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MINI-REVIEW

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Basic and applied aspects in the microbial degradation of azo dyes

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Abstract Azo dyes are the most important group of synthetic colorants. They are generally considered as xenobiotic compounds that are very recalcitrant against biodegradative processes. Nevertheless, during the last few years it has been demonstrated that several microorganisms are able, under certain environmental conditions, to transform azo dyes to non-colored products or even to completely mineralize them. Thus, various lignolytic fungi were shown to decolorize azo dyes using ligninases, manganese peroxidases or laccases. For some model dyes, the degradative pathways have been investigated and a true mineralization to carbon dioxide has been shown. The bacterial metabolism of azo dyes is initiated in most cases by a reductive cleavage of the azo bond, which results in the formation of (usually colorless) amines. These reductive processes have been described for some aerobic bacteria, which can grow with (rather simple) azo compounds. These specifically adapted microorganisms synthesize true azoreductases, which reductively cleave the azo group in the presence of molecular oxygen. Much more common is the reductive cleavage of azo dyes under anaerobic conditions. These reactions usually occur with rather low specific activities but are extremely unspecific with regard to the organisms involved and the dyes converted. In these unspecific anaerobic processes, low-molecular weight redox mediators (e.g. flavins or quinones) which are enzymatically reduced by the cells (or chemically by bulk reductants in the environment) are very often involved. These reduced mediator compounds reduce the azo group in a purely chemical reaction. The (sulfonated) amines that are formed in the course of these reactions may be degraded aerobically. Therefore, several (laboratory-scale) continuous anaerobic/aerobic processes for the treatment of wastewaters containing azo dyes have recently been described.

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Introduction

Azo dyes are characterized by the presence of one or more azo groups (-N=N-). They are the largest and most versatile class of dyes, and more than half of the annually produced amount of dyes (estimated for 1994 worldwide as 1 million tons) are azo dyes. Presumably more than 2,000 different azo dyes are currently used to dye various materials such as textiles, leather, plastics, cosmetics, and food. The largest amount of azo dyes is used for the dyeing of textiles, and it had been estimated that about 10% of the dye-stuff used during these dyeing processes does not bind to the fibers and is therefore released into sewage treatment systems or the environment (Anliker 1979; Chudgar 1985; Clarke and Anliker 1980; Reisch 1996; Zollinger 1991). In particular, the soluble reactive dyes, which are being used in increasing quantities, are known to hydrolyze during application without a complete fixation, which may result in an even larger proportion of these dyes being released into the environment (Carliell et al. 1994; Jeckel 1997; Weber and Stickney 1993).

There is only a single example for the presence of an azo group in a natural product (4,4′-dihydroxyazobenzene; Gill and Strauch 1984) and the industrially produced azo dyes are therefore all xenobiotic compounds. It is thus not surprising that azo dyes usually resist biodegradation in conventional aerobic sewage-treatment plants (Pagga and Brown 1986; Shaul et al. 1991). The recalcitrance of the azo dyes to biological degradative processes results in severe contamination of the rivers and ground water in those areas of the world with a high concentration of dyeing industries (Maguire and Tkacz 1991; Namasivavayam and Yamuna 1992; Ràfols and Barceló 1997; Riu et al. 1998; Tincher and Robertson 1982).

The current state of the art for the treatment of wastewaters containing dyes are physicochemical techniques, such as adsorption, precipitation, chemical oxidation, photodegradation, or membrane filtration (e.g. Churchley 1994; Panswed and Wongehaisuwan 1986; Yeh and

Thomas 1995; Yoshida et al. 1991). All of these have serious restrictions as economically feasible methods for decolorizing textile wastewaters (such as high cost, formation of hazardous by-products or intensive energy requirements). This has resulted in considerable interest in the use of biological systems for the treatment of these wastewaters. In the present review the fundamental biological reactions that allow the transformation of azo dyes are discussed and a short survey of possible technical applications of these reactions to the treatment of wastewaters from the textile industry is given.

Aerobic decolorization of azo dyes by lignin-degrading fungi

The first report of aerobic degradation of azo dyes by lignolytic fungi appeared in 1990, when Cripps et al. demonstrated that nitrogen-limited cultures of *Phanerochaete chrysosporium* decolorized the azo dyes Acid Orange 7 (Orange II), Acid Orange 6 (Tropaeolin O), or Direct Red 28 (Congo Red) (Fig. 1 A–C). Subsequent work demonstrated that cultures of *P. chrysosporium* also decolorized several other azo dyes (Banat et al. 1996; Young and Yu 1997). Currently, there is no correlation known between the structure of the azo dyes and the ability of *P. chrysosporium* to degrade the dyes (Pasti-Grigsby et al. 1992; Paszczynski et al. 1992). Experiments using ^{14}C-labeled azo dyes demonstrated that simple non-sulfonated azo dyes (e.g. Disperse Yellow 3; Fig. 1D) and also sulfonated dyes containing radiolabeled sulfanilic acid (4-aminobenzenesulfonic acid) as structural elements (e.g. Acid Orange 7, Fig. 1A, or Acid Yellow 9, Fig. 1E) were degraded by *P. chrysosporium* to $^{14}CO_2$ (Paszczynski et al. 1992; Spadaro et al. 1992).

More recently, it has been shown that not only *P. chrysosporium* but also several other fungi (mainly white rot fungi) (e.g. *Geotrichum candidum*, *Trametes versicolor*, *Bjerkandera adusta*, *Penicillium* sp., *Pleurotus ostreatus*, *Pycnoporus cinnabarinus*, and *Pyricularia oryzae*) are able to decolorize rather complex azo dyes, such as Direct Blue 1 (Chicago Sky Blue 6B) (Fig. 1F) or the reactive dye Reactive Black 5 (Fig. 1G). Recent comparisons of different fungi suggested that other fungi (e.g. *Trametes* or *Bjerkandera* species) are superior compared to *P. chrysosporium* for the decoloration of different dyes (Chivukula and Renganathan 1995; Heinfling et al. 1997; Kim et al. 1995; Knapp et al. 1995; Rodríguez et al. 1999; Schliephake et al. 2000; Shin and Kim 1998; Swamy and Ramsay 1999a; Zheng et al. 1999).

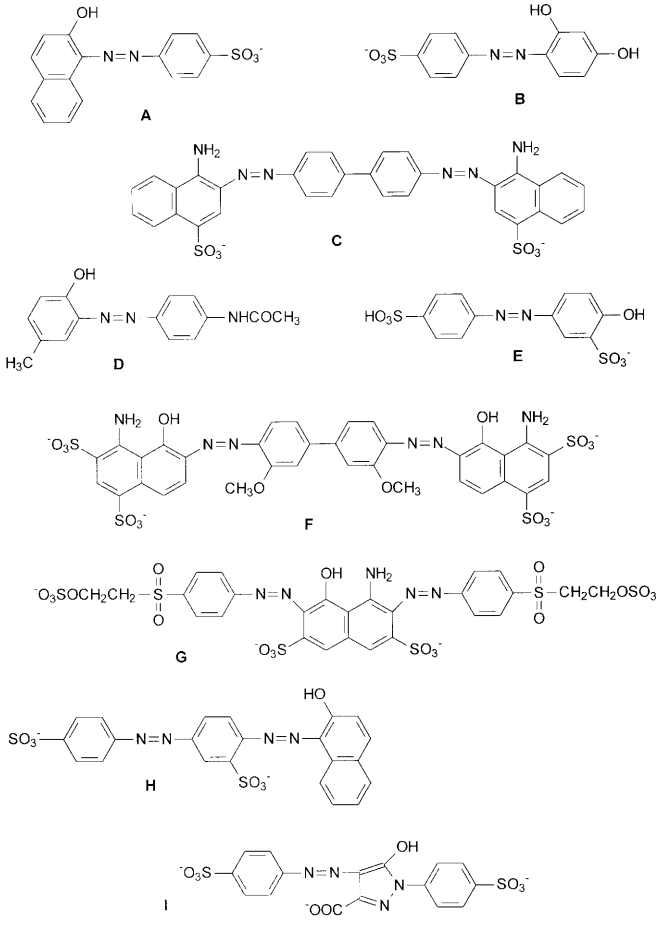

Fig. 1A–I Examples of azo compounds that are decolorized by (lignolytic) fungi. **A** Acid Orange 7 (Orange II); **B** Acid Orange 6 (Tropaeolin O); **C** Direct Red 28 (Congo Red); **D** Disperse Yellow 3; **E** Acid Yellow 9; **F** Direct Blue 1 (Chicago Sky Blue 6B); **G** Reactive Black 5; **H** Acid Red 66 (Biebrich Scarlet); I Acid Yellow 23 (Tartrazine) (Cripps et al. 1990; Heinfling et al. 1998; Paszczynski and Crawford 1992; Paszczynski et al. 1992; Schliephake et al. 2000; Spadaro et al. 1992)

Function of lignin and manganese peroxidases and laccases in the fungal degradation of azo dyes

The ability of *P. chrysosporium* and other fungi to degrade azo dyes is generally correlated with the ability of these organisms to synthesize lignin-degrading exoenzymes such as lignin- and manganese peroxidases or laccases (Chivukula and Renganathan 1995; Heinfling et al. 1998; Kim and Shoda 1999; Schliephake et al. 2000). Lignin and manganese peroxidases show a similar reaction mechanism and are oxidized during their catalytic cycle by H_2O_2 to an oxidized state which is reduced by the substrates (e.g. azo dyes) in two subsequent one-electron transfer steps to the native form of the enzyme. While lignin peroxidases are able to oxidize nonphenolic aromatic compounds, manganese peroxidases preferentially oxidize Mn^{2+} to Mn^{3+}, and the Mn^{3+} is responsible for the oxidation of many phenolic compounds. Laccases are copper-containing enzymes produced by a number of plants and fungi which oxidize phenols and anilines in the presence of oxygen (Barr and Aust 1994; Glenn et al. 1986; Thurston 1994).

It was shown for *P. chrysosporium* that lignin peroxidase and manganese peroxidase (in the presence of Mn^{2+}) were both able to decolorize azo dyes and that both enzymes showed differences in substrate specificity towards different azo dyes (Pasti-Grigsby et al 1992;

Paszczynski et al. 1991). The activity of the lignin peroxidase from *P. chrysosporium* with certain azo dyes [such as Acid Red 66 (Biebrich Scarlet), Fig. 1H, and Acid Yellow 23 (Tartrazine), Fig. 1I] was significantly enhanced by the addition of the mediator compound veratryl alcohol. Similar increases in the reaction rates have also been observed for the oxidation of other organic substrates by this enzyme (Bumpus 1995; Ollikka et al. 1993; Paszczynski and Crawford 1991).

It was originally assumed that manganese peroxidases and laccases would only convert a rather limited spectrum of azo dyes and preferentially convert dyes which carry a phenolic substituent in *para*-position to the azo bond and additional methyl- or methoxy-substituents in 2- or 2,6-position in relation to the hydroxy-group (Chivukula and Renganathan 1995; Pasti-Grigsby et al. 1992). More recently it was shown that certain manganese peroxidases (e.g. from *Bjerkandera adusta*) or laccases (e.g. from *Pycnoporus cinnabarinus*) are also able to decolorize complex industrially relevant azo dyes, such as Reactive Black 5 (Fig. 1G) or Direct Blue 1 (Fig. 1F) (Heinfling et al. 1998; Schliephake et al. 2000).

Elucidation of the degradative pathways utilized by white rot fungi for the decoloration of azo dyes

The oxidation of the non-sulfonated azo dye 1-(4′-acetamidophenylazo)-2-naphthol (a structural analogue of the industrially relevant azo dye Disperse Yellow 3) by the lignin peroxidase from *P. chrysosporium* resulted in the formation of 1,2-naphtoquinone and acetanilide (Fig. 2). This suggested that the oxidized form of the lignin peroxidase abstracted two electrons from the phenolic ring of the dye, which resulted in formation of the corresponding carbonium ion on the C-1 carbon of the naphthol ring. This carbonium ion can then be hydrated by a nucleophilic attack of water to an intermediate that breaks down to the naphthoquinone and an unstable phenyldiazene. It was suggested that this phenyldiazene could be oxidized by molecular oxygen to the corresponding radical, which finally splits off molecular nitrogen under formation of the phenyl radical, which is stabilized by the abstraction of a hydrogen radical from its surroundings (Spadaro and Renganathan 1994).

The enzymatic mechanism for the oxidation of sulfonated azo dyes by fungal peroxidases has been studied independently by two different groups who presented slightly different results. Goszczynski et al. (1994) incubated 3,5-dimethyl-4-hydroxyazobenzene-4′-sulfonic acid and 3-methoxy-4-hydroxyazobenzene-4′-sulfonamide with a crude peroxidase preparation from *P. chrysosporium* and analyzed the products formed using mass spectroscopy. From the identified products, they also suggested an initial oxidative activation of the dyes with the formation of a carbonium ion followed by a nucleophilic attack of water on this cationic species. From the metabolites observed, it was suggested that this unstable tetrahedral intermediate could either break down by a symmetric cleavage of the azo group (which produces a quinone imine and a nitroso compound) or an asymmetric cleavage (resulting in a quinone and a phenyldiazene). These direct oxidation products should finally undergo various spontaneous reactions that finally result in the formation of various secondary products.

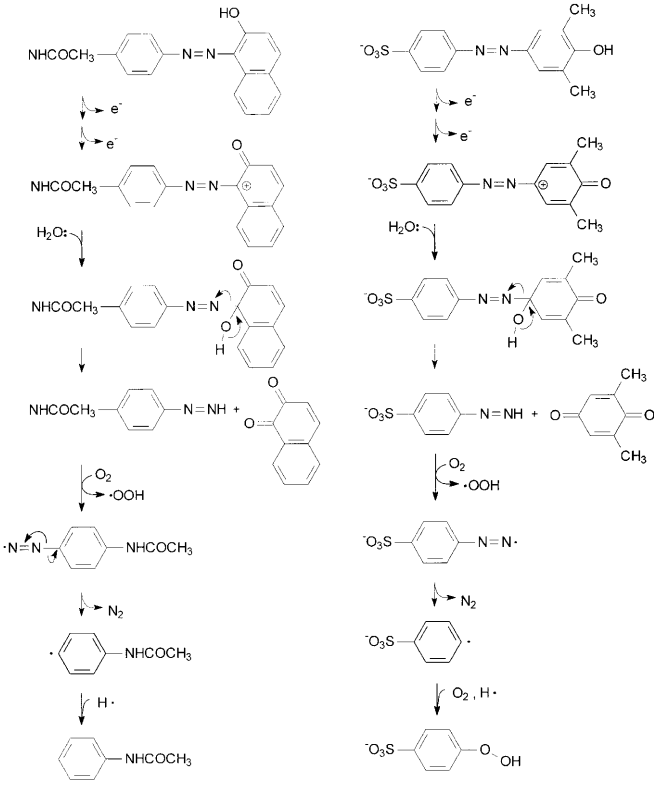

Fig. 2 Proposed reaction mechanisms for the oxidation of 1-(4′-acetamidophenylazo)-2-naphthol (*left*) and 3,5-dimethyl-4-hydroxyazobenzene-4′-sulfonate (*right*) by the lignin peroxidase from *Phanerochaete chrysosporium* (Chivukula et al. 1995; Goszczynski et al. 1994; Spadaro and Renganathan 1994)

In the second study, a purified lignin peroxidase preparation from *P. chrysosporium* was used for the oxidation of 3,5-dimethyl-4-hydroxyazobenzene-4′-sulfonic acid and Acid Orange 7 (Chivukula et al. 1995). In contrast to the previous study, a 4-sulfophenylhydroperoxide was found as major product formed from 3,5-dimethyl-4-hydroxyazobenzene-4′-sulfonic acid and Acid Orange 7. The second aromatic system of the azo dyes was also converted according to these authors to the corresponding quinones. It was suggested that the differences in the products formed from the non-sulfonated and the sulfonated azo dyes were due to differences in the reactivity between phenyl radicals and sulfophenyl radicals (Chivukula et al. 1995). The formation of the same products (2,6-dimethoxybenzoquinone and 4-sulfophenylhydroperoxide) was also described for the oxidation of 3,5-dimethyl-4-hydroxyazobenzene-4′-sulfonic acid by a laccase from *Pyricularia oryzae* (Chivukula and Renganathan 1995).

Degradation of azo dyes by bacterial peroxidases

In the course of investigating the degradation of azo dyes by lignolytic fungi, it was discovered that also some peroxidase-producing bacterial strains (mainly *Streptomyces* species, but also gram-negative bacteria such as *Sphingomonas chlorophenolicus*="*Flavobacterium* ATCC 39723") decolorize azo dyes (Cao et al. 1993; Paszczynski et al. 1992). The oxidation of azo dyes by *Streptomyces chromofuscus* A11 involved an extracellular peroxidase that showed a restricted substrate specificity similar to that of the manganese peroxidase from *P. chrysosporium* or horseradish peroxidase (Pasti-Grigsby et al. 1992, 1996). In contrast to the lignolytic fungi, the peroxidase-producing bacteria studied produced only insignificant amounts of $^{14}CO_2$ from industrially relevant ^{14}C-labeled azo dyes (Paszczynski et al. 1992).

Cometabolic reductive cleavage of azo dyes by aerobic bacteria

During the last years, several bacterial strains have been described that aerobically decolorize azo dyes by reductive mechanisms (for an overview of these the organisms, see Banat et al. 1996). Many of these isolates decolorize the azo compounds only in the presence of other carbon sources and therefore presumably do not use the azo dyes as carbon or energy sources. Thus a *Bacillus subtilis* strain was studied that reductively cleaved *p*-aminoazobenzene (Fig. 3A) to aniline (and presumably *p*-phenylendiamine) during aerobic growth on glucose (Zissi et al. 1997). Similarly, strains of *Pseudomonas stutzeri*, *Acetobacter liquefaciens*, and *Klebsiella pneumoniae* were able to reductively cleave 4′-dimethylaminoazobenzene-2-carboxylic acid [Acid Red 2 (Methyl Red), Fig. 3B] during aerobic growth on Nutrient Broth or glucose (Wong and Yuen 1996; Yatome et al. 1993). Furthermore, the reductive decolorization of sulfonated azo dyes (e.g. Acid Orange 7, Fig. 1A, Acid Orange 10, Acid Red 88, Acid Red 4, Acid Orange 8 Fig. 3C–F) by different bacterial strains (*Bacillus* sp., *Pseudomonas* sp., *Sphingomonas* sp., *Xanthomonas* sp.) under aerobic conditions in the presence of additional carbon sources has been reported (Coughlin et al. 1997, 1999; Dykes et al. 1994; Jiang and Bishop 1994; Sugiura et al. 1999).

In many reports on the "aerobic" metabolism of azo dyes, the bacterial strains (e.g. *Aeromonas* sp., *Bacillus subtilis*, *Proteus mirabilis*, *Pseudomonas pseudomallei* 13NA, *Pseudomonas luteola*) were grown aerobically with complex media or sugars and then incubated (often using high cell densities) without shaking in the presence of different azo dyes (Chang and Lin 2000; Chen et al. 1999; Hayase et al. 2000; Horitsu et al. 1977; Idaka et al. 1978; Ogawa et al. 1986; Yatome et al. 1981). These resting cell cultures presumably become rapidly oxygen-depleted, and the reactions observed should therefore be viewed as an anaerobic incubation of azo dyes (see below).

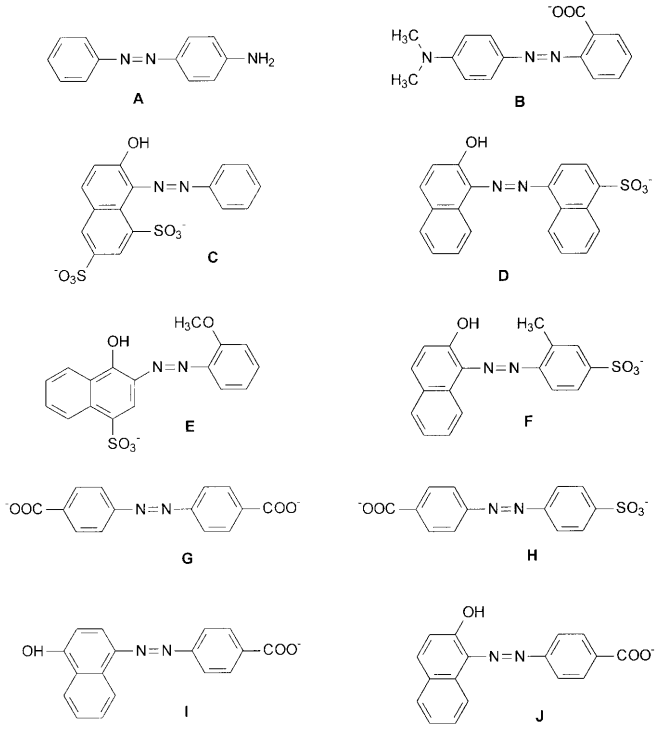

Fig. 3A–J Examples of azo compounds that are decolorized by aerobic bacteria. **A** *p*-Aminoazobenzene; **B** 4′-dimethylaminoazobenzene-2-carboxylic acid (Methyl Red); **C** Acid Orange 10; **D** Acid Red 88; **E** Acid Red 4; **F** Acid Orange 8; **G** 4,4′-dicarboxyazobenzene; **H** 4-carboxy-4′-sulfoazobenzene; **I** 1-(4′-carboxyphenylazo)-4-naphthol ("carboxy-Orange I"), **J** 1-(4′-carboxyphenylazo)-2-naphthol ("carboxy-Orange II") (Blümel et al. 1998; Coughlin et al. 1999; Kulla 1981; Overney, 1979; Yatome et al. 1993; Zissi et al. 1997)

Aerobic growth of bacteria with azo dyes as sole source of carbon and energy

There are several claims in the literature that bacteria with the ability to reduce azo dyes aerobically in a cometabolic fashion can also use these dyes as sole source of carbon and energy (e.g. Dykes et al. 1994; Yatome et al. 1993); however, there are very few studies that unequivocally demonstrate the utilization of azo compounds as sole source of carbon and energy under aerobic conditions. The ability of bacteria to grow with simple carboxylated azo compounds as sole source of carbon and energy was first shown by Overney (1979), who isolated a "*Flavobacterium*" that was able to grow aerobically with the simple model compound 4,4′-dicarboxyazobenzene (Fig. 3G). In a later study it was demonstrated that after enrichments with 4,4′-dicarboxyazobenzene a wide range of bacterial strains could be readily isolated from different inocula. These strains were classified according to the Biolog test system and found to belong to different genera, such as *Sphingomonas*, *Comamonas*, *Pseudomonas*, *Xanthomonas*, or *Alcaligenes* (Hausser 1995).

In a now almost classical study on the potential of bacteria to acquire novel metabolic traits, Kulla, Leising-

er and coworkers demonstrated that a mixed bacterial culture which degraded 4,4′-dicarboxyazobenzene could be adapted to the degradation of more complex azo compounds such as 1-(4′-carboxyphenylazo)-4-naphthol ("carboxy-Orange I") (Fig. 3I) or 1-(4′-carboxyphenylazo)-2-naphthol ("carboxy-Orange II") (Fig. 3J). From these adaptation processes in continuous cultures, strain "*Pseudomonas*" K22 was obtained after cultivation with "carboxy Orange I" and strain KF46 from an enrichment with "carboxy Orange II" (Kulla 1981; Kulla et al. 1984). A recent taxonomic study, which was performed with two direct descendants of these strains, which are currently still available (strain K24 and strain KF46F), demonstrated that both strains belong to two new genera in different families within the β-subgroup of the Proteobacteria. Thus strain K24 was described as a member of the *Alcaligenaceae* (*Pigmentiphaga kullae*) and strain KF46F as a member of the *Comamonadaceae* (*Xenophilus azovorans*) (Blümel et al. 2001a, b).

The subsequent attempts of Kulla and coworkers to adapt the "carboxy-Orange"-degrading bacterial strains K22 and KF46 to grow with the structurally analogous sulfonated dyes Acid Orange 20 (Orange I) and Acid Orange 7 (Orange II) were not successful, and it was suggested that the intermediate formation of 4-aminobenzenesulfonate (sulfanilate) somehow interfered with the central metabolism of the bacteria (Kulla et al. 1983). Therefore it was later attempted to adapt the sulfanilate-degrading strain *Hydrogenophaga palleronii* strain S1 (recently reclassified as *H. intermedia*, Contzen et al. 2000) to grow with the sulfonated azo compound 4-carboxy-4′-sulfoazobenzene (Fig. 3H) as sole source of carbon and energy. This resulted finally in the isolation of a mutant strain of strain S1 (called strain S5) that grew with the simple sulfonated azo dye as sole source of carbon and energy. Strain S5 metabolized 4-carboxy-4′-sulfoazobenzene reductively to 4-aminobenzoate and sulfanilate, which were mineralized by previously established degradative pathways (Blümel et al. 1998; Feigel and Knackmuss 1993). Recently, evidence has been presented that *Sphingomonas* 1CX, which cometabolically decolorized several sulfonated azo dyes (see above), also grew with (low concentrations) of Acid Orange 7 (Coughlin et al. 1999).

The aerobic azoreductases

During the aerobic, "semi-aerobic" (in static culture) or anaerobic incubation of bacteria with azo compounds, amines were often detected that originated from a reductive cleavage of the azo bond. The aerobic reductive metabolism of azo dyes requires specific enzymes ("aerobic azoreductases") that catalyze these reactions in the presence of molecular oxygen. The aerobic azoreductases from the "carboxy-Orange"-degrading strains K22 and KF46 were purified, characterized and compared with each other (Zimmermann et al. 1982, 1984). Both azoreductases were monomeric flavin-free enzymes that preferentially used NADPH (and only with significantly higher K_m values NADH) as cofactors and reductively cleaved not only the carboxylated growth substrates of the bacteria but also the sulfonated structural analogues. Both enzymes significantly differed in size (21 kDa vs 30 kDa) and substrate specificity. The azoreductase from strain KF46 (Orange II azoreductase) strictly required the presence of a hydroxy-group in *ortho*-position to the azo bond. In contrast, the Orange I azoreductase from strain K22 required a hydroxy-group in *para*-position to the azo bond for catalytic activity. Surprisingly, neither of the purified enzymes exhibited immunological cross-reaction with each other, which suggests that the two enzymes are evolutionary significantly different (Zimmermann et al. 1982, 1984).

More recently, the purification and characterization of enzymes from *Shigella dysenteriae* and *Escherichia coli* with flavin-dependent aerobic azoreductase activities has been reported (Ghosh et al. 1992, 1993). Unfortunately, the authors used assay conditions that did not allow a clear distinction between a true aerobic azoreductase (the existence of which would be very surprising in these well-characterized bacterial species, given the long search by different groups for aerobic azoreductases) and a reaction caused by the intermediate formation of reduced flavins by flavin reductase activities, which then could unspecifically reduce the azo dyes (see below) (Russ et al. 2000).

Anaerobic reduction of azo dyes by bacteria

In contrast to the few reports of aerobic decolorization of azo dyes, a wide range of organisms are able to reduce azo compounds under anaerobic conditions. This has been shown for purely anaerobic (e.g. *Bacteroides* sp., *Eubacterium* sp; *Clostridium* sp.), facultatively anaerobic (e.g. *Proteus vulgaris*, *Streptococcus faecalis*), and aerobic (e.g. *Bacillus* sp., *Sphingomonas* sp.) bacteria, yeasts, and even tissues from higher organisms (Adamson et al. 1965; Bragger et al. 1997; Dieckhues 1960; Dubin and Wright 1975; Mecke and Schmähl 1957; Rafii et al. 1990; Scheline et al. 1970; Walker 1970; Wuhrmann et al. 1980). 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 unspecificity of this reaction is also demonstrated by the many reports of decolorization of azo dyes by anaerobically incubated sewage sludge (e.g. Bromley-Challenor et al. 2000; Brown and Laboureur; 1983a; Carliell et al. 1994; Delée et al. 1998; Ganesh et al. 1994). It appears that almost every azo compound that has been tested is biologically reduced under anaerobic conditions, although there are some indications that metal-ion-containing dyes sometimes have reduced decolorization rates (for a survey of compounds tested see Brown and DeVito 1993; Chung et al. 1978, 1992; Delee et al. 1998; Diekhues 1960).

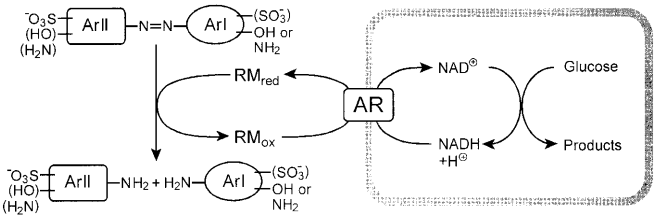

Fig. 4 Proposed mechanism for the redox-mediator-dependent reduction of azo dyes by *Sphingomonas xenophaga* BN6. *AR* Azoreductase, *RM* redox mediator (Keck et al. 1997)

Mechanisms for the unspecific reduction of azo dyes under anaerobic conditions

The physiology of the possible reactions that result in a reductive cleavage of azo compounds under anaerobic conditions differs significantly from the situation in the presence of oxygen, because several redox active compounds (e.g. reduced flavins or hydroquinones) rapidly react either with oxygen or with azo dyes. Therefore, under aerobic conditions oxygen and the azo compounds compete for the reduced electron carriers. The spontaneous reactions of the reduced forms of these electron carriers (or mediator compounds) with the azo dyes allows for very unspecific reduction processes, which are mainly governed by the redox potentials of the redox mediators and the azo compounds. In earlier studies with facultatively anaerobic bacteria, it was repeatedly suggested that reduced flavins generated by cytosolic flavin-dependent reductases (flavin reductases) were responsible for the unspecific reduction of azo dyes (Roxon et al. 1967; Walker 1970). The ability of cytosolic flavin reductases to act in vitro as azoreductases was recently demonstrated by experiments using a recombinant flavin reductase in different genetic backgrounds (Russ et al. 2000). Also, the addition of other putative redox mediators (e.g. benzyl viologen or quinones) to cultures of strictly anaerobic bacteria significantly increased the reduction rate of azo dyes (Bragger et al. 1997; Brown 1981; Chung et al. 1978). Cell extracts show generally much higher rates for the anaerobic reduction of azo dyes than do preparations of resting cells (Mechsner and Wuhrmann 1982; Walker 1970; Wuhrmann et al. 1980). This has generally been explained by the low permeability of the cell membranes for the highly polar sulfonated azo compounds. Therefore, it appears reasonable that, in vivo, intracellular enzymes like flavin reductases are of little importance for the reduction of sulfonated azo compounds (Russ et al. 2000).

A different model for the unspecific reduction of azo dyes by bacteria which does not require transport of the azo dyes or reduced flavins through the cell membranes was recently suggested for *Sphingomonas xenophaga* BN6. The anaerobic reduction of azo compounds by this strain was significantly increased after the addition of different quinones, such as anthraquinone-2-sulfonate or 2-hydroxy-1,4-naphthoquinone. It was suggested that in this system the quinones acted as redox mediators which were enzymatically reduced by the cells of *S. xenophaga* BN6 and that the hydroquinones formed reduced the azo dyes in the culture supernatants in a purely chemical redox reaction (Fig. 4). Cell fractioning experiments demonstrated that the quinone reductase activity was located in the cell membranes of *S. xenophaga* BN6 and that therefore no transport of the sulfonated azo compounds or of the hydroquinone/quinone redox mediator via the cell membrane was necessary (Kudlich et al. 1997). Furthermore, it was demonstrated that (probably quinoide) redox mediators, active in the reduction of azo dyes, were also formed by *S. xenophaga* BN6 during growth with naphthalenesulfonates (Keck et al. 1997).

The involvement of membrane-bound enzyme systems (e.g. NAD(P)H-cytochrome *c* reductase or the cytochrome P_{450} system) in the anaerobic reduction of azo dyes has also been described for mammalian cells (Brown and deVito 1993; Hernandez et al. 1967a, 1967b; Zbaida 1995).

Yet another model for the reduction of sulfonated azo compounds, one which also does not require membrane transport of the dyes, has been suggested for certain strictly anaerobic bacterial strains from the intestine. Rafii and coworkers isolated different bacteria from the human intestine (e.g. *Eubacterium* sp., *Clostridium* sp., *Butyrvibrio* sp., *or Bacteroides* sp.) that decolorized sulfonated azo dyes during growth on solid or liquid complex media. It was shown that at least part of the azoreductase activities were extracellular, because the culture supernatants were able to decolorize the dyes under anaerobic conditions (Rafii et al. 1990, 1995). The azoreductase activity from *Clostridium perfringens* was described as being independent of added flavins; furthermore, the enzyme was rapidly and irreversibly inactivated by oxygen (Rafii et al. 1990). It is still unclear in this system how the supposed extracellular azoreductases gain the NADH necessary for the reduction of the azo dyes in their extracellular environment and if there are some effects that are caused by the complex growth media of the cells.

Another possibility for the extracellular reduction of azo compounds by microorganisms is the action of reduced inorganic compounds (e.g. Fe^{2+}, H_2S) that are formed as end-products of certain strictly anaerobic bacterial metabolic reactions on the azo bond. Thus it has been recently shown that the formation of H_2S by sulfate-reducing bacteria resulted in the reduction of the azo dye Reactive Orange 96 (Libra et al. 1997; Yoo et al. 1999). In the environment, presumably also "bulk reductants" such as Fe^{2+} or H_2S will show significantly increased reaction rates in the presence of mediator compounds (Schwarzenbach et al. 1990; Perlinger et al. 1996).

In summary, it appears that under anaerobic conditions in the environment or in sewage treatment systems, specific azoreductases (if they exist at all) are probably only of limited importance for the reduction of azo dyes. This is in sharp contrast to the requirement for true azo-

reductases under aerobic conditions and readily explains the ubiquitous spread of the ability of microorganisms to reduce azo compounds under anaerobic conditions.

Possible applications of microorganisms for the treatment of dye-containing waste waters

It is generally observed that in conventional aerobic sewage-treatment plants most azo dyes are not degraded by the bacteria, but that a certain percentage (usually about 40–80%) of the dyes physically adsorb to the sewage sludge (Clarke and Anliker 1980; Dohányos et al. 1978; Hitz et al. 1978; Shaul et al. 1991; Pagga and Brown 1986; Pagga and Taeger 1994; Shaul et al. 1991). This correlates well with the observed difficulties when the isolation of bacteria with "aerobic azoreductase" activity is attempted (see above). Therefore, conventional aerobic sewage-treatment systems are not useful for the decolorization of effluents containing azo dyes and various advanced chemo-physical techniques are necessary for the treatment of textile wastewater (Schönberger 1997).

In the textile processing industry, a wide range of structurally diverse dyes is used within short time periods in one and the same factory, and therefore effluents from the textile industry are extremely variable in composition (Correia et al. 1995). This underlines the need for a largely unspecific process for the treatment of textile wastewater. From the currently known biological systems, the required unspecifity may be obtained by using either the lignin peroxidases from lignolytic fungi or the unspecific reduction processes catalyzed by various bacteria under anaerobic conditions.

Although Zhang et al. (1999) recently demonstrated that a white rot fungus was able to stably decolorize Acid Orange 7 (Orange II) in a bioreactor for 2 months, it appears that currently there are severe problems which interfere with the utilization of lignolytic fungi for the treatment of dye-containing wastewaters:

- Wastewater treatment plants are not the natural habitat of lignolytic fungi and therefore special care has to be taken to establish these fungi in a wastewater treatment system.
- The lignolytic enzymes of the white rot fungi are thought to be expressed in most cases only during secondary metabolism following growth when carbon and/or nitrogen sources become limiting. Neither lignin nor any of the pollutants degraded by the enzymes has been shown to be utilized as a carbon or energy source, and a separate carbon source is required for the cultivation of the organisms (Swamy and Ramsay 1999b).
- The observed degradation rates are usually rather low. In typical experiments about 50–150 mg of the respective dyes/l are decolorized within 5–10 days (Chao and Lee 1994; Pasti-Grigsby et al. 1992; Paszczynski et al. 1992; Hardin et al. 2000; Swamy and Ramsay 1999a, b).
- Lignin peroxidases are very unspecific for the oxidation of aromatic and xenobiotic compounds. Therefore, in the presence of complex substrate mixtures such as those observed in industrial sewage-treatment systems, also other substrates will be oxidized by lignin peroxidases.
- Lignin peroxidases exhibit a pH-optimum at pH 4.5–5. Therefore a rather acid pH of the wastewater treatment system is required, which may inhibit the growth of several other useful microorganisms (Swamy and Ramsay 1999a).

Based on our current knowledge, anaerobic reduction of the azo bond by bacteria seems to be better suited for the decolorization of azo dyes in sewage treatment systems. The putative advantages of this method are:

- The depletion of oxygen is easily accomplished in static cultures and enables anaerobic, facultatively anaerobic, and aerobic bacteria to reduce the azo dyes.
- The reactions take place at neutral pH values and are expected to be extremely unspecific when low-molecular redox mediators are involved.
- The reduction rates generally increase in the presence of other carbon sources. The reduction equivalents that are formed during anaerobic oxidation of these carbon sources are finally used for the reduction of the azo bond.

The main restriction to the anaerobic treatment of azo compounds is that the amines that are formed are usually not further metabolized under anaerobic conditions (Brown and Hamburger 1987) and there is only one example demonstrating the growth of a (methanogenic) anaerobic consortium on a model azo compound (azodisalicylate) (Razo-Flores et al. 1997). The accumulation of these reduction products from the azo dyes is especially relevant if the amines are presumed carcinogens (e.g. naphthylamine or benzidine derivatives). This problem is of serious concern for human health, because the relevant amines are also formed in the body in the anaerobic compartment of the lower intestine after ingestion of these dyes and may be even formed by skin bacteria (Brown and DeVito 1993; Chung et al. 1992; Platzek et al. 1999). Therefore the relevant dyes have been banned from the market in some countries (e.g. Germany) and the problem may be solved by regulatory efforts (Reife and Freeman 2000).

Anaerobic/aerobic treatment of azo dyes

Since certain aromatic amines and also sulfonated aminoaromatics are aerobically degraded by bacteria (Brown and Laboureur 1983b; Feigel and Knackmuss 1993; Locher et al. 1989; Nörtemann et al. 1986, 1994; Ohe and Watanabe 1986; Thurnheer et al. 1986, 1988), it has been repeatedly suggested to combine the anaerobic cleavage of the azo dyes with an aerobic treatment

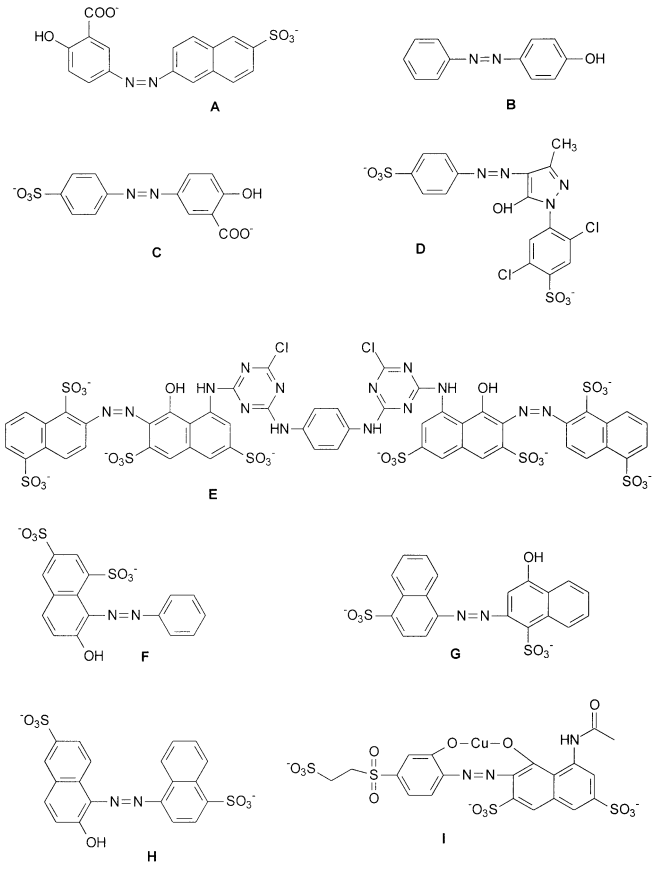

Fig. 5A–I Examples of azo compounds that have been studied in anaerobic/aerobic treatment systems. **A** Mordant Yellow 3; **B** 4-phenylazophenol; **C** Mordant Yellow 10; **D** Acid Yellow 17; **E** Reactive Red 141 (Procion Red H-E7B); **F** Acid Orange 10; **G** Acid Red 14; **H** Acid Red 18; **I** Reactive Violet 5 (An et al. 1996; Fitzgerald and Bishop 1995; Glässer et al. 1992; Haug et al. 1991; O'Neill et al. 2000a, b; Sosath and Libra 1997; Tan et al. 1999)

system for the amines formed. The feasibility of this strategy was first demonstrated for the sulfonated azo dye Mordant Yellow 3 (Fig. 5A) (Glässer et al. 1992; Haug et al. 1991). The anaerobic/aerobic treatment can be carried out either sequentially or simultaneously. Sequential processes may combine the anaerobic and the aerobic step either alternately in the same reaction vessel or in a continuous system in separate vessels (Glässer et al. 1992). The simultaneous treatment systems utilize anaerobic zones within basically aerobic bulk phases, such as observed in biofilms, granular sludge or biomass immobilized in other matrices (Field et al. 1995; Jiang and Bishop 1994; Kudlich et al. 1996; Tan et al. 1999; Zhang et al. 1995). In the sequential and simultaneous treatment systems, auxiliary substrates are required, which supply the bacteria in the anaerobic zones with a source of carbon and energy and a source of reduction equivalents for the cleavage of the azo bond.

Although at least certain azo dyes can be mineralized by anaerobic/aerobic treatment systems, also this strategy has serious drawbacks. The fact that many of the amines which are formed during the anaerobic reduction of the azo dyes (which are very often *ortho*-aminohydroxynaphthalenes) are rather unstable under aerobic conditions and undergo auto-oxidation reactions is a serious problem if a true mineralization of the azo dyes is the aim of the treatment. A recent analysis of these auto-oxidation reactions suggested that the fate of differently substituted *ortho*-aminohydroxynaphthalenes varies and that depending on the degree of sulfonation either dimers, disulfonated cinnamic acid derivatives or naphthoquinone imines are formed as major reaction products (Kudlich et al. 1999). It will require further work in order to analyze if a biological degradation of the aminohydroxynaphthalenes can compete with these auto-oxidation reactions and whether the products of the auto-oxidation reactions are accessible for a biological mineralization.

During the last few years, different reactor designs have been proposed in order to obtain an effective continuous anaerobic/aerobic treatment of azo dyes: an anaerobic and an aerobic rotating biological contactor (Zaoyan et al. 1992), an anaerobic fixed-film fluidized bed reactor followed by an aerobic suspended-bed activated sludge reactor (Fitzgerald and Bishop 1995; Seshadri et al. 1994), a combination of anaerobic and aerobic rotating-drum reactors (Harmer and Bishop 1992; Sosath and Libra 1997), and an anaerobic up-flow fixed bed column together with an aerobic agitated tank (An et al. 1996; O'Neill et al. 2000a, b; Rajaguru et al. 2000). It is very difficult to compare the efficiencies of these treatment systems because of differences in the dyes and conditions used, the presence of auxiliary carbon sources, and the difficulties in the analysis of the biological or spontaneous reactions of the (auto-oxidizable) amines formed during the anaerobic reactions (for an overview of the azo dyes that have been analyzed in anaerobic/aerobic treatment systems, see Fig. 5). In general, it may be concluded that, in continuous anaerobic/aerobic systems which are fed with substrate mixtures with a high biological and chemical oxygen demand (BOD, COD) and low dye concentrations to the anaerobic stage, a complete decolorization of the dyes and a significant BOD and COD removal can be achieved in the anaerobic stage. In the subsequent aerobic step, the remaining BOD from the auxiliary substrates may be mineralized. There are several examples demonstrating COD removal in the anaerobic/aerobic processes of 70–95% [e.g. for the treatment of Reactive Red 141 (Procion Red H-E7B) (Fig. 5 E) in a simulated textile effluent containing modified starch, O'Neill et al. 2000a, b]. Similar results have also been described for the treatment of wastewater from a dyeing factory on a laboratory scale (Zaoyan et al. 1992). Because the concentrations of the azo dyes are generally much lower than those of the auxiliary substrates, the fate of the aromatic amines formed (especially if they are auto-oxidizable) in the aerobic treatment process is still unclear and some contradicting results have been published. For the treatment of the copper-containing dye Reactive Violet 5

(Fig. 5I) in an anaerob/aerobic system with three rotating-disc reactors, no indications for a mineralization of the amines in the aerobic stage were detected by Sosath and Libra (1997). In contrast, the analysis of the fate of nitrogen-containing compounds (presumed amines) in the aerobic step of Reactive Red 141 (Fig. 5E) treatment suggested a decrease in the concentration of nitrogen-containing metabolites (O'Neill et al. 2000a). It is clear that the fate of the reduction products of the azo dyes will vary significantly depending on their tendency to be subject to auto-oxidation processes and/or biodegradation. Preliminary results suggested that the aerobic incubation of certain *ortho*-aminohydroxynaphthalenes with activated sludge resulted in a reduced number of products formed from the *ortho*-aminohydroxynaphthalenes compared to the abiotic auto-oxidation processes, and indications for a biological conversion of some products have been found (Kudlich et al. 1999).

Thus, encouraging results have been obtained in laboratory experiments, which demonstrated that the anaerobic disintegration of azo dyes results in products that are significantly more available for subsequent aerobic processes. This resulted recently in the decision to build, for the first time, a full-scale anaerobic/aerobic treatment plant for the treatment of wastewater from the textile processing industry. The plant is scheduled to treat more than 1,000 m^3 of dye-containing wastewater per day (Krull et al. 2000).

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