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Microbial decolorization of azo dyes by *Proteus mirabilis*

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A bacterium identified as *Proteus mirabilis* was isolated from acclimated sludge from a dyeing wastewater treatment plant. This strain rapidly decolorized a deep red azo dye solution (RED RBN). Features of the decolorizing process related to biodegradation and biosorption were also studied. Although *P. mirabilis* displayed good growth in shake culture, color removal was best in anoxic static cultures. For color removal, the optimal pH and temperature were 6.5–7.5 and 30–35°C, respectively. The organism exhibited a remarkable color removal capability, even at a high concentration of azo dye. More than 95% of azo dye was reduced within 20 h at a dye concentration of 1.0 g L^{-1}. Decolorization appears to proceed primarily by enzymatic reduction associated with a minor portion, 13–17%, of biosorption to inactivated microbial cells.

Keywords: azo dyes; biodegradation; biosorption; enzymatic reduction; *Proteus mirabilis*

Introduction

Azo dyes consist of a diazotized amine coupled to an amine or a phenol, and contain one or more azo linkages. Azo dyes are the largest class of dyes with the greatest variety of colors. At least 3000 different varieties of azo dyes are extensively used in the textile, paper, food, cosmetics and pharmaceutical industries. A related topic having received considerable interest is the presence of color in effluents associated with production. Dyes are recalcitrant molecules which are difficult to degrade biologically. In addition, some azo dyes are either toxic or mutagenic and carcinogenic [1]. Treatment of dye waste water involves chemical/physical methods, among which are coagulation, precipitation, adsorption by activated carbon, oxidation by ozone, ionizing radiation and ultrafiltration. Chemical or physical-chemical methods are generally costly, produce wastes which are difficult to dispose of, are less efficient and of limited applicability. As a viable alternative, biological processes have received increasing interest owing to their cost effectiveness, ability to produce less sludge, and environmental friendliness. Therefore, a practical bioprocess for dye-containing wastewater needs to be developed.

Microorganisms capable of degrading azo dyes include *Proteus* spp [10], *Enterococcus* spp [12], *Streptococcus* spp [13], *Bacillus cereus* [15], *Streptomyces* spp [9], and the white rot fungus *Chanerochaetes chrysosporium* [4,5]. Under aerobic conditions, most azo dyes are not degradable by bacteria [4,8]. However, under anaerobic conditions, the azo linkage in the dye molecule can be reduced to form colorless aromatic amines which are occasionally toxic and carcinogenic [17]. The reduction of azo dyes in the intestine of humans and other animals has been known for quite some time. The azo reduction of tartrazine mediated by azo reductase was first demonstrated by a pure culture of *Proteus vulgaris* isolated from the feces of rats [10,11]. Besides azo reductase, lignin peroxidase was also considered to be involved in the bioreduction of azo dyes [4]. Biological azo reduction and metabolism of azo compounds by the intestinal microorganisms are biological [3]. In addition to biological degradation of azo dyes, the uptake or accumulation of dyes by biomass, termed biosorption, can also be involved [14]. The biosorption of azo dye to *B. subtilis* and *E. coli* reached an adsorption equilibrium in 8–12 h [7]. In addition, strains of *Actinomyces* have been used as an effective absorbent in decolorization of industrial effluents containing reactive dyes, such as anthoquinone, phthalocyanine and azo dyes [16].

RED RBN (Figure 1) is an azo dye used in textile and dyestuff industries. RED RBN has a deep red color, and its presence in effluents poses an environmental threat. We isolated a bacterium with the ability to decolorize high concentrations of RED RBN. In addition, characteristics of the decolorization process involving biodegradation and biosorption were examined to establish a feasible color removal process for a wastewater treatment system.

Materials and methods

Screening of decolorizers

Sludge obtained from a dyeing wastewater treatment plant was acclimated for 1 month and then served as the source of the bacterium. The screening medium (SM medium) contained: peptone, 10 g; meat extract, 10 g; NaCl, 5 g in 1 liter of distilled water with 0.1 g of RED RBN. RED RBN dye was sterilized by passing it through a 0.45-μm pore size filter, while other components were sterilized at 121°C for 20 min. Ten ml of sludge solution was then added to a 500-ml Erlenmeyer flask containing 100 ml of SM medium. The cultures were incubated at 30°C on a

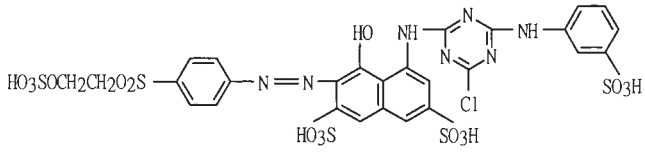

Figure 1 Chemical structure of azo dye RED RBN.

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rotary shaker at 130 rpm. Next, the broth of the decolorized flask culture was transferred to fresh SM medium to screen the strain having color removing ability. The screening procedure in the liquid culture was conducted repeatedly until a decolorized culture occurred. A small amount of decolorized broth was then poured into an agar plate containing SM medium and it was incubated at 30°C. Colonies surrounded by decolorized zones were selected. Isolates were then tested for their color removal ability in a submerged culture and the best isolate was selected. Finally, identification of the isolate was entrusted to the Culture Collection and Research Center, Food Industry Research and Development Institute (Hsinchu, Taiwan).

Dye assays

RED RBN (R-198) was obtained from Everlight Chemical Industrial Co, Taoyuan, Taiwan. The dye had maximum absorbance at 515 nm, and had a chemical structure as illustrated in Figure 1. To ensure that pH change in the dye solution did not influence decolorization, the visible absorption spectra were recorded between pH 3 and 9 and pH did not affect spectrum. Dye concentration was measured spectrophotometrically at 515 nm. The efficiency of color removal was expressed as the percentage ratio of the decolorized dye concentration to that of the initial one, ie, the difference between the initial dye concentration, *Dye(i)*, and the residual dye concentration, *Dye(r)*, of the sample:

$$\text{Color removal (\%)} = \frac{Dye(i) - Dye(r)}{Dye(i)} \times 100\%$$

Decolorizing cultivation

The ability of *P. mirabilis* to decolorize the dye was tested using shake culture and static culture. The cells were precultured for 12 h at 30°C on a rotary shaker at 130 rpm. Ten ml of the culture was inoculated into a 500-ml Erlenmeyer flask containing 200 ml of SM medium. The culture was incubated on the shaker. Samples (5 ml) of culture broth were taken at regular intervals and suspended particles were removed by centrifugation, the pH and dye concentrations (after appropriate dilution) were then measured. The decolorizing cultivation was also performed under static conditions at 30°C for an appropriate time period to observe the progression of color removal. Anoxic conditions were achieved by allowing nitrogen to flow in the flask and topping the mouth with a rubber stopper.

Results and discussion

Isolation and identification

This study was started by screening the novel dye-decolorizing bacteria and then identifying the specific isolate. Colonies having orange, yellow and white colors surrounded by a nearly decolorized zone were isolated and then tested for dye removal capability using submerged culture. One strain isolated from white colonies was inoculated in 100 ml of SM medium in a 500-ml flask and cultivated at 30°C under static conditions. After 24 h, the isolate displayed an excellent ability in clearing dye color to a degree undetected by the naked eye. The isolate was a Gram-negative rod, it was motile, catalase-positive and facultatively anaerobic. The strain was identified as *Proteus mirabilis* based on the GN microplate (Biolog, CA, USA), fatty acid analysis (Hewlett-Packard, CA, USA), API 20 E (BioMerieux SA, Marcy l'étoile, France) and API 50 CHE (BioMerieux SA).

Characteristics of microbial decolorization

P. mirabilis was propagated in SM medium in a shake culture and in anoxic static culture to observe cell growth and decolorization. Growth in shake culture was much better than in anoxic static culture (Figure 2). After 12-h cultivation, the biomass produced in shake culture was around five times that produced in anoxic static culture. Only 20% of color removal was observed in the shake culture but more than 95% of the dye was eliminated in the anoxic static culture, even when associated with a low level of cell growth. Next, the effect of surface aeration on decolorization in static culture was examined by gassing the flasks with or without pure nitrogen before cultivation. At the beginning of cultivation, a slight amount of dissolved oxygen existing inside the flask supported cell growth. As the cells exhausted the dissolved oxygen, color removal was better under anoxia. Roxon *et al* demonstrated biodegradation of azo dyes by pure cultures of *Proteus* species. Microbial reduction of azo dyes is an enzymatic reaction linked to anaerobiosis because it is inhibited by oxygen [10,11]. Therefore, facultative or obligate anaerobes are necessary for azo dye reduction. Bacteria are seldom able to decolorize azo compounds in the presence of oxygen [15].

The optimal pH for decolorization ranged from 6.5–7.5 (data not shown). The rate of decolorization decreased with decreasing temperature. At temperatures of 30–35°C, the color reduction rate was two times faster than that at a range of 20–25°C, but nearly the same level of dye removal was finally reached at both temperatures. Consequently, the following decolorization experiments were performed at 30°C, pH 7.0 in anoxic static cultures.

Peptone and meat extract were nitrogen sources for cell

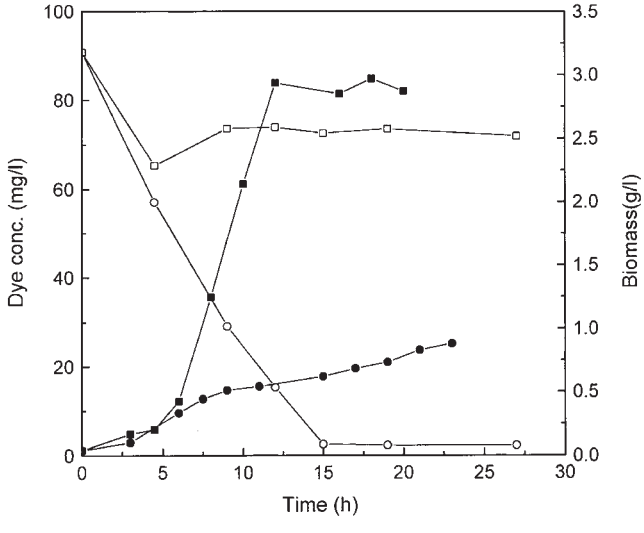

Figure 2 Comparison of shake culture with static culture on cell growth and dye removal in SM medium. Biomass: (■) shake culture, (●) static culture. Dye concentration: (□) shake culture, (○) static culture.

growth. When the meat extract was omitted, a better color reduction efficiency was observed (Figure 3). The effect of peptone concentration was then investigated. The color reduction rate during the first 10 h of incubation increased with increasing peptone concentration up to 40 g L^{-1}. A higher peptone concentration did not further increase the color reduction rate. After 33-h cultivation, cultures containing varying amounts of peptone showed no significant difference in their biomass concentrations (approximately 1.0 g L^{-1}).

Substitution of inorganic nitrogen (NH_4Cl) for peptone gave poor cell growth and low color reduction efficiency (data not shown). Roxon *et al* demonstrated that glucose stimulated the reduction of tartrazine by *P. vulgaris* [10]. Chung *et al* reported that glucose inhibited reduction of tartrazine by *B. fragilis* [2]. Therefore, an additional carbon source was tested. Color reduction was not improved by the addition of either glucose or sucrose to the culture containing inorganic nitrogen, although the biomass reached a similar level (approximately 1.0 g L^{-1}) as the control culture (Figure 4).

Figure 5 depicts the effect of dye concentration at a range of 100–1000 mg L^{-1} on color removal by *P. mirabilis*. Even with the dye concentration as high as 1000 mg L^{-1}, nearly 85% of the dye was removed after 10 h and nearly complete color loss was observed after 20 h. *P. mirabilis* also showed excellent dye removal capability for azo dye BK-5 (data not shown), which is a deep black dye conventionally used in the textile industry and has a structure similar to RED RBN. These results suggest that *P. mirabilis* isolated in this work is highly promising for applications involving biodegradation of azo dyes at high concentrations.

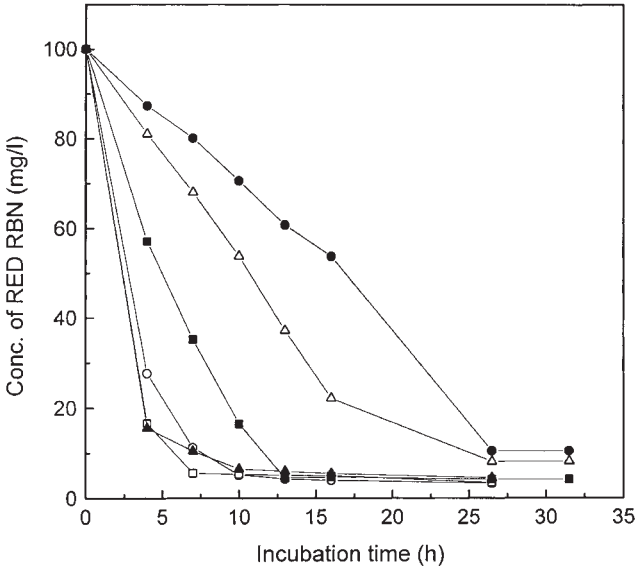

Figure 3 Effect of peptone concentration on decolorization. Medium composition (each contained 0.1 g L^{-1} of RED RBN dye): (●) peptone 10 g L^{-1} and NaCl 5 g L^{-1}; (■) peptone 20 g L^{-1} and NaCl 5 g L^{-1}; (○) peptone 30 g L^{-1} and NaCl 5 g L^{-1}; (□) peptone 40 g L^{-1} and NaCl 5 g L^{-1}; (▲) peptone 30 g L^{-1} and NaCl 5 g L^{-1}; (△) peptone 10 g L^{-1}, meat extract 10 g L^{-1} and NaCl 5 g L^{-1}.

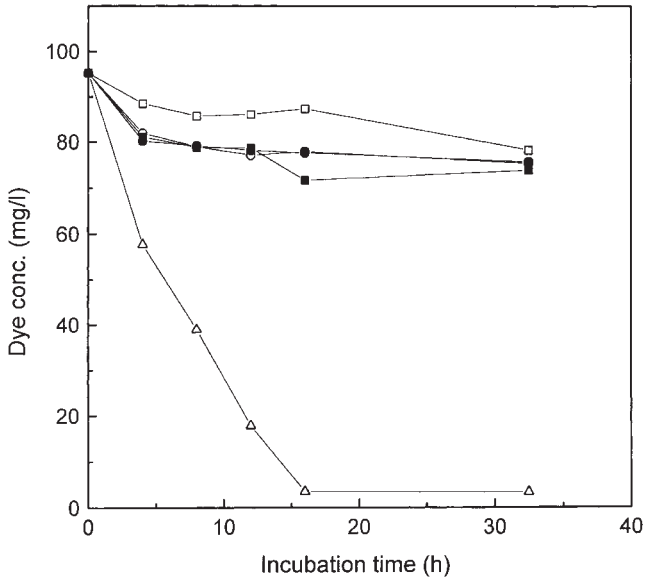

Figure 4 Effect of the concentration of glucose or sucrose on decolorization. Medium composition (each contained 95 mg L^{-1} of RED RBN dye): (□) sucrose 10 g L^{-1}, NH_4Cl 1 g L^{-1}, meat extract 10 g L^{-1} and NaCl 5 g L^{-1}; (●) glucose 10 g L^{-1}, NH_4Cl 1 g L^{-1}, meat extract 10 g L^{-1} and NaCl 5 g L^{-1}; (○) sucrose 20 g L^{-1}, NH_4Cl 1 g L^{-1}, meat extract 10 g L^{-1} and NaCl 5 g L^{-1}; (■) sucrose 5 g L^{-1}, NH_4Cl 1 g L^{-1}, meat extract 10 g L^{-1} and NaCl 5 g L^{-1}; (△) peptone 10 g L^{-1}, meat extract 10 g L^{-1} and NaCl 5 g L^{-1}.

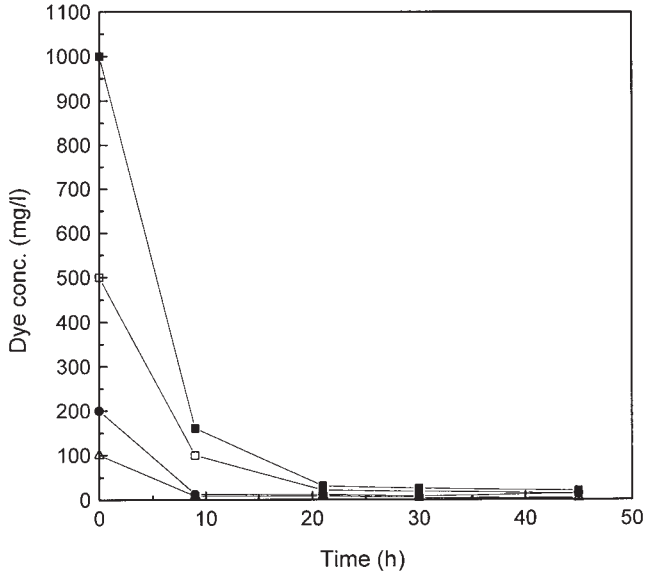

Figure 5 Effect of dye concentration on decolorization in SM medium. Concentration of RED RBN: (■) 1000 mg L^{-1}; (□) 500 mg L^{-1}; (●) 200 mg L^{-1}; (△) 100 mg L^{-1}.

Mechanism of microbial decolorization

Decolorization of the dye solution by bacteria could be due to adsorption to microbial cells or to biodegradation. In adsorption, examination of the absorption spectrum would reveal that all peaks decreased approximately in proportion to each other. If dye removal is attributed to biodegradation, either the major visible light absorbance peak would completely disappear or a new peak would appear. Dye adsorption would result in cell mats which are deeply colored

because of adsorbed dyes, whereas those retaining their original colors are accompanied by the occurrence of biodegradation [6]. Figure 6 displays the change of UV-visible spectra of RED RBN (R-198), using the supernatant fluid of the culture, before and after 20-h decolorizing cultivation with *P. mirabilis*. The absorbance peak at 515 nm completely disappeared after cultivation for decolorization. In addition, as the azo dye was reduced, the broth returned to its original white color. These results indicate that the color removal by *P. mirabilis* may be largely attributed to biodegradation.

Enzymes in microbial cells can be permanently inactivated by $HgCl_2$. In this work, various concentrations of $HgCl_2$ were added to decolorization cultures to study dye removal by *P. mirabilis*. Dye removal decreased with increased concentrations of $HgCl_2$ (Figure 7). At concentrations of $HgCl_2$ higher than 500 mg L^{-1}, a slight color reduction (around 13–17%) was observed in the first 3 h and the cell surface became red. Thereafter, no more dye removal was observed. Moreover, cell growth nearly ceased during incubation with a high concentration of $HgCl_2$. Therefore, color reduction in the initial 3 h of cultivation is attributed to adsorption to the biomass. The cell surface became red when decolorization was proceeding in the cells which were inactivated by $HgCl_2$. When living bacteria were used, the cell surface retained its original white color and only a trace amount of azo dye covered the cell surface. Moreover, no significant color change of the cell surface was observed in aerobic cultures. Therefore, microbial decolorization by *P. mirabilis* was preceded primarily by biological degradation. Even at a dye concentration as high as 1000 mg L^{-1}, *P. mirabilis* degraded the azo dye almost completely. Therefore, it is postulated that the mechanism of the color reduction by *P. mirabilis* is: (1) the dye is adsorbed to the cell surface; (2) some amounts of the dye were transferred through the cell membrane; (3) the transferred dye was degraded enzymatically inside the cells; and (4) the degraded products diffused out to the bulk solution. At a low dye concentration, almost all of the dye could be degraded completely by the reductase inside the cells. When the dye concentration was increased up to a critical value, the color-reduction capability of the cells would not be enough to degrade all the transferred dye through the cell membrane and then the dye could be accumulated on the cell surface.

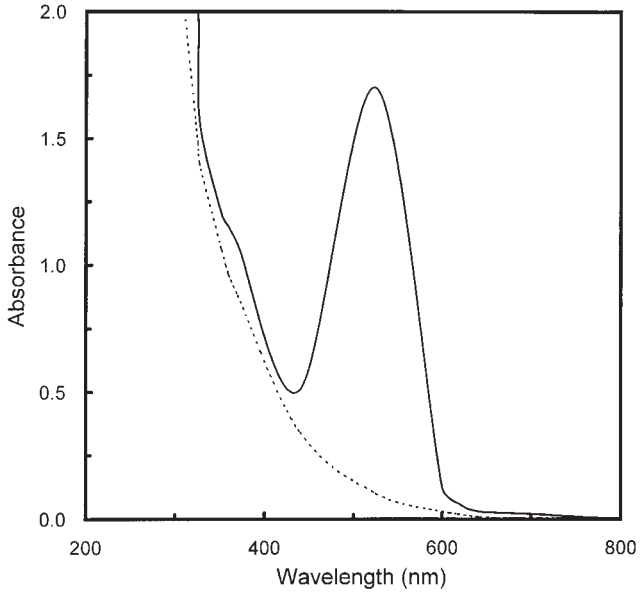

Figure 6 Variation of UV-visible spectra of azo dye solution after decolorizing cultivation with *P. mirabilis*. (——) Original dye solution; (-----) decolorized dye solution.

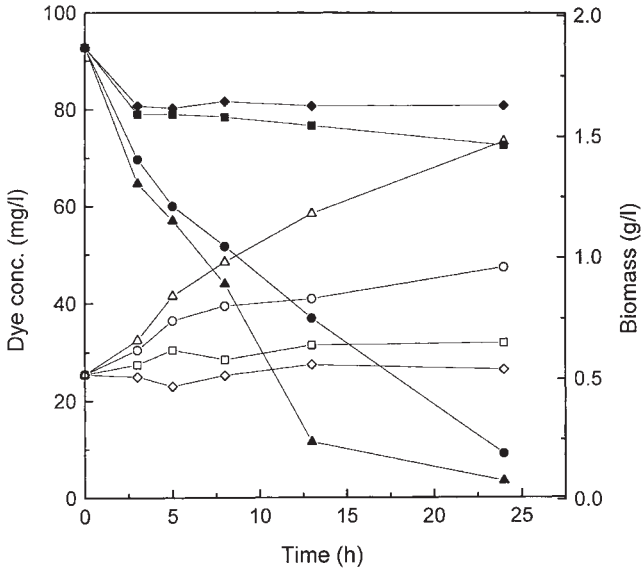

Figure 7 Effect of $HgCl_2$ concentration on biosorption during decolorizing cultivation in SM medium. The $HgCl_2$ concentration: (◆, ◇) 1000 mg L^{-1}; (■, □) 500 mg L^{-1}; (●, ○) 100 mg L^{-1}; (▲, △) control. Filled symbols: dye concentration. Open symbols: biomass.

Figure 8 depicts the effect of antibiotics on growth of

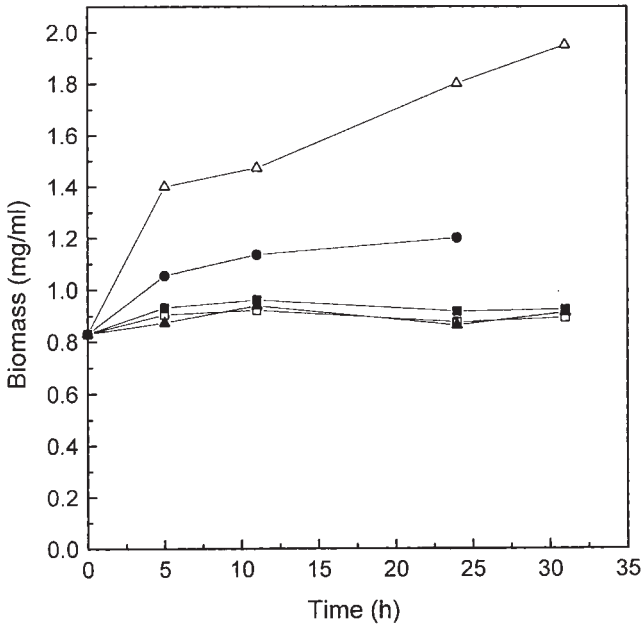

Figure 8 Effect of antibiotics on cell growth of *P. mirabilis* in SM medium. (△) Control; (●) penicillin-G, 1000 mg L^{-1}; (■) chloramphenicol, 1000 mg L^{-1}; (□) chloramphenicol, 3000 mg L^{-1}; (▲) chloramphenicol, 5000 mg L^{-1}.

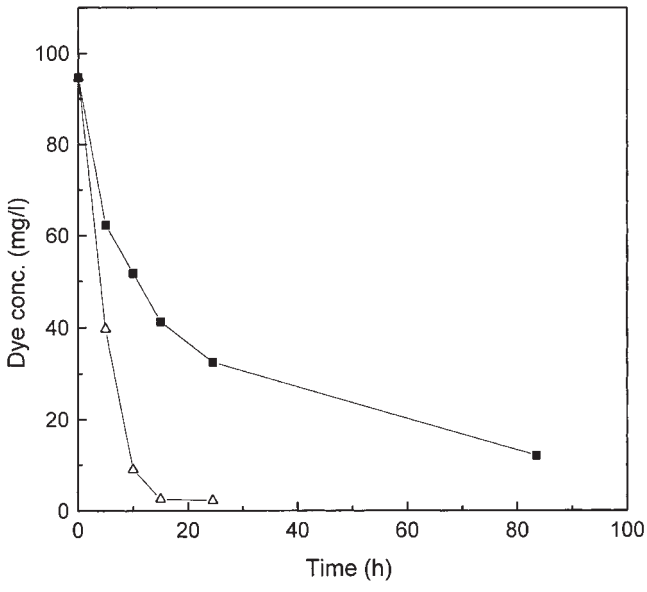

Figure 9 Effect of chloramphenicol on decolorization in SM medium. (△) Control; (■) chloramphenicol, 1000 mg L^{-1}.

P. mirabilis. Penicillin G inhibits cell wall synthesis and chloramphenicol inhibits protein synthesis. Cell growth was completely stopped as the concentration of chloramphenicol added exceeded 1000 mg L^{-1}. The cells grew slightly with the addition of Penicillin G. Based on this finding, cell growth was initially allowed to reach a sufficient biomass. Then dye and chloramphenicol (1000 mg L^{-1}) were added to conduct a decolorization experiment. Figure 9 reveals that about 85% of dye removal was observed in the decolorization culture in the presence of chloramphenicol. Thus, biodegradation in the decolorization process may result from the action of azo reductase inside the resting cell [10,11].

Conclusions

A novel strain having a high capacity for rapid decolorization of azo dyes, including a deep red color dye (RED RBN) and a deep black color dye (BK-5), was isolated from an activated-sludge system. The isolate was identified as *Proteus mirabilis*. The effects of oxygen, pH, temperature, and dye concentration on the decolorization of RED RBN dye were investigated. Examination of the mechanism of the decolorization process by *P. mirabilis* indicated that it proceeded primarily by biological degradation associated with a minor portion of the dye adsorbing onto the cell surface. A four-step mechanism was postulated based on these experimental observations. Toxicity study of the products from the degradation of RED RBN dye by *P. mirabilis* is now in progress.

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