Color removal ability of a streptomycin resistant decolorizing strain *Rhodococcus erythropolis* (ATCC 4277.1)

T.-L. Hu

Dept Environ Eng & Sci, Feng Chia University, Taichung, Taiwan 407 (E-mail: *tlhu@fcu.edu.tw*)

Abstract *Rhodococcus erythropolis* (ATCC 4277.1) is a streptomycin resistant mutant of ATCC 4277, which can decolorize the sulfonated azo dye of Orange II and Amido Black. *Pseudomonas luteola* is *a* decolorizing strain, which was isolated from sludge resulting from the treatment of dyeing wastewater. This study had two purposes: 1) to determine the color removal capacity of *R. erythropolis* (ATCC 4277.1) for Red 22, V_2RP and RP_2B dyes; and 2) to compare the decolorization capability of *R. erythropolis* (ATCC 4277.1) to that of *P. luteola,* a wild type decolorizing strain.

R. erythropolis (ATCC 4277.1) grew well in broth containing the azo dyes of Red 22, V_2RP or RP_2B, and the color of azo dyes could be removed within five days of incubation. The total percentage of color removal was 70%, 30% and 23%, respectively. Color removal by *R. erythropolis* occurred through a process of degradation, since after five day's incubation in a dye containing broth, the color of *R. erythropolis* cells remained the same as their original pinkish white color. Comparison of the color removing ability between *R. erythropolis* and *P. luteola* showed that the specific color removal (SCR) of *R. erythropolis* was 1.52 mg of RP_2B degraded per gram of dried cells, and that of SCR of *P. luteola* was 31.7 mg, which was 30 fold higher than that of *R. erythropolis*.

Keywords Degradation; *R. erythropolis*; specific color removal

Introduction

Azo dyes, having the desired characteristics of bright color, water fastness, and simple application techniques, are extensively used in the textile industry (Carliell *et al*., 1994). The biodegradation of azo dye has been intensively studied with a focus mainly on fungal systems. Many bacteria are capable of degrading azo dyes aerobically or anaerobically (Banat *et al*., 1996). The degradation of azo dyes with bacterial cultures is related to the number of azo bonds (Hu, 1998) and the complexity of the dye structure (Pasti-Grigsby *et al*., 1992). In addition to those decolorizing bacteria reported (Banat *et al*., 1996), certain genetically modified decolorization strains, such as *Sphingomonas* sp. (Russ *et al*., 2000), *E. coli* (Chang and Kuo, 2000) and *Rhodococcus* sp. (Dabbs, 1998), have been investigated for the purposes of studying the mechanism of azoreductase.

Members of the genus *Rhodococcus* have a wide metabolic versatility, including the ability to break down haloalkanes, aromatic hydrocarbons, pesticides and azo dyes (Dabbs, 1998). *Rhodococcus erythropolis* (ATCC 4277.1), a streptomycin resistant mutant of ATCC 4277, could efficiently decolorize sulfonated azo dye of Orange II and Amido Black (Dabbs *et al*., 1990). *Pseudomonas luteola,* which was isolated from sludge treating dyeing wastewater (Hu, 1994), could also effectively remove several azo dyes (Hu, 1998, 2000).

There were two aims in this study: 1) to determine whether *R. erythropolis* (ATCC 4277.1) could degrade azo dyes other than Orange II and Amido Black and to determine its color removal ability; 2) to compare the decolorizing capability of *R. erythropolis* (ATCC 4277.1) to *P. luteola* and therefore assess the decolorizing ability and potential application of this genetically modified strain.

Water Science and Technology **Vol 47 No 10 pp 169–174** © IWA Publishing 2003

Downloaded from http://iwaponline.com/wst/article-pdf/47/10/169/421919/169.pdf
by guest

Materials and methods

Bacterial strains

Rhodococcus erythropolis (ATCC 4277.1) is a streptomycin resistant mutant of the azo dye degrading bacteria *R. erythropolis* (ATCC 4277), which could efficiently degrade the sulfonated azo dyes, Orange II and Amido Black (Hess *et al.*, 1992). *Pseudomonas luteola* is a decolorizing strain isolated from an activated sludge resulting from the treatment of dyeing wastewater (Hu, 1994).

Medium

The medium used for the growth of *R. erythropolis* (ATCC 4277.1) was MM as described in Hess *et al.* (1992). In addition to carbon and energy sources, MM contained streptomycin 20 μg/ml and vitamin B1 1 μg/ml and glutamate 100 μg/ml as supplements. Tryptic Soy Broth (TSB, Difco) containing streptomycin (20 μg/ml) was also used for comparison. Media containing 10 mg/l of sterilized azo dyes was used to perform experiments on the ability of decolorization.

Glucose–YE medium (Hu, 1994) was used for the growth of *P. luteola*, and YE containing 10 mg/l of sterilized azo dyes was used for the determination of decolorization.

During the experiment, concentrated cells were prepared by centrifuging (7,000 rpm, 4°C, 10 min, Hitachi, SCR20B) fresh cells (overnight cultivation), and the pellet was collected as the inoculum.

Dyes

Azo dyes Red 22, RP_2B and V_2RP were chosen. The structure and maximal absorbency of these dyes are shown in Table 1. Since the concentration of the dyes in the wastewater from dye houses ranged from 0.01–7 mg/l (O'Nell *et al.*, 1999), a concentration of 10mg/l was used to determine the ability of decolorization.

Table 1 Structures and maximal absorbency of azo dyes used in this study

Dye	Structure	Maximal absorbency (nm)
Red 22	OCH_3; OH; N=N; O_2S; SO_3Na	508
RP_2B	SO_3Na; H; N; N=N; N; Cl; N; N; Cl; Na SO_3; SO_3 Na	533
V_2RP	O–Cu; O; N=N; H; N; N; Cl; N; N; R; SO_3Na; SO_3Na; (C_6H_5 or NH_3)	551

Downloaded from http://iwaponline.com/wst/article-pdf/47/10/169/421919/169.pdf
by guest

Effect of culture broth on the growth of *R. erythropolis* (ATCC 4277.1)

Fresh cells of *R. erythropolis* (24 h, 1%, v/v) were separately inoculated into MM and TSB broth. The culture broths were incubated in a shaker at 28°C and 100 rpm. During the incubation, broth was withdrawn every 12 h for the determination of OD_{560} (Shimadzu 12A). Total cell count was carried out at the same time by standard plate count.

Decolorization test

Concentrated cells (1% v/v) were inoculated into broth containing 10 mg/l of Red 22, RP_2B or V_2RP. The cultures were incubated in a shaker at 28°C (100 rpm). At the beginning and after incubated for 5 days, the OD_{560} of the cell cultures was determined, and the same broth was centrifuged (7,000 rpm, 4°C, 10 min) and the pellet discarded. The absorbency of the supernatant was determined for each azo dye. The color removal ability was determined as the absorbency at day 0 (A_0) minus the absorbency at day n (A_n), and divided by A_0. Culture broth was taken for the determination of dried weight.

Effect of streptomycin on the color removal of *R. erythropolis*

Concentrated cells of *R. erythropolis* (1%) were inoculated into dye containing MM broth with 20 µg/ml of streptomycin and without streptomycin. Culture broth was withdrawn every 24 h for the detection of maximal absorbency of the supernatant.

Results and discussion

Effect of media composition on the growth of *R. erythropolis* (ATCC 4277.1)

The growth medium for *R. erythropolis* (ATCC 4277.1) was a minimal medium (MM), which contained glucose (5.0 g/l) as the carbon and energy source, and vitamin B1 and glutamate as growth factors (Hess *et al*., 1992). Whether *R. erythropolis* (ATCC 4277.1) could grow in a commercially made medium such as TSB was investigated. The growth curve of *R. erythropolis* (ATCC 4277.1) in MM and TSB in Figure 1 shows that a 24 h lag phase was required for *R. erythropolis* (ATCC 4277.1) to reach the log phase when MM was used and the cells were incubated for 36 h in the stationary phase. *R. erythropolis* (ATCC 4277.1) grew faster in TSB, with the lag phase ending at 12 h of inoculation, and the log phase lasting for 24 h (Figure 1). Although *R. erythropolis* (ATCC 4277.1) showed higher total counts in TSB than in MM, the t_d (doubling time) of *R. erythropolis* (ATCC 4277.1) in MM, 5.14 h, was shorter than in TSB, 9.18 h, indicating that *R. erythropolis* (ATCC 4277.1) grew faster in MM than in TSB. The optical density (OD_{560}) was also analyzed to determine the growth of *R. erythropolis*. Since the OD_{560} could not differentiate between live and dead cells, the stationary phase was not observed.

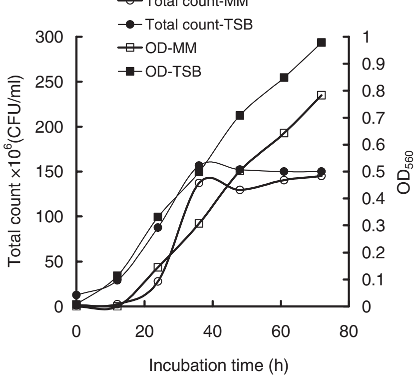

Figure 1 Growth curve of *R. erythropolis* (ATCC 4277.1) in TSB and MM broth

Downloaded from http://iwaponline.com/wst/article-pdf/47/10/169/421919/169.pdf
by guest

Since the t_d of *R. erythropolis* (ATCC 4277.1) in MM was long, after incubation for one day there were still not enough cells for inoculation. Therefore, the following decolorization experiments were performed using concentrated cells as inoculate.

Decolorization ability of *R. erythropolis* (ATCC 4277.1) in MM and TSB containing dyes

Three azo dyes (Red 22, RP_2B, V_2RP; 100 mg/l) were added into MM and TSB individually, and concentrated cells of *R. erythropolis* (ATCC 4277.1) were inoculated. The color removal ability of *R. erythropolis* (ATCC 4277.1) is shown in Figure 2. After 5 days of incubation, the maximal absorbance of each dye in the culture broth dramatically decreased. The color of *R. erythropolis* (ATCC 4277.1) remained pinkish white after 5 days of incubation, suggesting that the decolorization of Red 22, RP_2B and V_2RP occurred through degradation rather than adsorption (Hu, 1998). The color removal ability of *R. erythropolis* (ATCC 4277.1) was higher in MM than in TSB (Table 2), i.e. 23%–70% in MM, and 5.4%–65% in TSB. During the preparation of dye containing culture broth of V_2RP in TSB, the color of V_2RP changed from purple to yellow brown, and the maximal absorbency of V_2RP also shifted from 551 nm to 520nm (Figure 2f), indicating that V_2RP reacted with components in the TSB and interfered with the ability of *R. erythropolis* (ATCC 4277.1) to remove color.

The mechanism of decolorization of azo dyes is mainly due to the chromogen of azo bond (–N=N–) being broken or transformed to the single bond (–N–N–) (Hu, 1994). Many factors affect the decolorization of azo dyes, such as the number of azo bonds, and the struc-

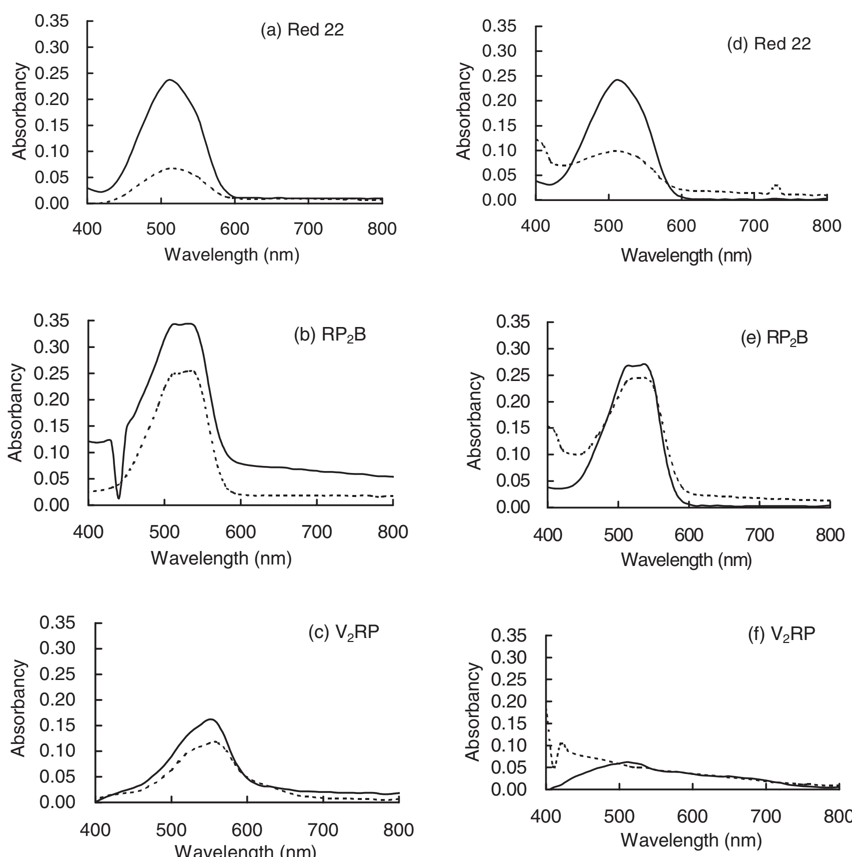

Figure 2 UV spectra of azo dyes before (–) and after (..) treatment of *R. erythropolis*. Left panel was MM broth, right panel was TSB broth

Downloaded from http://iwaponline.com/wst/article-pdf/47/10/169/421919/169.pdf
by guest

Table 2 Effect of medium composition on color removal of *R. erythropolis* (ATCC 4277.1)

Medium	Color removal* (%)		
	Red 22	V_2RP	RP_2B
MM	70	30	23
TSB	65	5.4	10

* after five-days incubation

tural features of dyes (Urushigawa and Yonezawa 1977). Red 22, RP_2B and V_2RP are monoazo dyes; therefore the color removal ability is unrelated to the number of azo bonds (Hu, 2000). Urushigawa and Yonezawa (1977) discovered that azo compounds with a hydroxy (–OH) or amino (–NH_2) group are more likely to be reliably degraded than those with a methyl, methoxy, and sulfo or nitro group. Red 22 contains more hydroxyl groups than V_2RP and RP_2B and the decreasing in absorbance in Figure 2 supports this finding.

It took *R. erythropolis* (ATCC 4277.1) 5 days to decolorize Red 22, RP_2B and V_2RP. However, color removal of Red 22 took *P. luteola*, a wild type decolorizing strain isolated from the sludge, was over 95% after 2 days (Hu, 1998). It is obvious that both microbes can degrade the azo dyes and the susceptibility of the dyes to degradation has the same trend; the more complicated the structure of dyes the harder it is to be degraded (Liu and Liu, 1992).

Comparison of color removal capability of *R. erythropolis* (ATCC 4277.1) and *P. luteola*

The color removal ability of the genetically modified decolorization strain *R. erythropolis* (ATCC 4277.1) was compared to a wild type decolorizing strain *P. luteola* on the Red 22, RP_2B and V_2RP dyes (Table 3). The specific color removal (SCR) capacity for *R. erythropolis* on Red 22, V_2RP and RP_2B was 11.5 mg/mg, 3.63 mg/mg, and 1.52 mg/mg, respectively, and the SCR for *P. luteola* on the same dyes was 45.8 mg/mg, 18.6 mg/mg and 31.7 mg/mg, respectively. The SCR of *P. luteola* was higher than that of *R. erythropolis*, and *P. luteola* could remove color of Red 22 and V_2RP within 48 h or 96 h, which was least 24 h shorter than the time required for *R. erythropolis*. The color removal capacity of *P. luteola* on RP_2B was about 20 fold higher than that of *R. erythropolis* for the same period of biodegradation (120 h). The lower efficiency of *R. erythropolis* in removing color of Red 22, RP_2B and V_2RP might have been because it was screened on Orange II and Amido Black, which are structurally simple dyes (Hess *et al.*, 1992), and the azoreductase from *R. erythropolis* was specific to these two dyes. If the activity of azoreductase was specific, it would not be useful for molecular cloning on the target of azoreductase. Another possible reason for the inefficiency of *R. erythropolis* in color removal was the instability of cloned azoreductase.

Effect of streptomycin on the color removal ability of *R. erythropolis*

Antibiotics are usually used as a marker in mutant or recombinant selection. *R. erythropolis* (ATCC 4277.1) is a streptomycin resistant mutant of the decolorizing strain ATCC 4277

Table 3 Comparison of the specific color removal of *R. erythropolis* ATCC 4277.1) and *P. luteola*

Strain	Specific color removal (mg /mg)		
	Red 22	V_2RP	RP_2B
R. erythropolis	11.5 (120 h)	3.63 (120 h)	1.52 (120 h)
P. luteola	45.8 (48 h)	18.6 (96 h)	31.7 (120 h)

Numbers in the parentheses are the total incubation time

Downloaded from http://iwaponline.com/wst/article-pdf/47/10/169/421919/169.pdf
by guest

Table 4 Effect of streptomycin on the color removal* of *R. erythropolis* (ATCC 4277.1)

Medium	Color removal (%)		
	Red 22	V_2RP	RP_2B
MM + streptomycin	64	25	11
MM	75	24	9

*after five-days incubation

(Dabbs *et al.*, 1990). In order to confirm the decolorization of *R. erythropolis* (ATCC 4277.1) is unrelated to the antibiotic. The effect of streptomycin on the color removal of *R. erythropolis* (ATCC 4277.1) is shown in Table 4. There was no difference in color removal for *R. erythropolis* inoculated in broth containing streptomycin or without it, indicating that the presence of streptomycin in the culture broth had no effect on the ability of *R. erythropolis* to remove color.

Acknowledgements

This work was supported by grant NSC 89-2815-C035-042-RE from the National Science Council. I would like to thank Ms. F. L. Chiu for her technical analysis.

References

Banat, I.M., Nigam, P., Singh, D. and Marchant, R. (1996). Microbial decolorization of textile-dye-containing effluents: A review, *Bioresource Tech.*, **54**, 217–227.

Carliell, C.M., Barclay, S.J., Nidoo, N., Buckley, C.A., Muholland, A. and Senior, E. (1994). Anaerobic decolorization of reactive azo dyes in conventional sewage treatment processes. *Water S. A.*, **20**, 341–344.

Chang, J.S. and Kuo, T.S. (2000). Kinetics of bacterial decolorization of azo dye with *Escherichia coli* NO3, *Bioresource Tech.*, **75**, 107–111.

Dabbs, E.R., Gowan, B. and Andersen, S.J. (1990). *Nocardioform* arsenic resistance plasmids and construction of *Rhodococcus* cloning vectors, *Plasmid*, **23**, 242–247.

Dabbs, E.R. (1998). Cloning of genes that have environmental and clinical importance from *rhodococci* and related bacteria, *Antonie van Leeuwenhoek*, **74**, 155–168.

Heiss, G.S., Gowan, B. and Dabbs, E.R. (1992). Cloning of DNA from a *Rhodococcus* strain conferring the ability to decolorize sulfonated azo dyes, *FEMS Microbiol. Lett.*, **99**, 221–226.

Hu, T.L. (1994). Decolourization of reactive azo dyes by transformation with *Pseudomonas luteola*, *Bioresource Tech.*, **49**, 47–51.

Hu, T.L. (1998). Degradation of azo dye RP2B by *Pseudomonas luteola*, *Wat. Sci. Tech.*, **38**(4–5), 299–306.

Hu, T.L. (2000). Kinetics of azoreductase and assessment of toxicity of metabolic products from azo dyes *Pseudomonas luteola*, *Wat. Sci. Tech.*, **43**(2), 261–269.

Kulla, M.G. (1981). Aerobic bacterial degradation of azo dyes. Microbial degradation of xenobiotics and recalcitrant compounds. In: Leisinger, T., Cook, A.M., Hutter, R., Nuesch, J. (eds), *FEMS Symposium, 12*, Academic Press, London, pp. 387–399.

Liu, J. and Liu, H. (1992). Degradation of azo dyes by algae, *Enviro. Poll.*, **75**, 237–278.

O'Neill, C., Hawkes, F.R., Hawkes, D.L., Lourenco, N.D., Pinheiro, H.M. and Delee, W. (1999). Review colour in textile effluents-sources, measurement, discharge consents and simulation: a review, *J. Chem. Tech. Biotech.*, **74**, 1009–1018.

O'Neill, C., Hawkes, F.R., Hawkes, D. Esteves, L.S. and Wilcox, S.J. (2000). Anaerobic-aerobic biotreatment of simulated textile effluent containing varied ratios of starch and azo dye, *Wat. Res.*, **34**, 2355–2361.

Russ, R., Rau, J. and Stolz, A. (2000). The function of cytoplasmic flavin reductases in the reduction of azo dyes by bacteria, *Appl. Environ. Microbiol.*, **66**(4), 1429–1434.

Urushigawa, Y. and Yonczawa, Y. (1977). Chemo-biological interactions in biological purification system. Biodegradation of azo compound by activated sludge, *Bull. Environ. Contam. Toxicol.*, **17**, 214–218.

Downloaded from http://iwaponline.com/wst/article-pdf/47/10/169/421919/169.pdf
by guest