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Biodegradation of azo dyes by genetically engineered azoreductase

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Abstract: A azoreductase gene with 537 bp was obtained by PCR amplification from *Rhodobacter sphaeroides* AS1.1737. The enzyme, with a molecular weight of 18.7 kD, was efficiently expressed in *Escherichia coli* and its biodegradation characteristics for azo dyes were investigated. Furthermore, the reaction kinetics and mechanism of azo dyes catalyzed by the genetically engineered azoreductase were studied in detail. The presence of a hydrazo-intermediate was identified, which provided a convincing evidence for the assumption that azo dyes were degraded via an incomplete reduction stage.

Keywords: genetically engineered microorganisms; azoreductase; biodegradation; azo dyes

Introduction

The conventional biological treatment systems often fail to achieve high efficiency in removing refractory compounds. Numerous strategies have been proposed to deal with these toxic compounds, one of which bioaugmentation adding specialized biocatalyst(microorganisms or enzyme) could be a powerful tool to improve treatment performance of these chemicals(Watanabe, 2002; Wang, 2002).

The key of bioaugmentation is to obtain efficient biocatalyst for target pollutant, of which, genetical engineering as an innovative and promising technology has received considerable attention in wastewater treatment (Watanabe, 2002). Although potential ecological safety problems exist, it could be solved by using the products, enzymes or intact cells containing overexpressed enzymes of genetically modified organisms.

Azo dyes are widely used in printing, dyeing and textile for their versatility. Industrial effluents often contain residual dyes, which not only affect water quality, but also become highly toxic and genotoxic. Azo dyes are generally considered to be recalcitrant in conventional wastewater treatment processes (Chung, 1993). It has been found that some microorganisms can transform azo dyes into colorless products, which is often initiated by an enzymatic biotransformation step that involves cleavage of azo linkages with the aid of an azoreductase and an electron donor (Zimmermann, 1984). As the azoreductase in some microorganisms can catalyze the reductive cleavage of azo groups, they have potential advantages in developing biotreatment methods of wastewater loading azo compounds.

A few genes encoding azoreductase were cloned from some bacteria, such as *Bacillus stearothermophilus* OY1-2 (Suzuki, 2001), *Xenophilus azovorans* KF46F (Blumel, 2002) and *Escherichia coli*(Nakanishi, 2001).

Generally, it is assumed that the first step in the biodegradation of azo compounds is the reduction to the corresponding amines, a reaction catalyzed by azoreductase. Then the resulting aromatic amines are further degraded by multiple-step bioconversion, aerobically or anaerobically (Seshadri, 1994; Flores, 1997). Recently, it was found that *o*-aminohydroxybenzenes and *o*-aminohydroxynaphthalenes, common products of the anaerobic reduction of azo dyes, are oxygen-sensitive and can be decomposed under aerobic condition (Kudlich, 1999). It is suspected that a hydrazine might be an intermediate in the reduction of azo dyes to the corresponding amines(Hu, 1994). However, the assumed mechanism needs more evidences.

Our earlier study showed that a non-sulfur phototrophic bacterium, *Rhodobacter sphaeroides* AS1.1737, was able to decolorize several azo dyes(Song, 2003). Recently, we have been cloned a azoreductase gene (GenBank accession number: AY 150311) from *R. sphaeroides* AS1.1737 using PCR-electronic extension method. In order to produce efficient bioaugmentant for azo dyes biodegradation, it is necessary to investigate biodegradation characteristics of genetically engineered azoreductase.

The objective of this paper was to obtain high expression of the gene and to investigate biodegradation characteristics of azo dyes by the genetically engineered azoreductase. As a part of our results, a partially reduced intermediate by mass spectrometry was identified.

1 Materials and methods

1.1 Bacterial strains, plasmids and culture conditions

R. sphaeroides AS1.1737 was obtained from the Culture Collection Center of the Institute of Microbiology of the Chinese Academy of Sciences. It was grown in aerobic medium(Luria Bertani) or anaerobic medium(NH_4Cl 0.1 g, $NaHCO_3$ 0.1 g, K_2HPO_4 0.02 g, $MgSO_4 \cdot 7H_2O$ 0.02 g, NaCl 0.05—0.2 g, peptone 4 g, distilled water 100 ml, pH 7.0) at 30℃. *E. coli* strain BL21(DE3) was used as host strain for recombinant DNA work. Strain BL21 was routinely cultured at 37℃ in LB medium. The plasmid pGEX 4T-1 (Amersham Pharmacia) was used for recombinant expression. PCR fragment recovery kit, restriction endonucleases and T4 ligase were obtained from Takara Biotechnology(Dalian, China).

1.2 Azo dyes

The chemical structures of azo dyes used in this study are shown in Table 1. All these compounds were provided by Dye Synthesis Laboratory, Dalian University of Technology.

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Table 1 The chemical structures of azo dyes

Azo dyes	Structures	λ_{max}, nm
Reactive Brilliant Red K-2BP		540
Reactive Brilliant Red X-3B		544
Methyl Red		430
Orange Ⅱ		482
Acid Red G		531
Acid Red B		513
Methyl Orange		463
Red Dye Ⅰ		525
Red Dye Ⅱ		525
Red Dye Ⅲ		525

1.3 Construction of recombinant plasmid and expression of the azoreductase in *E. coli*

For expression in *E. coli*, the azoreductase gene was inserted in to pGEX 4T-1 under the control of the lac promoter. The DNA segments were amplified by PCR with primers, P1, 5-GAATTC (*EcoR* Ⅰ) ATGAAACTAGTCG-TTATTAAC-3; P2, 5-CTCGAG (*Xho* Ⅰ) TCACTCCAC-TCCTAGTTGT-3. The amplification products were cleaved with *EcoR* Ⅰ and *Xho* Ⅰ, and ligated into pGEX 4T-1. The resulting recombinant plasmid was designated as pGEX-AZR. Strain BL21(DE3) was transformed with the plasmids pGEX-AZR. Positive clones were identified by PCR with primers

P1/P2 and DNA sequencing with primers M13-47/RV-M.

1.4 Preparation of cell extracts

The *E. coli* (pGEX-AZR) cells were suspended in 20 mmol/L sodium phosphate buffer (pH 7.0) and lysed by freezing and thawing followed by sonication. Cell debris was removed by centrifugation (9000 g, 30 min at 4℃). Protein concentration was determined by the modified Lowry protein assay (Markwell, 1978), using bovine serum albumin as the standard. The cell extracts of *R. sphaeroides* AS1.1737 was obtained with the method described previously (Song, 2003).

1.5 Purification of recombinant azoreductase

Recombinant azoreductase was purified by Glutathione Sepharose TM 4B (Amersham Pharmacia Biotech) (Smith, 1988). The *E. coli* BL21 (pGEX-AZR) cell extracts corresponding to 100 ml of culture medium (1—2 g of cells) were applied onto a glutathione-agarose column. Approximately 1 ml of sedimented bed was used for typical preparation. The cell extracts were passed three times through the column to ensure maximum binding of the fusion protein. After being eluted with 10 mmol/L reduced glutathione buffer and hyperfiltrated, purified fusion azoreductase was obtained. Non-fusion azoreductase was obtained by digestion of fusion azoreductase with thrombin. Purified enzymes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Park, 1997).

1.6 Determination of enzyme activities with cell extracts and purified enzyme

The total volume of the reaction mixture was 2.0 ml. If not noted otherwise, the mixture contained 40 μmol sodium phosphate buffer (pH 8.0), 200 nmol Methyl Red and 0.5 μg purified non-fusion azoreductase. The reaction mixture was preincubated at 40℃ for 10 min in a cuvette that was placed inside an UV-vis Spectrophotometer V-560 (JASCO, Japan). The temperature of the cuvette holder was held constant by means of circulating thermostatic oven. The decolorization experiments were performed under static condition (i.e., neither aeration nor agitation was employed). The typical dissolved oxygen (DO) level in the static systems was approximately 0.1—0.4 mg/L. The reaction was started by adding 40 μl 10 mmol/L NADH, and was spectrophotometrically assayed at 430 nm, with the corresponding buffer as control. The enzyme activity was defined as the amount of reduced dyes by per mg protein per minute. Each experiment was repeated five times, and the means of these data were statistically analyzed. Kinetics parameters of recombinant azoreductase were determined by varying the concentration of Methyl Red or NADH in the presence of a constant concentration of NADH or Methyl Red, respectively. Enzyme activities of cell extracts were measured with 1 μg lysates of *E. coli* (pGEX-AZR) or that of *R. sphaeroides* AS1.1737 in the reaction system described above. To determine the effect of DO on decolorization, the azoreductase activities were measured under aerobic (with agitation), static and anaerobic conditions. The anaerobic reaction was performed in gastight cuvettes by gassing with N_2. The effects of temperature and pH were studied at different temperatures (20—90℃) and pH values (5.0—9.0). The non-fusion azoreductase activities with other azo dyes were also investigated. Azo dye-reducing activity was analyzed by measuring the decrease of optical density at corresponding λ_{max} and calculated with the relevant molar extinction coefficients.

1.7 HPLC-MS method

Methyl Red (0.1 mmol/L) was reduced at 40℃ with purified non-fusion azoreductase. The enzyme was removed by hyperfiltration. The products were then analyzed with a HPLC-MS system equipped with a ODSC$_{18}$ column (2.0 mm × 250 mm) for liquid chromatography and a Finnigan LCQ^{DUO} ion trap mass spectrometer for mass spectrometry. The mobile phase was composed of A (94.4% acetonitrile + 0.6% formic acid + 5% water) and B (5% acetonitrile + 0.6% formic acid + 94.4% water), and gradient elution consisted of 10% A to 30% A over 30 min at a flow rate of 0.25 ml/min. Compound confirmation was achieved by MS/MS using m/z 272 as the parent ion. Finnigan LCQ^{DUO} ion trap mass spectrometer worked under the following conditions: electrospray ionization source (+ESI), spray voltage 4.5 kV, sheath-gas 40 ml/min, Aux-gas 10 ml/min, and MS/MS collision energy 27%.

2 Results and discussion

2.1 Construction of recombinant plasmid and expression of the azoreductase in *E. coli*

The cloned azoreductase gene (GenBank accession number: AY 150311) from *R. sphaeroides* AS1.1737 contains 537 bp with a complete open reading frame (ORF). Construction strategy for expression plasmid pGEX-AZR is shown in Fig.1. Strain BL21 (DE3) was transformed with the plasmids pGEX-AZR. Clones with recombinant plasmid were identified by PCR with primers P1/P2 and DNA sequencing with primers M13-47/RV-M. Fig.2 and Fig.3 show expression activity of crude recombinant azoreductase. SDS-PAGE analysis revealed a large amount of recombinant azoreductase in the *E. coli* BL21 (pGEX-AZR) cell extracts (Fig.2). Red dye I was decolored completely in 5 min (Fig.3), while *E. coli* BL21 had no activity for Red dye I (data not shown). The azoreductase activity towards Acid Red B in the *E. coli* BL21 (pGEX-AZR) cell extracts and in that of *R. sphaeroides* AS1.1737 was 5.21 and 0.41 μmol/(mg protein

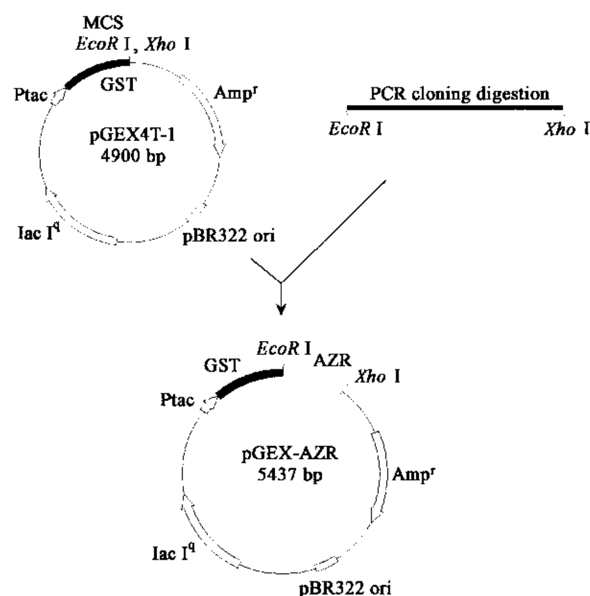

Fig.1 Construction strategy of expression plasmid in *E. coli*

· min), respectively, The former was 12 more times than the latter.

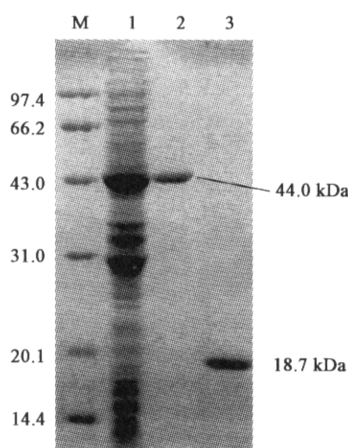

Fig.2 SDS-PAGE of azoreductase
Lane M: protein markers; lane 1: cell extract(1.5 μg); lane 2: purified GST-azoreductase(70 ng); lane 3: azoreductase(0.2 μg).

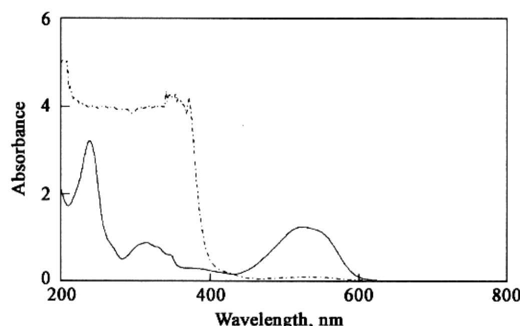

Fig.3 Spectral scans (200—800 nm) of the Red Dye I solution.
— Before reaction; ---- after reaction for 300 s

2.2 Effect of dissolved oxygen on azoreductase activity

The non-fusion azoreductase activities for Methyl Red under aerobic condition(DO = 4.38 mg/L), static condition (DO = 0.1 mg/L) and anaerobic condition, were 48.8, 48.9 and 51.2 μmol/(mg protein · min), respectively. These results suggested that the azoreductase was insensitive to oxygen. Thus, the following experiments were all performed under static conditions.

2.3 Effect of temperature and pH on azoreductase activity

The effects of temperature on the recombinant azoreductase activity are shown in Fig. 4. The optimum temperature for GST-azoreductase was 40℃ (79.1 μmol/(mg protein · min)), while it was 50℃ (57.3 μmol/mg protein · min)) for non-fusion azoreductase. The maximal specific activity of GST-azoreductase was 38% higher than that of non-fusion azoreductase. The effects of pH on the activity of two azoreductases were also examined for pH values ranging from 5.0 to 9.0 and the maximal activity occurred at pH 8.0 (Fig. not shown).

2.4 Kinetic analysis

Double-reciprocal plots of initial velocity versus concentration of NADH or Methyl Red resulted in parallel lines, which of nonfusion azoreductase are shown in Fig.5 and Fig.6. Such patterns suggested that the azoreductase catalysis corresponded to a pingpong mechanism, which was

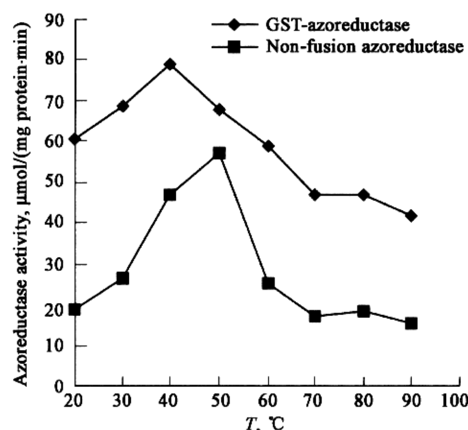

Fig.4 Effect of temperature on GST-azoreductase and nonfusion azoreductase activity

consistent with the mode of ACP phosphodiesterase responsible for azo reduction (Blumel, 2002). However, a flavin cofactor was required by ACP phosphodiesterase (Blumel, 2002), which should belong to a different protein family. This mechanism can be described using the following equation:

$$\nu = (V_{max}[A][B])/(K_{mA}[B] + K_{mB}[A] + [A][B]), \quad (1)$$

where K_{mA} and K_{mB} are the Michaelis-Menten constants for Methyl Red and NADH, respectively. $[A]$ is the concentration of Methyl Red, $[B]$ is the concentration of NADH, and ν is the initial velocity. Michaelis-Menten constants were determined according to Lineweaver-Burk. The calculation showed that the kinetics parameters for non-fusion azoreductase was K_{mA} = 0.42 mmol/L and K_{mB} = 2.3 mmol/L, and that for GST-azoreductase was K_{mA} = 0.28 mmol/L and K_{mB} = 2.1 mmol/L. The V_{max} for nonfusion azoreductase was 65.2 μmol/(mg protein · min), and that for GST-azoreductase was 87.3 μmol/(mg protein · min).

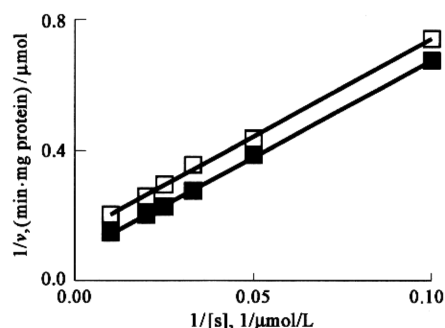

Fig.5 Double-reciprocal plot of initial velocity (ν) versus concentration of Methyl Red[s] with constant initial NADH concentration (■: 250 μmol/L, □: 500 μmol/L)

2.5 Azoreductase activities with different azo dyes as substrates

The activities of non-fusion azoreductase with different azo dyes were determined and are shown in Fig.7. All the tested azo dyes could be decolorized by the azoreductase. Among these azo dyes, Orange II has the highest reduction rate of 82.9 μmol/(mg protein · min) at concentration of 200 μmol/L, whereas X-3B has the lowest rate of 16.2 μmol/(mg

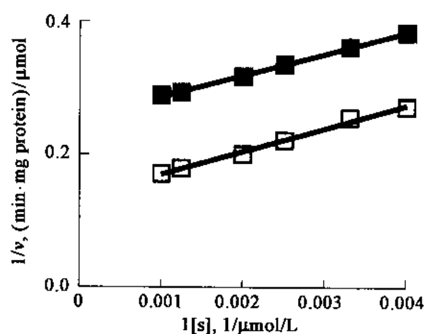

Fig.6 Double-reciprocal plot of initial velocity (ν) versus concentration of NADH [s] with constant initial Methyl Red concentration (■: 20 μmol/L, □: 40 μmol/L)

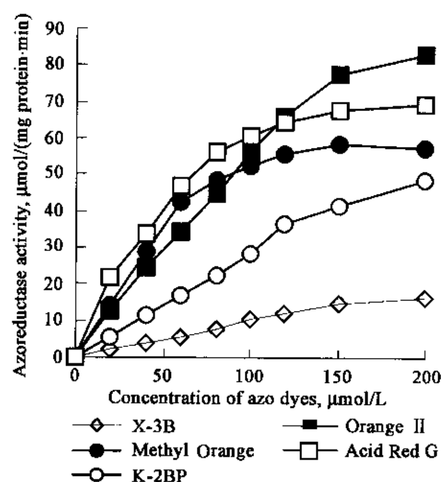

Fig.7 Azoreductase assays with different azo dyes as the substrates

protein · min) at the same concentration. The results suggested that the simpler the structures of azo dyes were, the easier they were degraded. In the same reaction system, with 50 μmol/L Red Dye I, Red Dye II or Red Dye III as substrates, the effect of the molecular weight on the reduction of a series of azo dyes was analyzed. The specific enzyme activities with Red Dye I, Red Dye II and Red Dye III were 20.3, 12.6 and 1.1 μmol/(mg protein·min), respectively. This result indicates that, given that the structures of dyes were similar, the larger the molecule mass was, the more difficult the biodegradation would be.

2.6 Biodegradation mechanism analysis

Hu pointed out that decolorization of an azo dye(Red G) by *P. luteola* might involve the incomplete reduction of the azo compound to a hydrazine(Hu, 1994). It seems that the biodegradation of azo dyes under reductive condition proceeds via an incomplete reduced intermediate. This hypothesis was also proposed by Nakanishi for Methyl Red reduction based on stoichiometry calculations(Nakanishi, 2001). However, the assumed intermediate product has never been clearly observed and analyzed so far. Here, we report supporting evidence for such an intermediate.

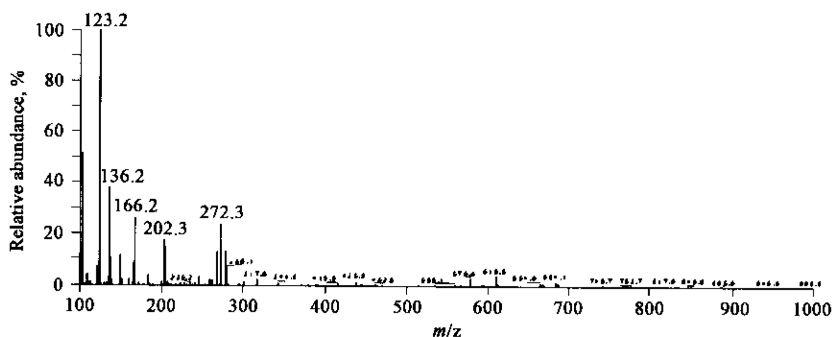

Fig.8 Mass spectra of retention time from 3.01 to 3.57 min

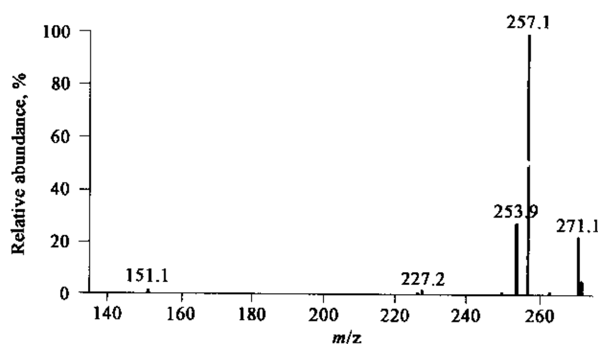

Fig.9 Mass spectra of m/z 272

HPLC analysis gave peaks for the complete reducing products, N; N'-dimethyl-*p*-phenylenediamine (retention time 2.51—3.01 min) and 2-aminobenzoic acid (retention time 8.45—9.27 min). These two compounds were identified by mass spectra (data not shown). A substance with a mass-to-charge ratio of 272 was observed in mass spectra, which corresponded to a peak(retention time 3.01—3.57 min) in HPLC(Fig.8). It was separated and analyzed by ion trap mass analyzer. Compound confirmation was achieved by MS/MS using m/z 272 as the parent ion. Collision-induced dissociation reactions for this partially reduced intermediate save several fragmen tions. They were $[M+H]^+$, $[M]^+$, $[M+1-CH_3]^+$, $[M+1-H_2O]^+$, $[M+1-COOH]^+$ and $[M+1-\text{benzoic acid}]^+$, with molecular weight 272, 271, 257, 254, 227 and 151, and abundances 5%, 22%, 100%, 28%, 2% and 2%, respectively(Fig.9). The data agree with the hypothesis that the intermediate was the hydrazine from the reduction of Methyl Red. These results provide additional evidences for the assumed mechanism of azo dyesbiodegradation(Fig.10). They are a strong indication for existence of hydrazointermediate. This experiment was repeated several times. We found it difficult to detect the hydrazo-product during the reduction process of Methyl Red, for its abundance was always low in mass spectra. This experience

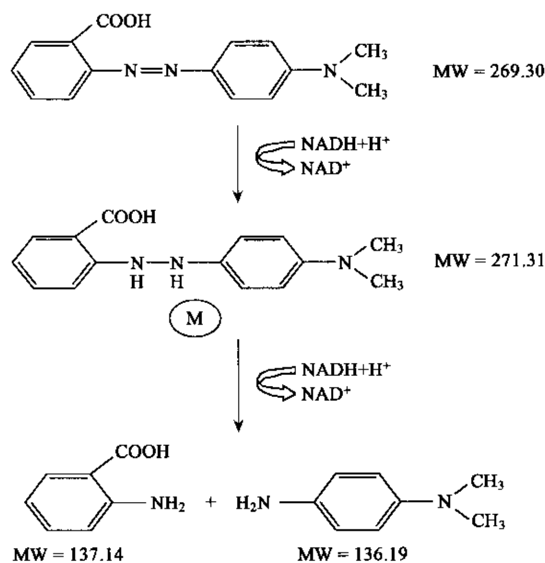

Fig.10 The mechanism for azo dyes biodegradation. M, partially reduced intermediate

suggested that step 1 (from azo dye to hydrazoproduct) might be a limiting process. The partially reduced intermediate was not stable, being reduced quickly after forming.

References:

Blumel S, Knackmuss H, Stolz A, 2002. Molecular cloning and characterization of the gene coding for the aerobic azoreductase form *Xenophilus azovorans* KF46F[J]. Appl Environ Microbiol, 68: 3948—3955.

Chung K T, Stevens J S, 1993. Degradation of azo dyes by environmental microorganisms and helminths[J]. Environ Toxicol Chem, 12: 2121—2132.

Flores E R, Luijten M, Donlon B A *et al*., 1997. Complete biodegradation of the azo dye azodisalicylate under anaerobic conditions[J]. Environ Sci Technol, 31: 2098—2103.

Hu T L, 1994. Decolourization of reactive azo dyes by transformation with *Pseudomonas luteola*[J]. Bioresour Technol, 49: 47—51.

Kudlich M, Hetheridge J M, Knackmuss J H *et al*., 1999. Autoxidation reactions of different aromatic *o*-aminohydroxynaphthalenes that are formed during the anaerobic reduction of sulfonated azo dyes[J]. Environ Sci Technol, 33: 895—901.

Markwell M A, Hass S M, Buber L *et al*., 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples[J]. Anal Biochem, 87: 206—210.

Nakanishi M, Yatome C, Ishida N *et al*., 2001. Putative ACP phosphodiesterase gene(acpD) encodes an azoreductase[J]. J Biol Chem, 276: 46394—46399.

Park C S, Chang C C, Kim J Y, 1997. Expression, secretion, and processing of rice amylase in the yeast *Yarrowia lipolytica*[J]. J Biol Chem, 272: 6876—6881.

Seshadri S, Bishop P L, Agha A M, 1994. Anaerobic/aerobic treatment of selected azo dyes in wastewater[J]. Waste Manage, 14: 127—137.

Smith D B, Johnson K S, 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione *s*-transferase[J]. Gene, 67: 31—40.

Song Z Y, Zhou J T, Wang J, 2003. Decolorization of azo dyes by *Rhodobacter sphaeroides*[J]. Biotechnol Lett, 25: 1815—1818.

Suzuki Y, Yoda T, Ruhul A *et al*., 2001. Molecular cloning and characterization of the gene coding for azoreductase from *Bacillus* sp. OY1-2 isolated from soil[J]. J Biol Chem, 276: 9059—9065.

Wang J L, Quan X C, Wu L B, 2002. Bioaugmentation as a tool to enhance the removal of refractory compound in coke plant wastewater[J]. Process Biochemistry, 38: 777—781.

Watanabe K, Teramoto M, Harayama S, 2002. Stable augmentation of activated sludge with foreign catabolic genes harboured by an indigenous dominant bacterium[J]. Environmental Microbiology, 4: 577—583.

Zimmermann T, Gasser F, Kulla H G *et al*., 1984. Comparison of two bacterial azoreductases acquired during adaptation to growth on azo dyes[J]. Arch Microbiol, 138: 37—43.

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