



Title	EXPRESSION, PURIFICATION, AND ENZYMATIC CHARACTERIZATION OF TWO RECOMBINANT AZOREDUCTASES OF BACILLUS SP. B29
Author(s)	Ooi, T; Shibata, T; Kinoshita, S et al.
Citation	Annual Report of FY 2007, The Core University Program between Japan Society for the Promotion of Science (JSPS) and Vietnamese Academy of Science and Technology (VAST). 2008, p. 421-426
Version Type	VoR
URL	https://hdl.handle.net/11094/12961
rights	
Note	

The University of Osaka Institutional Knowledge Archive : OUKA

<https://ir.library.osaka-u.ac.jp/>

The University of Osaka

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 μ M MR, 100 μ 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 μ 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 Sephacryl S-200 column. Active fractions were pooled and use 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:  MSKVLFEVKANDRPAEQAVSSKMYETTFVSTYKEANPNTETITELD
Azr8:  MTKVLFEITANPNSAEGSFGMAVGAEFIEAYKNEHEODEVVTID
Azr18: MATVLEFVKANNRPAEQAVSVKLYEAFLANKNEANENDTVVELD
AcpD:  MSKVLVLKSSILAGY-SQSNQLSDYFVEQWRK-HSADEITVRD
AzoA:  S L VV HPLTK ESR VRAL T LAS RETN S IEIL

Azr6:  LFALDLEYYGNIAIS----GGYNSSQGMETAEEEKAVATVD
Azr8:  LFNTTTPAIDADVFA--AWGKFAAGECFEATTEVQQQKVAAMN
Azr18: LYKEELPYVGVDMIN----GTFKVGKCFD-ITEEEAKAVAVAD
AcpD:  LAANPIEVLDELV----GALRPSDAP--TPRQOEALALSD
AzoA:  VYAPETNMPIDEELLSAWGALRAGAAFETISENQOQKVARFN

Azr6:  QYNQLEADKVVFAFLNFTVPAPLITVSYLSQAGKTFKY
Azr8:  TNEETFMNADRYVEVTMWNFSYPPVVKAYLDNVAIAGKTFKY
Azr18: KYNQLEADKVVFGFLNLTIPAVLHTYIDYLNRAKTFKY
AcpD:  ELIAELKAHDVIVIAAPMYNFNISTOLKNFIDLVARAGVTFRY
AzoA:  ELTDQELSDAKVVIANEMWLNVPETRLKAWLDTINVAGKTFQY

Azr6:  TANGPEGLVGGKKVVVLGARGSDYSSEQMAPMEMAVNVTTTVE
Azr8:  TENGPVGLLEGKKALHIQATGGVYSEGAYAVDFGRNHLKTVL
Azr18: TPEGPVGLIGDKKIALLNARGGVYSEGPAREVEMAVKVVASMM
AcpD:  TENGPGLVTKKKAIVITSRGGI--HKD-GPTDLVTPVYSTFTE
AzoA:  TAECKPLTSGKKALHIQSNGGFVEGKDFAS-Q----VIKAIL

Azr6:  GFWGITNPETVVI EGHNOYE DRSQOIVEEGLNVKKVAAKF*
Azr8:  GFVGVNDTEYIAYEGMNANPEKAOEIKEAAIANARELAKRF*
Azr18: GFFGATNMETVVI EGHNOFEDKAEETIAAGLEEAAKVASKE*
AcpD:  GFI GITDVKFVFAEGIAYGPEMAAKAQS DAKAAIDSIVSA*
AzoA:  NFI GVDQVDGLFI EGIDHFE DRAEELLNTAMTKATEYGKTS*

```

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 *M_r* of the *Azr8* and *Azr18* under denatured conditions were estimated to be 23 kDa. The *M_r* 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 (%)
Azr8				
Crude enzyme	5,600	5,810	1.04	100.0
DEAE cellulose	654	3,750	5.73	64.5
Buthyl toyopearl	240	3,370	14.0	58.0
Sephacryl S-200	65.8	2,520	38.3	43.3
Azr18				
Crude enzyme	8,290	5,430	0.64	100.0
DEAE cellulose	1,330	4,870	3.66	91.1
Buthyl toyopearl	188	3,160	16.8	59.2
Sephacryl S-200	81.1	2,140	26.4	40.0

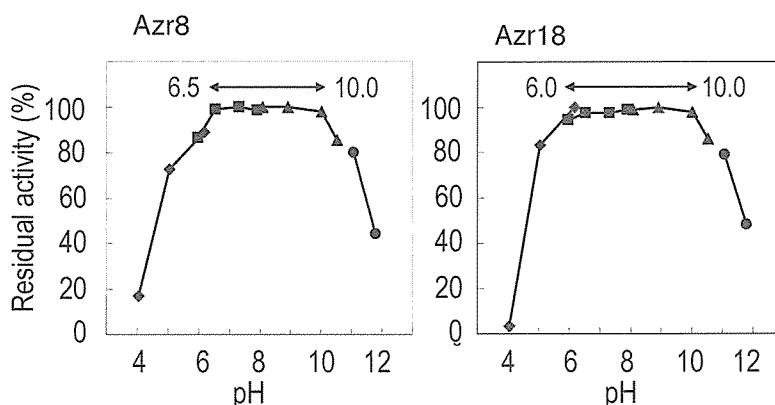
Table 2. Quantitation of FMN extracted from Azr8 and Azr18

	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.

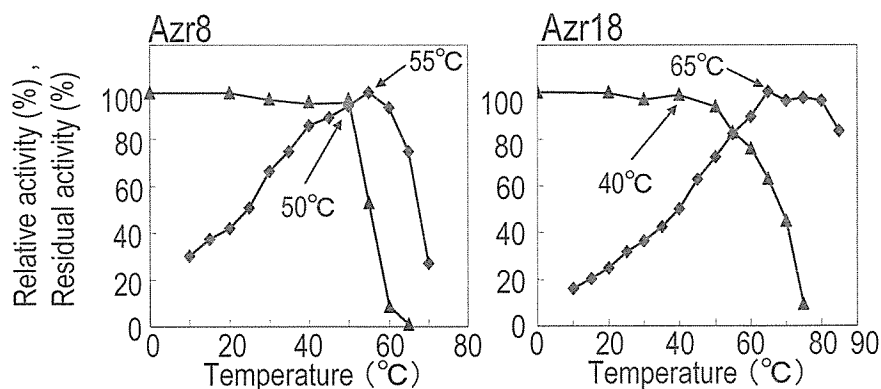
The pH stabilities of purified azoreductases were determined at various pHs as shown in Fig. 2.

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 Azr18as 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 of Azr8 and Azr18

Azo dye	Structure	Relative activity (%)		Azo dye	Structure	Relative activity (%)	
		Azr8	Azr18			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.

REFERENCES

- Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1987) Current protocols in molecular biology, vol 1, chapter 2.4. John Wiley & Sons, New York
- Bradford M (1976) A rapid and sensitive methods for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* 72:248-254
- Chen H, Woug R-F, Cerriglia CE (2004) Molecular cloning, overexpression, purification, and characterization of an aerobic FMN-dependent azoreductase from *Enterococcus faecalis*. *Protein Expr Purif* 34:302-310
- Green FJ (1990) The sigma-aldrich handbook of stains, Dyes and indicators. Aldrich chemical company, Milwaukee WI
- Griffith J (1984) Developments in the light absorption properties of dyes-color and photochemical degradation reactions. In: Griffith J (ed) *Developments in the Chemistry and Technology of Organic Dyes*. Blackwell, Oxford, pp 1-30
- Ivanova N, Sorokin A, Anderson I, Galleron N, Candelon B, Kapatral V, Bhattacharyya A, Reznik G., Mikhailova N, Lapidu, A, Chu L, Mazur M, Goltsman E, Larsen N, D'Souza M, Walunas T, Grechkin Y, Pusch G, Haselkorn R, Fonstein M, Ehrlich SD, Overbeek R, Kyrpides N (2003) Genome sequence of *Bacillus cereus* and comparative analysis with *Bacillus anthracis*. *Nature* 423: 87-91
- Nakanishi M, Yatome C, Ishida N, Kitade Y (2001) Putative ACP phosphodiesterase gene (*acpD*) encodes an azoreductase. *J Biol Chem* 176:46394-46399