (2009) reported that the inducer such as sodium acetate, sodium formate and sodium citrate improve the decolourisation efficiency of C.I. Reactive Orange 16 by the reduced activity of NADH-DCIP reductase produced by the isolated *Bacillus* sp. So far, the concentration of inducers and their effect on azo dye decolourisation were not well understood. Hence, the choice of inducers and their optimizations are more important factors for the colour removal of dyes from the wastewater. Microbes cannot easily uptake azo dyes inside the cell membrane due to their high degree of complexity and also for the wide range of redox potentials (-180 to -430 mV) when they are present in the effluent (Dos Santos et al. 2004). Further, the azo dye, which has a more electron-dense region in the azo bond, is found to be highly resistant to the biological decolourisation process. The redox mediator is the substance which enhances the transfer of reducing equivalents from the co-substrate to the azo dyes. Due to this electron transfer mechanism, steric hindrance of the dye molecule will be reduced, thus facilitating the dye molecule to the bacterial cell membrane (Rau & Stolz 2003). It

has been observed that the redox mediator increases the rate of degradation by improving the substrate specificity of oxidoreductive enzymes (Canas & Camarero 2010). Nowadays, the natural mediators have been extensively used in bioremediation technology when compared to the synthetic mediator because they are less toxic, and eco-friendly. However, the possible concentration of natural mediators and their impact on the oxidoreductive enzymes for effective degradation of azo dye was not well studied.

IMMOBILIZATION OF MICROBIAL CELLS FOR AZO DYE DEGRADATION

Nowadays, immobilization of bacteria/fungi/bacterial-fungal cells has received much attention from biotechnologists due to its wide range of applications in the industrial sector (Ahamad & Kunhi 2011) Further, immobilization has several advantages over freely suspended cells in wastewater treatment because it has higher stability, low-cost, reuse, decrease the cell surface area volume, protect the cells from direct exposure of toxic substances, and eco-friendly (El-Naas et al. 2009). The choice of the supporting materials is an important factor to be considered for the process of cell immobilization (Zacheus et al. 2000). Natural and synthetic gels are the two types of immobilization matrices which have been extensively used in the process of decolourisation of azo dyes. However, the synthetic gel has higher stability, but it does not allow the dye to penetrate the matrix and also will reduce the viability of cells. The natural gels such as alginate offers a wide range of applications in entrapment methods because it has a mild effect on the microbial cells when compared to synthetic gels (Tal et al. 2001). Immobilized biocatalysts act as promising tool to eradicate azo dyes continuously in a controlled manner with low cost and in an eco-friendly way.

CONCLUSIONS

Owing to the high demand for fabrics, the industrial production of azo dyes has drastically increased worldwide. Unfortunately, every year 15% of azo dyes are discharged into the environment from the textile industries and pose a serious problem in aquatic habitats. These effluents contain a mixture of dyes with high complexity of structure and found to have a high alkaline condition, which does not favour the microbial degradation of dyes by monoculture. In the present times, most wastewater treatment plants are obsolete and in need of modern technology to improve the degradation of azo dyes in an eco-friendly manner with low cost. So, there is an urgent need for treating wastewaters using novel methods for complete mineralization of azo dyes. Microbial strain development through random mutations for the production of oxidoreductive enzymes is a primary initiative for enhanced decolourisation and degradation of azo dyes. Further, bacterial-fungal consortium mediated decolourisation and degradation of azo dyes have significant potential to address this issue in an eco-friendly manner without generating a secondary disposal problem in the form of sludge. The mutated consortium, able to produce different types of oxidoreductive enzymes at an increased level, enhances the degradation rate of mixed dyes. In addition to the above, the immobilization and optimization of parameters for the accelerated decolourisation and degradation of azo dyes are also important. It is well-understood now that the bacterial-fungal consortium developed from the mutated strain increases the production rate of oxidoreductive enzymes. Hence, in near future, the mutated bacterial-fungal consortia may be considered as biological weapons for the effective decolourisation and degradation of azo dyes.

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