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Isolation and Characterization of *Klebsiella pneumoniae* WL-5 Capable of Decolorizing Triphenylmethane and Azo Dyes

Jing Wu and Young-Choon Lee*

Department of Biotechnology and Brain Korea 21 Center for Silver-Bio Industrialization, College of Natural Resources and Life Science, Dong-A University, Busan 604-714, South Korea

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A *Klebsiella pneumoniae* WL-5 with the capability of decolorizing several recalcitrant dyes was isolated from activated sludge of an effluent treatment plant of a textile and dyeing industry. This strain showed a higher dye decolorization under static condition and color removal was optimal at pH 6-8 and 30-35°C. More than 90% of its color of Congo Red were reduced within 12 hr at 200 μM dye concentration. Malachite Green, Brilliant Green and Reactive Black-5 lost over 85% of their colors at 10 μM dye concentration, but the percentage decolorization of Reactive Red-120, Reactive Orange-16, and Crystal Violet were about 46%, 25%, and 13%, respectively. Decolorizations of Congo Red and triphenylmethane dyes, such as Malachite Green, Brilliant Green, and Crystal Violet were mainly due to adsorption to cells, whereas azo dyes, such as Reactive Black-5, Reactive Red-120, and Reactive Orange-16 seemed to be removed by biodegradation through unknown enzymatic processes.

Key words : Decolorization, *Klebsiella pneumoniae*, azo dye, triphenylmethane dye

Introduction

Triphenylmethane and azo dyes are used widely in textile, paper, leather, cosmetics, and food industries. These dyes are generally xenobiotic compounds which are rather recalcitrant to conventional sewage treatment systems [2,3]. Since most of them have mutagenic or carcinogenic effects [4], industrial effluent containing these dyes must be treated before it is discharged into the environment. The biological processes by microorganisms have received considerable attention for the treatment of dye-containing wastewater, because they are cost-effective and environmentally friendly, and don't produce large amount of sludge, compared with physical and chemical methods [3].

To date, many bacterial strains capable of decolorizing triphenylmethane and azo dyes have been reported and their characteristics have been reviewed [2,3,7-11]. With exception of the decolorization of dyes by *Pseudomonas pseudomallei* 13NA [16], *Citrobacter* sp. [1], and *Aeromonas hydrophila* strain [11], there are no reports on the decolorization of both triphenlymethane and azo dyes by a single species of bacterium. Therefore, much work is still required to isolate new microorganisms applicable to biodegradation of a wide range of structurally different dyes and to study their physiology, in order not only to understand the mechanisms underlying dye biodegradation, but also for biotechnological purposes.

In the course of our screening programs capable of decolorizing azo as well as triphenylmethane dyes, we have isolated a bacterium, identified as the *Klebsiella pneumoniae*, which is capable of decolorizing these dyes. This paper describes the isolation and characterization of *Klebsiella pneumoniae* WL-5 with the capability of decolorizing triphenylmethane and azo dyes.

***Corresponding author**
Tel : +82-51-200-7591, Fax : +82-51-200-6536
E-mail : yclee@dau.ac.kr

Materials and Methods

Dye and chemicals

Reactive Black 5 (RB-5), Reactive Red-120 (RR-120), Reactive Orange-16 (RO-16), Congo Red (CR), Crystal Violet (CV), Malachite Green (MG), and Brilliant Green (BG) were purchased from Sigma Chemical Co. (St Louis, MO, USA). The stock solutions of each dye were prepared by membrane filtration. 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS), DCIP (2,6-dichlorophenolindophenol), and pyrocatechol were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). All other chemicals used were of analytical grade.

Isolation and identification of dye-decolorizing microorganism

Activated sludge was collected from the effluent treatment plant of a textile and dyeing industry in Busan, South Korea and screened for dye decolorization microorganisms. The screening of the strains for dye decolorization was performed on NM9 agar plate containing (g/l): beef extract 0.3; peptone 0.5; Na_2HPO_4 6; KH_2PO_4 3; NH_4Cl 1; NaCl 0.5, agar 20; RB-5 0.1. Microorganisms were selected on the basis of clear zone on agar plate. The strains growing on the plates and decolorizing the dye were selected and grown in the same liquid medium. A bacterial strain with high RB-5 decolorization ability was identified by physiological tests using Biolog GN2 microplate (Biolog, CA, USA) and analysis of 16S rDNA sequences as described previously [1].

Decolorization of dyes by growing cells

The cells were aerobically grown at 35°C for 15 hr in LB medium. Precultured cells were inoculated at 1% (v/v) into 500 ml flasks containing 100 ml LB medium and cultured at 37°C in rotary shakers running at 200 rpm (shaking condition) or without shaking (static condition). When A_{660} reached 0.6, different dyes were added aseptically to separate flasks. After incubation for 12 hr, the cells were centrifuged at 10,000× *g* for 20 min and the precipitated cells, after being washed twice with sterile water, were dried at 80°C for 24 hr to measure dry weight of the cells. The supernatants were used as samples for decolorization assay. Decolorization assay was performed using a scanning spectrophotometer (Ultrospec 3000 UV/VIS, Pharmacia, Sweden) and expressed in terms of the percentage decolorization by the same method as described previously [1]. All experiments were conducted in triplicate. Cell growth was monitored by measurement of optical density of culture medium at 600 nm.

Effects of physicochemical factors on decolorization

Precultured cells were inoculated at 1% (v/v) into 500 ml flasks containing 100 ml LB medium with 50 μM RB-5, and incubated at various temperatures or in the same medium adjusted to different pH values under static condition. Also, the cultures were incubated under static condition in M9 medium containing various carbon or nitrogen sources at 35°C for 48 hr. The decolorization rate was calculated at λ_{max} (597 nm) of culture supernatant after centrifugation at 10,000× *g* for 20 min. The uninoculated sterile medium was used as a control.

Preparation of cell free extract and enzyme assay

After cultivation for 48 hr at 35°C under static condition, cells collected from LB medium without and with 50 μM RB-5 were used as the control and induced samples, respectively. After centrifugation at 10,000× *g* for 20 min. the supernatants were used as the extracellular enzyme sources. The harvested cells were suspended in 0.1 M potassium phosphate buffer (pH 7.4) and disrupted by ultrasonication. The debris from the disrupted cells was removed by centrifugation at 10,000× *g* for 10 min and the supernatant containing intracellular enzymes, together with extracellular enzyme sources, were used for enzyme assay.

Lignin peroxidase activity was determined by monitoring the formation of propanaldehyde at 300 nm containing 100 mM 1-propanol, 250 mM tartaric acid, 10 mM H_2O_2 in 2.5 ml reaction mixture [12]. Laccase activity was determined spectrophotometrically as the absorbance increase at 420 nm of 10% ABTS in 100 mM sodium acetate buffer, pH 4.9 [6]. Tyrosinase activity was determined by the formation of catechol quinone at 410 nm in a reaction mixture containing 0.01% catechol in 0.1 M potassium phosphate buffer (pH 7.4) in 2 ml reaction mixture [17]. Azoreductase activity was determined spectrophotometrically by monitoring NADH disappearance at 340 nm as described previously [18]. NADH-DCIP reductase activity was determined by the procedure reported previously [8].

Results and Discussion

Isolation and characterization of dye-decolorizing bacteria

Three bacterial strains capable of decolorizing RB-5 were isolated from activated sludge of the effluent treatment plant of a textile and dyeing industry. Among these bacteria, one strain (no. WL-5), which had the highest decolorization ability against RB-5, was selected for further study. This isolate was a gram-negative, straight rod, motile and physiological test indicated that the strain WL-5 was a member of the genus *Klebsiella pneumoniae*. Moreover, the phylogenetic analysis of strain WL-5 using its 16S rDNA sequence data showed this strain had the highest homology (98%) with *K. pneumoniae* (X87276). Therefore, this strain was named *K. pneumoniae* WL-5.

K. pneumoniae WL-5 showed a higher dye decolorization

under static condition although cell growth was comparatively faster under shaking condition (Fig. 1). This is consistent with the recent reports describing decolorization of azo dye by *Shewanella decolorations* S12 [15] and *S. putrefaciens* AS96 [9]. At temperatures of 30-35°C, decolorization rate was two times faster than that at a range of 20-25°C and the optimal pH for decolorization ranged from 6 to 8. *K. pneumoniae* WL-5 showed the most effective decolorization rate in the presence of yeast extract or peptone, like *S. putrefaciens* AS96 [9], while glucose resulted in lower decolorization activity, like *S. decolorations* S12 [15] (Fig. 2).

Decolorization of triphenylmethane and azo dyes by *K. pneumoniae* WL-5

Decolorization of various dyes under static condition by *K. pneumoniae* WL-5 is shown in Table 1. The cell mass of *K. pneumoniae* WL-5 cultures grown for 12 hr after addition of each dye was almost similar by about 0.6-0.8 g/l (dry weight). In the case of azo dyes tested, *K. pneumoniae* WL-5 showed the highest decolorization capability against Congo Red (diazo group): even at 500 μM, more than 80% of color was removed in 12 hr. In addition, *K. pneumoniae* WL-5 was able to decolorize RB-5, RR-120 (diazo group) and RO-16 (monoazo group) by more than 88%, 46% and 25%, respectively, at 10 μM. This strain completely decolorized 50 μM of RB-5 within 72 hr under static conditions at 37°C (Fig. 3). Khalid et al. [11] reported that *S. putrefaciens* AS96 completely decolorized 100 μM of RB-5 within 6 hr under static conditions at 35°C. On the other hand, in the case of

Table 1. Decolorization of triphenylmethane and azo dyes by *K. pneumoniae* WL-5 under static condition

Dye Conc. (μM)	Decolorization (%) after 12 hr[a]						
	RB-5	RR-120	RO-16	CR	BG	MG	CV
10	88.1	46.9	25.3	96.5	96.6	87.9	13.5
20	61.2	17.7	24.5	95.9	96.2	87.8	13.2
50	46.7	7.8	15.2	93.7	95.8	0.8	11.5
100	45.9	3.7	13.8	93.2	95.6	0.4	3.1
200	4.8	2.4	7.4	92.3	0.4	0.3	1.5
500	3.1	1.1	3.1	81.8	0.3	0.2	0.7

RB-5, Reactive Black 5; RR-120, Reactive Red 120; RO-16, Reactive Orange 16; CR, Congo Red; BG, Brilliant Green; MG, Malachite Green; CV, Crystal Violet; ND, not decolorized. [a]The variation in decolorization between three replicas was 0.01 to 0.16%.

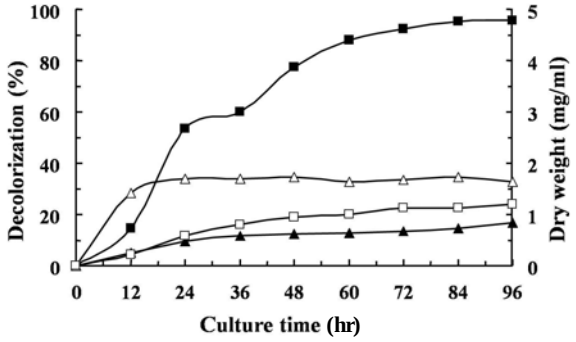

Fig. 1. Effect of aeration on the cell growth and the decolorization of 20 μM RB-5. *K. pneumoniae* WL-5 was incubated at 37°C under shaking condition (△, ▲) or under static condition (□, ■). The cell growth and the decolorization are shown as *open* and *closed* symbols, respectively. Each point represents mean±SD of triplicates

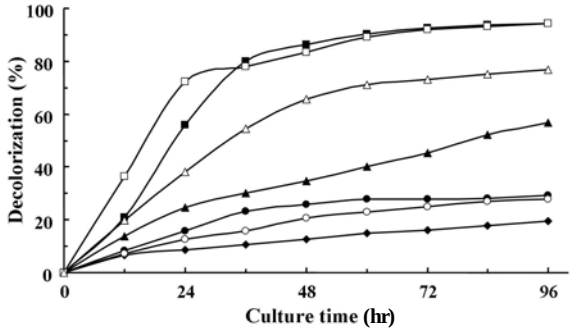

Fig. 2. Effects of carbon and nitrogen sources on decolorization of 20 μM RB-5. *K. pneumoniae* WL-5 was cultured at 37°C under static condition and harvested at the indicated time. (■), 0.5% yeast extract; (□), 1% peptone + 0.5% yeast extract; (◆), 1% glucose; (▲), 1% peptone; (●), 0.5% beef extract; (△), 1% peptone + 0.5% beef extract; (○), none.

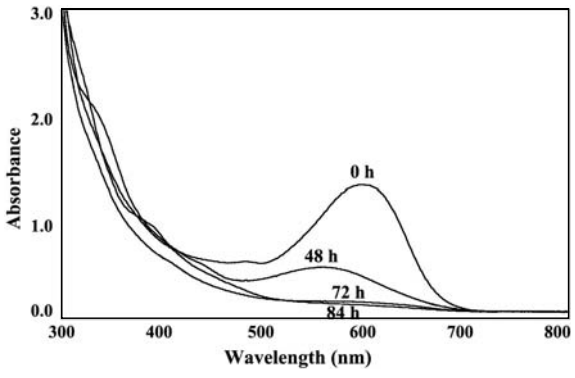

Fig. 3. UV-visible spectral scans (300-800 nm) showing color removal of crystal violet by *K. pneumoniae* WL-5. After cultivation for defined intervals in LB medium containing 50 μM RB-5 under static condition, samples were centrifuged at 10,000× *g* for 10 min and then decolorization of supernatants was monitored using a scanning spectrophotometer.

triphenylmethane dyes tested, *K. pneumoniae* WL-5 was able to decolorize BG and MG by more than 96% and 87%, respectively, at 10 μM, whereas less than 13% of CV was decolorized by this strain at the same concentration. This may be due to differences in the chemical structure of the dyes, as reported in other bacteria [1-3,11]. Especially Crystal Violet was found to be toxic to cell growth even at a dye concentration as low as 10 μM.

Mechanism of microbial decolorization

Decolorization of the dye solution may take place in two ways, either adsorption on the microbial biomass or biodegradation of the dyes by the cells [1-3]. Dye adsorption may be evident from inspection of the bacterial growth; those adsorbing dyes will be deeply colored, whereas those causing degradation will remain colorless. While *K. pneumoniae* WL-5 cells cultured under static condition for 12 hr with CR, MG, BG, and CV were stained the respective colors. This result indicates that decolorization of these dyes was mainly due to adsorption to cells. On the contrary, the cells cultured with RB-5, RR-120, and RO-16 under the same conditions remained colorless during the process of decolorization, suggesting *K. pneumoniae* WL-5 removed these dyes through biodegradation, as reported previously [2,3].

Previous studies on bacterial decolorization of azo dyes have primarily focused on the biodegradation of dyes by azoreductase that catalyzes reductive cleavage of azo bonds (-N=N-) [3,13]. Very recently several reports have shown that oxidative enzymes (lignin peroxidase and laccase), tyrosinase and NADH-DCIP reductase are responsible for dye decolorization in bacteria [5,7,8]. The result of enzyme assay using cell free extract of *K. pneumoniae* WL-5 did not show the activities of these enzymes (data not shown). Only intracellular NADH-DCIP reductase was found to be present in the control cells without dye addition, but induction of the enzyme activity was not observed in the cells after decolorization. This indicates that unknown enzymatic processes except these enzymes are involved in decolorization process by *K. pneumoniae* WL-5.

In conclusion, the present study have shown here that *K. pneumoniae* WL-5 isolated from activated sludge of an effluent treatment plant of a textile and dyeing industry is capable of decolorizing triphenylmethane and azo dyes. Decolorization efficiency was higher under static condition. Mechanisms of decolorization were different according to dye structures.

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References

1. An, S. Y., S. K. Min, I. H. Cha, Y. L. Choi, Y. S. Cho, C. H. Kim and Y. C. Lee. 2002. Decolorization of triphenylmethane and azo dyes by *Citrobacter* sp. *Biotechnol. Lett.* **24,** 1037-1040.
2. Azmi, W., R. K. Sani and U. C. Banerjee. 1998. Biodegradation of triphenylmethane dyes. *Enzyme Microb. Technol.* **22,** 185-191.
3. Banat, I. M., P. Nigam, D. Singh and R. Marchant. 1996. Microbial decolorization of textile-dye-containing effluents: a review. *Bioresour. Technol.* **58,** 217-227.
4. Chung, K. T. and C. E. Cerniglia. 1992. Mutagenicity of azo dyes: structure-activity relationships. *Mutat. Res.* **277,** 201-220.
5. Dawkar, V. V., U. U. Jadhav, S. U. Jadhav and S. P. Govindwar. 2008. Biodegradation of disperse textile dye Brown 3REL by newly isolated *Bacillus* sp. VUS. *J. Appl. Microbiol.* **105,** 14-24.
6. Hatvani, N. and I. Mecs. 2001. Production of laccase and manganese peroxidase by *Lentinus edodes* on malt containing by product of the brewing process. *Process Biochem.* **37,** 491-496.
7. Kalme, S. D., G. K. Parshetti, S. U. Jadhav and S. P. Govindwar. 2007. Biodegradation of benzidine based dye Direct Blue-6 by *Pseudomonas desmolyticum* NCIM 2112. *Bioresour. Technol.* **98,** 1405-1410.
8. Kalyani, D. C., P. S. Patil, J. P. Padhavand and S. P. Govindwar. 2008. Biodegradation of reactive textile dye Red BLI by an isolated bacterium *Pseudomonas* sp. SUK1. *Bioresour. Technol.* **99,** 4635-4641.
9. Khalid, A., M. Arshad and D. E. Crowley. 2008. Accelerated decolorization of structurally different azo dyes by newly isolated bacterial strains. *Appl. Microbiol. Biotechnol.* **78,** 361-369.
10. Parshetti, G., S. Kalme, G. Saratale and S. Govindwar. 2006. Biodegradation of malachite green by *Kocuria rosea* MTCC 1532. *Acta Chim. Slov.* **53,** 492-498.
11. Ren, S., J. Guo, G. Zeng and G. Sun. 2006. Decolorization of triphenylmethane, azo, and anthraquinone dyes by a newly isolated *Aeromonas hydrophila* strain. *Appl. Microbiol. Biotechnol.* **72,** 1316-1321.
12. Shanmugam, V., M. Kumari and K. D. Yadav. 1999. n-propanol as a substrate for assaying the lignin peroxidase activity of *Phanerochaete chrysoporium*. *Ind. J. Biochem. Biophys.* **36,** 39-43.
13. Stolz, A. 2001. Basic and applied aspects in the microbial

degradation of azo dyes. *Appl. Microbiol. Biotechnol.* **56,** 69-80.
14. Xu, M., J. Guo and G. Sun. 2007. Biodegradation of textile azo dye by *Shewanella decolorationis* S12 under microaerophilic conditions. *Appl. Microbiol. Biotechnol.* **76,** 719-726.
15. Xu, M., J. Guo, G. Zeng, X. Zhong and G. Sun. 2006. Decolorization of anthraquinone dye by *Shewanella decolorationis* S12. *Appl. Microbiol. Biotechnol.* **71,** 246-251.
16. Yatome, C., T. Ogawa, D. Koga and E. Idaka. 1981. Biodegradability of azo and triphenylmethane dyes by *Pesudomonas pseudomallei* 13NA. *J. Soc. Dyers Col.* **97,** 166-169.
17. Zhang, X. and W. H. Flurkey. 1997. Phenol oxidases in Portabella Mushrooms. *J. Food Sci.* **62,** 97-100.
18. Zimmerman, T., H. G. Kulla and T. Leisinger. 1982. Properties of purified Orange II azoreductase, the enzyme initiating azo dye degradation by *Pseudomonas* KF46. *Eur. J. Biochem.* **129,** 197-203.

초록 : 트리페닐메탄계와 아조계 색소를 탈색할 수 있는 *Klebsiella pneumoniae* WL-5의 분리 및 특성

우징 · 이영춘*
(동아대학교 생명자원과학대학 생명공학과)

여러 가지 난분해성 색소에 대하여 탈색능을 나타내는 *Klebsiella pneumoniae* WL-5이 염색폐수처리장의 활성슬러지로부터 분리되었다. 이 세균은 정치배양과 at pH 6-8 및 30-35°C에서 높은 탈색능을 나타내었다. Congo Red색소에 대해서는 200 μM 농도에서 12시간 배양하였을 때 90% 이상이 탈색되었고, Malachite Green, Brilliant Green, Reactive Black-5에 대해서는 10 μM 농도에서 80% 이상이 탈색되었지만, Reactive Red-120, Reactive Orange-16, Crystal Violet에 대해서는 10 μM 농도에서 각각 46%, 25%, 13%의 비교적 낮은 탈색능을 나타내었다. 트리페닐메탄계 색소는 세포표면에의 흡착에 의한 탈색을 나타내었고, 아조계 색소는 지금까지 알려져 있지 않는 새로운 효소반응계에 의해서 탈색된다는 것을 제시하였다.