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# EXPRESSION, PURIFICATION, AND ENZYMATIC CHARACTERIZATION OF TWO RECOMBINANT AZOREDUCTASES OF *BACILLUS* SP. B29

T. Ooi\*, T. Shibata\*, S. Kinoshita\*, S. Taguchi\*, and TL Thuoc\*\*

\* Division of Biotechnology and Macromolecular Chemistry, Graduate School of Engineering, Hokkaido University, Sapporo, 060-8628, Japan

\*\* Faculty of Biology, University of Science, Vietnam National University-HCMC, Vietnam

## ABSTRACT

Two recombinant azoreductases (Azr8 and Azr18) were purified to homogeneity from the cell extracts of *Escherichia coli* transformants containing *azr8* and *azr18*. Both azoreductases were homodimer of identical subunits of 211 aa (Azr8) and 208 aa (Azr18). Both were flavoproteins, each containing 1 mol of FMN per mol of subunits. NADH functioned as electron donor for the azoreductases. Both recombinant azoreductase showed higher activity to Methyl Red and showed different substrate specificities.

## KEYWORDS

Azoreductase, Azo dyes, *Bacillus* sp. Decolorization

## INTRODUCTION

Synthetic water-soluble azo dyes are the most common colourants, which are characterized by the presence of azo group. The largest class of dyes in industrial use is known as azo dyes in number and amount produced (Griffiths 1984). Most of the azo dyes and their metabolic intermediates have been proved to be genotoxic. Therefore, azo dyes are of significant concern as pollutants of the environment. It is now well-established that the reductive cleavage of the azo linkage is an important pathway in the metabolism of azo dyes. One of the most interesting approaches of the ability of the bacteria is to promote the degradation of these compounds. During the course of our research, we have isolated a bacterial strain, identified as *Bacillus* sp. B29 belonging to *B. cereus* group, that highly decolorized the toxic azo dye methyl red (MR) from soil samples by an enrichment culture. The *B. cereus* genome encodes at least four annotated azoreductase genes, an azoreductase and other three FMN-dependent NADH azoreductases, but there is no information about enzymatic characteristics of their encoded proteins (Ivanova *et al*, 2003). Here we described the cloning, expression and the purification to homogeneity of two recombinant azoreductases to analyze to analyze enzymatic characterization.

## MATERIALS AND METHODS

### *Isolation and identification of MR-decolorizing bacterium*

The screening was carried out to isolate the MR-decolorizing by repeated enrichment culture using the medium containing MR. The growth and decolorization were monitored visually and the aliquot of the cultures showing efficient decolorization of MR was streaked onto a agar plate. An isolate strain was identified as *Bacillus* sp. B29 based on 16S rDNA sequence.

### *Cloning of two azoreductase genes from *Bacillus* sp. B29*

The nucleotide sequence of a predicted azoreductase genes of *B. cereus* strain ATCC 14579 were obtained from DDBJ database (<http://srs.ddbj.nig.ac.jp/index-j.html>, accession number AE017015). Genomic DNA from *Bacillus* sp. strain B29 was isolated essentially as described elsewhere (Ausbel et al. 1987). Amplification of the gene was performed by PCR. Two oligonucleotide primer pairs were designated to amplify the each azoreductase genes (*azr8* and *azr18*). The PCR products amplified from the genomic DNA were directly cloned into the pGEM-T vector (Promega, Madison, WI, USA) and nucleotide sequences were determined. For heterologous expression of both *azr8* and *azr18* in *E. coli*, pET3a was used as an expression vector and *E. coli* BL21 (DE3) pLysS (Novagen, Madison, WI, USA) as a host strain.

### *Enzyme assay for the activity and protein measurement*

Azoreductase activity was assayed by measuring the decrease in optical density at suitable wavelengths. The extinction coefficients and absorption maxima of all azo dyes tested in this study are referred as described by Green (1990). The standard reaction mixture containing 25 mM Tris-HCl buffer (pH 7.4), 25  $\mu$ M MR, 100  $\mu$ M NADH, and suitable amount of enzyme in 3 ml of reaction mixture, was incubated at 30°C. Reaction mixture without MR was preincubated for 3 min and MR decolorization was followed by monitoring initial rate of the decrease in absorbance at 430 nm. One unit of enzyme activity was defined as the amount of enzyme required to decolorize 1  $\mu$ mol of dye per min under the assay conditions. Protein concentration was measured by the Bradford method, using a bovine serum albumin as the standard (1976). During the chromatographic purification steps, protein concentration in the fractions was monitored by measuring its absorbance at 280 nm.

### *Purification of recombinant azoreductases*

Recombinant *E. coli* having expression vector (pET3a-*azr8* and pET3a-*azr18*, respectively) were grown in LB medium with shaking. After induction by IPTG, growing cells were harvested and disrupted by sonication. Resulting crude extract was applied onto a DEAE-cellulose column. After washing the column, the protein was eluted by a linear gradient of NaCl. Active fractions were brought to 20% saturation with ammonium sulfate, and then applied to a Buthyl toyopearl column for Azr8 and Phenyl toyopearl column for Azr18. Elution was carried out a liner gradient of ammonium sulfate concentration (20% to 0%). Active fractions were concentrated and then applied

to a Sephadex S-200 column. Active fractions were pooled and used as a purified enzyme.

## RESULTS AND DISCUSSION

### Cloning of two azoreductase genes

Two azoreductase genes corresponding to a predicted azoreductase genes from *B. cereus* ATCC 14579 (accession number AE016877), designated *azr8* and *Azr18*, were amplified from *Bacillus* sp. B29 genomic DNA by PCR, using a pair of oligonucleotides, to yield an about 1 kbp DNA bands on agarose gel (data not shown). Nucleotide sequence revealed that each DNA fragment contained a complete ORF encoding a protein (Azr8 and Azr18). The *azr8* encoded a protein consisting of 211 amino acids, while *azr18* encoded a protein consisting 208 amino acids. Nucleotide sequences of *azr8* and *azr18* showed very high identity to those of corresponding genes from *B. cereus* ATCC 14579 (data not shown).

Deduced amino acid sequences of Azr8 and Azr18 showed the homology to those of azoreductases from *E. coli* AcpD (Nakanishi *et al*, 2001) and from *Enterococcus faecalis* AzoA (Chen *et al*, 2004).

Azr6:	MSKVLFVKANDRPAEQAVSSKMYETEVSTVKEANENTEITELD
Azr8:	MTKVLFITANPNSAEGSGFGMAVGEEAFIEAYKNEHPODEVVTTID
Azr18:	MAIVLFLVKANNRPAEQAVSVKLYEAEELANVKEANPNDTIVVLD
AcpD:	MSKVLVLKSSILAGY-SQSNQLSDYEVQWRK-HSADEITVRLD
AzoA:	SLLVVHPLTKESRVRALTTLASRETNSIEIL
Azr6:	LFALDLPYYGNIAIS-----GGYNSSQGME-LTAEEEKAATVTD
Azr8:	LFNTTVPIDADEVFA---AWCKFAAGEGFEAIIIEVQQQKVAAMN
Azr18:	LYKEELPYVGVDMIN-----GTFKVGKCFD-LTEEEAKAVAVAD
AcpD:	LAANPIPVLDGELV-----GALRPSDAP--LTPRQQEALALSD
AzoA:	VYAPETNMPEIDEELLSAWCALRAGAAFETISENQQQKVARFN
Azr6:	QYIINOFLADKVVEAFELWNFTVPPALIITYISYLSQAGKTFKY
Azr8:	TNLETFMNADRYVEVTMWNFSYPPVVKAYLDNVIAAGKTFKY
Azr18:	KYIINOFLADKVVEGFPLWNLTIPAVIHTYIDYLNRAAGKTFKY
AcpD:	ELIAELKAHDVIVITAAEMYNFNISTOKNEDLVARAGVIFRY
AzoA:	ELTDQELSADKVWIANPMWLNVPTRIKAWVDTINVAGKTFQY
Azr6:	TANGPEGLVGGKKVVVLGARGSDYSSQMAPMEMAVNVTTVIL
Azr8:	TENGPVGLLEGKKKALHIQATGGVYSEGAYTAVDFGRNHLKTVI
Azr18:	TPEGPVGLTGDKKKIALLNARGGVYSEGPAEEVEMAVKVASMM
AcpD:	TENGPVGLTGGKAIVITSRGGI--HKD-GPTDLVTPVLSFTI
AzoA:	TAEGPKPLTSGKKALHIQSNGFVEGKDFAS-Q-----MIKAIT
Azr6:	GFWGICTNPETVVIECHNQYPDRSOOTVEEGLEENVKKVIAKF*
Azr8:	GEVGVNDTEYIAVECMNNAPKKAQEIKEAAAIANARELAKRF*
Azr18:	GFFGATNMETVVIECHNQFPDKAEEITIAAGLEEEAKVLSKE*
AcpD:	GGIGITDVVKVFAEGIAYGPEMAKAQOSDAKAIAIDSIVSA*
AzoA:	NEIGVQDVQDGLFIEGIDHFPDRAEELLNTAMTKATEYGKT*

Azr6 : *Bacillus* sp. B29

Azr8: *Bacillus* sp. B

Azr18: *Bacillus* sp. B29

AcpD: *E. coli*

AzoA: *E. faecalis*

Fig. 1 Alignment of bacterial azoreductases

### Purification and Enzyme characterization of recombinant Azr8 and Azr18

Two recombinant azoreductases (Azr8 and Azr18) were purified homogeneity with high recovery (43% for Azr8 and 40% for Azr18, Table 1). The Mrs of the Azr8 and Azr18 under denatured conditions were estimated to be 23 kDa. The Mrs of the native form of the both Azr8 and Azr18 were calculated to be 48 kDa by size exclusion chromatography, indicating that recombinant AzrA exists as a homodimeric protein (Data not shown).

The purified Azr8 and Azr18 exhibited typical flavoprotein absorption maxima at 377 and 463 nm.

HPLC analyses of released flavin from the proteins indicated that the enzymes contained FMN. Quantitation analysis of FMN and protein, it was calculated that both Azr8 and Azr18 contained 2 mol of FMN /mol subunit. (Table 2)

Table 1. Purification summaries of Azr8 and Azr18 from recombinant *E. coli*

Strps	Protein (mg)	Total activity (U)	Specific act. (U/mg)	Recovery (%)
<b>Azr8</b>				
Crude enzyme	5,600	5,810	1.04	100.0
DEAE cellulose	654	3,750	5.73	64.5
Butyl toyopearl	240	3,370	14.0	58.0
Sephacryl S-200	65.8	2,520	38.3	43.3
<b>Azr18</b>				
Crude enzyme	8,290	5,430	0.64	100.0
DEAE cellulose	1,330	4,870	3.66	91.1
Butyl toyopearl	188	3,160	16.8	59.2
Sephacryl S-200	81.1	2,140	26.4	40.0

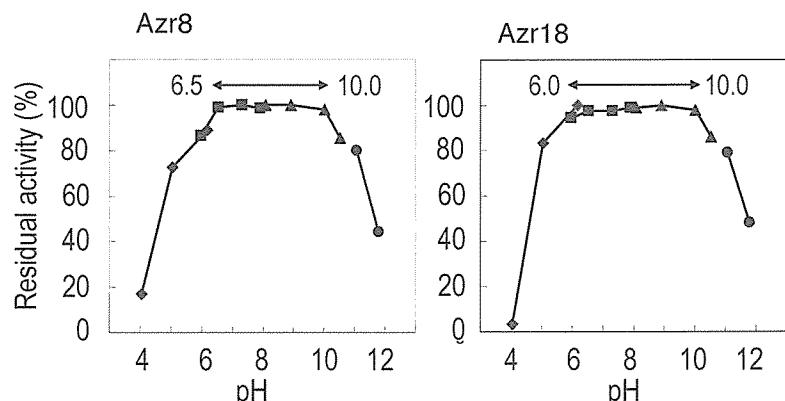
Table 2. Quantitation of FMN extracted from Azr8 and Azr18

	mol/mol monomer protein	
	Azr8	Azr18
FMN	0.96	1.13

Analysis of coenzyme requirement for the azoreductase activity showed that NADH promoted 25 times higher than NADPH for the reduction of MR. (data not shown). The capability of azoreductases to reduce MR was evaluated by measuring NADH consumption. Initial rates of both MR reduction and NADH oxidation in the presence of each enzymes were 15.6 and 34.8  $\mu\text{M}\cdot\text{min}^{-1}$  for Azr8, and 11.0 and 23.1  $\mu\text{M}\cdot\text{min}^{-1}$  for Azr18, respectively. HPLC analysis of degradation products from MR during the enzyme reaction showed that the two peaks corresponding ABA and DMPD were detected. These results clearly indicate that the azoreductases catalyzed the reductive cleavage at azo linkage of MR with NADH at a molar ratio of 1 to 2 to generate ABA and DMPD.

The pH stabilities of purified azoreductases were determined at various pHs as shown in Fig. 2.

Fig. 2 pH stabilities of Azr8 and Azr18



The enzymes were stable between 6.5 and 10.0 for Azr8 and 6.0 and 10.0 for Azr18. The effect of temperature of the enzyme activity was determined. Enzyme was stable up to 50°C for Azr8 and 40 °C for Azr18 and the maximal activity was 55°C for Azr8 and 65 °C for Azr18 as shown in Fig. 3.

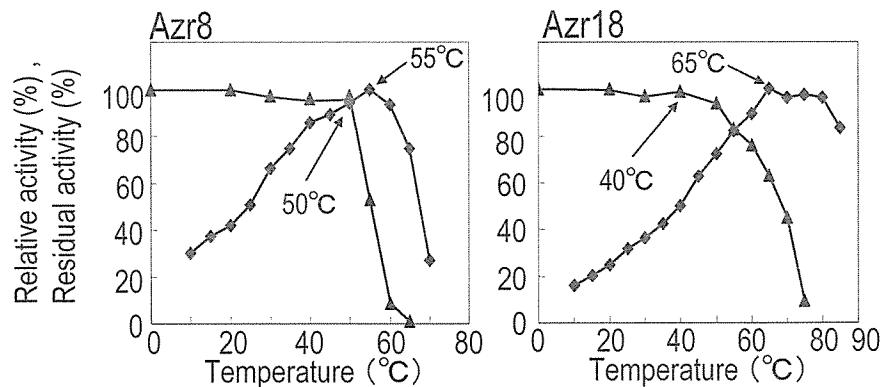


Fig. 3 Effect of temperature on the enzyme activity

#### Kinetic analysis of recombinant Azr8 and Azr18

Double-reciprocal plots of initial reaction velocity against concentration of NADH and MR resulted in parallel lines (data not shown). These results suggested that the reaction mechanism were a ping-pong type.

#### Substrate specificities of recombinant Azr8 and Azr18

Both azoreductases showed the highest activity against MR. Interestingly, 1-(2-pyridylazo)-2-naphthol and Orange 1 were also good substrate for Azr18 but not Azr8. Although Azr8 showed the activity against Sudan Black B, Azr18 was not reacted as shown in Table 3.

Table 3 Substrate specificities od Azr8 and Azr18

Azo dye	Structure	Relative activity (%) Azr8 Azr18		Azo dye	Structure	Relative activity (%) Azr8 Azr18	
Methyl red		100	100	Sunset Yellow FCF		0.16	0.26
Methyl orange		0.93	0.69	Orange G		ND	ND
1-(2-pyridylazo)-2-naphthol		23.2	91.4	Congo red		0.16	1.64
Orange I		1.26	43.1	Sudan black B		0.31	ND
Orange II		0.29	14.1	Reactive black 5		0.14	3.62
Acid red 88		12.2	27.8	ND : Not detected.			

## CONCLUSIONS

We demonstrated that the mesophilic gram-positive *Bacillus* sp. B29 contained genes encoding azoreductases, and enzymatic properties of Azr8 and Azr18 are characterized. High stable physicochemical properties of the azoreductases may contribute to azo dyes degradation and passively making for biotechnological application for treatment of azo dyes containing industrial waste water.

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