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Biotechnological treatment of textile dye effluent

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9.1 Introduction

Pollution of communal water bodies by waste dyestuff released from textile plants and dyehouses represents a major environmental concern. Although presently a wide range of physical and chemical methods is available to decolorize dye-contaminated effluents (Hao *et al*., 2000), alternative processes based on biotechnological principles are attracting increasing interest (Kandelbauer and Gübitz, 2005) since they often avoid consumption of high quantities of additional chemicals and energy. In this chapter, a short overview is given of such biotechnological approaches. Their advantages and disadvantages and hence their range of applicability are outlined.

In Section 9.2.1, there is a discussion of biological treatment processes based on living and proliferating cell populations. These may consist either of well-defined species of special micro-organisms or of various kinds of different micro-organisms that have established an ecosystem suitable for dye elimination. One major advantage of such systems is the complete mineralization often achieved due to synergistic action of different organisms (Stolz, 2001). However, the actual biodegradation is always a stepwise chemical transformation consecutively catalyzed by different enzymes. Therefore, enzymes may be used as such for the treatment process in some cases. Some key information on enzyme remediation is given in Section 9.2.2.

Finally, some conclusions are drawn about potential future applications of bioremediation techniques in the treatment of textile effluent and partial process streams contaminated with residual dyestuff.

9.2 Biotechnology and dye effluent treatment

9.2.1 Microbial processes

General aspects

Most biotreatment systems are based on living micro-organisms. The common

method for the treatment of wastewater in the textile finishing industry is physicochemical flocculation in combination with subsequent biological treatment (Krull *et al.*, 1998). Like most organic materials of animal and vegetable origin, dyes can be degraded into simpler compounds and are finally mineralized to water and carbon dioxide by a wide variety of aerobic or anaerobic organisms (Binkley and Kandelbauer, 2003; McMullan *et al.*, 2001). Biodegradation can take place in the presence of oxygen (aerobic degradation) or in the absence of oxygen (anaerobic degradation).

Biological treatment can be carried out directly at the site of an industrial plant or in a communal sewage treatment plant. In both cases, living whole cell systems are typically used in mixed cultures of various types of micro-organisms. Mixed populations are much more commonly applied than isolated cultures of single organisms because of their relative robustness and versatility against xenobiotic compounds. They are more resistant towards unexpected or sudden changes in environmental conditions. They are self-establishing little ecosystems and adapt continuously via natural selection. In particular, industrial on-site effluent plants develop very specialized micro-organism populations that are very powerful in the degradation of the specific waste produced at the plant. Usually the plants simply benefits from their presence.

However, studies have been published where such naturally evolved strains have been isolated and optimized further under chemostat conditions in the laboratory in order to create even more powerful species (Zimmermann *et al.*, 1984, Nigam *et al.*, 1996). The complete mineralization of specific xenobiotic compounds upon action of single organisms has been reported (Blümel *et al.*, 1998) but this is, however, not a typical result and thus is of limited use. Furthermore, by conventional methods this is very time-consuming and may take up to a year or more. By applying genetic methods, however, such super strains may be developed much faster in future and may be returned to the mixed culture again as a boosting inoculum assisting the overall biotreatment system.

In order to yield successful biotreatment, some requirements must be met. The micro-organisms must be kept healthy and active. It is important to keep type and concentration of potentially toxic substances at a level that does not cause any serious damage to the micro-organism population. Since dye degradation is attributed to secondary metabolic pathways, appropriate growth conditions have to be accomplished by addition of a nutritional supply. Sufficient amounts of nitrogen- and phosphorus-containing nutrients must be present in the effluent. Typical conditions necessary to ensure reliable performance of a biological mixed culture system are pH between 6.5 and 9, temperature at around 35 °C (or higher in the case of anaerobic systems), ratio of the biological oxygen demand (BOD) to nitrogen and BOD to phosphorus of approximately 17:1 and 100:1, respectively (Binkley and

Kandelbauer, 2003). At sewage treatment plants where domestic effluent is mixed with industrial effluents, C, N and P sources typically appear in quantities high enough to maintain the micro-organism population. Industrial on-site treatment plants on the other hand are limited to effluents containing the rather small range of solutes corresponding to the product range. It may thus be necessary to add supplemental N and P sources to a dye house on-site facility. This leads to additional loads of the effluent with chemicals.

The effect of structural parameters of the dye on bioelimination is generally linked to the mechanism of the treatment process. For example, in adsorption processes, where biomass – dead or alive – is basically used as a filter material, a large molecular size is typically of advantage for satisfactory decolorization (Cooper, 1993). Since, in this case, poor solubility is of advantage, functional groups that confer high water solubility, such as, for example, sulfonic acid groups, are unfavourable. On the other hand, for biodegradation processes involving bacteria where intracellular digestion takes place, solubility and suitable polarity seem to be of advantage. Furthermore, it is important that the dye molecule is able to penetrate the cell wall and thus it should be of relatively smaller size. Large functional groups such as, for example, sulfonic acid groups may again prove unfavourable if too many are present. In general, sufficient bioavailability must be guaranteed in order to efficiently eliminate dye molecules.

Aerobic treatment

Activated sludge

Aerobic treatment is carried out in stabilization ponds, aerated lagoons, activated sludge or percolating filters. In aerobic treatment, micro-organisms in the activated sludge utilize dissolved oxygen to convert the wastes into more biomass and carbon dioxide. Organic matter is partially oxidized and some of the energy produced is used for generating new living cells under the formation of flocs. The flocs are allowed to settle and are then removed as sludge. A proportion of the sludge removed is recycled back to the aeration tank to maintain the micro-organism population. The remainder of the sludge can be fed back in a subsequent anaerobic treatment. Combinations of anaerobic/aerobic pilot plant for the treatment of coloured textile effluents are very powerful (for instance, Sarsour *et al.,* 2001), especially for the elimination of azo dyes (Stolz, 2001).

Aerobic bacteria have been described that oxidatively decolorize many dyes from several classes, among which azo dyes always turned out to be the most recalcitrant compounds (Kandelbauer and Gübitz, 2005). The ease of elimination of a dye is strongly related to its solubility. The more sulfonic acid groups are present in the dye structure, the more soluble and, therefore, the less responsive to treatment is the dye to the activated sludge process.

Insoluble vat and disperse dyes can be removed in quite high proportions by primary settlement. Basic and direct dyes respond well to treatment in the activated sludge process. However, reactive dyes and some acid dyes seem to cause more of a problem. It is generally considered that the activated sludge process removes only low levels of these dyes.

If only aerobic treatment is performed, the sludge can be disposed of in landfill or by drying and incineration. Disposal through agricultural use as a fertilizer is mostly prohibited by law in many countries because of the presence of heavy metals from dye residues.

Fungi

In nature, the class of white-rot fungi is able to degrade complex substrates like lignin via oxidative radical pathways. They can also degrade textile dyes due to the unspecific nature of their lignin degrading enzymatic system. The enzymes responsible for this action are peroxidases and laccases. The enzymes show broad substrate specificities (see Section 9.2.2) and are excreted by the fungi. Since extracellular digestion of dyestuff takes place, physical separation of living organism and toxic waste can be accomplished. This makes fungi especially interesting for bioremediation. One drawback with fungal cultures is that they require rather long growth phases before actually producing high amounts of active enzymes.

A huge number of scientific papers show the versatility of white-rot fungi for decolorization (Fu and Viraraghavan, 2001) and consequently, much is known about their potential in treating dye contaminated (model) waste water and their resistance towards dye toxicity under more or less native conditions. The list of white-rot fungi known to degrade the various types of dyes is long: amongst others, various *Trametes* sp. (Abadulla *et al.*, 2000, Campos *et al.*, 2001, Kandelbauer *et al.* 2004a, b, and 2006, Shin and Kim, 1998b), *Trametes versicolor* (Swamy and Ramsey, 1999a,b), *Irpex lacteus* (Novotny *et al.*, 2001), *Pleurotus ostreatus* (Shin and Kim, 1998a), *Pycnoporus sanguineus* (Pointing and Vrijmoed, 2000), *Pycnoporus cinnabarinus* (Schliephake *et al.*, 2000), *Phlebia tremellosa* (Kirby *et al.*, 2000), *Geotrichum candidum* (Kim *et al.*, 1995), or *Neurospora crassa* (Corso *et al.*, 1981), and *Phanerochaete chrysosporium* (Martins *et al.*, 2001; Tatarko and Bumpus, 1998) seem to be the most extensively investigated fungi for dye decolorization working on dyes of all classes. The genus of *Penicillium* has been shown to degrade various polymeric dyes (Zheng *et al.*, 1999). *Trametes versicolor, Pleurotus ostreatus, Phanerochaete chrysosporium, Piptoporus betulinus, Laetiporus sulphureus* and several *Cyathus* species) have been described in literature to degrade triphenylmethane dyes (Azmi *et al.*, 1998). For an informative compendium of recent literature describing fungi able to decolorize dyes, see Fu and Viraraghavan (2001).

Living fungi can decolorize dye solutions by means of real biodegradation (Chagas and Durrant, 2001; Swamy and Ramsay, 1999b). Typically, dyestuff is added to either a more or less purified enzyme solution, culture filtrate or fermentation broth, which may still contain the living organism. The cultivation can be optimized with respect to dye decolorization by stimulation with inducing substances, which induce increased formation of active enzymes (Robinson *et al.*, 2001; Tekere *et al.*, 2001; Bakshi *et al.*, 1999).

Various types of reactor systems based on fungi have been described (Fu and Viraraghavan, 2001; Nicolella *et al.*, 2000) such as static biofilms on rotating discs (Kapdan and Kargi, 2002) and drum reactors (Dominguez *et al.*, 2001), biological aerated filter cascades (Basibuyuk and Forster, 1997), packed-bed bioreactors (Schliephake *et al.*, 1996), or fed-batch and continuous fluidized bed reactors (Zhang *et al.*, 1999).

Many methods use the adsorption of dye contaminants on biomass which is commonly referred to as biosorption. Such processes take place, for example, in the course of activated sludge treatment (see Section 9.2.1 General aspects). Cells of white-rot fungi are preferably used for biosorption on both growing cells and on dead biomass (Fu and Viraraghavan, 2001). Decolorization without any transformation readily takes place only physically via adsorption onto their mycelia (Assadi and Jahangiri, 2001; Robinson *et al.*, 2001). With living fungi, adsorption may be accompanied by concomitant biodegradation (Aretxaga *et al.*, 2001, Sumathi and Manju, 2000). Pellets consisting of activated carbon and mycelium of *Trametes versicolor* were used for effective textile dye decolorization (Zhang and Yu, 2000). Azo dyes have been shown to quickly bind onto the mycelium of active *Aspergillus niger* resulting in extensive colour removal higher than 95% (Sumathi and Manju, 2000). Evidently, decolorization with active biomass is highly effective. High decolorization rates were also achieved with a combination of biodegradation by bacteria and adsorption using carbon black as a carrier material (Walker and Weatherly, 1999).

Coloured substances may be adsorbed onto many materials like sawdust (Khattri and Singh, 1999), charcoals, activated carbon, clays, soils, diatomaceous earth, activated sludge, compost, living plant communities, synthetic polymers, or inorganic salt coagulants (Slokar and Marechal, 1998). When biodegradable materials which provide good growth substrates for white rot fungi such as agricultural residues are used for biosorption, the physical adsorption can be used to rapidly decolorize the effluent and preconcentrate the dye stuff in a first step. Subsequently, for complete mineralization of dyes solid state fermentation can be performed on the dried adsorbent using white rot fungi. (Robinson *et al.*, 2001, Nigam *et al.*, 2000).

The removal of acid dyes by biosorption onto the biomass rather than biodegradation was found to be related not to the number of sulfonic groups

(and thereby the solubility), but to the size of the molecule. It is thought that the greater the molecular size, the greater the degree of adsorption (Cooper, 1993).

Anaerobic treatment

Anaerobic treatment occurs in sealed tanks and converts the waste into methane and carbon dioxide. Where nitrogenous and sulfide-containing pollutants are present, ammoniacal substances and hydrogen sulfide are produced. At some municipal sewage treatment plants, the sludge formed by the aerobic treatment process passes into tanks for anaerobic treatment. Considerable heat is produced from anaerobic treatment. By using heat exchangers to extract the heat, the bioenergy can be utilized to heat buildings. The methane produced is collected, compressed and then used in generators to produce electricity. The electricity produced can power site processes and the surplus is sold to the national grid. The production of this power not only reduces the running costs of the treatment plant but also provides a welcome income, thus reducing the costs further.

Under anaerobic conditions, the decolorization of many azo dyes via reduction of the azo bond has long been shown for anaerobic (e.g. *Bacteroides* sp., *Eubacterium* sp., and *Clostridium* sp.) and facultative anaerobic (e.g. *Proteus vulgaris* and *Streptococcus faecalis*) bacteria (Bragger *et al.*, 1997; Rafii *et al.*, 1990; Wuhrmann *et al.*, 1980; Gingell *et al.*, 1971). The main interest in this field has been focused on bacteria from the human intestine that are involved in the metabolism of azo dyes ingested as food additives (Chung *et al.*, 1992). The fecal enzyme activity of azoreductase is commonly considered a marker for procarcinogenic activity (Haberer *et al.*, 2003). The nonspecificity of the azoreductase reaction is demonstrated by many reports on the decolorization of azo dyes by sewage sludge under anaerobic conditions (Carliell *et al.*, 1994; Pagga and Brown, 1986). It seems that almost all azo compounds tested are biologically reduced under anaerobic conditions, although there are some indications that metal-ion-containing dyes sometimes have reduced decolorization rates (Chung and Stevens, 1993).

The conventional treatment of coloured effluents produces a lot of sludge, but does not remove all dyes, thus preventing recycling of the treated wastewater. In activated sludge treatments, dyeing effluents, e.g. reactive azo dyes and naphthalene-sulfonic acids as well as aromatic amino derivatives, represent an extensive nonbiodegradable class of compounds (Krull *et al.*, 1998) and can even inhibit activated sludge organisms. Such dyes often will respond better to anaerobic conditions than aerobic conditions. Many dyes are not biodegraded but only adsorbed under aerobic conditions. Studies have found that many azo dyes can be degraded under anaerobic conditions by reductive cleavage of the N=N double bond yielding the corresponding

aromatic amines. Some of these amines are carcinogenic and thus pose a considerable potential health risk when released into the environment. However, as shown already, aromatic amines are most likely to be further degraded under anaerobic conditions (Laing, 1991). Specialized strains of micro-organisms can be conditioned to fully degrade azo dyes (Razo-Flores *et al.*, 1997a, b).

9.2.2 Enzymic processes

General aspects

The concept of using isolated and partly purified enzyme preparations has several advantages over whole cell approaches. The expression of the enzymes involved in dye degradation is not constant with time but dependent on the growth phase of the population when living organisms are used. This can be circumvented by using isolated enzymes. Instead of maintaining living cultures of micro-organisms at the site of pollution, the production, downstreaming and preparation of stabilized biocatalysts or enzyme cocktails is provided off-site by specialized production technologies. Enzymes are easier to handle than living organisms and can be regarded more as speciality chemicals. Enzymes can be produced on a large scale and may already be applied in crude form (Moreira *et al.*, 1998; Linko, 1988; Fahreus and Reinhammar, 1967) in order to keep the costs considerably low. For industrial applications, immobilization of enzymes allows the reuse of the enzyme and thus further reduces the cost for such a process.

Tailor-made enzymes can be optimized independently by exploring induction reagents or using genetic engineering methods. This results in specialized biocatalysts, which may be superior to their naturally evolved counterparts. Efficiency may be increased upon combination with suitable additives and stabilizers and their application in much higher concentrations and in clearly defined quantities is possible unlike with naturally grown systems, which are much more susceptible towards variations. Thus, constant performance may be easier achieved.

Since isolated enzymes are protein molecules, they do not metabolize dyes like living whole cell organisms do. They only catalyze a specific type of transformation. Mineralization of dyes can therefore never be achieved by only using enzymes. However, enzymatic modification of dyes may often be sufficient at a certain stage in the process. Decolorization may be readily achieved by enzymatic destruction of the chromophoric centre of the dye. Detoxification may already be achieved after enzyme treatment by the transformation of the functional group conferring toxicity.

The major potential for enzyme reactors lies in special treatments of specific partial process streams of relatively constant and known composition.

In such cases, biological processes other than defined enzymatic systems may not be applicable at all. For example, the selective biological removal of hydrogen peroxide at high pHs and temperatures from partial process streams within the plant is only possible by using an immobilized catalase enzyme system specifically designed for this purpose. Such a reactor system has already been successfully tested in industry (Paar *et al.*, 2001).

Although the enzymes used for dye remediation display broad substrate specificities and practically all the different structural patterns such as the triphenylmethane, anthraquinonoid, indigoid, and azodyes can be degraded, the molecular structure of the waste dyestuff plays a considerable role on the rate and extent of transformation. Dyes are generally designed to exhibit high stability. They must resist irradiation with UV light, they must survive numerous washing processes and, of course, they have to resist microbial attack while in use on a textile fabric.

Various structural parameters of the dye molecule are to be taken into consideration when its potential degradation in bioremediation processes is discussed. No single model is currently available that would describe all the observations of structural effects on biodegradability since too many different aspects are to be taken into consideration. With redox active enzymes, the redox potential of the dye plays a central role (Xu, 1996; Xu and Salmon, 1999). While electron-withdrawing substituents enhance the reductive biodegradation of azo dyes (Maier *et al.*, 2004), the opposite trend was observed for the oxidative pathway (Kandelbauer *et al.*, 2004a, b and 2006). The redox potentials of textile dyes were successfully used for the quantitative prediction of the biodegradability of a wide structural variety of different textile dyes (Zille *et al.*, 2004). For a more detailed discussion of substrate specifities and various observations on structural effects with dyes of very different molecular structure, see, for instance, Kandelbauer *et al.* (2004a, b, and 2006) Knackmus (1996) and the references given therein.

Oxidative enzyme remediation

In general, there are two kinds of classes of oxido-reductases that are involved in dye degradation: electron transferring enzymes and hydroxy-group inserting enzymes. Peroxidases and laccases act via electron transfer and yield an oxidized dye species. They are the most important types of enzymes involved in enzymatic dye degradation. Both are secreted by lignolytic fungi (Mester and Tien, 2000; Duran and Esposito, 2000).

The second type of oxido-reductive enzymes is the oxygenases. They insert hydroxy-groups into a substrate. Depending on the number of hydroxy-groups transferred by the enzymes they are classified as mono- or dioxygenases. Oxygenases rely on complicated organic cofactors such FAD(H), NAD(P)(H) or cytochrome P450. For efficient regeneration of the catalytic system, living

organisms are needed and, thus they are not used for enzyme reactors as such. Oxygenases are not only present in whole cell systems and mixed cultures used for dye degradation, but are ubiquitously found within the cell walls of every living organism. They play a vital role in the breakdown of aromatic ring systems. Upon hydroxylation, the subsequent cleavage of the aromatic ring yields carboxylic acids, which are further metabolized.

Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) catalyze the removal of one hydrogen atom from the hydroxyl group of *ortho*- and *para*-substituted mono- and polyphenolic substances and from aromatic amines by one-electron abstraction. Thereby, free radicals are formed which are capable of undergoing further degradation or coupling reactions, demethylation or quinone formation (Thurston 1994, Yaropolov *et al.*, 1994). In contrast to other types of enzymes, such as hydrolytic enzymes like cellulases or lipases, laccases exhibit very broad substrate specificities. By using additional low molecular compounds such as ABTS, HBT and TEMPO which act as redox mediators (Fabbrini *et al.*, 2002; Almansa *et al.*, 2004), polyoxometalates (Tavares *et al.*, 2004) or osmium-based redox polymers, their substrate specificity can further be expanded. Redox mediating compounds are also secreted by lignolytic fungi in order to assist the extracellular digestion process (Eggert *et al.*, 1996; Johannes and Majcherczyk, 2000a, b). Laccases contain active copper centres. Hence, traces of copper may be introduced into the effluent upon excessive addition of laccase.

Peroxidases (e.g. EC 1.11.1.9) are enzymes that catalyze the transfer of two electrons from a donor molecule to hydogen peroxide or organic peroxides. The oxidized substrate may be a textile dye. Peroxidases are a much more diverse group of enzymes than laccases and the structure of the electron donor may limit the choice of peroxidases. Most commonly, manganese peroxidases and lignin peroxidases from ligninolytic fungi are employed in the degradation of textile dyes (Mester and Tien, 2000). The presence of low molecular substances may enhance the performance of peroxidases as well. Thus, the addition of veratryl alcohol was shown to positively influence decolorization of azo and anthraquinone dyes catalyzed by lignin peroxidase. However, this effect may either be attributed to the protection of the enzyme against being inactivated by hydrogen peroxide or to the completion of the oxidation-reduction cycle of the lignin peroxidase rather than to redox-mediation (Young and Yu, 1997).

Laccases and peroxidases may exhibit different substrate specificities. For example, the laccase treatment of the three different triphenylmethane dyes malachite green, crystal violet and bromophenol blue resulted in overall decolorizations of 100, 20, and 98%, respectively (Pointing and Vrijmoed, 2000). In contrast, an analogous experiment using the same dyes in a treatment with a peroxidase yielded a different ratio of reactivities of 46, 74, and 98%, respectively (Shin and Kim, 1998a).

The major advantage in using laccases lies in that they just require molecular oxygen as a co-substrate. Such treatment systems therefore only require sufficient aeration of the system and are therefore relatively simple. Peroxidases require hydrogen peroxide as a co-substrate in order to oxidize the dye molecules and catalyze degradation. Thus, here additional chemical load is required in order to gain catalytic transformation. Consequently, in the past decade, the focus on designing enzyme reactors for dye decolorization has been on laccase systems.

Laccases decolorize a wide range of industrial dyes (Rodriguez *et al.*, 1999; Reyes *et al.*, 1999; Campos *et al.*, 2001; Kandelbauer *et al.*, 2004 a, b). In the presence of redox mediators, this range was extended (Reyes *et al.*, 1999; Soares *et al.*, 2001a, b; Almansa *et al.*, 2004) or the extent of decolorization of degradable dyes was significantly enhanced (Abadulla *et al.*, 2000). The presence of low molecular weight compounds like 2,2′-azino-*bis*-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was in some cases necessary to initiate the actual electron transfer steps of laccases (Wong and Yu, 1999). Dye degradation intermediates may have an enhancing effect on decolorization due to redox mediation. Anthraquinonoid dyes act as redox mediators (Kandelbauer *et al.*, 2004) and they may assist in the laccase catalyzed remediation of dye mixtures.

For technical applications, enzymes are immobilized. Various applications of laccases immobilized on different types of supports have been reviewed recently (Duran *et al.*, 2002). Immobilization of fungal laccases on various carrier materials such as activated carbon (Davis and Burns, 1992), sepharose (Milstein *et al.*, 1993), or porosity glass (Rogalski *et al.*, 1995 and 1999) has been shown to increase stabilities of the enzyme at higher pH and tolerance to elevated temperatures and to make the enzyme less vulnerable to inhibitors such as Cu chelators. Membrane enzyme reactors containing laccases from *Neurospora crassa* (Luke and Burton, 2001) or *Pyricularia oryzae* (Lante *et al.*, 2000) have been employed for the bioremediation of phenols. Laccase bound to Eupergit (Hublik and Schinner, 2000; D'Annibale *et al.*, 2000) has also been used for this purpose. Reyes *et al.* (1999) reported the application of a bioreactor based on *Coriolopsis gallica* laccase immobilized on activated agarose. A laccase from *Lentinula edodes* was immobilized on a chitosan solid support (D'Annibale *et al.*, 1999).

An important issue for the industrial application is the long-term stability of biocatalytic systems. The application of *Trametes hirsuta* laccase upon covalent immobilisation to a γ-Al_2O_3-carrier was described for the efficient use in the detoxification and degradation of structurally diverse dyes. Reactors containing such laccase preparations were run in ten repeated batch decolorizations for about 15 h while still retaining 85% of their initial activity (Abadulla *et al.*, 2000). Model dye house effluents containing a wide variety of structurally different textile dyes such as triphenyl methane dyes, heterocyclic

azo dyes, anthraquinonoid dyes and Indigo Carmine were successfully decolorized by using a similarly immobilized enzyme reactor based on a laccase from *Trametes modesta* (Kandelbauer *et al.,* 2003 and 2004b). Here, the simulated effluent was pumped continuously through a reaction cell and dye loads were added in regular intervals yielding dye concentrations of 50 mg l^{-1} and more. Mainly due to mechanical abrasion of the enzyme from the support, the reactor had lost 50% of its activity after 10 h and within five decolorization cycles. Other experiments with an authentic textile effluent caused a loss in laccase activity resulting in 14% retained activity (Reyes *et al.*, 1999). The authors investigated all known components of the effluent like salts, soap, and dispersant and their mixtures for laccase inactivation but none of them was identified as detrimental to the enzyme.

Thus, one major problem with enzyme reactors is currently their limited lifetime under harsh conditions. Especially when real-life effluent streams are approached, enzyme reactors seem currently no useful solution for end of pipe operations. Here, whole cell populations with mixed cultures will always perform much more reliably.

However, within partial process streams at more defined loads and reaction conditions, promising results have been presented with enzymes. After treatment with immobilized enzyme, decolorized dyeing water was recycled within the dyeing process (Abadulla *et al.*, 2000). This is not possible with effluents treated with micro-organisms since they require additional components to support growth, which cause substantial changes in the process water composition. Both nutrients added to the effluent and metabolites secreted by micro-organisms can cause problems in the recycling of effluents (Abadulla *et al.*, 2000).

The lifetime and resistance of enzyme reactors can possibly significantly be enhanced by applying enzymes from other sources. Currently, practically all laccases used for dye remediation are derived from white-rot fungi. Despite promising efficiency in dye decolorization in general, with these enzymes, one is typically restricted to reaction conditions which correspond to the natural environment of the fungi in which they are formed. Thus, no decolorization activities at all are observed at values higher than 7 and temperatures well above 45 °C (e.g. Kandelbauer *et al.*, 2004b and 2006).

A very potent candidate for future high-performance enzyme sources is therefore the class of extremophilic micro-organisms. Since they live under extreme heat and pH conditions such as on the sea bottom or in hot sulfate-containing springs, their enzymatic systems are more likely to withstand extreme environmental conditions. Consequently, screening for suitable enzymes expressed by thermo-alkalophilic organisms is of special interest with respect to their potential applicability at elevated temperatures and pHs. Organisms of this kind are impossible to cultivate using conventional

fermentation protocols. However, this is not required. The enzymes of interest could be cloned, genetically transferred and expressed by organisms for which efficient standard production methods are already well established (Kruus *et al.*, 2001).

However, it might not even be necessary to go for extremophiles in the first place. The universally present class of bacteria might still hold unexplored potential that is more readily available. One example for the extension of the range of reaction conditions is the bacterial laccase from a *Bacillus* species (Held *et al.*, 2004 and 2005).

In contrast to fungal laccases, which have been known for many years, laccase activity in bacteria was only discovered in 1993 for *Azospirillum lipoferum* and was only recently characterized in more detail (Diamantidis *et al.*, 2000). Interestingly, the spore coat protein CotA of *Bacillus subtilis* was identified as a laccase (Hullo *et al.*, 2001). Bacterial laccases seem to be involved in pigment formation with some bacterial spores (Solano *et al.*, 2001) and, in this function, they are assumed to be widespread among that class of bacteria (Alexandre and Zhulin, 2000). Since spores serve micro-organisms to survive drastic conditions, spore coat enzymes are likely also to withstand high temperatures or extreme pH values, which would be advantageous for industrial applications.

When using preparations of bacterial laccase immobilized on spores for the treatment of 50 mg l^{-1} of Indigo Carmine, Diamond Black PV 200 or Diamond Fast Brown (Held *et al.*, 2005), complete decolorization was achieved at pH 9 within two hours. While with fungal laccases, the optimum temperature for dye decolorization was never above 40 °C, the best results with bacterial laccase were found at 60 °C. Within a pH range of 5.0–7.0 the half-life was more than 120 h, compared with a half-life of 13 h under similar conditions as previously measured for a laccase from *Trametes hirsuta* (Abadulla, 2000); this is a ten-fold increase in stability.

Most studies in the past decade have been focused on the laccase-catalysed degradation of dye molecules. However, since laccases are primarily involved in the initial formation of reactive radicals, the same enzymes could also advantageously be used to increase the molecular weight of waste dyestuff.

In a recent study, it was found that, upon laccase catalysis, the acid dye Indigo Carmine was either completely degraded or transformed to another product of higher molecular weight. Which pathway the reaction followed depended strongly on the reaction conditions, especially on the pH: at higher pHs (pH 5–6), the predominant product formed was that of higher molecular weight (Kandelbauer *et al.*, 2006).

In an earlier study using a peroxidase in the Indigo Carmine transformation, the observation of an unidentified by-product in small quantities was reported that might also have been formed by an polymerization pathway as well (Podgorny *et al.*, 2001). This might indicate that the strategy of increasing

the molecular weight is not limited only to laccase reactors. And it is certainly not limited to indigoid dyes. By-products of higher molecular weight than the starting material were observed in the laccase-catalyzed transformation of the azo dye Diamond Black PV 200 and various structurally related azo dyes (Kandelbauer *et al.*, 2004a). *Trametes villosa* laccase in solution was used for the degradation of azodyes over a period of 72 h of oxidative conditions, demonstrating not only degradation of the dyes but also polymerization to some degree (Zille *et al.*, 2005).

The consequent application of this idea would lead to a strategy opposite to the one pursued so far. Instead of aiming at biodegradation, polymerized dye stuff of high enough molecular weight could be produced and subsequently readily removed from the effluent by filtration. Thereby, the chance of producing potentially toxic residual by products as in the degradation process would be minimized.

Laccases are already used industrially in hair dyeing formulations, as described in various patents for the dyeing of keratinous fibres (Lang and Cotteret, 2002; Onuki *et al.*, 2000; Aaslyng *et al.*, 1999) and a number of publications have been published on laccase-catalyzed coupling reactions meaning that much useful knowledge may be already available from other research fields.

Reductive enzymes

Unlike the oxidative enzymes laccase and peroxidase, the application of reductases or oxidases requires cofactors like NAD(H), NADP(H), or FAD(H), which are extremely expensive compounds. Enzyme remediation systems based upon such enzymes is therefore economically not feasible and industrially very difficult to implement. Most decolorizations in connection with reductive enzymes usually take place in whole cell applications. Bacterial degradation of azo dyes may be attributed to unspecific reduction of dyes (Yoo, 2002; Nam and Renganathan, 2000) or the action of azoreductase activity (Ramalho *et al.*, 2005; Maier *et al.*, 2004; Zimmermann *et al.*, 1984).

9.3 Future trends

Although enzyme remediation of dyestuff successfully removes its colour, a potentially harmful organic load may remain in the process waters. Thus, an interesting future perspective in the application of laccases for the treatment of process waters containing waste dyestuffs is the coupling of phenolic dye fragments rather than their oxidative breakdown. If such polymerized fragments were of sufficiently increased molecular weight, they could readily be removed by a subsequent filtration step (laccase-assisted dye precipitation). Consequently, future research activities should focus on optimization of the

reaction conditions in order to achieve maximum oxidative coupling of primarily formed dye fragments.

Exploring the potential of different or newly discovered organisms such as various species of extremophiles or bacteria will lead to more powerful organismic and enzymatic treatment systems. Genetic methods will be of increasing interest in designing and tailoring new super-enzymes with expanded potential not only in effluent treatment but in all fields of industrial microbiology. They will also help to optimize fermentation technology in order to further reduce the costs and increase availability of enzymes.

Application of the present knowledge about the relationships between the chemical structure and its influence on biodegradability could possibly lead to the design of novel dyes that are perfectly stable while in use on the textile but that provide sufficient affinity towards bioremediation systems to be completely biodegradable.

Intelligent combination of different treatment techniques will provide more powerful integrated tools. For instance, the adsorption of dyestuff on living material paired with subsequent enzymatic degradation is not new, but is a very good illustration of the concept for using the advantages and avoiding the disadvantages of two different methods. Another example is the combination of laccase systems with ultrasound (Rehorek *et al.,* in press) in order to enhance the biodegradation process. The use of peroxidases depends on the availability of hydrogen peroxide as a second substrate. Here, a combination of enzyme remediation with conventional chemical regeneration processes relying on a combination of UV and hydrogen peroxide could prove beneficial in the future. In both cases, the enzymes have to be protected against too high an energy input since they easily may be destroyed. Therefore, sequential application of the different techniques may provide an industrially feasible solution. Physicochemical pretreatment of waste loads in order to facilitate subsequent biological treatments is presently well established.

9.4 References

Aaslyng D, Sorensen N H and Rorbaek K, (1999) 'Laccases with improved dyeing properties', United States Patent, US 5,948,121.

Abadulla E, Tzanov T, Costa S, Robra K H, Cavaco-Paulo A and Gübitz G M, (2000) 'Decolorization and detoxification of textile dyes with a laccase from *Trametes hirsuta*', *Appl Environ Microbiol*, **66** 3357–3362.

Alexandre G and Zhulin I B, (2000) 'Laccases are widespread in bacteria', *Tibtech*, **18** 41–42.

Almansa E, Kandelbauer A, Pereira L, Cavaco-Paulo A and Gübitz G M, (2004) 'Influence of structure on dye degradation with laccase mediator systems', *Biocatal Biotrans*, **22** 315–324.

Aretxaga A, Romero S, Sarr M and Vicent T, (2001) 'Adsorption step in the biological degradation of a textile dye', *Biotechnol Prog*, **17** 664–668.

Assadi M M and Jahangiri M R, (2001) 'Textile wastewater treatment by *Aspergillus niger*', *Desalination*, **141** 1–6.

Azmi W, Sani R K and Banerjee U C, (1998) 'Biodegradation of triphenylmethane dyes', *Enzyme Microb Technol*, **22** 185–191.

Bakshi D K, Gupta K G and Sharma P, (1999) 'Enhanced biodecolorization of synthetic textile dye effluent by *Phanerochaete chrysosporium* under improved culture conditions', *World J Microbiol Biotechnol*, **15** 507–509.

Basibuyuk M and Forster C F (1997) 'An examination of the treatability of a simulated textile wastewater containing Maxilon Red BL-N', *Process Biochem*, **32**(6) 523–527.

Binkley J and Kandelbauer A (2003) 'Effluent treatment – Enzymes in activated sludge', in: Cavaco-Paulo A; Gübitz G M, editors *Textile processing with enzymes.* Woodhead, Cambridge, 199–222.

Blümel S, Contzen M, Lutz M, Stolz A and Knackmuss H J (1998) 'Isolation of a bacterial strain with the ability to utilize the sulfonated azo compound 4-carboxy-4′-sulfoazobenzene as the sole source of carbon and energy', *Appl Environ Microbiol*, **64** 2315–2317.

Bragger J L, Lloyd A W, Soozandehfar S H, Bloomfield S F, Marriott C and Martin G P (1997) 'Investigations into the azo reducing activity of a common colonic microorganism', *Int J Pharm*, **157** 61–71.

Campos R, Kandelbauer A, Robra K H, Cavaco-Paulo A and Gübitz G M (2001) 'Indigo degradation with purified laccases from *Trametes hirsuta* and *Sclerotium rolfsii*', *J Biotechnol*, **89** 131–139.

Carliell C M, Barclay S J, Naidoo N, Buckley C A, Mulholland D A, and Senior E (1994) 'Anaerobic decolorisation of reactive dyes in conventional sewage treatment processes', *Water SA,* **20**(4) 341–344.

Chagas E P and Durrant L R (2001) 'Decolorization of azo dyes by *Phanerochaete chrysosporium* and *Pleurotus sajorcaju'*, *Enz Microb Technol*, **29** 473–477.

Chung K T, Stevens S E and Cerniglia C E (1992) 'The reduction of azo dyes by the intestinal microflora', *CRC Crit Rev Microbiol*, **18**(3) 175–190.

Chung K T and Stevens S E (1993) 'Degradation of azo dyes by environmental microorganisms and helminths', *Environ Toxicol Chem*, **12** 2121–2132.

Cooper P (1993) 'Consequences of U K and E C environmental legislation on textile finishing', *J Text Inst,* **84**(4) 553–576.

Corso C R, de Angelis D F, de Oliveira J E and Kiyan C (1981) 'Interaction between the diazo dye Vermelho Reanil P8B, and *Neurospora crassa* strain 74A', *Eur J Appl Microbiol Biotechnol,* **13** 64–66.

D'Annibale A, Stazi S R, Vinciguerra V and Sermanni G G (2000) 'Oxirane-immobilized *Lentinula edodes* laccase: stability and phenolics removal efficiency in olive mill wastewater', *J Biotechnol*, **77**(2–3) 265–273.

D'Annibale A, Stazi S R, Vinciguerra V, Di Mattia E and Sermanni G G. (1999) 'Characterization of immobilized laccase from *Lentinula edodes* and its use in olive-mill wastewater treatment. *Process Biochem*, **34** 697–706.

Davis S and Burns R G (1992) 'Decolorization of phenolic effluents by soluble and immobilized phenol oxidases', *Appl Microbiol Biotechnol*, **37** 474–479.

Diamantidis G, Effosse A, Potier P, and Bally R (2000) 'Purification and characterization of the first bacterial laccase in the rhizospheric bacterium *Azospirilium lipoferum*', *Soil Biol Biochem,* **32** 919–927.

Dominguez A, Rivela I, Rodriguez Couto S and Sanroman M A (2001) 'Design of a new rotating drum bioreactor for ligninolytic enzyme production by *Phanerochaete chrysosporium* grown on an inert support', *Process Biochem*, **37** 549–554.

Duran N and Esposito E (2000) 'Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment: a review', *Appl Cat B Environ*, **28** 83–99.

Duran N, Rosa M A, D'Annibale A and Gianfreda L (2002) 'Applications of laccases and tyrosinases (phenoloxidases) immobilized on different supports: a review', *Enzyme Microb Technol*, **31** 907–931.

Eggert C, Temp, U and Eriksson K E (1996) 'Laccase-producing white-rot fungus lacking lignin peroxidase and manganese peroxidase', *ACS Symp Ser*, **655** 130–150.

Fabbrini M, Galli C and Gentili P (2002) 'Comparing the catalytic efficiency of some mediators of laccase', *J. Mol. Catal. B: Enzymatic*, **16**(5–6) 231–240.

Fahreus G and Reinhammar B (1967) 'Large scale production and purification of laccase from cultures of the fungus *Polyporus versicolor* and some properties of laccase A', *Acta Chem Scand*, **21** 2367–2378.

Fu Y and Viraraghavan T (2001) 'Fungal decolorization of dye wastewaters: a review', *Biores Technol,* **79** 251–262.

Gingell R and Walker R (1971) 'Mechanisms of azo reduction by *Streptococcus faecalis*. II. The role of soluble flavins', *Xenobiotica*, **1** 231–239.

Haberer P, du Toit M, Dicks L M, Ahrens F and Holzapfel W H (2003) 'Effect of potentially probiotic lactobacilli on faecal enzyme activity in minipigs on a high-fat, high-cholesterol diet – a preliminary in vivo trial', *Int J Food Microbiol*, **87**(3) 287–291.

Hao O J, Kim H and Chiang P C (2000) 'Decolorization of wastewater', *Crit Rev Environ Sci Technol*, **30** 449–505.

Held C, Kandelbauer A, Schroeder M, Cavaco-Paulo A and Gübitz G M (2004) 'Eine Laccase enthaltender Biokatalysator', European Patent Application EP 1468968 A1.

Held C, Kandelbauer A, Schroeder M, Cavaco-Paulo A and Gübitz G M (2005) 'Biotransformation of phenolics with laccase containing bacterial spores', *Environ. Chem. Lett*, **2** 74–77.

Hublik G and Schinner F (2000) 'Characterization and immobilization of the laccase from *Pleurotus ostreatus* and its use for the continuous elimination of phenolic pollutants', *Enzyme Microb Technol*, **27** 330–336.

Hullo M F, Moszer I, Danchin A and Martin-Verstraete I (2001) 'CotA of *Bacillus subtilis* is a copper-dependent laccase', *J Bacteriol*, **183** 5426–5430.

Johannes C and Majcherczyk A (2000a) 'Natural mediators in the oxidation of polycyclic aromatic hydrocarbons by laccase mediator systems', *Appl Environ Microbiol*, **66**(2) 524–528.

Johannes C and Majcherczyk A (2000b) 'Laccase activity tests and laccase inhibitors', *J Biotechnol*, **78**(2) 193–199.

Kandelbauer A, Erlacher A, Cavaco-Paulo A and Gübitz G M (2004a) 'Laccase-catalyzed decolorization of the synthetic azo dye Diamond Black PV 200 and of some structurally related derivatives', *Biocatal Biotrans*, **22** 331–339.

Kandelbauer A and Gübitz G M (2005) 'Bioremediation for the decolorization of textile dyes, a review' in: Lichtfouse E, Dudd S and Robert D (Eds.) *Environmental Chemistry* Springer-Verlag, Berlin Heidelberg New York, 269–288.

Kandelbauer A, Maute O, Kessler R W, Erlacher A and Gübitz G M (2004b) 'Study of dye decolorization in an immobilized laccase enzyme reactor using online spectroscopy', *Biotechnol Bioeng*, **87** 552–563.

Kandelbauer A, Maute O, Kimmig M, Kessler R W and Gübitz G M (2006) 'Laccase catalyzed Indigo Carmine transformation', *J Nat Fibres*, **3**(2–3) 131–53.

Kandelbauer A, Schnitzhofer W, Kessler R W, Cavaco-Paulo A and Gübitz G M (2003) 'Production and immobilisation of a laccase from the white rot fungus *Trametes modesta* for application in the decolorization of textile dyes', in Hardin I R, Akin D E and Wilson S J (ed). *Advances in biotechnology for textile processing*. University of Georgia, Athens, Georgia, 1–13.

Kapdan I K and Kargi F (2002) 'Simultaneous biodegradation and adsorption of textile dye stuff in an activated sludge unit', *Process Biochem,* **37** 973–981.

Khattri S D and Singh M K (1999) 'Colour removal from synthetic dye wastewater using a bioadsorbent', *Water Air Soil Pollut*, **120** 283–294.

Kim S J, Ishikawa K, Hirai M and Shoda M (1995) 'Characteristics of a newly isolated fungus, *Geotrichum candidum* Dec 1, which decolorizes various dyes', *J Ferment Bioeng,* **79** 601–607.

Kirby N, Marchant R and McMullan G (2000) 'Decolourisation of synthetic textile dyes by *Phlebia tremellosa*', *FEMS Microbiol Lett*, **188** 93–96.

Knackmus H J (1996) 'Basic knowledge and perspectives of bioelimination of xenobiotic compounds', *J Biotechnol* **51** 287–295.

Krull R, Hemmi M, Otto P and Hempel D C (1998) 'Combined biological and chemical treatment of highly concentrated residual dyehouse liquors', *Water Sci Technol*, **38** 339–346.

Krull R, Hempel D C A and Alfter P (2000) 'Konzept zur technischen Umsetzung einer zweistufigen anoxischen und aeroben Textilabwasserbehandlung', *Chem Ing Tech*, **72** 1113–1114.

Kruus K, Kiiskinen L L, Raettoe M, Viikari L and Saloheimo M (2001) 'Purification, characterization and use of *Melanocarpus albomyces* laccase and cloning and sequencing of the laccase gene', *PCT Int Appl WO* 01/92498 1–59.

Laing I G (1991) 'The impact of effluent regulations on the dyeing industry', *Rev Prog Color*, **21** 56–71.

Lang G, and Cotteret J (2002) 'Dyeing composition containing a laccase and keratinous fiber dyeing methods using same', United States Patent US 6,471,730 B1.

Lante A, Crapisi A, Krastanov A and Spettoli P (2000) 'Biodegradation of phenols by laccase immobilised in a membrane reactor', *Proc Biochem*, **36** 51–58.

Linko S (1988) 'Production and characterization of extracellular lignin peroxidase from immobilized *Phanerochaete chrysosporium* in a 10-l bioreactor', *Enzyme Microb Technol*, **10** 410–417.

Luke A K and Burton S G (2001) 'A novel application for *Neurospora crassa*: Progress from batch culture to a membrane bioreactor for the bioremediation of phenols', *Enz. Microb. Technol*, **29** 348–356.

Maier J, Kandelbauer A, Erlacher A, Cavaco-Paulo A and Gübitz G M (2004) 'A new alkali-thermostable azoreductase from *Bacillus* sp. Strain SF', *Appl Environ Microbiol*, **70** 837–844.

Martins M A M, Cardoso Ferreira I C, Santos I M, Queiroz M J and Lima N (2001) 'Biodegradation of bioaccessible textile dyes by *Phanerochaete chrysosporium*', *J Biotechnol*, **89** 91–98.

McMullan G, Meehan C, Conneely A, Kirby N, Robinson T, Nigam P, Banat I M, Marchant R and Smyth W F (2001) 'Microbial decolourization and degradation of textile dyes', *Appl Microbiol Biotechnol,* **56** 81–87.

Mester T and Tien M (2000) 'Oxidative mechanism of ligninolytic enzymes involved in the degradation of environmental pollutants', *Int Biodeter Biodeg*, **46** 51–59.

Milstein O, Huettermann A, Majcherczyk A, Schulze K, Fruend R and Luedermann H D (1993) 'Transformation of lignin-related compounds with laccase in organic solvents', *J Biotechnol*, **30** 37–47.

Moreira M T, Palma C, Feijoo G and Lema J M (1998) 'Strategies for the continuous production of lignolytic enzymes in fixed and fluidised bed bioreactors', *J Biotechnol*, **66** 27–39.

Nam S and Renganathan V (2000) 'Non-enzymatic reduction of azo dyes by NADH', *Chemosphere*, **40** 351–357.

Nicolella C, van Loosdrecht M C M and Heijnen J J (2000) 'Wastewater treatment with particulate biofilm reactors', *J Biotechnol*, **80** 1–33.

Nigam P, Armour G, Banat I M, Singh D and Marchant R (2000) 'Physical removal of textile dyes from effluents and solid-state fermentation of dye-adsorbed agricultural residues', *Biores Technol*, **72** 219–226.

Nigam P, McMullan G, Banat I M and Marchant R (1996) 'Decolourisation of effluent from the textile industry by a microbial consortium', *Biotechnol Lett*, **18** 117–120.

Novotny C, Rawal B, Manish B, Patel M, Sasek V and Molitoris H P (2001) 'Capacity of *Irpex lacteus* and *Pleurotus ostreatus* for decolorization of chemically different dyes', *J Biotechnol*, **89** 113–122.

Onuki T, Noguchi M and Mitamura J (1999) 'Hair Dye Compositions', European Patent Application EP 1 142 561 A1.

Paar A, Costa S, Tzanov T, Gudelj M, Robra K-H, Cavaco-Paulo A and Gübitz G M (2001) 'Thermoalkali stable catalases from newly isolated *Bacillus* sp. for the treatment and recycling of textile bleaching effluents', *J Biotechnol*, **89** 147–154.

Pagga U and Brown D (1986) 'The degradation of dyestuffs: Part II. Behaviour of dyestuffs in aerobic bioegradation tests', *Chemosphere*, **15**(4) 478–491.

Podgornik H, Poljansek I and Perdih A (2001) 'Transformation of Indigo Carmine by *Phanerochaete chrysosporium* ligninolytic enzymes', *Enzyme Microb Technol*, **29** 166–172.

Pointing S B and Vrijmoed L L P (2000) 'Decolorization of azo and triphenylmethane dyes by *Pycnoporus sanguineus* producing laccase as the sole phenoloxidase', *World J Microbiol Biotechnol*, **16** 317–318.

Rafii F, Franklin W and Cerniglia C E (1990) 'Azoreductase activity of anaerobic bacteria isolated from human intestinal microflora', *Appl Environ Microbiol*, **56** 2146–2151.

Ramalho P A, Paiva S, Cavaco-Paulo A, Casal M, Cardoso M H and Ramalho M T (2005) 'Azo reductase activity of intact *Saccharomyces cerevisiae* cells is dependent on the Fre1p component of plasma membrane ferric reductase', *Appl Environ Microbiol*, **71**(7) 3882–3888.

Razo-Flores E, Donlon B, Lettinga G and Field J A (1997a) 'Biotransformation and biodegradation of N-substituted aromatics in methanogenic granular sludge', *FEMS Microbiol Rev*, **20** 525–538.

Razo-Flores E, Luijten M, Donlon B, Lettinga G and Field J A (1997b) 'Complete biodegradation of the azodye azodisalicylate under anaerobic conditions', *Environ Sci Technol*, **31** 2098–2103.

Rehorek A, Hoffmann P, Kandelbauer A and Gübitz G M (2007) 'Sonochemical substrate specificity and reaction pathway of systematically substituted azo dyes', *Chemosphere*, in press.

Reyes P, Pickard M A and Vazquez-Duhalt R (1999) 'Hydroxybenzotriazole increases the range of textile dyes decolorised by immobilised laccase', *Biotechnol Lett*, **21** 875–880.

Robinson T, Chandran B and Nigam P (2001) 'Studies on the production of enzymes by white-rot fungi for the decolourisation of textile dyes', *Enzyme Microb Technol*, **29** 575–579.

Rodriguez E, Pickard M A and Vazquez-Duhalt R (1999) 'Industrial dye decolorization by laccases from ligninolytic fungi', *Curr Microbiol*, **38** 27–32.

Rogalski J, Dawidowicz A L, Jozwik E and Leonowicz A (1999) 'Immobilization of laccase from *Cerrena unicolor* on controlled porosity glass', *J Mol Catal B Enzym*, **6** 29–39.

Rogalski J, Jozwik E, Hatakka A and Leonowicz A (1995) 'Immobilization of laccase from *Phlebia radiata* on controlled porosity glass', *J Mol Catal A Chem*, **95** 99–108.

Sarsour J, Janitza J and Göhr F (2001) 'Biologischer Abbau farbstoffhaltiger Abwässer', Abw*asser/Abwassertechnik*, **11**(12) 44–46.

Schliephake K and Lonergan G T (1996) 'Laccase variations during dye decolourisation in a 200L packed bed reactor', *Biotechnol Lett*, **18** 881–886.

Schliephake K, Mainwaring D E, Lonergan G T, Jones I K and Baker W L (2000) 'Transformation and degradation of the disazo dye Chicago Sky Blue by a purified laccase from *Pycnoporus cinnabarinus*', *Enzyme Microb Technol*, **27** 100–107.

Shin K S and Kim C J (1998a) 'Decolorization of artificial dyes by peroxidase from the white-rot fungus, *Pleurotus ostreatus*', *Biotechnol Lett*, **20** 569–572.

Shin K S and Kim H M (1998b) 'Properties of laccase purified from nitrogen limited culture of white-rot fungus *Coriolus hirsutus'*, *Biotechnol Technol*, **12**(2) 101–104.

Slokar Y M and Marechal A M L (1998) 'Methods of decoloration of textile wastewaters', *Dyes Pigments*, **37** 335–356.

Soares G M, Costa-Ferreira M and Pessoa d'A (2001a) 'Decolorization of an anthraquinone-type dye using a laccase formulation', *Biores Technol*, **79** 171–177.

Soares G M, Pessoa d'A and Costa-Ferreira M (2001b) 'Use of laccase together with redox mediators to decolorize Remazol Brilliant Blue R', *J Biotechnol*, **89** 123–129.

Solano F, Eljo P, López-Serrano D, Fernández E and Sanchez-Amat A (2001) 'Dimethoxyphenol oxidase activity of different microbial blue multicopper proteins', *FEMS Microbiol Lett*, **204** 175–181.

Stolz A (2001) 'Basic and applied aspects in the microbial degradation of azo dyes', *Appl Microbiol Biotechnol*, **56** 69–80.

Sumathi S and Manju B S (2000) 'Uptake of reactive textile dyes by *Aspergillus foetidus*', *Enzyme Microb Technol*, **27** 347–355.

Swamy J and Ramsey J A (1999a) 'Effects of glucose and NH_4^+-concentrations on sequential dye decolorization by *Trametes versicolor*', *Enzyme Microb Technol,* **25** 278–284.

Swamy J and Ramsey J A (1999b) 'The evaluation of white-rot fungi in the decolorization of textile dyes', *Enzyme Microb Technol*, **24** 130–137.

Tatarko M and Bumpus J A (1998) 'Biodegradation of Congo Red by *Phanerochaete chrysosporium*', *Water Res*, **32** 1713–1717.

Tavares A P M, Gamelas J A F, Gaspar A R, Evtuguin D V and Xavier A M R B (2004) 'A novel approach for the oxidative catalysis employing polyoxometalate-laccase system: application to the oxygen bleaching of kraft pulp', *Catal Comm*, **5** 485–489.

Tekere M, Mswaka A Y A and Read J S (2001) 'Growth, dye degradation and ligninolytic activity studies on Zimbabwean white rot fungi', *Enz Microb Technol,* **28** 420–426.

Walker G M and Weatherly L R (1999) 'Biological activated carbon treatment of industrial wastewater in stirred tank reactors', *Chem Eng J*, **75** 201–206.

Thurston C F. (1994) 'The structure and function of fungal laccases', *Microbiol,* **140** 19–26.

Wong Y and Yu J (1999) 'Laccase-catalyzed decolorization of synthetic dyes', *Water Res*, **33** 3512–3520.

Wuhrmann K, Mechsner K and Kappeler T (1980) 'Investigation on rate determining factors in the microbial reduction of azo dyes', *Eur J Appl Microbiol Biotechnol*, **9** 325–338.

Xu F (1996) 'Oxidation of phenols, anilines, and benzenethiols by fungal laccases: correlation between activity and redox potentials as well as halide inhibition', *Biochem*, **35** 7608–7614.

Xu F and Salmon S I (1999) 'Enzymatic methods for dyeing with reduced vat and sulfur dyes', United States Patent US 5,948,122.

Yaropolov A I, Skorobogatko O V, Vartanov S S and Varfolomeyev S D (1994) 'Laccase Properties, Catalytic Mechanism, and Applicability', *Appl Biochem Biotechnol*, **49** 257–280.

Yoo E S (2002) 'Kinetics of chemical decolorization of the azo dye C.I. Reactive Orange 96 by sulfide', *Chemosphere*, **47** 925–931.

Young L and Yu J (1997) 'Ligninase-catalysed decolorization of synthetic dyes', *Water Res*, **31** 1187–1193.

Zhang F and Yu J (2000) 'Decolourisation of Acid Violet 7 with complex pellets of white rot fungus and activated carbon', *Bioprocess Eng*, **23** 295–301.

Zhang T C, Fu Y C, Bishop P L, Kupferle M, FitzGerald S, Jiang H H and Harmer C (1999) 'Transport and biodegradation of toxic organics in biofilms', *J Hazard Mat*, **41** 267–285.

Zheng Z, Levin R E, Pinkham J L and Shetty K (1999) 'Decolorization of polymeric dyes by a novel *Penicillium isolate*', *Process Biochem*, **34** 31–37.

Zille A, Gornacka B, Rehorek A, and Cavaco-Paulo A (2005) 'Degradation of azo dyes by *Trametes villosa* laccase over long periods of oxidative conditions', *Appl Environ Microbiol*, **71**(11) 6711–6718.

Zille A, Ramalho P, Tzanov T, Millward R, Aires V, Cardoso M H, Ramalho M T, Gübitz G M and Cavaco-Paulo A (2004) 'Predicting dye biodegradation from redox potentials', *Biotechnol Prog*, **20** 1588–1592.

Zimmermann T, Gasser F, Kulla H G and Leisinger T (1984) 'Comparison of two bacterial azoreductases acquired during adaptation to growth on azo dyes', *Arch Microbiol*, **138** 37–43.