Isolation and Characterization of Soil *Streptomyces* Involved in 2,4-Dichlorophenol Oxidation

KANG, MINJIN, JA KYOUNG KANG, AND EUNG-SOO KIM*

Department of Environmental Science, Hankuk University of Foreign Studies, Yongin, Kyunggi-Do 449-791, Korea

Abstract Over 50 morphologically distinctive soil *Streptomyces* were isolated from various locations in the Yongin area in Korea and visually screened for dye-decoloring activities on an agar plate. Two *Streptomyces* species (AD001 and ND002) showed strong dye-decoloring activities on the plate containing congo-red and new-fuchin dyes, respectively. Also, the liquid culture supernatants of these species showed 2,4-dicholophenol (DCP) oxidation activities only in the presence of hydrogen peroxide, a characteristic of Actinomycetes lignin-peroxidase (ALiP)-P3 isoform found in dye-degrading *S. viridosporus* T7A and *S. badius* 252. Based on their dye-decoloring capabilities and the 2,4-DCP oxidation kinetic data, it is suggested that these *Streptomyces* secrete not-yet-characterized extracelluar enzyme(s), whose activities are very similar to the ALiP-P3 enzyme.

Key words: 2,4-DCP oxidation, *Streptomyces*, Actinomycetes lignin-peroxidase

The microbial biodegradation of recalcitrant xenobiotic compounds has become increasingly important as a research subject not only for improving our understanding of microbial metabolism, but also for applying this knowledge to bioremediation. Accordingly, the isolation of environmentally-beneficial microorganisms possessing outstanding xenobiotic-degrading activities, followed by the characterization of degrading microorganisms at both the biochemical and molecular genetic levels have become the most important topics in environmental microbiology. Among xenobiotic-degrading microorganisms, lignin peroxidase (LiP)-producing white rot fungus such as *Phanerochaete chrysosporium* has been extensively studied for many years as the best candidate for the biodegradation of recalcitrant xenobiotic compounds such as azo-dyes, chlorinated compounds, and polyaromatic hydrocarbon

(PAH) [3]. LiP is believed to be a heme protein that catalyzes H_2O_2-dependent oxidation of lignin as well as various recalcitrant hazardous toxic compounds [4]. Although diverse research projects related to fungal LiP-mediated bioremediation have been pursued [6], unfortunately, very few have been focused on other LiP-producing microorganisms including soil bacteria.

Streptomyces is a gram-positive filamentous soil bacteria which undergoes a unique morphological differentiation [1]. For many years, *Streptomyces* has been considered as one of the most useful industrial microorganisms, mainly because they produce many valuable secondary metabolites including antibiotics, anti-cancer drugs, immunosuppresors, enzyme inhibitors, and dyes [4]. In addition, some *Streptomyces* species have also been recognized as being ecologically and environmentally valuable microorganisms due to their superior capabilities of degrading many recalcitrant substances with diverse structures [7, 8, 11, 14]. *S. viridosporus* T7A, one of the best characterized xenobiotic-degrading *Streptomyces*, was proved to secrete four different isoforms (P1 to P4) of extracellular Actinomycetes lignin-peroxidase (ALiP) [9]. Like LiP-producing *P. chrysosporium*, ALiP-secreting *Streptomyces* has also been confirmed to degrade recalcitrant xenobiotics of various structures including azo-dyes and chlorinated phenols [10]. Among the four ALiP isoforms found in *S. viridosporus*T7A, only P3 exhibits 2,4-DCP oxidation activity only in the presence of hydrogen peroxide, indicating its usefulness as the most promising bacterial enzyme for xenobiotic-biodegradation due to its broad substrate specificity [2].

In order to achieve more effective bioremediation, it would seem to be critical to isolate novel xenobiotic-degrading microorganisms from various natural environments, and characterize mechanisms involved in degrading recalcitrant hazardous toxic compounds of diverse structures. This note presents a very simple and efficient way of isolating soil *Streptomyces* involved in recalcitrant dye-degradation. Newly-isolated dye-degrading soil *Streptomyces* apparently

*Corresponding author
Phone: 82-335-330-4350; Fax: 82-335-333-1696;
E-mail: eungsoo@san.hufs.ac.kr

secretes a putative extracellular peroxidase-like enzyme(s), the 2,4-DCP oxidation activities, which seem to be very similar to the ALiP-P3 isoform characterized in *S. viridosporus* T7A.

Soil samples (about 1 g) taken at various locations in the Yongin area near Hankuk Univeristy of Foreign Studies (HUFS) were dried at room temperature for about a week, followed by dilution with 10 ml of double-distilled water. The diluted soil samples were vigorously vortexed, and then the supernatants were spread on *Streptomyces*-isolation agar (casein 0.4 g, starch 1.0 g, potassium nitrate 0.5 g, potassium phosphate dibasic 0.2 g, magnesium phosphate 0.1 g, calcium carbonate 0.1 g, and agar 15 g in 1 l of distilled water) plates followed by incubation at 30°C for over a week, which resulted in approximately 10^7 *Streptomyces* cfu/g of soil. Over 50 morphologically-distinct colonies were selected and they were then restreaked on CF agar [13] plates containing 200 ppm of congo-red dye or 50 ppm of new-fuchin dye followed by visual screening for dye-decolorization around the *Streptomyces* colonies. Two *Streptomyces* species (named AD001 and ND002) showed a very distinct decolorization phenotype around the colonies on the plates containing congo-red and new-fuschin, respectively (Fig. 1). Two previously characterized ALiP-producing *Streptomyces* (*S. viridosporus* T7A and *S. badius* 252) also exhibited the decolorization phenotype of congo-red and new-fuchin, respectively (data not shown). Based on the spore pigment colors and colony morphologies, *Streptomyces* AD001 and ND002 are believed to be different from the previously characterized ALiP-producing *S. viridosporus* T7A and *S. badius* 252. This observation implies that both *Streptomyces* AD001 and ND002 secrete unknown extracellular enzyme(s), which may be involved in dye-decolorization. This screening method seems to be a very easy and efficient means to isolate environmentally-valuable, dye-degrading soil *Streptomyces*.

**Streptomyces AD001**   **S. lividans TK21**
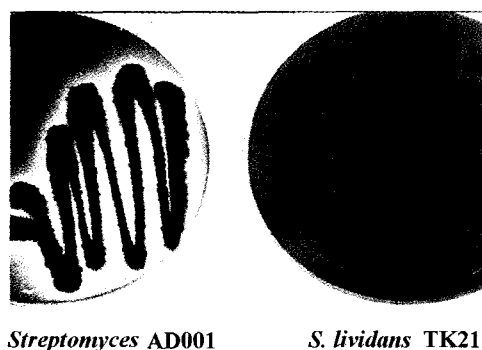
Fig. 1. Congo-red decolorization by *Streptomyces*.
Streptomyces AD001 (left) and dye non-degrading *Streptomyces lividans* (right) were streaked on CF media containing 200 ppm of congo-red azo dye and incubated at 30°C for 2 weeks. Clear decolorization of the congo-red azo-dye around the colony was detected only in the plate streaked with *Streptomyces* AD001.

Based on previous observations of dye-degradation with the well-characterized *S. viridosporus* T7A and *S. badius* 252 along with the isolated *Streptomyces* in this study, it is suggested that the *Streptomyces* AD001 and ND002 may secrete extracellular ALiP-like enzymes involved in dye-degradation. In order to verify and quantify this putative ALiP activity, especially the P3 isoform activity which is believed to possess a very broad substrate specificity including 2,4-DCP oxidation in the presence of hydrogen peroxide [9], five *Streptomyces* species (*S. lividans*, *S. viridosporus* T7A, *S. badius* 252, AD001, ND002) were grown in liquid CF and YEME media [5], followed by ALiP-P3 activity assays [9] after various time points. The ALiP-P3 enzyme assay conditions were slightly modified from the previously reported method, which utilized a UV-spectrophotometric assay using 4-aminoantipyrine [2]. One ml of an ALiP-P3 assay reaction mixture consisting of a 50 mM potassium phosphate buffer (pH 7.0), 3 mM 2,4-DCP, 0.164 mM 4-aminoantipyrine, 0.4 mM H_2O_2, and 100 μl of the culture supernatant (assay method #1). This enzyme assay was further optimized by changing to 50 mM potassium phosphate buffer (pH 9.5) and 0.518 mM 4-aminoantipyrine (assay method #2). The reaction was initiated by adding hydrogen peroxide at 37°C and an increase of absorbance was recorded at 510 nm for 2 min (one ALiP-P3 unit is an absorbance difference at 510 nm in one minute). As shown in Fig. 2, the supernatants of all four dye-decoloring *Streptomyces* species cultured in both CF and YEME media showed 2,4-DCP
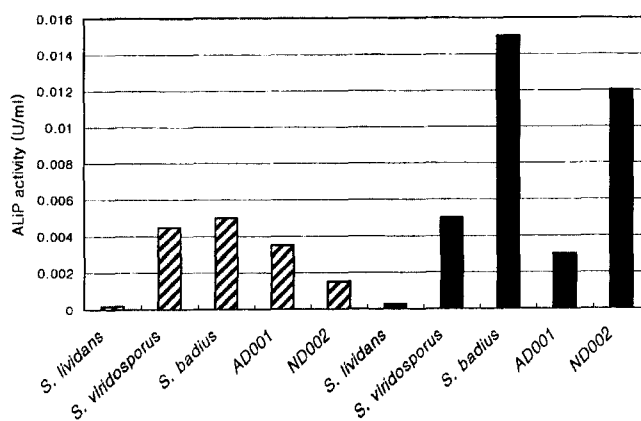
Fig. 2. 2,4-DCP oxidation activities by different *Streptomyces* species cultured in CF and YEME media.
One ml samples of five different *Streptomyces* species (*S. lividans*, *S. viridosporus* T7A, *S. badius* 252, AD001, ND002) cultured in 25 ml YEME in a 250-ml baffeled flask at 30°C were taken every 24 h for 5 days and the 2,4-DCP oxidation activity (assay method #1) was determined using the culture supernatant after centrifugation at 15,000 rpm for 10 min. The highest 2,4-DCP oxidation activity during the 5-day-culture of each *Streptomyces* was compared (strip bars for CF media and dark bars for YEME media). These experiments were repeated more than twice and produced very similar results. All data points are averages of the duplicated assay results.
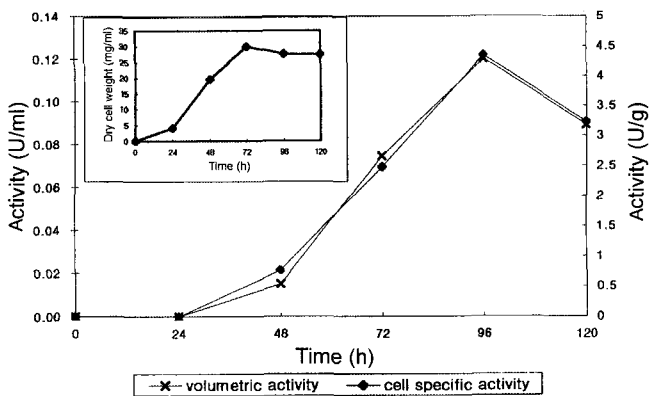
Fig. 3. Growth-associated 2,4-DCP oxidation activities in *Streptomyces* ND002.

Six ml of *Streptomyces* ND002 cultured in 100 ml YEME in a 1-l baffeled flask at 30°C was taken every 24 h for 5 days. One ml was used for measuring 2,4-DCP oxidation activity (assay method #2) and a GF/C-filtered five ml culture was dried at 105°C for 2 h for a dry cell weight measurement (inset figure). These experiments were repeated more than twice and produced very similar results. All data points are the average of duplicated assay results.

oxidation activities, usually starting from day 2 to day 5 of the cultures. All four *Streptomyces* species showed different amounts of ALiP-P3 activities, but generally higher activities in the YEME media than in the CF media, suggesting that *Streptomyces* ALiP-P3 activities are dependent on both the *Streptomyces* species as well as the culture media conditions. In order to further characterize the ALiP-P3 activity, *Streptomyces* ND002 was cultured in 100 ml YEME media for 5 days, followed by measuring ALiP-P3 activities based on both the dry cell weight and culture volume. The volumetric- and cell specific-activities of the 2,4-DCP oxidation from ND002 cultured in YEME media both exhibited almost identical patterns, suggesting that the 2,4-DCP oxidation activity is under the control of a growth-associated cell metabolism rather than a growth-arrested one in the YEME media (Fig. 3).

Considering the fact that 2,4-DCP oxidation activity in the culture supernatant of ND002 occurs only in the presence of hydrogen peroxide, it is very likely that ND002 may secrete a putative extracellular ALiP-P3 enzyme. In fact, the 2,4-DCP oxidation activity increased proportionally with the amount of the culture supernatant in the enzyme assay mixture, and no activity was observed either in the absence of the culture supernatant or with 10-min boiled culture supernatant (data not shown). In order to further verify this putative enzyme activity, kinetic analysis of 2,4-DCP oxidation was performed. With a fixed amount of the ND002 culture supernatant (100 μl of a 3-day-old ND002 culture) and hydrogen peroxide (0.4 mM), 2,4-DCP oxidation assay was conducted at various concentrations of 2,4-DCP (1 to 16 mM) and K_m of 0.221 mM was observed. This K_m value for 2,4-DCP is within a similar range as the previously reported K_m (0.372 mM) of
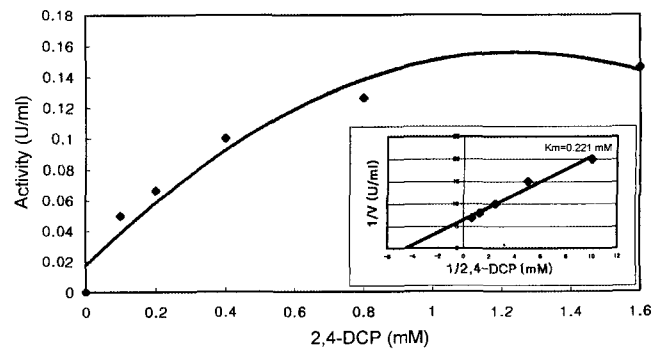
Fig. 4. Estimation of K_m for 2,4-DCP using the culture supernatant from *Streptomyces* ND002.

2,4-DCP oxidation activities (assay method #2) were determined with various concentrations of 2,4-DCP (1 to 16 mM) in the presence of 100 μl of a 3-day-old ND002 culture and hydrogen peroxide (0.4 mM). The Lineweaver-Burk plot (inset figure) shows a K_m of 0.221 mM for 2,4-DCP with *Streptomyces* ND002.

the ALiP-P3 enzyme in *S. viridosporus*T7A [2]. Although it has not been confirmed, it is highly probable that a newly-isolated, dye-decoloring soil *Streptomyces* such as ND002 produces something similar to an extracellular ALiP-P3 enzyme that plays a major role in 2,4-DCP oxidation in the presence of hydrogen peroxide. Currently, this putative enzyme is under investigation using several different biochemical and molecular genetic approaches. In conclusion, a direct visual screening assay method of dye-decolorization with soil samples can provide a very easy and efficient means to isolate environmentally-valuable xenobiotic-degrading *Streptomyces* species such as the 2,4-DCP oxidizing (probably ALiP-producing) *Streptomyces* species in natural environments.

Acknowledgments

References

1. Chater, K. F. and M. J. Merrick. 1979. Developmental biology of prokaryotes, pp. 93- 114. *In* J. H. Parish (ed.). Univ. of California Press, Berkeley and Los Angeles.
2. Dennis C. Y. and T. K. Wood. 1997. 2,4-Dichlorophenol degradation using *Streptomyces viridosporus*T7A lignin peroxidase. *Biotechnol. Prog.* **13:** 53- 59.
3. Higson, F. K. 1991. Degradation of xenobiotics by white rot fungi. *Rev. Environ. Contam. Toxicol.* **122:** 111- 152.
4. Hopwood, D. A. 1988. Towards an understanding of gene switching in *Streptomyces*, the basis of sporulation and

antibiotic production. *Proc. Roy. Soc. Lond. Ser.* **B235:** 121–138.

5. Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrempf. 1985. *Genetic Manipulation of Streptomyces. A Laboratory Manual.* John Innes Foundation Norwich, U.K.

6. Kim, M.-S., E.-J. Huh, H.-K. Kim, and K.-W. Moon. 1998. Degradation of polycyclic aromatic hydrocarbons by selected white-rot fungi and the influence of lignin peroxidase. *J. Microbiol. Biotech.* **8:** 129–133.

7. Kumar, S., K. G. Mukerji, and R. Lal. Molecular aspects of pesticide degradation by microorganisms. *Crit. Rev. Microbiol.* **22:** 1–26.

8. Li, X. and P. Gao. 1996. Isolation and partial characterization of cellulose-degrading strain of *Streptomyces* sp. LX from soil. *Lett. Appl. Microbiol.* **22:** 209–213.

9. Ramachandra, M., D. L. Crawford, and G. Hertel. 1988. Characterization of an extracellular lignin peroxidase of the lignocellulolytic Actinomycete *Streptomyces viridosporus. Appl. Environ. Microbiol.* **54:** 3057–3063.

10. Paszczynski, A., M. B. Pasti-Grigsby, S. Goszczynski, R. L. Crawford, and D. L. Crawford. 1992. Mineralization of sulfonated azo dyes and sulfanilic acid by *Phanerochaete chrysosporium* and *Streptomyces chromofuscucs. Appl. Environ. Microbiol.* **68:** 3598–3604.

11. Shelton, D. R., S. Khader, J. S. Karns, and B. M. Pogell. 1996. Metabolism of twelve herbicides by *Streptomyces. Biodegradation* **7:** 129–136.

12. Tien, M. and T. K. Kirk. 1984. Lignin-degrading enzyme from *Phanerochaete chrysosporium*: Purification, characterization, and catalytic properties of a unique H_2O_2-requiring oxygenase. *Proc. Natl. Acad. Sci. USA* **81:** 2280–2284.

13. Wang, Z., B. H. Bleakley, D. L. Crawford, G. Hertel, and F. Rafii. 1990. Cloning and expression of a ligin peroxidase gene from *Streptomyces viridosporus* in *Streptomyces lividans. J. Biotechnol.* **13:** 131–144.

14. Yee D. C. and T. K. Wood. 1997. 2,4-Dichlorophenol degradation using *Streptomyces viridosporus* T7A lignin peroxidase. *Biotechnol. Prog.* **13:** 53–59.

15. Zaborina, O., M. Latus, J. Eberspacher, L. A. Golovleva, and F. Lingens. 1995. Purification and characterization of 6-chlorohydroxyquinol 1,2-dioxygenase from *Streptomyces rochei* 303: Comparison with an analogous enzyme from *Azotobacter* sp. strain GP1. *J. Bacteriol.* **177:** 229–234.