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Review Article

Molecular approaches for bacterial azoreductases

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Abstract

Azo dyes are the dominant types of synthetic dyes, widely used in textiles, foods, leather, printing, tattooing, cosmetics, and pharmaceutical industries. Many microorganisms are able to decolorize azo dyes, and there is increasing interest in biological waste treatment methods. Bacterial azoreductases can cleave azo linkages (-N=N-) in azo dyes, forming aromatic amines. This review mainly focuses on employing molecular approaches, including gene manipulation and recombinant strains, to study bacterial azoreductases. The construction of the recombinant protein by cloning and the overexpression of azoreductase is described. The mechanisms and function of bacterial azoreductases can be studied by other molecular techniques discussed in this review, such as RT-PCR, southern blot analysis, western blot analysis, zymography, and mutagenesis in order to understand bacterial azoreductase properties, function and application. In addition, understanding the regulation of azoreductase gene expression will lead to the systematic use of gene manipulation in bacterial strains for new strategies in future waste remediation technologies.

Keywords: azoreductase, azo dyes, molecular techniques, recombinant strain

1. Introduction

Synthetic dyes are defined as colored substances which are resistant to fading upon exposure to light, water, sweat, many chemicals and microbial attack (Robinson *et al.*, 2001; Saratale *et al.*, 2011). Due to their chemical structure, many dyes are difficult to decolorize (Stolz, 2001). They are classified as acidic, basic, disperse, azo, diazo, anthroquinone based and metal complex dyes (Robinson *et al.*, 2001). Azo dye compounds, the most used synthetic dyes, account for approximately half of the dyes used in the textile industry. They are the most common synthetic colorants released into the environment (Baiocchi *et al.*, 2002; Saratale *et al.*, 2011).

Azo dyes are characterized by the presence of one or more azo group (-N=N-) that are chromophores, associated with aromatic and other groups such as hydroxyls (-OH), chloro (-Cl), methyl (-CH_3), nitro (-NO_2), amino (-NH_3), carboxyl (-COOH) and sulfonic groups (-SO_3H), which give various types of azo dyes (Figure 1) (Stolz, 2001; Forgacs *et al.*, 2004; Saratale *et al.*, 2011). They are widely used in textiles, foods, industrial, printing, tattooing, cosmetics and for clinical purposes (Suzuki *et al.*, 2001; Bin *et al.*, 2004; Chen *et al.*, 2005). These dyes are usually recalcitrant to conventional wastewater treatment (Forgacs *et al.*, 2004). Several physico-chemical methods like adsorption, electrocoagulation, chemical treatment, photocatalysis, oxidation and ion pair extractions, have been adopted and proven to be costly and to produce large amounts of sludge (Robinson *et al.*, 2001; Forgacs *et al.*, 2004; Saratale *et al.*, 2011). More studies focus on biological treatment methods (Supaka *et al.*, 2004; Jadhav *et al.*, 2007; Pandey *et al.*, 2007; Mabrouk and Yusef, 2008; Gopinath *et al.*, 2009; Parshetti *et al.*, 2010).

A wide range of microorganisms including bacteria (Song *et al.*, 2003; Dafale *et al.*, 2008; Jadhav *et al.*, 2010; Telke *et al.*, 2010), yeast, (Jadhav and Govindwar, 2006; Jadhav *et al.*, 2007; Tastan *et al.*, 2010), fungi (Gou *et al.*, 2009, Kaushik and Malik, 2009) and algae (Daneshvar *et al.*, 2007), are able to reduce azo compounds to non-colored products

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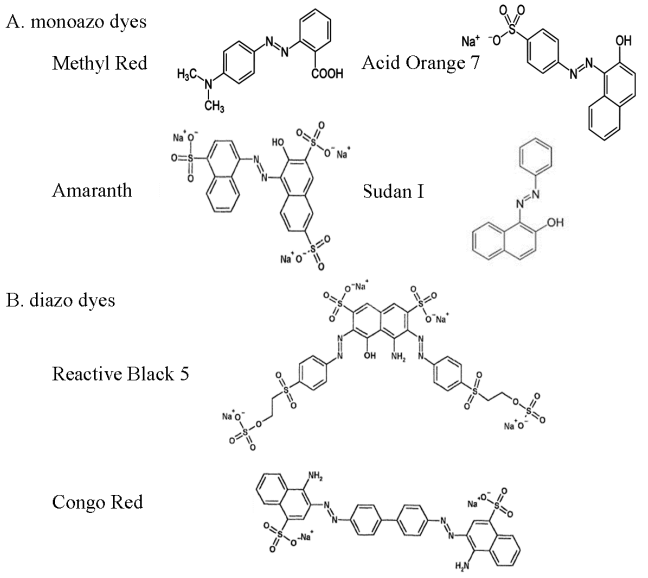

Figure 1. The chemical structures of most frequently studied azo dyes.

or even to completely mineralize them (Stolz, 2001; Chen *et al.*, 2004; Mohanty *et al.*, 2006; Ooi *et al.*, 2007). Various microorganisms are able to metabolize azo dyes by biosorption and biodegradation, involving enzymatic mechanisms such as those associated with lignin peroxidases, manganese peroxidases, laccases and azoreductases, (Stolz, 2001; Jadhav *et al.*, 2007; Bafana *et al.*, 2008; Kaushik and Malik, 2009; Mendes *et al.*, 2011; Saratale *et al.*, 2011). Therefore, the biological degradation and use of microbial or enzymatic treatment methods for removal of these dyes have potential important advantages: less sludge, environmental friendliness and cost competitiveness (Stolz, 2001; Forgacs *et al.*, 2004; Pandey *et al.*, 2007; Saratale *et al.*, 2011).

There are several reviews on treatment of waste effluents containing synthetic dyes by physicochemical and microbiological methods, including bacterial decolorization of azo dyes (Robinson *et al.*, 2001; Stolz, 2001; Forgacs *et al.*, 2004; Pandey *et al.*, 2007; Saratale *et al.*, 2011). However, the current review focuses on bacterial azoreductases and their characterization by molecular biology approaches, in relation to wastewater treatment. In addition, gene manipulation and the recombinant strains with higher biodegradation capacity are included, because they can significantly benefit the future technologies for dye removal.

2. Decolorization of azo dyes by bacterial azoreductases

2.1 Isolation and identification of dye decolorizing bacterium

In recent years, there has been an increasing interest in the use of biological systems, especially bacteria, for the treatment of wastewaters containing dyes (Kumar *et al.*, 2006; Dafale *et al.*, 2008; Sandhya *et al.*, 2008; Liu *et al.*, 2009b; Mendes *et al.*, 2011a). Numerous studies have aimed to isolate good dye-decolorizing species, either in pure cultures or in consortia (Maier *et al.*, 2004; Mohanty *et al.*, 2006; Dafale *et al.*, 2008; Telke *et al.*, 2010). It has been reported that microbial consortia have considerable advantages over pure cultures in the decolorization of azo dyes (Khehra *et al.*, 2005; Junnarkar, 2006; Saratale *et al.*, 2009; Jadhav *et al.*, 2010).

Bacteria responsible for decolorization have been collected from many sites such as activated sludge from the textile effluent treatment plant (Mohanty *et al.*, 2006), wastewater from textile finishing company (Maier *et al.*, 2004), soil samples from effluent contaminated site of dyestuff industries (Junnarkar, 2006; Telke *et al.*, 2010; Anjaneya *et al.*, 2011), polluted sediment samples (Mabrouk and Yusef, 2008), dairy wastewater treatment plant (Seesuriyachan *et al.*, 2007), marine environment (Liu *et al.*, 2013) and hot springs (Deive *et al.*, 2010). A plate assay was used to detect decolorizing activity of bacteria, by observing clear zones appearing around a few bacterial colonies on an azo dyed agar plate (Rafii *et al.*, 1990; Mohanty *et al.*, 2006). The isolated strains including *Pseudomonas aeruginosa*, *Bacillus curculans* (Dafale *et al.*, 2008), *Pseodomonas* sp. SU-EBT (Telke *et al.*, 2010), *Bacillus* sp. strain SF, *Bacillus* sp. strain LF, *B. pallidus, B. subtilis* HM (Maier *et al.*, 2004; Mabrouk and Yusef, 2008), *Lactobacillus casei* TISTR 1500 (Seesuriyachan *et al.*, 2007) and *B. velezensis* strain AB (Bafana *et al.*, 2008), showed a clearing zone on plate, and also decolorized a wide range of azo dyes in liquid cultures.

Identification of bacteria based on 16S ribosomal RNA gene (16S rRNA) sequences has been used extensively for molecular taxonomic studies as an attractive alternative to the methods following traditional standard references such as Bergey's Manual of Systematic Bacteriology or the Manual of Clinical Microbiology (Clarridge, 2004; Woo *et al.*, 2008; Anjaneya *et al.*, 2011). The rRNA genes are highly conserved (least variable) DNA in all cells (Boye *et al.*, 1999). The 16S rRNA gene is now most commonly used for bacterial taxonomic purposes (Tortoli, 2003; Clarridge, 2004; Seesuriyachan *et al.*, 2007; Woo *et al.*, 2008). Using 16S rRNA sequencing, bacterial identification is more robust, reproducible, accurate and less subjective test results (Clarridge, 2004; Woo *et al.*, 2008).

2.2 Decolorization mechanism

Many microorganisms such as bacteria, fungi and yeast have been found to be able to decolorize azo dyes by bioadsorption or degradation (Mabrouk and Yusef, 2008; Gou *et al.*, 2009). A new fungal isolate, *Penicillium* sp. QQ could aerobically decolorize Reactive Brilliant Red X-3B by bioadsorption rather than biodegradation due to the adsorption of azo dyes by many functional groups located on the surface of microbial cells (Gou *et al.*, 2009). Mabrouk and Yusef (2008) showed that the decolorization of Fast Red was achieved by *B. subtilis* HM due to degradation rather than adsorption as indicated by the uncolored biomass.

Bacterial decolorization has been associated with various oxidoreductive enzymes, including laccase, azoreductase and NADH-DCIP reductase (Stolz, 2001; Kalme *et al.*, 2007; Parshetti *et al.*, 2010; Telke *et al.*, 2010; Kolekar *et al.*, 2013). In addition, the location of the reaction can be either intracellular or extracellular (Pandey *et al.*, 2007; Seesuriyachan *et al.*, 2007). This reaction may involve different mechanisms such as enzymes by direct enzymatic azo dye reduction, low molecular weight redox mediators, electron donor from the respiratory chain or a combination of these (Pandey *et al.*, 2007). The significance of oxidoreductive enzymes in decolorization of Congo Red was examined by Telke and coworkers. The observations demonstrated that laccase from *Pseudomonas* sp. SU-EBT was the key enzyme responsible for Congo Red docolorization (Telke *et al.*, 2010). To elucidate the RO16 decolorization mechanism, oxidative and reductive enzyme was observed after decolorization by bacterial consortium. The study showed that the laccase and azoreductase are involved in RO16 biodegradation (Jadhav *et al.*, 2010). The significant increase in the enzyme activities of azoreductase and NADH-DCIP reductase were observed by *Kocuria rosea* MTCC 1532 suggesting its involvement in decolorization of methyl orange (Parshetti *et al.*, 2010). The proposed pathway for degradation of methyl orange by *K. rosea* is reported. Since azoreductases require the addition of expensive cofactors as electron donors for the reductive reaction resulting in aromatic amines which are potential toxic (Stolz, 2001; Chen *et al.*, 2004; Mohanty *et al.*, 2006; Ooi *et al.*, 2007), a recent study from Mendes *et al.* (2011b) has constructed a co-expressing strain (azoreductase and laccase gene) which maximizes decolorization and detoxification of azo dye-containing wastewater.

Azoreductases are involved in the degradation of azo dyes and also found in intestinal microflora for activation of azo prodrugs in the treatment of inflammatory bowel disease (IBD) (Ryan *et al.*, 2010a; Wang *et al.*, 2010). Azoreducases have been shown to reduce azo compounds via a Ping Pong Bi Bi mechanism (Nakanishi *et al.*, 2001; Liu *et al.*, 2008a; Wang *et al.*, 2010; Mendes *et al.*, 2011a). The proposed mechanism for azo compound reduction requires two cycles of NAD(P)H-dependent reduction, which reduces the azo substrate to a hydrazine in the first cycle and reduces the hydrazine to two amines in the second cycle (Figure 2) (Blumel *et al.*, 2002; Deller *et al.*, 2008; Ryan *et al.*, 2010a; Ryan *et al.*, 2010b; Wang *et al.*, 2010). The detection of hydrazine intermediate by mass spectrometry has supported the mechanism (Bin *et al.*, 2004). By deducted amino acid sequence alignment, the NADH binding motif (GXGXXG) has been found in azoreductases from *B. cereus* ATCC 10987, *B. anthracis* Ames, *Geobacillus* sp. OY1-2, and *Bacillus* sp. OY1-2 (Suzuki *et al.*, 2001; Bin *et al.*, 2004).

In summary, azoreductases have been classified into three major groups based on structure, flavin dependency and dinucleotide preference: Group I, the polymeric flavin-dependent NADH-preferred azoreductases; Group II, the polymeric falvin-dependent NADPH-preferred azoreductases and Group III, the monomeric flavin-free NAD(P)H-preferred azoreductases (Seesuriyachan *et al.*, 2007; Chen *et al.*, 2010; Stingley *et al.*, 2010; Feng *et al.*, 2012).

3. Decolorization of azo dyes by recombinant azoreductases

3.1 Cloning and overexpression of azoreductase

The use of molecular tool has become increasingly integrated into understanding enzyme biochemical properties and characterization. Researchers have utilized a gene cloning method as a tool in producing recombinant strains for decolorizing dyes more efficiently. Recombinant strain is also used to study azoreductase activity and its mechanism (Chen *et al.*, 2005; Deller *et al.*, 2006; Ito *et al.*, 2008; Chen *et al.*, 2010; Ryan *et al.*, 2010a). Construction of recombinant expression vector is shown in Figure 3. Recently, genes coding for aerobic azoreductase have been cloned from *Escherichia coli* (Nakanishi *et al.*, 2001; Liu *et al.*, 2009a), *Bacillus* sp. OY1-2 (Suzuki *et al.*, 2001), *B. subtilis* (Deller *et al.*, 2006; Nishiya and Yamamoto, 2007), *Enterococcus faecalis* (Chen *et al.*, 2004), *E. faecium* (Macwana *et al.*, 2010), *Staphylococcus aureus* (Chen *et al.*, 2005), *Rhodobacter sphaeroides* (Bin *et al.*, 2004), *Xenophilus azovorans* KF46F (Blumel *et al.*, 2002), *Pigmentiphaga kullae* K24 (Chen *et al.*, 2010), *Pseudomonas aeruginosa* (Wang *et al.*, 2007, Ryan *et al.*, 2010b) and *Geobacillus stearothermophilus* (Matsumoto *et al.*, 2010) (Table 1).

Many researchers have chosen *E. coli* as a host to express azoreductases (Table 1). They have cloned the azoreductase gene from potential decolorizing bacteria and expressed it in *E. coli* to produce a recombinant *E. coli* strain followed by protein purification. The expression of proteins in *E. coli* has many potential advantages including the ease of growth and manipulation. Many vectors are available with different N- and C-terminal tags and many host strains have

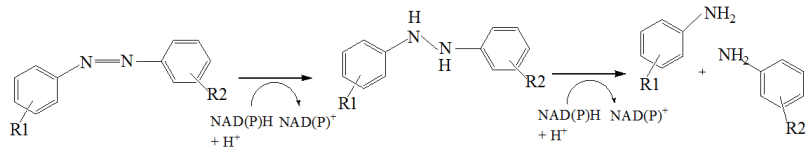

Figure 2. A proposed catalytic reaction of azoreductase. Azoreductase reduces the azo compound via Ping Pong Bi Bi mechanism, with two cycles consuming NAD(P)H, reducing the azo substrate to a hydrazine (partially reduced intermediate) in the first cycle and to two amines in the second cycle (Bin *et al.*, 2004, Ryan *et al.*, 2010b, Wang *et al.*, 2010).

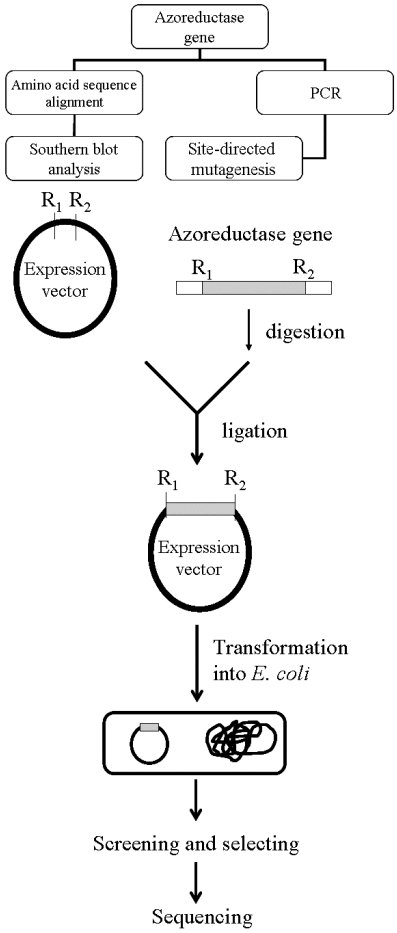

Figure 3. Construction of a recombinant expression vector. Amino acid sequence alignment and southern blot analysis enable finding azoreductase gene homologs in other bacterial stains. The selected azoreductase gene is amplified by PCR followed by restriction digestion. The gene sequence encoding the azoreductase is cloned into an appropriate expression vector in the correct reading frame. To create site-directed mutagenesis, the coding sequence can be modified by PCR. An overexpressed construct is performed by ligation and then transformation into *E. coli* host strain. The transformants are screened on plates with appropriate antibiotic(s), and selectively subjected to sequencing analysis. Sequencing allows monitoring of progress in this iterative selection process. R_1 and R_2 indicate restriction sites.

been developed for maximizing expression. Several chosen expression vectors are shown in Table 1. pET vector system contains several important elements including a *lacI* gene which codes for the *lac* repressor protein, a T_7 promoter which is specific to only T_7 RNA polymerase, multiple cloning site, selectable marker and protease cleavage site (e.g. thrombin site in pET-15b) in order to remove a tag or other fusion proteins. Many vectors encode additional optional components such as signal sequences (e.g. pET-22b) to direct secretion and/or short peptide tags that are added to the N- or C- terminus of the protein in order to improve expression, solubility, detection and purification (Appelbaum and Shatzman, 1999). Some expression systems require the use of specialized host strains which provide regulatory elements. Use of protease-deficient host strains (e.g. BL21) can sometimes enhance product accumulation by reducing degradation. The gene on the host cell chromosome usually has an inducible promoter (e.g. T_7-lac operator in pET vectors and P_L in pTrx vectors) so that protein expression can be induced by the addition of the proper inducer such as IPTG or by shifting the temperature (Appelbaum and Shatzman, 1999). The construction of expression vectors is generally straightforward by cloning gene sequence encoding the azoreductase sequence to be expressed into an appropriate vector in the same reading frame. Expression of recombinant azoreductase can be approached in general by starting with introducing the recombinant plasmid into the required host cell, growing the host cells and inducing expression, lysing the cells and analyzing by SDS-PAGE to verify the presence of the protein (Qiagen).

3.2 Characterization of recombinant azoreductase

Molecular cloning of the gene encoding azoreductase enzyme followed by protein purification is likely to be crucial for further characterization and application of this enzyme (Suzuki *et al.*, 2001; Wang *et al.*, 2007; Ryan *et al.*, 2010a; Wang *et al.*, 2010; Mendes *et al.*, 2011a). Physiochemical properties, enzyme characterization and kinetic studies can be investigated by obtaining purified azoreductase from whole cell extract from the source organism or recombinant cell extract (Nachiyar and Rajakumar, 2005; Wang *et al.*, 2007; Gopinath *et al.*, 2009; Punj and John, 2009; Mendes *et al.*, 2011a; Morrison *et al.*, 2012). Purification from whole cell extracts from the source organism employs classical purification procedures which require many steps such as ammonium sulfate precipitation followed by ion exchange and affinity chromatography methods (Maier *et al.*, 2004; Nachiyar and Rajakumar, 2005; Punj and John, 2009; Kolekar *et al.*, 2013). However, in most cases recombinant DNA techniques permit the construction of fusion proteins in which specific affinity tags are added to the protein sequence of interest (Bin *et al.*, 2004; Wang *et al.*, 2007). Therefore, the purification of the recombinant fusion proteins is simplified by employing affinity chromatography methods. In addition, the expression and purification of recombinant proteins facilitate the production and detailed characterization of virtually any protein. Native molecular weight of a protein can be determined by native gel electrophoresis and/or size exclusion chromatography (Moutaouakkil *et al.*, 2003; Deller *et al.*, 2006; Ooi *et al.*, 2007; Wang *et al.*, 2007).

Chen *et al.* (2010) described the cloning of azoreductase gene *azoB* from *Pigmentiphaga kullae* K24. The recombinant azoreductase expressed in *E. coli* exhibited optimal for activity of Orange I at pH 6.0 at temperatures between 37 and 45°C. Both NADH and NADPH can be used as an electron donor but NADPH is preferred. The gene

Table 1. Expression of recombinant azoreductase in *E. coli*

Source of organism	gene	expression vector	Molecular mass	Cofactors	Reference
Pseudomonas putida MET94	*ppAzoR*	pET-21a	Homodimer 40 kDa	FMN, NADPH	Mendes *et al.*, 2011
Pseudomonas aeruginosa	*paazor1*	pET-28b	Tetramer 110 kDa	FMN, NAD(P)H	Wang *et al.*, 2007
	paazor2	pET-28b	23 kDa*	NADH	Ryan *et al.*, 2010b
	paazor3	pET-28b	26 kDa*	NADH	Ryan *et al.*, 2010b
Pigmentiphaga kullae K24	*azoB*	pET-11a	Monomer 22 kDa	NADPH	Chen *et al.*, 2010
Geobacillus stearothermophilus	*azrG*	pET-3a	Homodimer 23 kDa	FMN, NADH	Matsumoto *et al.*, 2010
Brevibacillus latrosporus RRK1 (formerly *Bacillus latrosporus* RRK1)		pET-32a	58 kDa*	NADH	Sandhya *et al.*, 2008
Bacillus sp. B29	*azrA*	pET-3a	Homodimer 48 kDa	FMN, NADH	Ooi *et al.*, 2007
	azrB	pET-3a	Homodimer 48 kDa	FMN, NADH	Ooi *et al.*, 2009
	azrC	pET-3a	Homodimer 48 kDa	FMN, NADH	Ooi *et al.*, 2009
Bacillus sp. OY1-2		pTrx-Fus	20 kDa*	NADPH	Suzuki *et al.*, 2001
Bacillus subtilis	*yvaB* (*azoR2*)	pBluescript	Homodimer 45 kDa	NADH	Nishiya and Yamamoto, 2007
	yhdA	pET-12a	Tetramer 76 kDa	FMN, NADPH	Deller *et al.*, 2006
Escherichia coli	*acpD* (*azoR*)	pET-22b	Homodimer 46 kDa	FMN, NADH	Nakanishi *et al.*, 2001
Enterococcus faecium	*acpD*	pET-15b	23 kDa*	NAD(P)H	Macwana *et al.*, 2010
Enterococcus faecalis	*azoA*	PET-11a	Homodimer 43 kDa	FMN, NADH	Chen *et al.*, 2004
Xenophilus azovorans KF46F	*azoB*	pET-11a	Monomer 30 kDa	NADPH	Blumel *et al.*, 2002
Clostridium perfringens	*azoC*	pET-15b	Tetramer 90.4 kDa	FAD, NADH	Morrison *et al.*, 2012

* azoreductase determined by SDS-PAGE

azrA coding for an azoreductase from *Bacillus* sp. strain B29 was characterized (Ooi *et al.*, 2007). The recombinant azoreductase expressed in *E. coli* exhibited a broad pH stability between 6 and 10 with an optimal temperature of 60-80°C. AzrA effectively decolorized Methyl Red, Orange I, Orange II and Red 88. No enzyme activity was detected for Orange G and New Coccin. In addition, the enzyme activity of AzrA was oxygen insensitive and required NADH as electron donor for dye reduction. Similar results have also been described for azoreductase enzyme activity extracted from *B. velezensis* and *P. aeruginosa* (Nachiyar and Rajakumar, 2005, Bafana *et al.*, 2008). Furthermore, a gene encoding NADPH-flavin azoreductase (Azo1) from the skin bacterium *Staphylococcus aureus* ATCC 25923 overexpressed in *E. coli* demonstrated that this azoreductase is able to decolorize a wide range of structurally complex azo dyes (Chen *et al.*, 2005). The Azo1 cleaved the model azo dye Methyl Red and sulfonated azo dyes Orange II, Amaranth and Ponceau. However, no enzyme activity was observed when Orange G was used as substrate. Recently, the gene encoding an FMN-dependent NADH azoreductase AzrG from thermophilic *Geobacillus stearothermophilus* was cloned and expressed in recombinant *E. coli* (Matsumoto *et al.*, 2010). The optimal temperature of AzrG was 85°C for Methyl Red degradation and enzyme also showed a wide range of degrading activity towards several tenacious azo dyes such as Acid Red 88, Orange I and Congo Red. Therefore, the azoreductases expressed from different organisms are diverse and vary greatly. The purification and characterization experiments of enzymes were conducted and the results indicated that the enzyme activity differs in substrate specificity and preferential coenzymes serving as electron donors.

In conclusion, characterization of recombinant azoreductases provide information for understanding these azoreductases properties such as enzyme stability and activity, kinetic constants, cofactor requirement, substrate profile, structure and mechanism (Wang *et al.*, 2007, Ooi *et al.*, 2009, Macwana *et al.*, 2010, Ryan *et al.*, 2010b, Mendes *et al.*, 2011a). A broad range of substrate specificity and thermostability are important factors in determining the range of biologically degradable of azo dyes.

4. The genes encoding azoreductases and its other functions

Many azoreductase genes have been studied (Table 1). Azoreductase activity in azo dyes decolorization has been extensively examined to elucidate azo dye reduction mechanism (Chen *et al.*, 2005, Deller *et al.*, 2006, Wang *et al.*, 2007, Ryan *et al.*, 2010a, Ryan *et al.*, 2010b, Feng *et al.*, 2012). Only few reports have studied the regulation of azoreductase gene expression (Töwe *et al.*, 2007, Liu *et al.*, 2009a, Ryan *et al.*, 2010a). An increase of mRNA levels for azoreductase genes (ppazoR1, ppazoR2 and ppazoR3) from *P. aeruginosa* in the presence of azo dyes has been reported (Ryan *et al.*, 2010a). The effect of stressors on *E. coli* azoreductase gene *azoR* transcription was investigated (Liu *et al.*, 2009a). The results showed a significant induction of *azoR* transcription in the presence of electrophiles including 2-methylhydroquinine,

catechol, menadion and diamide. More significant increases in azoreductase mRNA levels including *azoR1* and *azoR2* have been observed in *B. subtilis* in the presence of quinones (Töwe *et al.*, 2007). It was reported that *azoR1* and *azoR2* are negatively regulated by redox-sensing transcription factors YodB and YkvE, respectively (Töwe *et al.*, 2007, Leelakriangsak *et al.*, 2008). Redox-sensing repressor YodB is a MarR/DUF-24 family repressor that directly senses and responds to quinone and diamide by thiol-disulfide switch (Leelakriangsak *et al.*, 2008, Chi *et al.*, 2010). Therefore, azoreductases AzoR1 and AzoR2 not only have azoreductase activity but also have quinone reductase activity that play a role in bacterial protection thiol-specific stress (Nishiya and Yamamoto, 2007, Töwe *et al.*, 2007, Leelakriangsak *et al.*, 2008, Leelakriangsak and Borisut, 2012).

More recently, evidence was presented that azoreductase possess quinone reductase and nitroreductase activity (Rafii and Cerniglia, 1993, Liu *et al.*, 2008a, Liu *et al.*, 2009a). The flavin-dependent azoreductases AZR, AzoR from *Rhodobacter sphaeroides* and *E. coli*, respectively, overexpressed in *E. coli* have quinone reductase activity by reducing quinone compounds as substrate. Moreover, the quinone compounds were better substrates for AzoR than the model azo dye substrate Methyl Red (Liu *et al.*, 2009a). Interestingly, the presence of quinone compound accelerated the azo dye decolorization of overexpressed azoreductase AZR (Liu *et al.*, 2009b). Parshetti *et al*. (2010) observed significant increase in the enzyme activities of azoreductase and NADH-DCIP reductase over a period of methyl orange decolorization by *K. rosea* MTCC 1532. A similar result of an increase in azoreductase and DCIP reductase activity was also observed when *Alishewamella* sp. KMK6 exposed to dyes (Kolekar *et al.*, 2013). Interestingly, a putative azoreductase gene (so3585) of *Shewanella oneidensis* is up-regulated in response to a heavy metal (Mugerfeld *et al.*, 2009). However, the results showed that azo dye reduction is not the primary function of the SO3585 protein in vivo.

In conclusion, the physiological role of bacterial azoreductases remains to be elucidated. Many researchers have investigated the toxicity of azo dyes and their metabolite products (aromatic amines) due to their high toxicity and potential carcinogenicity of some certain azo dyes or their metabolic intermediates (Stolz, 2001, Kumar *et al.*, 2006, Stingley *et al.*, 2010, Mendes *et al.*, 2011a, Kolekar *et al.*, 2013). Therefore, azoreductases may be involved in the detoxification of quinones (Liu *et al.*, 2008a, Liu *et al.*, 2009a, Ryan *et al.*, 2010a) and enhance bacteria survival (Liu *et al.*, 2008a).

5. Other molecular approaches in studying azoreductases and applications

5.1 RT-PCR

RNA levels of azoreductase genes are determined by the RT-PCR technique. To evaluate the effects of different stressors on the transcription of azoreductase gene, cells were cultured in media supplemented with different compounds (Liu *et al.*, 2009a, Mugerfeld *et al.*, 2009). The results showed that the transcription of *azoR* gene of *E. coli* is induced by 2-methylhydroquinone, catechol, menadione and diamide (Liu *et al.*, 2009a). AzoR is a quinone reductase providing resistance to thiol-specific stress caused by electophilic quinones. Similar results also have been described in *B. subtilis* (Töwe *et al.*, 2007). RT-PCR approach was also performed by Ryan *et al*. (2010a) to study the expression of azoreductase genes during growth on different azo compounds. Therefore, quinones were proposed to be the primary physiological substrate for azoreductases (Ryan *et al.*, 2010a). Also the co-transcription of a putative azoreductase gene in gene cluster of *Shewanella oneidensis* was determined by RT-PCR under heavy metal challenge (Mugerfeld *et al.*, 2009). The results suggested that a putative azoreductase gene responded to heavy metal stress by up-regulation of operon.

5.2 Southern blot hybridization

To look for new azoreductase genes from other bacteria, researchers have adopted southern blot hybridization techniques (Suzuki *et al.*, 2001, Sugiura *et al.*, 2006). Southern blot hybridization is a useful approach to search for azoreductase gene homologs in several bacterial strains. To clone genes similar to the azoreductase gene of known species from other bacteria, the DNA database is searched using TBLASTN software at NCBI. A pair of primers is designed according to the sequence data of the hypothetical ORF (open reading frames) for amplification of the whole ORF of azoreductase-like gene in other bacteria by PCR. Sugiura *et al*. (2006) chose a hypothetical ORF with lower identity found in bacterial genome due to expectation of altered substrate specificity. Genomic DNA fragments generated by restriction enzymes digestion are separated on agarose gel and are then transferred to a membrane which later is hybridized with digoxigenin labeled PCR products carrying the whole ORF of the azoreductase homolog. The bands observed in bacterial strains indicate that these strains carry azoreductase gene homologs. Therefore, researchers are able to amplify the DNA fragments carrying azoreductase gene homologs and are then cloned to an appropriate vector followed by nucleotide sequences analysis. More azoreductase genes are discovered by this approach.

5.3 Gel electrophoresis and Western blot analysis

Recently Stingley *et al*, (2010) has adopted western blot analysis to detect similar proteins in skin bacteria. Polyclonal antibodies against enzyme azoreductase are obtained by injecting small amounts of purified recombinant azoreductase into an animal such as a mouse, rabbit, sheep or horse. The sera are collected and used for western blot analysis. Proteins are extracted from several bacterial cultures

and separated by SDS-PAGE, transferred to membranes and then hybridized with polyclonal antibody. Proteins can be visualized by a variety of techniques including colorimetric detection, chemiluminescence or autoradiography (Pierce). The results showed the detection of similar proteins in several bacteria (Stingley *et al.*, 2010) and indicated that some human skin bacteria are capable of reducing azo dyes used in cosmetics, tattoo inks and other products that routinely contact skin and could potentially lead to the formation of carcinogenic aromatic amines. Moreover, several reports have demonstrated the application of zymography to detect azoreductase activity by native polyacrylamide gel (Rafii *et al.*, 1990, Maier *et al.*, 2004, Pricelius *et al.*, 2007). Whole proteins extracted from different bacteria and/or purified azoreductase are subjected into native polyacylamide gel. The location of clear bands on the gels indicates azoreductase activity after staining with azo dye (Rafii *et al.*, 1990, Maier *et al.*, 2004, Pricelius *et al.*, 2007). This approach can determine different forms of azoreductase expressed in each bacterium by the migration of the enzyme bands.

5.4 Mutagenesis approach

To improve biodegradation ability of microbial strain, a random mutagenesis technique is used to induce mutations in organisms and potential strains are selected based on their decolorization performance compared to wild type strain (Gopinath *et al.*, 2009). Mutagens including UV irradiation, ethyl methyl sulfonate (EMS) and ethidium bromide (EtBr) are used for inducing mutation (Gopinath *et al.*, 2009, Shafique *et al.*, 2010). Gopinath *et al.*, (2009) found that using EtBr was more effective than UV irradiation in mutagenesis. They selected mutants which showed the improvement of Congo red degradation and reduction of time requirement for complete degradation. Site-directed mutagenesis is an important approach to investigate enzyme mechanism and substrate specificity (Ito *et al.*, 2008, Liu *et al.*, 2008b, Wang *et al.*, 2010, Feng *et al.*, 2012). Single amino acid substitution of azoreductase reveals substrate binding sites (Liu *et al.*, 2008b, Feng *et al.*, 2012). Based on sequence and structure analysis of azoreductase, residues that are predicted to participate in the substrate binding site are chosen for site-directed mutagenesis (Ito *et al.*, 2008, Liu *et al.*, 2008b, Feng *et al.*, 2012). By using primers containing the corresponding mutations in PCR, the mutants are created. The mutant azorecductases are expressed and purified followed by azoreductase activity assays. Comparison of the kinetic parameters of wild type and mutant azoreductase indicate the residue which may affect the substrate binding and enzyme folding (Ito *et al.*, 2008, Liu *et al.*, 2008b, Wang *et al.*, 2010, Feng *et al.*, 2012).

6. Conclusion and future recommendations

Recent literature reviewed herein indicates that molecular approaches including gene cloning, PCR techniques, southern blot hybridization, gel electrophoresis, western blot analysis and mutagenesis have been extensively employed to understand bacterial azoreductase properties and function as well as in searching for potential azoreductase genes. In addition, the molecular techniques could also be used to improve bacterial strains which are capable of accelerating mineralization of the toxic aromatic amines. Scheme of bacterial azoreductase studies is summarized in Figure 4. However, the physiological role and gene regulation of azoreductase genes remain to be elucidated. Not surprisingly, few reports had indicated that azoreductases may be involved in detoxification due to the fact that some toxic aromatic

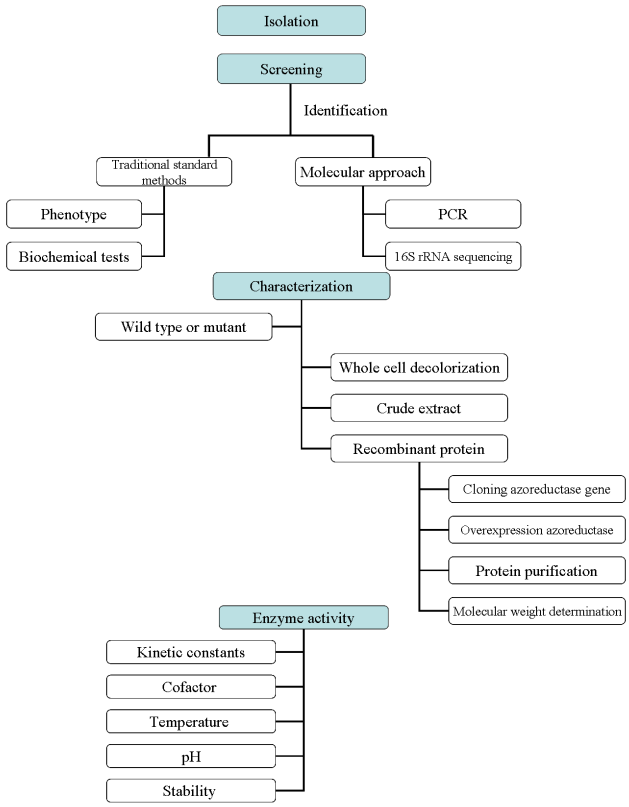

Figure 4. A high level scheme for bacterial azoreductase studies. The isolation of an efficient bacterial strain that decolorizes azo dyes is done by screening. The isolated bacteria are identified by traditional standard methods and/or molecular techniques. To study decolorization efficiency, whole cells, crude extract, and purified recombinant proteins can be used. Wild type and mutant azoreductase can be compared for dye decolorization and other characteristics. Mutant azoreductase is created by site-directed mutagenesis. Recombinant azoreductase is prepared by cloning the azoreductase gene, overexpressing it, and purifying the protein. Native molecular weight is determined by native polyacrylamide gel electrophoresis or size exclusion chromatography. Azoreductase is further characterized by examination of activity for model dye decolorization including kinetic constants, cofactor requirement, optimal temperature and pH, and enzyme stability.

amine intermediates are formed during decolorization. Therefore, understanding the regulation of azoreductase gene expression will lead to the use of gene manipulation of bacterial strains systematically with higher biotransformation in future technologies.

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