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Cloning and characterization of a robust recombinant azoreductase from *Shewanella xiamenensis* BC01

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ABSTRACT

Increasing interest in bio-treatment of azo dyes effluent requires more efforts on azoreductase exploration, which involves in the biodecolorization and bioremedimation. In this study, a full-length gene of 594 bp, *azoR*, encoding an aerobic azoreductase of 198 amino acids (designated as AzrS) was isolated from a *Shewanella xiamenensis* BC01. AzrS was heterologous recombinant into vectors of pET32a, pET28a or pET20bI, and further expressed in *E. coli* BL21(DE3) for the first time. The optimal condition was achieved using the pET28a vector, as more than 90% protein was soluble (i.e., 278 mg/L). AzrS was an oxygen-insensitive, FMN-dependent, robust against to organic solvent and had extremely high activity with stability at room temperature. It occupied strong activities in multiple substrates as a ranking of methyl red > methyl orange > congo red, that was consistent with the results of molecular docking. In conclusion, AzrS showed good potential for bioremediation, due to its high over-expression in *E. coli*, non-sensitivity to oxygen, and with robust activity against metals and solvents.

1. Introduction

Azo dyes are synthesized through the diazotization of aromatic amines and divided into monoazo, diazo, trisazo and polyazo dyes depending on the number of azo bonds (-N=N-), which have an extensive range of applications in the textile, cosmetic, plastic, food and pharmaceutical industries due to their chemical stability, ease of synthesis and versatility [1]. However, the azo dyes released into environment as effluent is not only cause pollution, but also will be a toxic and/or mutagenic element to many organisms [2]. The traditional physical and chemical methods, such as adsorption, precipitation and filtration would concentrate and remove azo dyes from a large amount of effluent. But both approaches have also introduced the secondary pollutant due to the abundant chemical involve in the process. An increasing numbers of researchers are working to establish a biological system via utilizing microbial or enzymatic decolorization under mild conditions to overcome the shortage of physical and chemical treatment of azo-dyes [3–5].

In the past 30 years, a number of bacteria capable of transforming various azo dyes into colorless metabolites have been isolated in gram-positive strains, such as, *Brevibacillus laterosporus* [3], *Enterococcus* [6,7], *Bacillus* [8–11] and *Geobacillus* [12] species and gram-negative strains such as *E. coli* [2], *Xenophilus* [13], *Rhodobacter* [14], *Pigmentiphaga* [15], *Shewanella* [16–18] species. Such strains are favorable for the development of biodegradation processes for azo dyes, and the microbial degradation of such dyes is commonly initiated by the reduction of the azo bonds, which is catalyzed by a group of NAD(P)H-dependent azoreductases (EC 1.7.1.6) with requiring of FMN or FAD as a cofactor [19]. Azoreductases can broadly be divided into two groups depending on cofactor requirement. The one is monomeric flavin-free enzymes containing a putative NAD(P)H binding motif [13,16], while the other is polymeric flavin-dependent enzymes [2,5]. Flavin-dependent azoreductase are further classified into NADH-preference, NADPH-preference and NAD(P)H enzymes. On the other hand, azoreductases also can be classified on requirement of oxygen. Most bacterial azoreductases are oxygen-insensitive, except for the azoreductase AzoC from a strictly anaerobic bacterium *Clostridium perfringens* [19]. To date, around 30 azoreductases obtained from bacteria have been verified for their enzymatic activity, and most of them were heterologously expressed in *E. coli*.

Recently, a novel bacterium, *S. xiamenensis* BC01, obtained from sediment collected near Xiamen, China, was reported to have

Abbreviations: BSA, bovine serum albumin; DMF, dimethyl formamide; DMSO, dimethyl sulfoxide; IAA, iodoacetamide; IPTG, isopropyl-β-D-thiogalactoside; POE-PCR, prolonged overlap extension PCR.

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a distinctive biodecolorization capability with regard to various azo dyes, especially methyl orange, reactive red 198, and recalcitrant dye congo red [20]. Moreover, *S. xiamenensis* BC01 was found to produce nanowires and nanoparticles under aerobic conditions [21]. To uncover the mysteries behind these properties of *S. xiamenensis*, enzymatic examinations of seven oxidoreductases, namely catalase, manganese peroxidase, laccase, NADH dehydrogenase, flavin reductase, azoreductase and Fe reductase, were carried out in our previous study [21]. *S. xiamenensis* showed the highest catalase activity but lowest azoreductase activity among the studied oxidoreductases. To explore the mechanism of decolorization and the potential for industrial applications of *S. xiamenensis* or its azoreductases, the azoreductase from *S. xiamenensis* BC01 was overexpressed in *E. coli* and biochemically characterized in this study. Due to many kinds of metal ions and organic solvents that exist in azo dye waste water, the effects of metal ions and organic solvents on AzrS activity and stability were determined. In addition, the dynamic parameters of AzrS with regard to methyl red, methyl orange and congo red were determined. Finally, the molecular docking was used to analyze the distance between enzyme and different substrates in order to characterize the affinities.

2. Materials and methods

2.1. Bacterial strains, plasmids, PCR primers and culture conditions

The wild-type strain *S. xiamenensis* BC01 has been deposited in the Bioresources Collection and Research Center as BCRC80598 [20]. It was routinely maintained in Luria–Bertani (LB) agar plate and grown LB medium at 30 °C and 150 rpm. The *E. coli* BL21(DE3) strain used for the cloning procedure of the plasmid, was grown in LB medium at 37 °C and 200 rpm, supplemented with ampicillin (100 μg/mL) or kanamycin (50 μg/mL) when needed. Plasmids pET32a and pET20bI were provided by Prof. Po Ting Chen in Southern Taiwan University of Science and Technology, Taiwan. Plasmid pET28a was provided by Prof. Yun-Peng Zhao in Feng Chia University, Taiwan. A list of detail information of all bacterial strains, plasmids and PCR primers used in this study is given in Table S1.

2.2. Cloning and construction of recombinant azoR in E. coli

The sequence of *azoR* was searched from the whole genome sequence of *S. xiamenensis* BC01 (Accession No., JGVI00000000) [22]. Genomic DNA was extracted from an overnight cultured broth of *S. xiamenensis* BC01 using the Bacterial Genomic DNA Miniprep Kit (Axygen). Recombinant *azoR* plasmids with different vectors of pET32a, pET28a and pET20bI were constructed using the "Simple Cloning" method, as previously described [23]. The procedure (Fig. 1) included three steps: (i) The insert gene BC01-*azoR* and linear vector (i.e., pET32a, pET28a or pET20bI) with two overlap ends were generated by regular high-fidelity PCR with primer pair IF/IR and VF/VR (Fig. 1B), respectively. (ii) The DNA multimers were generated based on using the equimolar insert gene and linear vector as template by applying prolonged overlap extension PCR (POE-PCR) without the addition of primers. (iii) Finally, the POE-PCR product was directly transformed into chemical competent *E. coli* BL21(DE3), yielding the desired plasmids pET32a-BC01-*azoR* (Fig. 1C), pET28a-BC01-*azoR* (Fig. 1D) and pET20bI-BC01-*azoR* (Fig. 1E). The DNA polymerase used in the above PCR was the New England BioLabs (NEB, UK) phusion polymerase.

2.3. Heterologous expression of recombinant azoreductase

The recombinant strains were inoculated in 5 mL Luria–Bertani (LB) medium supplemented with the corresponding antibiotics and cultured overnight. This seed culture was then diluted 1:100 into a 250-mL flask containing 50 mL fresh LB medium supplemented with the corresponding antibiotics. When the optical density at 600 nm ($OD_{600\ nm}$) reached to 0.6 ~ 0.8, 0.1 mM isopropyl-β-D-thiogalactoside (IPTG; Sangon Biotech, Shanghai, China) was added and incubated for another 5 h.

2.4. Protein preparation and SDS-PAGE analysis

The cells were collected and washed twice with 50 mM phosphate buffer (pH 7.0) via centrifugation at 4 °C, 8,000 × g for 10 min. The cell pellets were suspended in the same buffer and then disrupted via sonication at 125 W for 5 min (5 s on and 10 s off). Different samples were adjusted to the same $OD_{600\ nm}$. The supernatant was collected by centrifugation at 13,000 × g for 15 min under 4 °C and used to determine azoreductase activity, then the cell debris (i.e. pellet) was resuspended with same volume of 50 mM phosphate buffer (pH 7.0). The supernatant and pellet were used to visualize the expression result through SDS-PAGE, which was prepared with 0.1% SDS in 12% separating gel and 4% stacking gel. Tris-glycine buffer (pH 8.3) containing 0.1% SDS was used as the electrode buffer. Samples were treated with loading buffer (Solarbio, Beijing, China) and heated at 100 °C for 10 min prior to application to the gel. The concentration of the protein AzrS (i.e. protein amounts from band intensity) was determined by densitometry using Quantity One 4.6.2 analysis software (Biorad, USA) with bovine serum albumin (BSA) as the standard.

2.5. Purification of recombinant AzrS

Bacterium culture with recombinant AzrS was collected by centrifugation at 12,000 × g for 10 min at 4 °C and washed with deionized water twice. Cells were resuspended in a sodium phosphate buffer (pH 7.4) and disrupted by sonicator. The cell debris was removed by centrifugation at 14,000 xxg for 15 min at 4 °C and the supernatant was collected. The supernatant was applied to a His-Trap affinity chromatography column, (GE, Healthcare Biosciences AB, Sweden). Fractions containing recombinant AzrS were eluted with 250 mM imidazole and 300 mM NaCl made up in sodium phosphate buffer (pH 7.4).

2.6. Enzyme assay for azoreductase activity

Azoreductase activity was monitored by the decrease in absorbance of methyl red at 430 nm with a spectrophotometer (VersaMax™ microplate reader M2, Molecular Devices, CA). The 200 μl reaction mixture in a 96-well microplate contained 50 mM phosphate buffer (pH 6.5), 150 μM methyl red, 0.5 mM NADH and an appropriate amount of enzyme. The reaction was initiated by addition of NADH. All measurements were conducted in quadruplicate. One unit of azoreductase activity was defined as the amount of the azoreductase that catalyzed the reduction of 1 μM of methyl red per minute. The concentration of methyl red was calculated using the extinction coefficient of 23,360 $M^{-1}cm^{-1}$.

2.7. Effects of cofactors on azoreductase activity and phylogenetic analysis

Various concentrations of NADH or NADPH between 0.125 and 0.875 mM were used to test the preference for cofactors of AzrS. When 0.5 mM NADH was added, various concentrations of FMN