# PURIFICATION AND PROPERTIES OF AN AZO-REDUCTASE FROM *BACILLUS* SP.

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## ABSTRACT

An azo-reductase was purified 466-fold by column chromatographies on DEAE-cellulose, Toyo-pearl HW55, and Blue Sepharose from the cell extract of isolated strain, *Bacillus* sp. B29. The enzyme required an NADPH as a cofactor for the decolorization of Orange II. The purified enzyme showed a single band on the SDS-PAGE (Mr of 34 kDa). From the gel filtration on YMC-Pack Diol-200 it was found to be a dimmer protein. The optimal pH was pH 6.5 and the optimal temperature was 55°C. It was stable in the pH range of 6.0 to 7.0, and up to 60°C. It was inhibited strongly by 1 mM $Ag^{+}$ and 1 mM $Fe^{2+}$, and stimulated 2-fold by 1 mM $Mn^{2+}$. It had 3-fold higher activity toward Orange 16 and New Cossin than Orange II, but no activity toward Orange G, Trypan Blue and Congo Red, the last two of which were diazo compounds.

## KEYWARDS

azo dye, biodegradation, biodecolorization, azo-reductase, *Bacillus* sp.

## INTRODUCTION

A large amount of azo dyes are used in textile industries and printing factories. In textile industries, 10 to 15% of dyes are released after using (Vaidya, 1982). Some of them are recovered by adsorption but most of them are flow out from the factories without any treatment, where microbiological treatment is expected. The decolorization by anaerobic microbial process was reported (An, 1996), but whether it was complete degradation was not reported.

We tried to isolate a microbe to degrade aerobically Orange II completely. Here we found a bacterial strain B29, which was identified as *Bacillus* sp. Also we purified the first enzyme, azo-reductase to attack Orange II.

## MATERIALS AND METHODS

*Culture conditions*

For the isolation of microbe, a test tube (ϕ18×180 mm) containing 5 ml of the medium was shaken at 30°C. 500-ml conical flask and 3-L Sakaguchi flask for ordinary cultures, and 7-L fermentor for mass cultivation were used. The medium composition was 25 mg Orange II, 5.0 g glucose, 0.2 g $KH_2PO_4$, 0.3 g $K_2HPO_4$, 0.5 g ammonium sulfate, 0.1 g magnesium sulfate 7-hydrade, 0.5 g peptone, and 0.5 g yeast extract in 1 L (pH 6.8).

*Measurments*

The azo-reductase activity was determined spectrophotometrically at 30°C by monitoring Orange II decolorization at 484.4 nm. The reaction mixture consisted of 0.1 mM Orange II and 0.25 mM NADPH in 25 mM phosphate buffer, pH 7.0, and appropriate concentration of enzyme solution. One unit of the enzyme activity was defined as the amount of the enzyme that catalyses the decolorization of 1 μmol of Orange II/min.

Protein concentration during enzyme purification was estimated by monitoring at 280 nm.

## RESULTS AND DISCUSSION

*Isolation of microorganism and identification*

Microorganims to decolorize OrangeII were selected in an enrichment culture from soil samples. Among them strain B29 was isolated as the storongest decolorizer, which decolorized 25 mg/L Orange II for 24 hr. After 12 hr of the aerobic cultivation, it was further 12 hr in a static culture, and so it decolorized well, but the growth was ceased. The strain B29 was identified as *Bacillus* sp. by the taxonomic properties and homology of 16S rRNA gene.

*Purification of azo-reductase*

The cells were cultured in 5-L of the medium, disrupted by French pressure, and 40 ml of crude cell extract was prepared.

The enzyme was purified: (1) DEAE-cellulose column (ϕ34×210 mm) chromatography with 0 - 1 M NaCl gradient. Active fractions were pooled (67 ml), and concentrated to 13.5 ml by ultra-filtration (50-k cut off); (2) Blue Sepharose CL6B affinity chromatography with 0 - 1 M NaCl gradient. Active fraction (38 ml) was concentrated to 5.0 ml; (3) Toyopearl HW55F column (ϕ27×490 mm) chromatography. Active fractions were pooled (19 ml); (4) Blue Sepharose CL6B affinity chromatography with 0.5 mM NADH. Active fractions were pooled (60 ml). Up to this step the enzyme was purified 466-fold to have 0.75 units/mg of specific activity with the yield of 5.7% as shown in Table 1.

Table. 1 Summary of azoreductase purification from *Bacillus* sp. B29

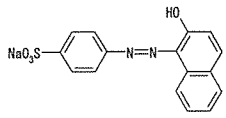
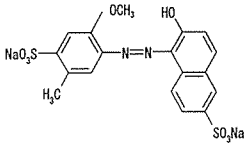
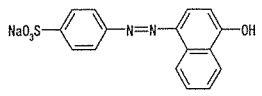
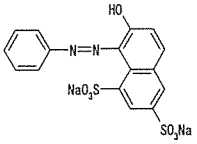
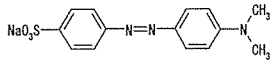
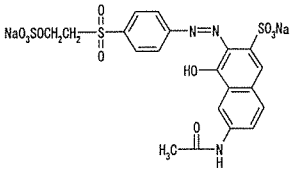
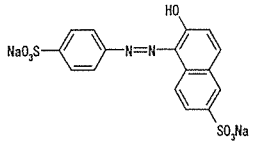
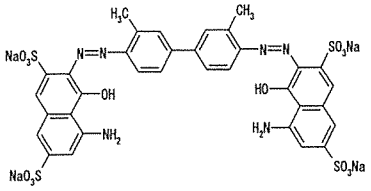
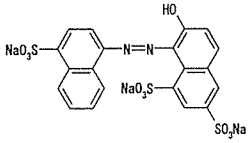
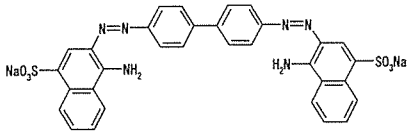
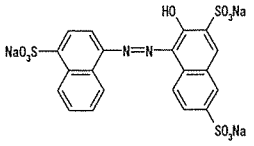
| Step | Total protein (A280) | Total activity (unit) | Specific activity (units/mg) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|
| Crude extract | 2030 | 3.3 | 0.0016 | 1 | 100 |
| DEAE-cellulose | 75 | 2.3 | 0.031 | 19 | 71 |
| Blue Sepharose CL6B | 3.2 | 1.3 | 0.42 | 262 | 41 |
| Toyopearl HW55F | 0.79 | 0.56 | 0.71 | 441 | 17 |
| Blue Sepharose CL6B | 0.25 | 0.19 | 0.75 | 466 | 5.7 |

*Properties of azo-reductase*

The activity of crude enzyme was negligible and NADPH was found to be only effective to measure the activity. NADH, FAD, and FMN were not effective. The ratio of decolorization of Orange II to utilized HNADP was about 3. We cannot answer why so much NADH is utilized for the decolorization, though the ratio of other case was reported to be 2 (Nakanishi, 2001).

SDS-PAGE was performed using 12.5% gel and it gave a single band. The Mr was estimated to be 34 kDa which differ from those of azo-reductase from *Bacillus* sp. (Suzuki, 2001). The Mr for native form of the purified enzyme was estimated to be 68 kDa by YMC-Pack Diol-200 column

Table 2 Substrate specificity of the azo reductase from *Bacillus* sp. B29

| Azo dye | Relative activity (%) | Azo dye | Relative activity (%) |
|---|---|---|---|
|  | |  | |
| Orange II | 100 | Allura Red AC | 55 |
|  | |  | |
| Orange I | 40 | Orange G | 0 |
|  | |  | |
| Methyl Orange | 107 | Orange 16 | 293 |
|  | |  | |
| Sunset Yellow FCF | 69 | Trypan Blue | 0 |
|  | |  | |
| New Cossin | 297 | Congo Red | 0 |
|  | | | |
| Amarnath | 66 | | |

chromatography. The results showed that the enzyme was a dimeric protein which is in accordance with those of azo-reductase from *Shigella dysenteriae* (Ghosh, 1992) and *Escherichia coli* (Nakanishi, 2001). However azo-reductase from *Pseudomonas* sp.