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REVIEW ARTICLE

Azo dye decolorization by halophilic and halotolerant microorganisms

Mohammad Ali Amoozegar · Mahbod Hajighasemi · Javad Hamedi · Sedigheh Asad · Antonio Ventosa

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Abstract Different types of microorganisms are capable of degrading azo dyes due to their high metabolic potentials. However, many of them cannot be used as degrading agents due to the harsh conditions of dye-polluted environments. Here, halophilic and halotolerant microorganisms can be the best candidates for a practical biodecolorization process as they are able to grow easily at high concentrations of salts. In addition, some of them can tolerate the presence of other stress factors such as toxic oxyanions and heavy metals which are so common in industrial wastewaters. In recent years, several studies have been focused on halophilic and halotolerant microorganisms and their abilities for decolorization of azo dyes. For example, *Shewanella putrefaciens* was determined to be capable of the complete removal of Reactive Black-5, Direct Red-81, Acid Red-88 and Disperse Orange-3 (all 100 mg l^{-1}) within 8 h in the presence of 40 g l^{-1} NaCl. Another halophilic example is *Halomonas* sp. GTW which has shown a remarkable performance in the removal of different azo dyes within 24 h in the presence of 150 g l^{-1} NaCl. Although these approaches need to be studied in more detail, some studies have designed different types of fermentation processes and even specific fermentors to provide a practical methodology for industrial wastewater remediation. Sequential anaerobic EGSB (expanded granular sludge blanket) and aerobic reactor was the result of an important attempt to design an effective approach to large-scale biodecolorization.

Keywords Azo dyes · Halophile · Halotolerant · Decolorization · Biodegradation

M. A. Amoozegar (✉) · M. Hajighasemi · J. Hamedi · S. Asad
Department of Microbiology, Faculty of Biology,
College of Science, University of Tehran,
P. O. Box 14155-6455, Tehran, Iran
e-mail: amozegar@khayam.ut.ac.ir

A. Ventosa
Department of Microbiology and Parasitology,
Faculty of Pharmacy, University of Sevilla,
Sevilla, Spain

Introduction

Over 100,000 commercially available dyes exist and more than 7×10^5 metric tons of dyestuff are produced annually worldwide (Zollinger 1987). In the textile and paper coloration industries, synthetic dyes from residual dye baths are released into waste streams. Dye concentrations that are used for processing vats are typically around 1,000 mg l^{-1} (Ince and Tezcanli 1999). Depending on the class of the dyes, their release into wastewaters can range from 2% of the original concentration for basic dyes to as high as 50% for reactive dyes (O'Neill et al. 1999; Tan et al. 2000; Boer et al. 2004; Khalid et al. 2008a), and these dye residues pose a large contaminant load that must be reduced for safe discharge to the environment (Khalid et al. 2008b).

Azo dyes constitute the largest and most versatile class of dyes, and more than half of the annually produced amounts of dyes (estimated for 1994 worldwide as 1 million tons) are azo dyes. More than 2,000 different azo dyes are currently used to dye various materials such as textiles, leather, plastics, cosmetics, and food. The largest amounts of azo dyes are used for dyeing of textiles, and it has 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).

Since the presence of dyes in water is highly visible and affects its transparency and aesthetics even in low concentrations, the azo dye-containing effluents have caused serious environmental pollution (Supaka et al. 2004). The color impedes light penetration and the dyes and/or their degradation derivatives can prove toxic to aquatic life (Junkins 1982; Kertell and Hill 1982; Grau 1991; Meyer 1981; Larson and Weber 1994; Georgiou et al. 2004). Therefore, industrial effluents containing dyes must be treated before their discharge into the environment (Hao et al. 2000; Supaka et al. 2004).

There is only a single example of the presence of an azo group in a natural product (4,4′-dihydroxyazobenzene) (Gill and Strauch 1984); the industrially produced azo dyes are therefore all xenobiotic compounds. Thus, it is not surprising that azo dyes usually resist biodegradation in conventional aerobic sewage-treatment plants (Pagga and Brown 1986; Shaul et al. 1991). The azo dyes recalcitrance to biological degrading 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; Namasivayam and Yamuna 1992; Ràfols and Barceló 1997; Riu et al. 1998; Tincher and Robertson 1982).

Several physical and chemical methods including adsorption, coagulation, precipitation, filtration, and oxidation have been used for the treatment of azo dye-contaminated effluents. These methods, however, may generate a significant amount of sludge or may easily cause secondary pollution due to excessive chemical usage. Moreover, their municipal treatment costs are high. Therefore, it may be economical to develop alternative means of dye decolorization, such as bioremediation due to its reputation as an environmentally friendly and publicly acceptable treatment technology (Hao et al. 2000; Supaka et al. 2004).

Possible applications of microorganisms for the treatment of dye-containing wastewaters

Many microorganisms belonging to the different taxonomic groups are capable of decolorizing azo dyes, including Gram-positive and Gram-negative bacteria (Sani and Banerjee 1999; Kodam et al. 2005; Moosvi et al. 2005; Kalyani et al. 2009; Wang et al. 2009) as well as fungi (Balan and Monteneiro 2001; Verma and Madamwar 2005; Taskin and Erdal 2010).

In the textile processing industry, a wide range of structurally diverse dyes are used simultaneously within short periods of time, and therefore effluents with an extremely variable composition are produced (Correia et al. 1994). This underlines the need for a largely unspecific process for the treatment of textile wastewater. Among the currently known biological systems, the required unspecificity may be obtained by using either the lignin peroxidases from lignolytic fungi or the unspecific reductase enzymes produced by various bacteria under anaerobic conditions. The degree of decolorization greatly depends on the type, molecular weight, and substitution groups of the dye. Azo compounds with an amino or hydroxyl group are more likely to be degraded than those with methyl, methoxy, sulfo or nitro groups (Nigam et al. 1996; Zimmermann et al. 1982; Supaka et al. 2004).

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:

1) The depletion of oxygen is easily accomplished in static cultures, which enables anaerobic, facultative anaerobic and aerobic bacteria to reduce the azo dyes.
2) The reactions take place at neutral pH values and are expected to be extremely unspecific when low-molecular redox mediators are involved.
3) 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 used finally for the reduction of the azo bond.

However, the main restriction on anaerobic treatment of azo compounds is that the amines that are formed are usually not further metabolized under anaerobic conditions (Brown and Hamburger 1987). There is only one example demonstrating the growth of an (methanogenic) anaerobic consortium on a model azo compound (azodisalicylate) (Razo-Flores et al. 1997). The accumulation of azo dyes reduction products 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).

Decolorization of azo dyes by halophilic and halotolerant bacteria

In recent years, research on halophilic microorganisms has become a hot subject in both basic research and biotechnology applications. Halophilic and halotolerant microorganisms are able to grow in saline and hypersaline environments such as hypersaline lakes and brines, saline and hypersaline soils, cold saline environments, alkaline hypersaline lakes (soda lakes) and salted fermented foods. The salinity of these habitats can vary from concentrations above 35 g l^{-1} total dissolved salts to concentrations near saturation. A great diversity of halophilic and halotolerant microorganisms from all three domains of life have been found in these environments (Oren 2002).

The potential for growth in different salt concentrations is a variable characteristic of microorganisms which greatly depends on their physiological properties. These properties have been used to classify different microorganisms. Non-halophilic microorganisms are defined as organisms growing optimally below 0.2 M NaCl, while halophilic microorganisms need concentrations of more than 0.2 M NaCl to reach their optimum growth. In addition, bacteria able to grow in the absence of salt as well as in the presence of relatively high salt concentrations are designated as halotolerant (DasSarma and Arora 2002).

Studies of such bacteria are of great importance, since they may produce compounds of industrial interest. These compounds have several applications of great potential in various fields of biotechnology, including polyhydroxyalcanoates, biosurfactants, exopolysaccharides, hydrolytic enzymes, compatible solutes and pharmaceutical compounds. Moreover, there are several important processes that can be performed through the usage of halophilic and halotolerant microorganisms such as the production of biofuels from renewable resources, enhanced oil recovery, cancer diagnosis, drug screening, and biodegradation and bioremediation of toxic organic and inorganic compounds (Margesin and Schinner 2001; Ventosa and Nieto 1995).

Many species and strains of bacteria have been isolated that can degrade azo dyes. Nonetheless, a limiting factor in the development of biotreatment methods has been the sensitivity of many azo dye-degrading bacteria to the high concentrations of salts that are also released into the wastewater (Carliell et al. 1994; Manu and Chauhari 2003). Salt concentrations up to 150–200 g l^{-1} have been measured in wastewaters from dye-stuff industries (U.S. Environmental Protection Agency). Sodium levels are also elevated when sodium hydroxide is used in the dye bath to increase the pH, which can be as high as pH 10 in the dye bath.

Generally, sodium concentrations above 3 g l^{-1} can cause moderate inhibition of most bacterial activities (De Baere et al. 1984; Guo et al. 2008a, b). Hypersaline wastewater usually causes plasmolysis and/or loss of activity of cells; therefore, some traditional aerobic- and anaerobic-biological treatments result in low BOD (biological oxygen demand) removal performance. In addition, the activated sludge will require a pretreatment of the effluent wastewater to dilute high salt concentrations before the biological treatment; thereby generating even larger volumes of wastewater. At the same time, many microorganisms can thrive in saline environments. The identification and characterization of halotolerant and halophilic bacteria that can degrade azo dyes may facilitate the development of biological treatment methods for treatment of saline azo dye solutions using bioreactors (Khalid et al. 2008b). Since externally added bacteria may have some deleterious effects on the ecosystem, applying or activating the indigenous microflora is preferred if possible (Margesin and Schinner 2001).

As mentioned above, isolation of microorganisms which are capable of azo dye decolorization under saline conditions is of great importance. There are several reports on azo dye degrading non-halophilic bacteria; however, the ability of halophilic organisms to eliminate industrial dyes from wastewaters has been studied just recently. Some halophilic or halotolerant bacteria which have been reported to degrade different azo dyes with high efficiency are outlined in Table 1.

The following includes a brief list of halophilic and halotolerant bacteria shown in Table 1 which have been recently reported to be capable of azo dye decolorization.

Salah Uddin et al. (2007) reported the decolorization of azo dye Acid Red B by growing and resting cells, as well as by extracted azo reductase of *Gracilibacillus* sp. GTY. Cells grown in media containing 150 g l^{-1} of NaCl showed the best performance in decolorization. Decolorization was observed by the UV–visible absorbance spectra. The maximum absorption peak in the visible area decreased to a minimum level after 96 h of incubation. The strain did not show a good performance in decolorization when grown in media containing very low or high concentrations of salt. This phenomenon suggests that the production of azo reductase can be highly influenced by the salt content of medium.

Ozdemir et al. (2008) isolated a halotolerant strain, designated TEMS1, from coastal seawaters of Izmir bay, Turkey. They studied the ability of the strain to decolorize five different azo dyes under saline conditions. The strain TEMS1 which was closely related to the bioluminescent bacterium *Vibrio harveyi*, was able to decolorize the dyes by adsorption of dye on its cell surface with a relatively high yield. Features like salt tolerance, high growth rate and resistance to different environmental conditions make this strain a potential organism for bioremediation of azo dye-polluted wastewaters.

Table 1 Recent reports on various halotolerant and halophilic microorganisms capable of azo-dye decolourization

Culture	Dye (concentration)	Percent (removal time)	Medium NaCl Content	Reference
Shewanella putrefaciens strain AS96	Reactive Black-5 (100 mg l^{-1})	100% (6 h)[a]	40 g l^{-1}	Khalid et al. (2008a, b);
	Direct Red-81(100 mg l^{-1})	100% (8 h)[a]	40 g l^{-1}	
	Acid Red-88(100 mg l^{-1})	100% (8 h)[a]	40 g l^{-1}	
	Disperse Orange-3(100 mg l^{-1})	100% (8 h)[a]	40 g l^{-1}	
Halomonas sp. GTW	Reactive Brilliant Red K-2BP (100 mg l^{-1})	100% (24 h)	150 g l^{-1}	Guo et al. (2008a, b);
	Acid Red G (50 mg l^{-1})	100% (24 h)	150 g l^{-1}	
	Acid Red B (50 mg l^{-1})	100% (24 h)	150 g l^{-1}	
	Acid Scarlet GR (50 mg l^{-1})	90% (24 h)	150 g l^{-1}	
	Acid Black 10B (50 mg l^{-1})	90% (24 h)	150 g l^{-1}	
	Reactive Brilliant Red X-3B (50 mg l^{-1})	60% (24 h)	150 g l^{-1}	
Shewanella decolorationis strain S12	Fast Acid Red GR (150 μM)	100% (10 h)[a]	5 g l^{-1}	Xu et al. (2007)
Gracilibacillus sp. GTY.	Acid Red B (100 mg l^{-1})	100% (96 h)	150 g l^{-1}	Salah Uddin et al. (2007)
Halomonas sp. D2	Remazol Black B (50 mg l^{-1})	72% (96 h)	50 g l^{-1}	Asad et al. (2007)
	Remazol Black N (50 mg l^{-1})	82% (96 h)	50 g l^{-1}	
	Sulphonyl Green BLE (50 mg l^{-1})	94% (96 h)	50 g l^{-1}	
	Sulphonyl Scarlet BNLE (50 mg l^{-1})	72% (96 h)	50 g l^{-1}	
	Sulphonyl Blue TLE (50 mg l^{-1})	56% (96 h)	50 g l^{-1}	
	Maxilon Blue (50 mg l^{-1})	37% (96 h)	50 g l^{-1}	
	Entrazol Blue IBC (50 mg l^{-1})	21% (96 h)	50 g l^{-1}	
	Mixture of above seven dyes (50 mg l^{-1})	100% (120 h)	50 g l^{-1}	
Vibrio harveyi strain TEMS1	Acid Black 210 (100 mg l^{-1})	93.9% (24 h)	5 g l^{-1}	Ozdemir et al. (2008)
	Acid Black 24 (100 mg l^{-1})	39.4% (24 h)	5 g l^{-1}	
	Acid Blue 7 (100 mg l^{-1})	4% (24 h)	5 g l^{-1}	
	Acid Green 20 (100 mg l^{-1})	16.3% (24 h)	5 g l^{-1}	
	Acid Yellow 36 (100 mg l^{-1})	1.5% (24 h)	5 g l^{-1}	
Halomonas sp. A3	Remazol Black B (50 mg l^{-1})	56% (96 h)	50 g l^{-1}	Asad et al. (2007)
	Remazol Black N (50 mg l^{-1})	87% (96 h)	50 g l^{-1}	
	Sulphonyl Green BLE (50 mg l^{-1})	97% (96 h)	50 g l^{-1}	
	Sulphonyl Scarlet BNLE (50 mg l^{-1})	60% (96 h)	50 g l^{-1}	
	Sulphonyl Blue TLE (50 mg l^{-1})	85% (96 h)	50 g l^{-1}	
	Maxilon Blue (50 mg l^{-1})	46% (96 h)	50 g l^{-1}	
	Entrazol Blue IBC (50 mg l^{-1})	41% (96 h)	50 g l^{-1}	
	Mixture of above seven dyes (50 mg l^{-1})	100% (120 h)	50 g l^{-1}	
Halomonas sp. Gb	Remazol Black B (50 mg l^{-1})	64% (96 h)	50 g l^{-1}	Asad et al. (2007)
	Remazol Black N (50 mg l^{-1})	82% (96 h)	50 g l^{-1}	
	Sulphonyl Green BLE (50 mg l^{-1})	95% (96 h)	50 g l^{-1}	
	Sulphonyl Scarlet BNLE (50 mg l^{-1})	74% (96 h)	50 g l^{-1}	
	Sulphonyl Blue TLE (50 mg l^{-1})	56% (96 h)	50 g l^{-1}	
	Maxilon Blue (50 mg l^{-1})	55% (96 h)	50 g l^{-1}	
	Entrazol Blue IBC (50 mg l^{-1})	32% (96 h)	50 g l^{-1}	
	Mixture of above seven dyes (50 mgl^{-1})	100% (120 h)	50 g l^{-1}	
Halomonas sp. IP8	Cibacron Black w-55 (50 mg l^{-1})	60% (8 h)[a]	50 g l^{-1}	Amoozegar et al. (unpublished data)

[a] Indicates just the decolorization time period, while all others are the elapsed time from media inoculation with bacteria

Xu et al. (2007) compared the decolorization yield of the bacterium *Shewanella decolorationis* strain S12 under aerobic, anaerobic and microaerophilic conditions. They showed that, although the maximum decolorization yield is achieved under anaerobic conditions, further degradation of aromatic products that resulted from the reduction of azo bonds during the first step needs small amounts of oxygen. Thus, the complete degradation of azo dye Fast Acid Red GR was only met by microaerophilic culture of decolorization media inoculated with this strain.

Asad et al. (2007) reported the isolation of three new *Halomonas* strains from textile effluents that were able to use a wide range of azo dyes as the sole source of carbon. These strains could decolorize the azo dyes in a wide range of NaCl concentrations (up to 200 g l^{-1}) and pH values (5–11), and decolorization occurred only under anaerobic conditions and in static cultures but not under aerobic conditions (cultures submitted to agitation). HPLC analysis of the decolorized media indicated that decolorization proceeded through reduction of the azo bond followed by cleavage of the reduced bond to produce aromatic amines. The wide range of salinities for dye decolorization is an interesting characteristic for an eventual application to the decolorization of textile effluents that are generally saline and slightly alkaline.

Guo et al. (2008a) have described the decolorization of several azo dyes under anaerobic conditions by a new member of the genus *Halomonas*, designated strain GTW, isolated from coastal sediments in Dalian Bay (China). Optimal decolorization occurred at 30°C, pH 6.5–8.5 and NaCl 100–200 g l^{-1} with yeast extract as the carbon source. Nevertheless, neither the precise anaerobic conditions nor the degradation products were described (Le Borgne et al. 2008).

Khalid et al. (2008a, b) investigated the decolorization of azo dyes by another member of the genus *Shewanella*: *Shewanella putrefaciens* strain AS96 under hypersaline conditions. In this experiment, the bacterium decolorized 100 mg dye l^{-1} at salt concentrations up to 60 g NaCl l^{-1} under static, low oxygen conditions. There was an inverse relationship between the rate of the decolorization reaction and salt concentration over the range between 5 and 60 g NaCl l^{-1} and at dye concentrations between 100 and 500 mg l^{-1}. The addition of either glucose (C source) or NH_4NO_3 (N source) to the medium strongly inhibited the decolorization process, while yeast extract (4 g l^{-1}) and Ca $(H_2PO_4)_2{\cdot}H_2O$ (1 g l^{-1}) both enhanced decolorization rates. High-performance liquid chromatography analysis demonstrated the presence of 1-amino-2-naphthol, sulfanilic acid and nitroaniline as the major metabolic products of the azo dyes, which could be further degraded by a shift to aerobic conditions.

Guo et al. (2008b) studied the incorporation of biotreatment technology of the bromoamine acid (BA) wastewater and azo dye wastewaters under high-salt conditions (NaCl, 150 g l^{-1}). They described the BA wastewater as a redox mediator in the biodecolorization of azo dye wastewaters. Decolorization of azo dyes was carried out experimentally using halotolerant bacteria grown on BA wastewater and high-salt conditions. The BA wastewater used as a redox mediator was able to increase the decolorization rate of wastewater containing azo dyes. The effects of various operating conditions such as dissolved oxygen, temperature, and pH on microbial decolorization were investigated experimentally. At the same time, BA was tested to assess the effects on the change of the Oxidation–Reduction Potential (ORP) values during the decolorization processes. The experiments explored a great improvement of the redox mediator application and the new bio-treatment concept.

Amoozegar et al. (unpublished data) have recently studied the decolorization of an azo dye, Cibacron Black w-55, by halophilic bacteria isolated from the salty effluents of textile industries. They isolated a halophilic strain, designated IP8, which was a member of the genus *Halomonas* based on polyphasic systematic studies. This strain showed a remarkable ability in decolorizing azo dyes over a wide range of pH values (7–11), temperature (25–45°C) and in the presence of NaCl and Na_2SO_4 (0.5–1.5 M) under aerobic and anaerobic conditions. These studies could be of great importance for application in large-scale decolorization plants. UV–Vis and HPLC analyses of decolorization products confirmed the active biodegradation of azo dye by this strain. Furthermore, the effect of metal compounds on decolorization showed that $AgNO_3$ and $NaAsO_4$ had higher and lower effects on decolorization, respectively.

Biochemical mechanisms of azo dye degradation by *Shewanella decolorationis* as a model halotolerant bacterium

A common characteristic of this group of chemicals is the presence of particular chemical bonds which lead to a general structure of R-N=N-R′ for the azo dyes. R and R′ can be either alkyl or aryl groups which are different groups in various dyes. N=N group which occurs the same in different azo dyes, is a di-imide bond named “azo”. The azo bond contributes its stability features to the related compound that contains this bond. Due to the electron-withdrawal character of these N=N groups, the containing chemicals are not very susceptible to oxidative metabolism (Le Borgne et al. 2008). Therefore, this stability can only be overcome by reductive cleavage of the double bond of the azo group. Azoreduction by halotolerant and halophilic bacteria has generally been considered a nonspecific reductive process mediated by their enzymes acting as

azoreductases (Zimmermann et al. 1982, 1984; Ghosh et al. 1992, 1993; Rafii and Cerniglia 1993; Suzuki et al. 2001; Moutaouakkil et al. 2003; Maier et al. 2004; Ramalho et al. 2005). Since the oxygen can be a potential electron acceptor, it must usually be excluded during enzymatic dye reduction in biological treatments.

Shewanella species have shown a great capability in biological decolorization of azo dyes under anaerobic conditions. This capability greatly depends on their potential anaerobic growth with a wide variety of terminal electron acceptors, including nitrate, insoluble Fe (III) and Mn (IV) oxides and oxyhydroxides, and toxic metals, such as chromium, arsenate, and uranium (Moser and Nealson 1996; Nealson and Saffarini 1994; Myers et al. 2000; Saltikov et al. 2003; Wade and DiChristina 2000). This remarkably diverse capability of using different electron acceptors for respiration is attributed in part to the abundant c-type cytochromes in *Shewanella*. The great performance of this strain in decolorization of azo dyes made it a good choice for future studies on the mechanisms used in this particular process.

Before the precise mechanism of anaerobic azoreduction was revealed, a study showed that the facultative anaerobe *S. decolorationis* S12 can reduce azo compounds under anaerobic conditions and couple its growth to this reduction (Hong et al. 2007a). Then, another experiment was designed with the usage of purified membranous, periplasmic, and cytoplasmic fractions from *S. decolorationis* S12 to decolorize azo dyes (Hong et al. 2007b). They realised that only the membranous fraction was capable of reducing azo dye in the presence of an electron donor, indicating that the enzyme system for anaerobic azoreduction was located on cellular membrane. In addition, by the usage of different respiratory inhibitors, they showed that the bacterial anaerobic azoreduction by strain S12 was a biochemical process that oxidizes the electron donors and transfers the electrons to the acceptors through a multi-component system related to electron transport chain. They reported in their research that dehydrogenases, cytochromes, and menaquinones were essential electron transport components for the azoreduction.

Several types of azo dyes are highly polar compounds, and this will make it impossible for them to penetrate into the cells through the cell membrane. On the other hand, the bacterial azoreductases are generally considered to be cytoplasmic enzymes. A relatively accepted hypothesis that can describe this controversy is that these azoreductase enzymes usually act in coordination with redox mediators shuttling electrons from the bacterial cytoplasm to the azo dyes under anaerobic conditions (Chen et al. 2010).

Chen et al. (2010) studied the electron pathways which may involve in azoreduction in *Shewanella decolorationis* S12. They found two different pathways which took part to deliver electrons to the azo bonds to reduce them. They revealed that one important pathway to transport electrons in azoreduction process is mediated by mature c-type cytochromes when electrons are generated from formate or NADH. Consistently, Brigé et al. (2008) had already pointed out that the dye molecules are most possibly reduced by the outer membrane cytochromes either directly or indirectly via melanin. In addition, Chen and colleagues showed that another pathway is mediated through NADH-dependent oxidoreductase without the involvement of mature c-type cytochromes inside the whole cells or cell extracts without bacterial membrane barriers. This pathway can reduce lowly polar sulfonated azo dyes which can easily penetrate into the cells or highly polar sulfonated azo dyes in the cell extracts.

Due to the reductive cleavage of the azo bond, azo dyes are reduced and, usually, aromatic amines are formed. Many of these aromatic amines have been reported to be powerful carcinogens and mutagens (Benigni and Passerini 2002), and therefore need to undergo further degradation. These aromatic amines can subsequently be mineralized aerobically. The reductive cleavage may result in the production of sulfonated aromatic amines if the biologically treated wastewater contains sulfonated azo dyes (Tan et al. 2005). This group of aromatic amines which have even more recalcitrant nature are not readily biodegraded.

A relatively comprehensive study was performed to evaluate the mechanism of reduction and partial mineralization of naphthylaminesulfonic azo dye, amaranth, by *S. decolorationis* S12 (Hong et al. 2007c). This study described that, under anaerobic conditions, strain S12, which has been grown aerobically in LB medium, can reductively transform amaranth into corresponding aromatic amines 1-AN-2-H-3, 6-DS and 1-AN-4-S by the way of cleaving azo bond. In addition, it was revealed that the reduction products cannot be degraded by strain S12 under anaerobic conditions. However, when aerobic conditions are provided, the aromatic amine 1-AN-2-H-3, 6-DS can be totally degraded. In contrast, the degradation of 1-AN-4-S was not observed (Fig. 1). The ability to both reduce and mineralize naphthylaminesulfonic azo dye with aerobic/anaerobic methods has been restricted to the *S. decolorationis* S12 and also a bacterial consortium (Haug et al. 1991).

Anaerobic/aerobic treatment of azo dyes

Since certain aromatic amines and also sulfonated amino-aromatics are aerobically degraded by bacteria (Brown and Laboureur 1983; 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

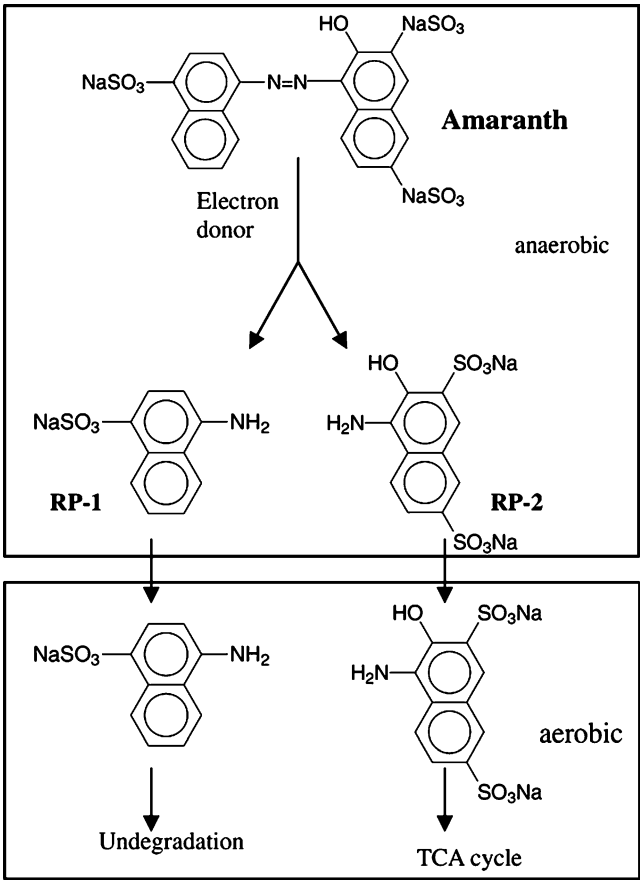

Fig. 1 The pathway of the reduction and partial mineralization of naphthylaminesulfonic azo dye amaranth by *S. decolorationis* strain S12 (Hong et al. 2007c). *RP-1* 1-aminenaphthylene- 4- sulfonic acid (1-AN-4-S), *RP-2* 1-aminenaphthylene-2-hydroxy- 3, 6-disulfonic acid (1-AN-2-H-3, 6-DS)

suggested to combine the anaerobic cleavage of the azo dyes with an aerobic treatment system for the amines formed. The feasibility of this strategy was first demonstrated for the sulfonated azo dye Mordant Yellow 3 (Fig. 2) (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 aerobic steps either alternately in the same reaction vessel as shown in Fig. 3a, or in a continuous system in separate vessels (Fig. 3b) (Wang et al. 2009; Glässer et al. 1992; Tan 2001; Lourenço et al. 2006).

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 (Tan 2001; Jiang and Bishop 1994; Kudlich et al. 1996, 1999; Tan et al. 1999). 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.

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(Fitzgerald and Bishop 1995), an anaerobic fixed-film fluidized bed reactor followed by an aerobic suspended-bed activated sludge reactor (Seshadri et al. 1994; Harmer and Bishop 1992), a combination of anaerobic and aerobic rotating-drum reactors (Sosath and Libra 1997; An et al. 1996), and an anaerobic up-flow fixed bed column together with an aerobic agitated tank (O'Neill et al. 2000a, b; Rajaguru et al. 2000; Stolz 2001). 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.

In general, it may be concluded that, in continuous anaerobic/ aerobic systems which are fed with substrate mixtures possessing a high biological and chemical oxygen demand (BOD, COD) and low dye concentrations, a complete decolorization and a significant BOD and COD removal can be achieved in the anaerobic stage.

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. 2e)] in a simulated textile effluent containing modified starch (O'Neill et al. 2000b; Rajaguru et al. 2000). Similar results have also been described for the treatment of wastewater from a dyeing factory on a laboratory scale (Fitzgerald and Bishop 1995; Krull et al. 2000). For the treatment of the copper-containing dye Reactive Violet 5 (Fig. 2i) in an anaerobic/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. 2e) treatment suggested a decrease in the concentration of nitrogen-containing metabolites (Chung et al. 1992). 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.

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 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).

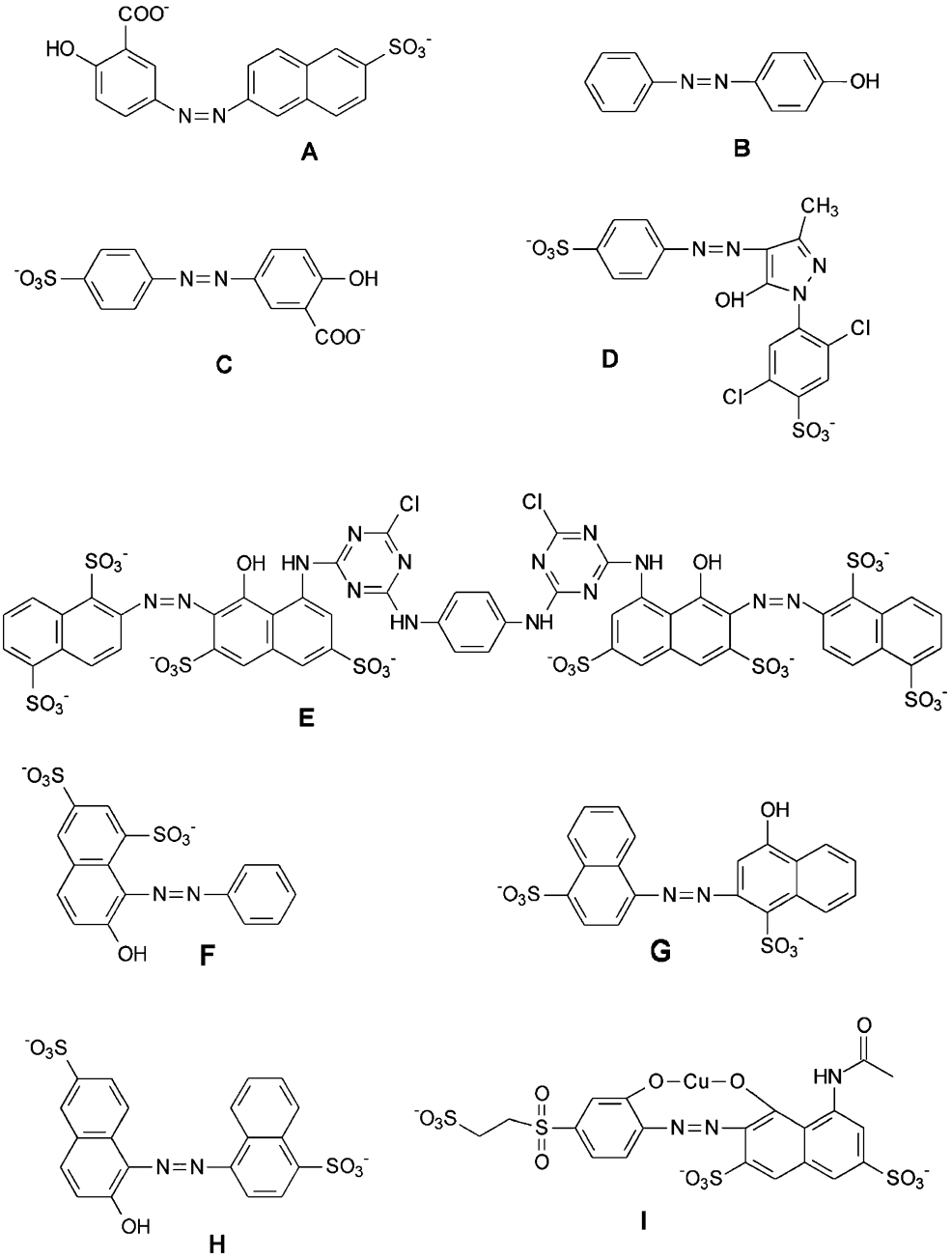

Fig. 2 Examples of azo compounds that have been studied in anaerobic/aerobic treatment systems. **a** Mordant Yellow 3; **b** 4- phenylazophenol; **c** Mordant Yellow 10; **d** Acid Yellow 17; **e** Reactive Red 141 (Procion Red H-E7B); **f** Acid Orange 10; **g** Acid Red 14; **h** Acid Red 18; **i** Reactive Violet 5 (Kudlich et al. 1999; Fitzgerald and Bishop 1995; O'Neill et al. 2000a; Stolz 2001; Margesin and Schinner 2001; Ventosa and Nieto 1995; Manu and Chauhari 2003; De Baere et al. 1984)

A conventional protocol used in different decolorization studies of halophilic bacteria

1) Sampling and culturing in complex media which best support the growth of halophilic bacteria. The culture media for growing of this physiological group of bacteria usually contain peptone from meat, peptone from casein, gelatine or yeast extract. The following medium has been widely used for the isolation of halophilic bacteria from environmental samples:

   MH medium composition (g l^{-1}): NaCl, 81; MgCl2, 7; MgSO4, 9.6; CaCl2, 0.36; KCl, 2; NaHCO3, 0.06; NaBr, 0.026; proteose-peptone no. 3 (Difco), 5; yeast extract (Difco), 10, and glucose, 1 (Ventosa et al. 1982).

   Environmental samples are simply added to the above medium and incubated under conditions (e.g., temperature, pH and aeration) similar to those of the environment they have been isolated from.
2) For maintenance of the isolates, MH medium supplemented with 1.2% (w/v) agar can be used.
3) In order to perform an objective screening for dye-degrading bacteria, the bacteria consortium grown in the above medium are inoculated onto primary screening media that contain low concentrations of the intended

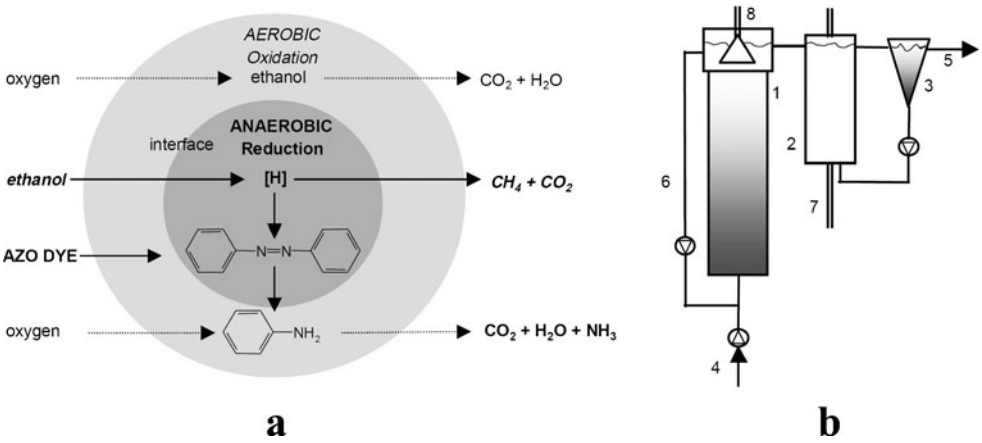

Fig. 3 **a** Theoretical representation of the mineralization of an azo dye under integrated anaerobic/aerobic conditions, with inside anaerobic and outside aerobic conditions. **b** Schematic drawings of the sequential anaerobic expanded granular sludge blanket (EGSB) and aerobic reactor (*1* anaerobic EGSB reactor; *2* aerobic reactor; *3* settler; *4* influent; *5* effluent; *6* EGSB recycle stream; *7* air supply; *8* gas liquid separator) (Wang et al. 2009)

dye. The primary screening medium is composed of MH agar medium supplemented with up to 50 mg l^{-1} of dye.

4) After sequential streaking and isolation of different bacterial strains in pure cultures, they should be transferred to decolorization broth medium in order to determine the optimum conditions for bacterial growth and degradation of the dye. Decolorization medium must contain few nutritional sources in order to minimize unfavorable interactions between optical absorbance of the dye and medium ingredients. The following medium can be used as decolorization medium:

: glucose, 10 g; yeast extract, 5 g; NaCl, 50 g; azo dye, 0.05 g dissolved in 1 l of distilled water (Asad et al. 2007).

Glucose and the dye are sterilized separately by autoclave or filtration and then added to the sterilized medium.

The above medium is usually inoculated with 1% of 1.5×10^8 CFU ml^{-1} of the bacterial suspensions in distilled water plus 30 g l^{-1} NaCl and then incubated at the suitable temperature. As mentioned before, bacterial decolorization of azo dyes is usually performed under static or anaerobic conditions and, as a result, it is better to seal the reaction tubes and put them in an anaerobic jar.

5) Different assay methods can be used to assess the decolorization efficiency. One most common and easy method is UV–Vis spectrophotometry. In this method, a standard graph of light absorbance versus dye concentration should be obtained for each dye. In order to have such a graph, different amounts of dye are separately dissolved in distilled water. Then, the corresponding maximum absorbance in the UV–Vis spectra is plotted for each dye concentration.

To measure decolorization, sampling must be done at different time intervals (Fig. 4) from the inoculated decolorizing media and clarified (centrifuged at 7,500 *g* for 4 min) in order to prevent absorbance interference from the cellular or other suspended debris. Uninoculated culture media with and without added dyes are used as negative controls. The decolorization efficiency of different isolates is calculated from Eq. (1) which is as follows:

$$Decolorization(\%) = \frac{(A_0 - A)}{A_0} \times 100 \quad (1)$$

A_0 is the initial absorbance while A is the absorbance of the medium after decolourization at the λ_{max} (nm) of each dye.

Turbidity (a measure of the bacterial growth) is calculated by formula (2) which determines the

Fig. 4 Decolorization of azo dye, Remazol Black B by *Halomonas* sp. D2. From *left to right*: the first tube contains decolorization medium without inoculation; the others are inoculated decolorization media at time intervals of 24, 48, 72 and 96 h of incubation, respectively

difference between the absorbance of culture samples before and after centrifugation at 600 nm (Dong et al. 2003).

$$\text{Turbidity} = \text{OD}_{(\text{before centrifugation})} - \text{OD}_{(\text{after centrifugation})} \quad (2)$$

The clarified samples from the decolorization media were used for determining the possible changes in the absorption spectra of the dye in the UV–Vis range against a baseline defined by the absorbance of clarified samples from dye-free media.

6) To determine the mechanism of dye degradation, other techniques are necessary. High Performance Liquid Chromatography (HPLC) is widely used in decolorization studies by halophilic organisms. For this purpose, the HPLC detector is set to λ_{max} (nm) following the above experiment by spectrophotometry. The absorbance in this wavelength is most probably due to the molecules or bonds which are not degraded through the reaction. For example, the reduction of azo bond (Fig. 5) results in formation of two aromatic amines which both have absorbance in the same wavelength that undegraded dye has.

   Samplings from decolorization media are performed at different time intervals during the completion of decolorization process. After centrifugation at 10,000 *g*, the supernatant is clarified by a 0.22-μm filter and then injected directly to the HPLC column. Different types of columns and conditions can be used based on the chemical nature of investigated dye. The emergence of peaks with different retention times comparing the negative control sample indicates that some reduced forms of molecules have been derived from degradation of primary dye. The production of reduced aromatic amines leads to a change in the retention time pattern of the investigated sample.

7) Other analytical methods can be used for determination of decolorization mechanisms. Thin Layer Chromatography (TLC) would be a suitable choice if the dye is hydrophobic so that its extraction becomes feasible. Especially when it is used in association with Mass Spectrometry (MS), other chromatography techniques like Gas Chromatography (GC) would also be a good choice for determination of the degradation mechanism. Nucleic Magnetic Resonance (NMR) has also been used to identify the molecular structure of products.

Conclusion and final remarks

Increasing industrial activities have caused the pollution of soil, water and atmosphere by several different chemicals such as petrochemical hydrocarbons, pesticides, herbicides, toxic metals and oxyanions and textile dyes. Azo dyes comprise the most frequent components of contaminated textile wastewaters. These compounds have chemical properties which make them recalcitrant and hard to eliminate from the environment. These dyes are accounted as hazardous environmental threats from at least two important points of view. First, the presence of dyes in water is highly visible and affects not only its aesthetics but impedes light penetration which inhibits photosynthetic activity in aquatic life. Second, they can be reduced by reductase activities in liver cells, skin surface microflora, kidney cells and intestinal bacteria and thus aromatic amines, with carcinogenic characteristics, are released. Several physico-chemical methodologies have been established for treatment of azo dye-contaminated effluents. The production of secondary pollutions and the high cost of these methods revealed the necessity of some alternative environmental friendly and publicly acceptable treatments. Many microorganisms belonging to different taxonomic groups are capable of decolorizing the azo dyes.

Azo dye-polluted wastewaters have particular physicochemical properties that may not be definitively adjusted to the optimal conditions required by most microorganisms. These environments are slightly alkaline, contain different toxic anions and organic solvents, and have a relatively high salinity. Halophilic and halotolerant bacteria seem to be the most compatible

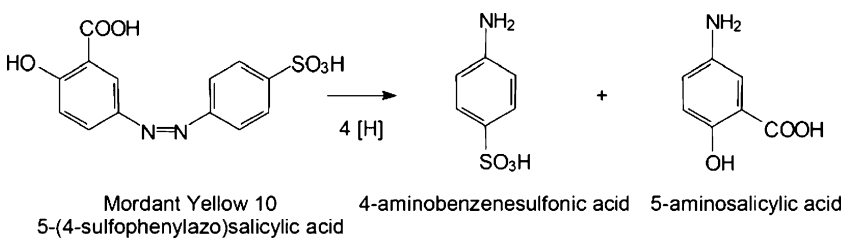

Fig. 5 Degradation of azo dye Mordant Yellow 10 via reduction of N=N bond

organisms in such harsh conditions. They can tolerate up to 5 molar or more salt concentrations near saturation. They have enzymes which are active in very low water activities. This pattern can be of great value when the enzyme needs to maintain its activity in the presence of organic solvents. They can also tolerate some toxic anions like chromate, selenite and tellurite which are common in industrial wastewaters (Kabiri et al. 2009; Amoozegar et al. 2007, 2008).

In addition to these specific properties, some other characteristics make halophilic microorganisms a good candidate for biological treatment of azo dye-containing wastewaters. The low level of contamination in their culture media, due to the high salt concentration is an asset for industrial application of halophiles (Ventosa et al. 1998). In addition, halophilic microorganisms are not known as human pathogens and they have not been reported to contain any kind of antibiotic-resistant plasmids, which minimizes the risk of their release in the environment.

In general, azo dye degradation is a two-step process: in the first step which is performed anaerobically, reductive cleavage of the azo bond makes the dye colorless and releases toxic aromatic intermediates. In the second step, aerobic mineralization occurs via hydroxylation and ring opening. Several aerobic/anaerobic systems have been designed including two-phase reactors, transit culture from anaerobic to aerobic conditions, immobilized cell/enzymes and fed-batch culturing which can be used practically in azo dye remediation. Due to the complexity of substrate and process, microbial consortia seem to perform more effectively than individual isolates in degradation of azo dyes.

In summary, several researches have been focused on developing a practical methodology to use halophilic and halotolerant microorganisms in the biodegradation of azo dyes. However, molecular mechanisms of these processes have been remained unknown in detail. Molecular biology techniques may be utilized in future studies to manipulate the production and activity of the responsible enzymes in azo dye biodegradation. Metagenomic approaches have provided promising findings in the studies related to the microbial enzymes and their related coding genes. These techniques can be used in finding robust reductases, hydroxylases and other participating enzymes in azo dye degradation through culture-independent processes. Moreover, the production of heterologous halophilic hosts which contain both reductate and ring opening enzymes simultaneously can be a significant achievement in the future azo dye decolorization studies. In addition, reverse genetic engineering to design specific enzymes to cleave these artificial human-made dyes may seem useful for these processes.

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