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Decolorization and biodegradation metabolism of azo dyes by *Pseudomonas S-42*

Liu Zhipei[1] and Yang Huifang[1]

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Abstract— A bacterial strain was isolated from activated sludge and has been identified as *Pseudomonas* sp. S-42 capable of decolorising azo dyes such as Diamira Brilliant Orange RR (DBO-RR), Direct Brown M (DBM), Eriochrome Brown R (EBR) and so on. The growing cells, intact cells, cell-free extract and purified enzyme of strain S-42 could decolorize azo dyes under similar conditions at the optimum pH 7.0 and temperature of 37°C. The efficiencies of decolorization for DBO-RR, DBM, EBR with intact cells stood more than 90%. When the cell concentration was 15mg (wet)/ml and the reaction time was 5 hours, the decolorizing activities of intact cells for above three azo dyes were 1.75, 2.4, 0.95 μg dye/mg cell, respectively. Cell-free extract and purified enzyme belonged to azoreductase with molecular weight about 34000 ± 2000 and Vmax and Km values for DBO-RR of 13μmol/mg protein/min and 54μmol, respectively. The results from the detection of the biodegradation products of DBO-RR by spectrophotometric and $NaNO_2$ reaction methods showed that the biodegradation of azo dyes was initiated by the reducing cleavage of azo bonds. The biodegradation metabolism path for DBO-RR by *Psued.* S-42 was hypothesized.

Keywords: *Pseudomonas* sp. S-42; azo dyes; decolorization; biodegradation; azoreductase.

INTRODUCTION

Almost all of dyes are artificial macromolecular compounds. Because of their artificiality, diversity and complicated structure, they are very difficult to be biodegraded, and some of them are toxic to high animals (Meyer, 1983). Wastewaters from dyes manufacturing factories, textile dyeing plants and food industry contain these pollutants and widely contaminate the environments, especially aquatic system. This has caused much attention to how to solve these problems.

As early as 1930s, it was found that the decolorization of dairy products dyed with azo dyes was caused by *Lactobacillus*. After that various bacteria had been found to be capable

[1]Institute of Microbiology, Academia Sinica, Beijing 100080, China.

of decolorizing azo dyes and most of the decolorizing bacteria were *Pseudomonas* and *Bacillus* (Roson, 1966). As Meyer reviewed, the biodegradation of azo compounds could occur in both aerobic and anaerobic systems. Anaerobic biodegradation was accomplished by a number of micro-organisms with rather non-specific enzymes. Kulla *et al.* (1983), Zimmermann *et al.* (1982) and Meyer *et al.* (1977) reported their investigations on the characteristics of azoreductases and the metabolites of azo dyes. In recent years, there were many reports on the isolation of decolorizing bacteria (Guizhou Institute of Environmental Protection Science, 1981), the decolorizations of dyes by immobilized cells (Hang, 1988), and the use of high efficient bacteria to treat dyeing wastewater (Yang, 1987) while no report has been found so far on the biodegradation of dyes in China.

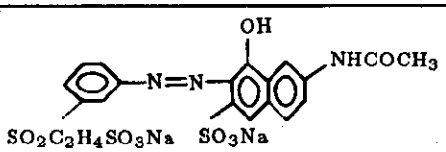
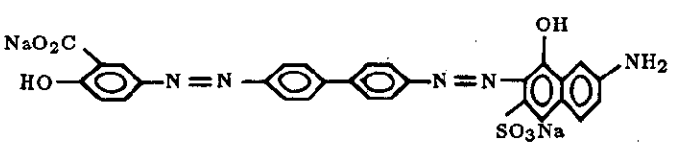
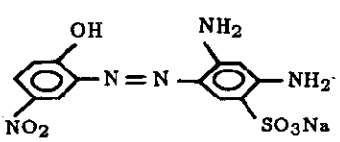
In this paper, some of the dyes mostly used in the dyeing plants in our country, such as DBO-RR, DBM and EBR, were selected as substrates to study the decolorization by *Pseud.* S-42, the decolorizing rate by the cell-free extract and purified enzyme, the properties of purified enzyme as well as the biodegradation and metabolism, of DBO-RR by *Pseudomonas* sp. S-42.

MATERIALS AND METHODS

Dyes

Dyes used in the experiments were listed in Table 1.

Table 1 Azo dyes and their structures

Azo dyes	Structures	$\lambda_{max,nm}$
Diamira brilliant orange RR (DBO-RR)		486
Direct brown M (DBM)		416
Eriochrome brown R (EBR)		430

Bacterial strain

Pseud. S-42 was isolated from activated sludge.

The composition of medium for culturing strain S-42 was as follows (g/L):

Beef broth 5.0, NaCl 5.0, peptone 10.0 and pH 7.0

Preparation of intact cell suspension and cell-free extract

Cells were harvested by centrifugation (at 5000r/min for 30min), washed twice with 1/15 mol/L potassium phosphate buffer of pH 7.0, suspended in the same buffer to make the intact cell suspension and stored at 0—4°C. Cell-free extracts were prepared by passing a 50%(w/v) cell suspension in 1/15 mol/L potassium phosphate buffer of pH 7.0 twice through a X-Press Pressure Cell at a pressure of 120 mPa. Cell debris was sedimented by centrifugation at 19000 r/min for 30min.

Enzyme purification

Solid ammonium sulfate was added to cell-free extract to make a 25% (w/v) concentration and the mixture allowed to stand overnight at 4°C. After removal of the precipitate by centrifugation at 6000 r/min for 30min, additional ammonium sulfate was added to the supernatant liquid up to 45% (w/v), and the mixture allowed to stand overnight. The precipitate thus formed was dissolved in a small volume of water, dialyzed against deionized water for 2 days, and lyophilized as the crude enzyme. This preparation was dissolved in 1/20 mol/L acetate buffer of pH 4.5, and placed on a DEAE-cellulose column (2.4 × 35cm) previously equilibrated with the same buffer. This column was washed with 250 ml of 1/20 mol/L acetate buffer of pH 4.5, and then eluted with a linear sodium chloride gradient increasing from 0 mol/L to 1.0 mol/L in 400 ml of the same buffer at a rate of 0.4ml/min. The effluent was collected at 4ml/tube. Decolorizing activity fractions were combined and concentrated. The concentrate was placed on a Sephadex G-200 column (1.6 × 100 cm) and filtration was performed with deionized water at 4°C at a rate of 0.4ml/min (4ml/tube). Fractions containing the decolorizing activity were combined and concentrated.

Determination of decolorizing activity

Intact cells assay

Mixture of dyes and strain S-42 intact cells were incubated at a given temperature without shaking. After a given period of time, the reaction mixture was centrifugated to remove the cells. The absorbance of the supernatant was measured at the wavelength in the visible band of spectrum for the absorption maximum of each dye, and this value referred to as the rate of decolorization of the dyes. Or the decolorization rate of dyes was presented by the consumption of dyes per mg cells.

Cell-free extract assay

The decolorizing assays of dyes by cell-free extract were performed as described by Walker (1971). The decolorization of dyes was presented by percent rate or by relative: μg dyes/mg protein/min.

Enzyme assay

The activity of purified enzyme was determined as described by Zimmermann *et al.* (1982). One unit (μ) of enzyme is defined as the amount yielding reduction of 1 μg of DBO-RR/min within 30 min.

Detection of aromatic amine

According to the properties of aromatic amine, Ar-NH_2 can be identified by $NaNO_2$ reaction (Zhou, 1984).

UV spectra of reaction products of DBO-RR

This assay was carried out as described by Chizuko *et al.* (1981). The mixture of DBO-RR solution and S-42 intact cells was incubated at 37°C without shaking. After a given period of time, the reaction mixture was extracted with n-butanol. The spectrum of n-butanol extract was measured using Beckman DU-7 spectrophotometer. The change in the ultraviolet absorption spectrum was used to detect reaction products.

Protein determination

Proteins were determined by the method of Lowry *et al.*

Determination of molecular weight

The molecular weight of the enzyme was determined by the method of SDS-polyacrylamide gel electrophoresis described by Zhang et al. (1981).

RESULTS

Decolorization of dyes by S-42 intact cells

The optimum pH and temperature

The concentrations of cells and dyes in reaction mixture were 15mg (wet)/ml and 50 ppm, respectively. The reaction mixture was incubated at 37°C for 8 hours, and then the decolorization was determined. The result (Table 2) showed that the optimum pHs for the decoloration of DBO-RR, DBM and EBR all were 7.0. The effect of temperature on decolorization of azo dyes by intact cells was carried out at a series of temperatures for reacting 8 hours at pH 7.0. Results in Table 3 indicated that optimum temperature all were 37°C.

Table 2 The optimum pH of decolorization of azo dyes by S-42 intact cells

Azo dyes	Decolorization rate, %				
	pH 5.0	pH 6.0	pH 7.0	pH 8.0	pH 9.0
DBO-RR	81.6	88.6	95.3	88.2	83.2
DBM	70.3	88.8	91.8	86.3	77.4
EBR	70.5	76.3	78.5	49.1	10.9

Table 3 The optimum temperature of decolorization of azo dyes by S-42 intact cells

Azo dyes	Decolorization rate, %						
	4°C	13°C	22°C	30°C	37°C	45°C	55°C
DBO-RR	0.2	6.3	38.3	59.0	94.8	81.5	61.4
DBM	0.6	9.7	33.5	60.7	90.6	67.3	44.2
EBR	4.6	11.2	31.3	51.6	75.8	60.3	31.3

The effects of cell concentration

Reaction mixtures containing various azo dyes and different concentration of cells were incubated at pH 7.0 and 37°C for 5 hours, and then the decolorizing activity of intact cells were determined. Cell concentration could affect the decolorizing activity of intact cells for azo dyes and the optimum cell concentration was 15 mg (wet)/ml (Fig. 1).

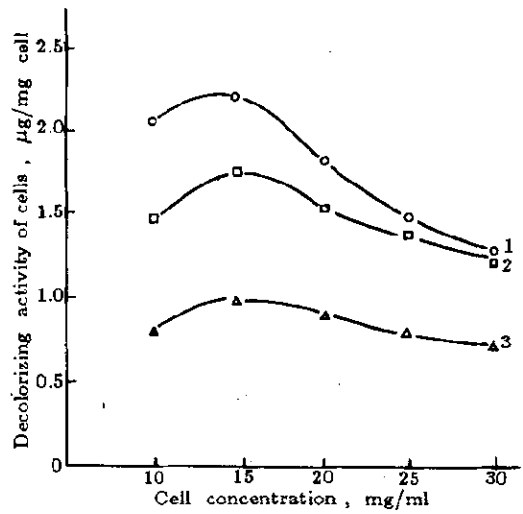


Fig. 1 The effect of cell concentration on the decolorization of various azo dyes by static culture of strain S-42 intact cells
Dye conc., 50ppm; pH, 7.0; temp., 37°C; 1: DBM 2: DBO-RR 3: EBR

The activity of intact cells for decolorization of azo dyes

Reaction mixture containing 15mg (wet)/ml cells and 50ppm azo dyes was incubated at pH 7.0 and 37°C. Then the decolorization of azo dyes were determined at different times. Results (Fig. 2) indicated that the decolorizing activities of strain S-42 intact cells were different with azo dyes: the best for DBO-RR, the secondly for DBM and the worst for EBR. This assay also showed that the decolorization of azo dyes by strain S-42 intact cells was fast at the initial of reaction, gradually decreased with the procedure of the reaction, and finally came to an end at about 10 hours.

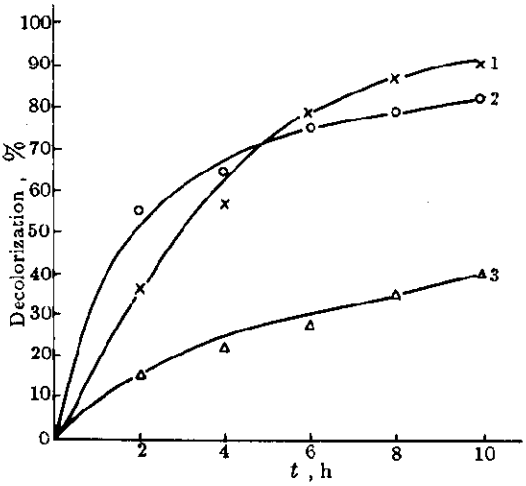


Fig. 2 The decolorizing activity of intact cells of strain S-42 dye conc., 50ppm; cell conc., 15mg (wet)/ml; pH, 7.0; temp., 37°C; 1: DBO-RR, 2: DBM, 3: EBR

The decolorization of DBO-RR by cell-free extract

The effects of oxygen and NADH

In the experiments of decolorizing activity of cell-free extract, the azo dye DBO-RR was selected as the substrate. The reactions were run under those conditions as described above. The effects of oxygen and NADH on the activities of intact cells and cell-free extract for decolorizing DBO-RR were tested for four different factors: gassing with nitrogen (anaerobic) and not gassing with nitrogen (aerobic) with adding NADH or no. The results in Table 4 showed that there were no effects of oxygen and NADH on the decolorizing activity of intact cells. However, Fig. 3 indicated that oxygen inhibited the decolorizing activity of cell-free extract for DBO-RR strongly while NADH improved this activity greatly. This result was similar to that reported by Kulla *et al.* (1983). It would supposed that strain S-42 cell-free extract also contained azoreductases.

Table 4 The influences of oxygen and NADH on the decolorizing activity of intact cells for azo dyes

Reaction conditions	Decolorization rate, %		
	DBO-RR	DBM	EBR
Gassing with N_2, adding NADH	91.6	87.5	87.0
Gassing with N_2, no NADH	90.4	86.2	85.2
No gassing with N_2, adding NADH	91.6	87.1	87.2
No gassing with N_2, no NADH	90.9	86.1	86.0

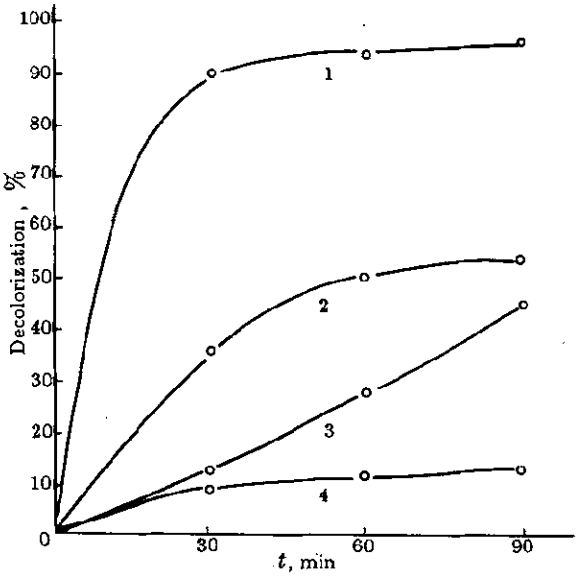


Fig. 3 The effect of oxygen and NADH on the decolorizational reaction of DBO-RR by cell-free extract of strain S-42
DBO-RR conc., 50ppm; pH, 7.0; temp., 37°C;
1. Gassing with N_2, adding NADH, 2. Not gassing with N_2, adding NADH, 3. Gassing with N_2, no NADH, 4. Not gassing with N_2, no NADH

The decolorizing activity of cell-free extract

The mixture of DBO-RR and cell-free extract was incubated under optimum conditions, and the decolorizing rate and relative decolorizing activity were determined at different times. The rate of decolorization was fast within the initial 15 min, then tended to decrease, and came to an end at about 40 min. The relative decolorizing activity decreased from 0.65 μg dye/mg protein/min at 5 min to 0.18 μg dye/mg protein/min at 35 min (Fig. 4).

Purification and properties of decolorizing enzyme

Purification of decolorizing enzyme

Purification of the decolorizing enzyme was performed by ammonium sulfate fractionation, DEAE-cellulose column chromatography, and Sephadex G-200 gel filtration. The results of purification steps are summarized in Table 5. Through these purification steps, the specific activity increased by 27.6-fold with an 8% recovery. Chromatograms of the final two steps, on DEAE-cellulose and Sephadex G-200, are shown in Fig. 5 and Fig. 6. Fraction Nos 114-117 from DEAE-cellulose column chromatography were subjected to the subsequent Sephadex G-200 gel fraction. There were three peaks of protein on Sephadex G-200 column chromatography, and the decolorizing activity was mainly seen in the second peak (No. 40—70). Electrophoretic analysis of purified enzyme in SDS-polyacrylamide gel proved it to be homogeneous (Fig.11).

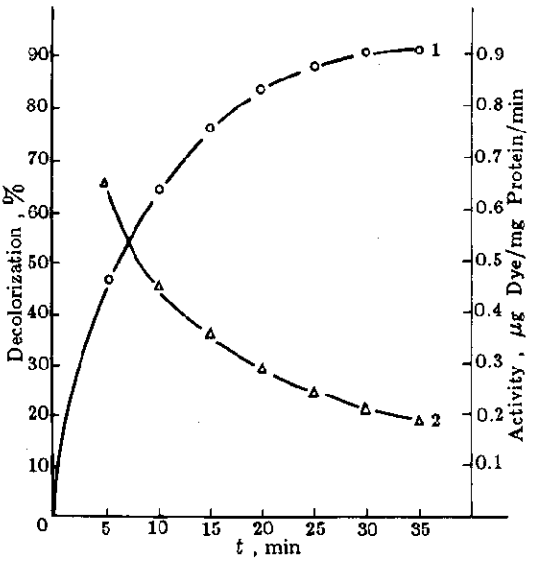


Fig. 4 The decolorization of strain DBO-RR by cell-free extract of strain S-42 DBO-RR conc., 50ppm; pH, 7.0; temp., 37°C; gassing with N_2 and adding NADH. 1: Decolorization rate, % 2: Relative activity

Table 5 The purification of DBO-RR decolorizing enzyme

Purification steps	Total protein, mg	Specific activity	Yield, %	Purification -fold
Cell-free extract	12000	0.51	100	1
$(NH_4)_2SO_4$ 25—45%, w/v	6240	0.94	95.5	1.84
DEAE-cellulose	320	2.34	12.2	4.59
Sephadex G-200	35	14.07	8.0	27.6

Properties of DBO-RR decolorizing enzyme

(1) Optimum pH

The effect of pH on DBO-RR decolorizing enzyme was examined in 1/15 mol/L potassium phosphate buffer of various pH by gassing with nitrogen and adding NADH. The enzyme was the best at pH 7.0 (Fig. 7) and the same as the other azoreductase reported by Zimmermann *et al.* (1982) and Wahrmann *et al.* (1980).

(2) Optimum temperature

The effect of temperature on DBO-RR decolorizing enzyme was examined over the range of 20°C to 40°C. As shown in Fig. 8, the optimum temperature was 37°C.

(3) Kinetic properties

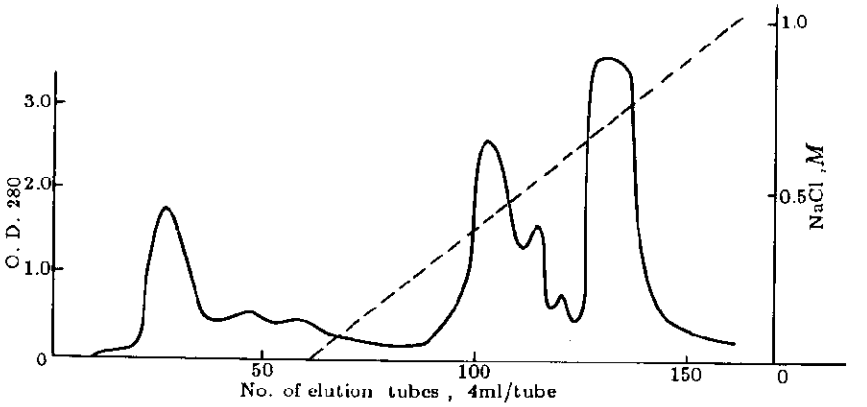


Fig. 5 Column chromatogram on DEAE-cellulose of DBO-RR decolorizing activity (activity mainly contained in tubes 114-117)

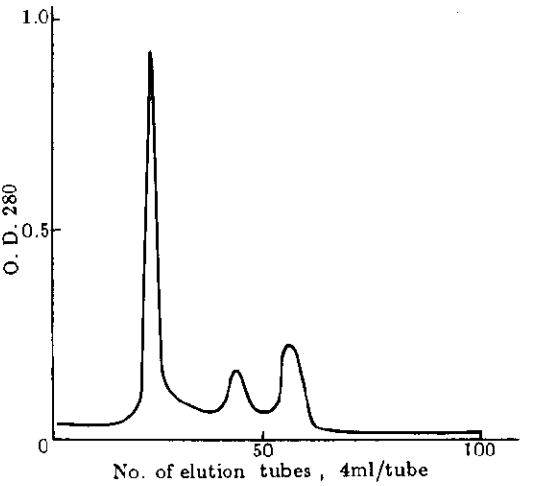


Fig 6 Sephadex G-200 gel filtration of DBO-RR decolorizing activity (activity mainly contained in tubes 40—70)

The Michaelis constant for decolorizing DBO-RR was determined with purified enzyme under standard assay conditions and in addition with NADH as the co-substrate and gassing with nitrogen to be anaerobic system. Fig. 9 indicated that the decolorizing reaction of BDO-RR by purified enzyme was the first order of reaction. With the substrate DBO-RR concentration increased from 16.5, 33.0, 58.0 to 83.0 μmol/L, the enzyme activities were examined. From the [S]/V vs.[S] plot (Fig. 10), Km value of 54 μmol and Vmax value of 13 μmol/mg protein/min

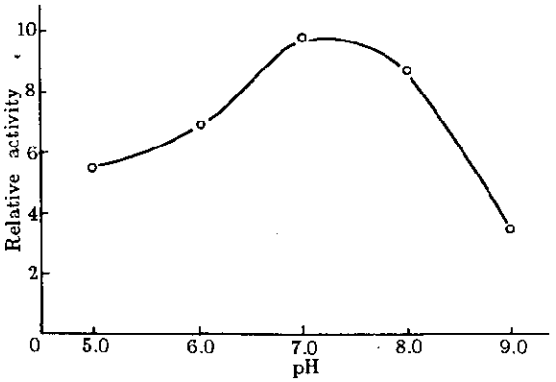


Fig. 7 The optimum pH of DBO-RR decolorizing enzyme

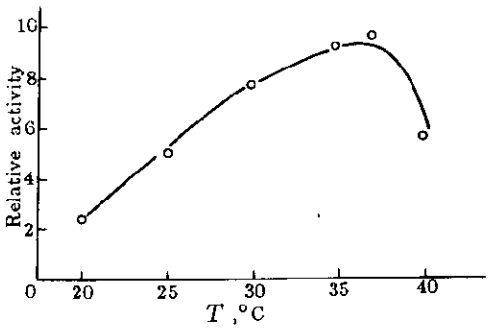


Fig. 8 Temperature-current curve

were obtained for DBO-RR, and this result indicated that DBO-RR was a good substrate for the enzyme. According to all above results, the decolorizing enzyme of strain S-42 for DBO-RR could be named as DBO-RR azoreductase.

(4) Molecular weight

From the distance of migration of the enzyme, located between Pepsin and Trypsinogen on the standard curve for the SDS-polyacrylamide gel electrophoresis, the molecular weight of the enzyme was calculated to be about 34000±2000 (Fig. 11 and Table 6), similar to that of Orange II azoreductase reported by Zimmermann *et al.* (1982).

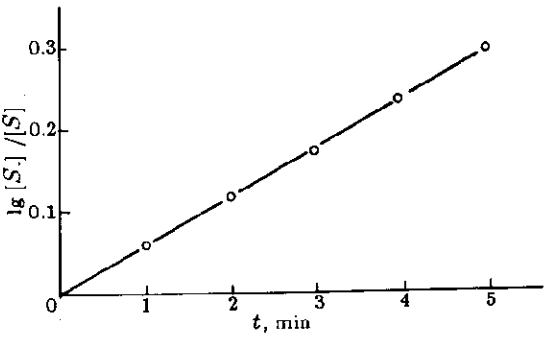


Fig. 9 First order of reaction

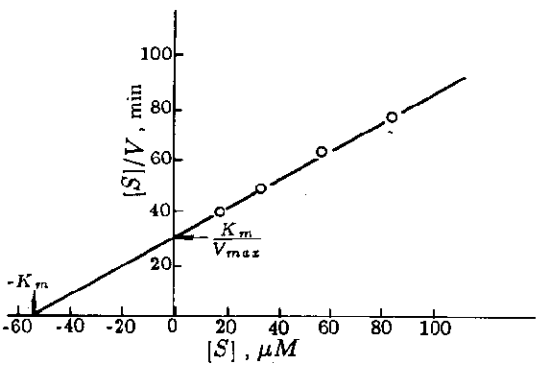


Fig. 10 The [S]/V vs. [S] plot for DBO-RR azoreductase

Table 6 The molecular weight of standard proteins and the relative mobility of standard proteins and sample

Proteins	Molecular weight	lgMW	Mr
Lysozyme	14300	4.16	0.80
Trypsinogen	24000	4.38	0.57
Pepsin	34700	4.54	0.43
Albumin, egg	45000	4.65	0.32
Albumin, bovine	66000	4.82	0.18
Unknown sample			0.44

Detection of biodegradation of DBO-RR

The mixture of DBO-RR solution and intact cells was centrifugalized after reaction to remove the cells and then the supernatant was detected by $NaNO_2$ reaction. Result in Table 7

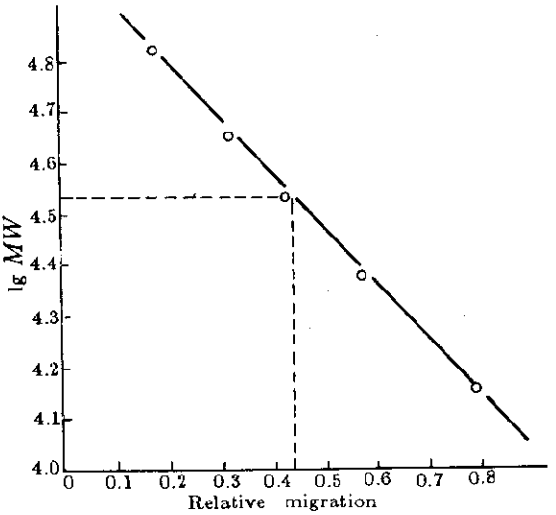


Fig. 11 Estimation of the molecular weight of DBO-RR azoreductase by SDS-polyacrylamide gel electrophoresis

showed that the DBO-RR had become aromatic amine products after the reaction.

Table 7 The $NaNO_2$ reaction of biodegrading products of DBO-RR by intact cells

Sample	DBO-RR	Biodegrading products	Benzidine
Result	Yellow precipitate	Orange-red precipitate	Orange-red precipitate

The ultraviolet-visible spectrum of the reaction products of DBO-RR in n-butanol is shown in Fig. 12. The spectrum of DBO-RR in n-butanol is characterized by 4 maxima at 486, 380, 304 and 252 nm. The slapes of these maxima varied with reaction time: the peak at 252 nm gradually shifted to 265 nm with an increse in absorbance. As the reaction proceeded, absorbance at other maxima gradually decreased. From this it appears that spectroscopic evidence for the presence of the reaction product is given by the 265 nm maximum. This result is similar to that reported by Chizuko *et al.* (1981).

DISCUSSION

The conditions of the decolorization of azo dye DBO-RR by *Pseud.* S-42, including not only the intact cells and cell-free extract but also the purified enzyme, were almost the same while the sensitivities to oxygen of different cell products were different: oxygen could inhibit the decolorizing activities of cell-free extract and purified enzyme but could not the intact cells.

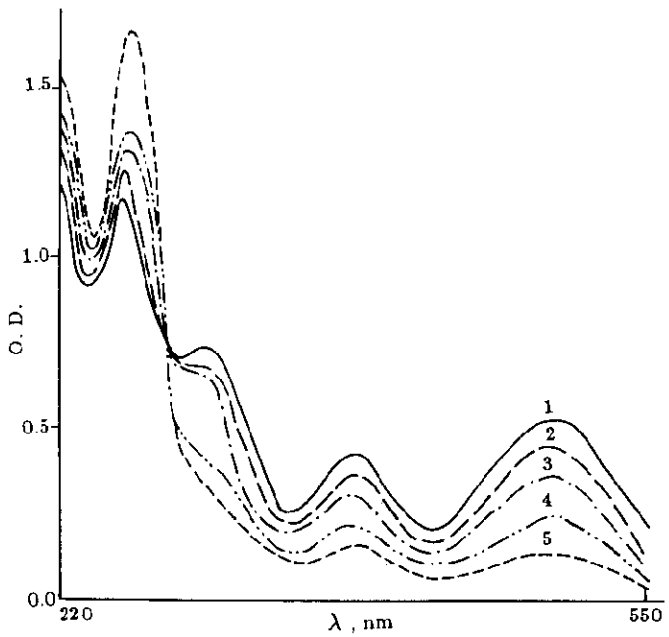
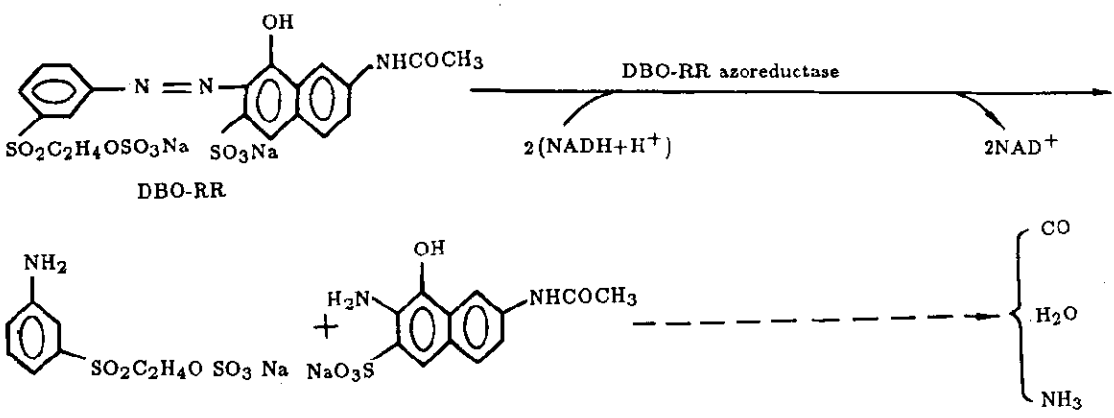


Fig. 12 The spectrum of DBO-RR products by intact cells of strain S-42 in n-butanol
[DBO-RR]=50 ppm (8.1×10^{-5} mol/L);
[Cell]=15mg(wet)/ml; pH=7.0; temp. =37°C. 1. reacted for 0 hr;
2. reacted for 1 hr; 3. reacted for 2 hrs; 4. reacted for 3 hrs; 5. reacted for 4 hrs

NADH improved the decolorizing activities of cell-free extract and purified enzyme for DBO-RR. The Vmax and Km values of purified enzyme were similar to those of Orange II azoreductase reported by Zimmermann *et al.* (1982). All these properties of the enzyme indicate that this enzyme belongs to azoreductase.

Based on the results of $NaNO_2$ reaction and the UV-visible spectrum of reaction products in n-butanol, it is assumed that the biodegradation of DBO-RR by *Pseud.* S-42 was initiated by the reducing cleavage of azo bonds catalyzed by azoreductase with NADH or NADPH as co-substrate to produce aromatic amine products. The biodegradation of DBO-RR was not thorough under anaerobic conditions. If the complete mineralization was needed, it must co-work with other enzymes in S-42 or other bacteria to open aromatic amine ring under aerobic conditions, and finally to produce inorganic compounds such as carbon dioxide, water, ammonia and so on. In terms of these, the biodegrading metabolism pathway of DBO-RR by *Pseud.* S-42

was hypothesized as follows:



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