# Decolorization of synthetic dyes by *Rhodopseudomonas palustris* P4

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## ABSTRACT

A newly isolated *Rhodopseudomonas palustris* P4 could decolorize various synthetic dyes containing different chromogenic groups such as azo linkage (Crocein Orange G, New Coccine, Chromotrope FB, Congo Red, Remazol Black B), anthraquinone Reactive blue 2, or indigo Indigo Carmine. Among them, the degradation rate of Black B was studied in detial. Degradation of Black B followed the Arrhenius equation in 25 - 40℃ with an activation energy of 7.79 kcal/mol. Optimum pH was 8. Glucose in the range of 5 - 50 g/l did not affect the Black B decolorization. When Black B increased from 25 mg/l to 2000 mg/l, decolorization activity increased almost linearly but the extent of decolorization was constant at about 86% irrespective of dye concentration. Analyses by HPLC revealed that the Black B molecules were partially degraded and some chromogenic intermediates were produced. These results indicate that *Rps. palustris* P4 has an outstanding capability to degrade various dyes.

## INTRODUCTION

Over $7 \times 10^5$ metric tones of synthetic dyes are produced worldwide every year . They have different chemical structures to meet various coloring requirements and all are usually classified by their chromophores such as azo, anthraquinone and indigo. Azo dyes constitute the largest group of over 10000 commercial dyestuffs. During the dyeing process, 5 - 10% of the dye used is lost and appears in the wastewater. The recalcitrant synthetic dyes are not easily removed from the effluents and cause a serious pollution problem.

In natural environment, azo dye can be transformed or degraded by a variety of microorganisms, including aerobic and anaerobic bacteria and fungi. Bacterial degradation of azo dyes is often initiated by an enzymatic cleavage of azo bond, and this reaction is catalyzed by azoreductase which utilizes NADH as an electron donor. The resulting aromatic amines are further degraded by other enzymes under aerobical or anaerobical conditions. Fungal species (such as *Phanerochaete*

*chrysosporium*) are known to use lignin peroxidase to degrade azo dyes under anaerobic conditions, and a process utilizing this pure fungal culture has been attempted. However, the slow growth of the fungal cells and moderate decolorization rate limited its application. In comparison, bacteria grow fast and decolorization is also fast. But, mixed cells of various bacteria are usually required to degrade azo dyes to completely harmless minerals.

Recently, a new chemoheterotrophic bacterium *Rps. palustris* P4 was isolated from a sludge digester[1]. It was a facultative anaerobe with a high-specific growth rate (0.347 /h) and produced $H_2$ and various organic acids from CO or sugars[1,2]. Since *Rps. palustris* P4 belongs to non-sulfur purple bacteria, many of which are known to have the capability to perform decolorization of synthetic dyes, P4 was studied for color degradation. Various synthetic dyes with different chromophores including monoazo, diazo, anthraquinone, and indigo were examined. Especially, the degradation of a reactive diazo dye, Remazol Black B was studied in detail, since it is commercially important in Korea and structurally complex. Important parameters studied included pH, temperature, glucose concentration, and dye concentration. Decolorizing capability of P4 was also compared to that of other bacteria.

## MATERIALS AND METHODS

### Dyes

Seven synthetic dyes (Table 1) with dye contents at 50% - 85% were purchased from Aldrich (Milwaukee, USA) and used as delivered. For convenience, the trade names are used in this study.

### Microorganism and cultivation

*Rps. palustris* P4 was used throughout this study. A mineral salt medium supplemented with 3 g yeast extract/l and 10 g glucose/l was employed for growing *Rps. palustris* P4. Cultivation was performed at 35℃ in a gyratory incubator with a shaking speed of 250 rpm. A serum bottle of 165 ml (working volume, 50 ml) was used. After inoculation, the bottle was flushed with argon (Ar) gas (99.999%) for 5 min to develop anaerobic condition and sealed with a 12 mm-thick butyl rubber septum and aluminum cap. The inoculum was cultivated in the same bottle and transferred anaerobically during late-exponential phase by a sterile hypodermic disposable syringe.

### Measurement of dye decolorization and $H_2$ production

Cells were harvested during late-exponential phase, washed once with a buffer solution (pH 7.0, 100 mM phosphate buffer), and placed in a 38 ml serum vial (working volume, 10 ml) to be 1.04 - 1.23 mg dry cell/ml. The vials was then sealed with a 12 mm-thick butyl rubber septum and

aluminum cap. All procedures, except for centrifugation, were conducted in an anaerobic chamber, and all the buffer solutions and media were carefully flushed with Ar gas before use. The dye decolorization was monitored for 2 h while being shaken at 100 strokes/min in a water bath at 35℃, the specific dye decolorization activity (mg dye/g cell · h) was determined from a plot of dye concentration vs time. For determining the pH dependence of the decolorization activity, several different buffer solutions were used as follows (all in 100 mM): acetate buffer (pH 5.0), phosphate buffer (pH 6.0, 7.0, 8.0), and Trizma base buffer (pH 9.0). The extents of dye decolorization were measured after 24 or 48 h incubation at 35℃ and pH 8. Initial dye concentration was 50 mg/L. $H_2$ production was measured after 24 h incubation using the same method as dye decolorization. Incubation temperature and pH were 35℃ and 8.0.

Analyses

Cell concentration was measured by a spectrophotometer. The $H_2$ content was measured by a gas chromatograph equipped with a thermal conductivity detector and a stainless steel column (6′ × 1/8″) packed with Molecular Sieve 5A. Dye concentration was determined spectrophotometrically at visible absorbance maxima of individual dyes (Refer to Table 1). A high-performance liquid chromatograph (HPLC) was used for analyzing the Black B and its degradation products. The HPLC was equipped with a diode array detector and a $C_{18}$ (200×4.6 mm, particle size 5 ㎛) reverse-phase column. Mobile phase was water for initial 10 min, varied linearly to 9:1 (v/v) mixture of water-methanol for 40 min and then, changed linearly to a pure methanol for the next 30 min. Ammonium acetate (30 mM) was always included in water used as eluent. The flow rate of eluent was 0.3 ml/min and the column was kept at 25℃. The absorption peaks of separated compounds were analyzed at 254 nm and 600 nm, respectively. Black B has a visible absorption maximum at 600 nm and many uncolored aromatic compounds derived from Black B have a clear absorption at around 254 nm.

## RESULTS AND DISCUSSION

Table 1 shows the decolorization of various synthetic dyes along with $H_2$ production by *Rps. palustris* P4. Many azo dyes were degraded at a fast rate, and their decolorization extent after 48 incubation was also very high as 88 - 99%. On the contrary, the decolorization rates for an anthraquinone dye, Reactive Blue 2 and an indigo dye, Indigo Carmine were much slower, especially for the latter. Decolorization extents for the anthraquinone and indigo were also low and that for the indigo was only 13%. $H_2$ production was not affected by the presence of dye. These results indicate that P4 can decolorize most monoazo and diazo dyes very efficiently, probably due

to the high activity of azoreductase.

Fig. 1 and 2 show the effect of temperature and pH on the specific Black B decolorization activity. *Rps. palustris* P4 could decolorize Black B in a relative wide range of temperature as shown Fig. 1. Specific dye decolorization activity ($q$) increased from 5.52 ± 0.93 to 9.97 ± 0.11 mg dye/g cell · h when the temperature was varied from 25℃ to 40℃. The activation energy was estimated as 7.79 kcal/mol from the Arrhenius plot (Insert in Fig. 1), which is comparable to that of *Escherichia coli* NO3 (6.57 kcal/mol)[3] or *Pseudomonas* sp. (16.87 kcal/mol)[4]. Fig. 2 shows that the activity of Black B decolorization by P4 is high in the range of pH 6 to pH 9 with an optimum at pH 8. However, at pH 5, the activity is greatly reduced. Since P4 produces many organic acids during fermentation of sugars and this can easily cause a pH drop below 5, it should be noticed that the maintenance of pH above 5 is very important for a successful dye decolorization with P4.

Glucose was used as energy source for decolorization in this study and the effect of glucose concentration on specific decolorization activity was examined in the range of 5 - 50 g/l. The activities were almost the same at 8.51 ± 0.72 mg dye/g cell · h irrespective of glucose concentration (data not shown).

Fig. 3 shows the effect of Black B concentration on specific dye decolorization activity, decolorization efficiency, and $H_2$ production activity. With increasing dye concentration, specific decolorization activity increased almost linearly and reached a maximum value of 170 mg dye/g cell · h at 2000 mg Black B/l. Decolorization extent decreased gradually with increasing dye concentration, but was still high as 86% even at 2000 mg Black B/l. $H_2$ production decreased with increasing dye concentration, but the decrease was only marginal. Considering the $H_2$ production rate is an indicator representing the overall metabolic activity in P4, it is suggested that P4 is not much inhibited by Black B up to 2000 mg/l and is a good microorganism to be used for the wastewater treatment containing Black B.

HPLC analyses exhibited that Black B was chemically broken by P4 treatment. The untreated Black B had a major peak at 66.8 min which has a strong absorption at both 600 nm and 254 nm. This peak disappeared completely after decolorization by P4, and several new and earlier peaks appeared. One major peak appearing at 10.2 min was further analyzed by a diode array detector in the range of 230 - 700 nm. The peak has strong absorptions at both 600 nm and 254 nm like the peak at 66.8 min of the untreated Black B, but the spectra of both peaks were considerably different from each other. This indicates that the original Black B molecules are degraded readily and completely by *Rps. palustris* P4. However, a degradation product giving the peak at 10.2 min seems to be resistant to a further degradation by P4 and become the major source of some residual

color. A subsequent process may be needed for complete decolorization/mineralization of Black B after P4 treatment. Identification of Black B degradation products are currently underway using LC/MSD.

## REFERENCES


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**Table 1.** Decolorization of synthetic dyes by *Rps. palustris* P4.

| Dyes[a] | Chromophore | $\lambda_{max}$ (nm) | Specific dye decolorization activity (mg dye/g cell · h)[b] | Decolorization extent (%)[c] | Relative $H_2$ production activity (%)[d] |
|---|---|---|---|---|---|
| Crocein Orange G | Monoazo | 482 | 5.57±0.98 | 97.0±0.1 | 98.6±0.8 |
| New Coccine | Monoazo | 506 | 6.12±0.08 | 97.2±0.3 | 98.4±0.6 |
| Chromotrope FB | Monoazo | 515 | 9.38±0.17 | 88.1±0.3 | 97.2±1.2 |
| Congo Red | Diazo | 497 | 10.70±0.47 | 91.2±3.3 | 94.4±2.1 |
| Remazol Black B | Diazo | 600 | 8.67±0.23 | 98.5±1.2 | 94.6±4.1 |
| Reactive Blue 2 | Anthraquinone | 607 | 3.75±0.37 | 48.3±0.9 | 100±0.8 |
| Indigo Carmine | Indigo | 608 | 1.02±0.16 | 12.5±6.9 | 96.7±1.1 |

[a] Dye concentration was 50 mg/l.

[b] The initial decolorization rate during 2 h incubation was determined.

[c] Decolorization extent was determined after 48 h incubation.

[d] $H_2$ production activity was determined after 24 h incubation and its relative activity was expressed as % activity to the activity obtained without dye. The 100% activity correspond to

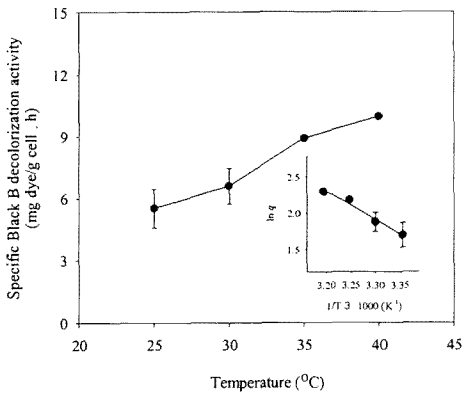


Fig. 1. Effect of temperature on specific dye decolorization activity ($q$) at pH 8.0. Insert shows the Arrhenius plot (ln $q$ vs. $1/T$) to determine the activation energy ($E_a$).

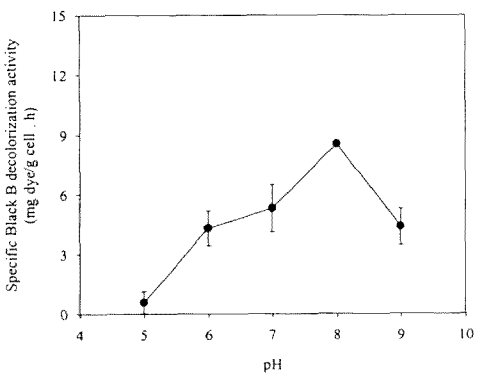


Fig 2 Effect of pH on specific dye decolorization activity at 35 °C

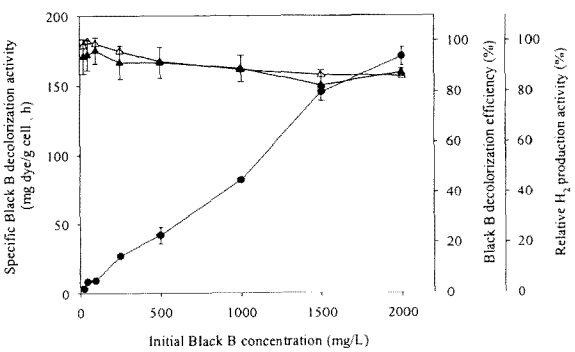


Fig. 3. Effect of initial Black B concentration on specific dye decolorization activity ( ● ), dye decolorization efficiency ( ▽ ), and relative $H_2$ production activity ( □ ). Decolorization efficiency and $H_2$ production activity were determined after 24 h incubation. Relative $H_2$ production activity was expressed as % activity to the activity obtained without dye.