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Microbial Biodegradation and Toxicity of Vinclozolin and its Toxic Metabolite 3,5-Dichloroaniline

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Vinclozolin, an endocrine disrupting chemical, is a chlorinated fungicide widely used to control fungal diseases. However, its metabolite 3,5-dichloroaniline is more toxic and persistent than the parent vinclozolin. For the biodegradation of vinclozolin, vinclozolin- and/or 3,5-dichloroaniline-degrading bacteria were isolated from pesticide-polluted agriculture soil. Among the isolated bacteria, a *Rhodococcus* sp. was identified from a 16S rDNA sequence analysis and named *Rhodococcus* sp. T1-1. The degradation ratios for vinclozolin or 3,5-dichloroaniline in a minimal medium containing vinclozolin (200 μg/ml) or 3,5-dichloroaniline (120 μg/ml) were 90% and 84.1%, respectively. Moreover, *Rhodococcus* sp. T1-1 also showed an effective capability to biodegrade dichloroaniline isomers on enrichment cultures in which they were contained. Therefore, these results suggest that *Rhodococcus* sp. T1-1 can bioremediate vinclozolin as well as 3,5-dichloroaniline.

Keywords: Vinclozolin, 3,5-dichloroaniline, bioremediation, biodegradation, *Rhodococcus* sp., endocrine disruptors

Recent concern about the possible effects of endocrine disruptors (EDs) on humans and wildlife has resulted in considerable interest in environmental contaminants, such as pesticides, as regards their effect on reproduction and early development [1]. The widespread use of pesticides in agrochemistry has already led to well-known environmental problems. Vinclozolin [3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-1,3-oxazolidin-2,4-dione], a known ED, is a chlorinated dicarboximide fungicide that has been widely used in Europe and the United States to control fungal diseases caused by *Botrytis cinerea*, *Sclerotinia sclerotiorum*, and *Monilinia* sp. in fruits, vegetables, ornamental plants, and turfgrass [19]. However, it has since been detected in rivers and in foods of plant origin [20], while reports have surfaced that vinclozolin has been found to influence sex differentiation in male rats because of its anti-androgen potential and that it fosters the formation of kidney stones [10]. Furthermore, the carcinogenic properties of vinclozolin in the liver, kidneys, and lung microsomes of mice [18] and in human peripheral blood lymphocytes cultured *in vitro* have also been reported [11].

Dichloroanilines (DCAs) are degradation products formed in natural environments by the degradation of various phenylcarbamate, phenylurea, and acylanilide herbicides, including vinclozolin [14]. Moreover, DCAs have been found to be cytotoxic *in vitro* to rat renal cortical and hepatic slices, and a heterogeneity was identified as regards the severity of the renal toxicity among the various DCA structural isomers [11, 31], with the following order of *in vivo* toxicity: 3,5-DCA>2,5-DCA>2,4-DCA, 2,6-DCA, 3,4-DCA>2,3-DCA [14]. As such, 3,5-DCA as a degradation product of vinclozolin is more toxic and persistent than the parent vinclozolin, yet most recent studies have mainly focused on the degradation of other DCA isomers [2, 5, 13, 15, 23, 33]. Meanwhile, recent research on vinclozolin has focused on its effect as an ED on the human body [3, 6, 12, 16, 35]. Therefore, there have been no reported studies on 3,5-DCA degradation by microorganisms.

Several studies have investigated the utilization of vinclozolin as a carbon source by a number of bacteria and fungi, including *Pseudomonas* sp., *Bacillus* sp., *Corynebacterium* sp., *Cunninghamella elegans*, and a mixed bacterial culture system [2, 8, 13]. However, the co-

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degradation of vinclozolin and 3,5-DCA, its most toxic isomer, has yet to be reported. Additionally, since the major degradation products of 3,5-DCA are more toxic and persistent than the parent vinclozolin, recent research has only focused on the degradation of DCA isomers [5, 13, 15, 28].

Accordingly, this study would appear to be the first investigation of the biodegradation of 3,5-DCA, and includes the isolation and identification of a soil bacterium, the concurrent degradation of vinclozolin and 3,5-DCA, the effect of bioremediation, and the evaluation of *Rhodococcus* sp. T1-1 as a potential biocatalyst for bioremediation.

Materials and Methods

Chemicals and Media

The vinclozolin (purity, 99%) was purchased from Supelco Co. (U.S.A.), and the dichloroaniline isomers (purity, 98%) were purchased from Aldrich Chem. Co. (U.S.A.). Stock solutions of the vinclozolin, 3,5-DCA, and dichloroaniline isomers were prepared by dissolving 10 mg of each compound in 1 ml of acetonitrile. These solutions were then stored at -20°C. The ethylacetate and acetonitrile used in the extraction and HPLC analysis solvent were obtained from J. T. Baker (U.S.A.).

The liquid minimal salt medium (MSM) contained 1 g NH_4NO_3, 1 g K_2HPO_4, 1 g KH_2PO_4, 0.4 g $MgSO_4$, 0.05 g $FeSO_4$, and 0.02 g $CaCO_3$ (Junsei Co., Japan) per liter of distilled water. The MSM was sterilized by autoclaving for 15 min at 121°C, and the final pH adjusted to 5.0. To create a solid medium, 1.8% (w/v) Bacto agar (Difco, U.S.A.) was added to the MSM. The vinclozolin and 3,5-DCA were dissolved in acetonitrile at 10 mg/ml and used in the medium at appropriate concentrations after sterilization.

Enrichment and Isolation of Vinclozolin- and 3,5-DCA-Degrading Bacterium

The vinclozolin-degrading bacteria were initially isolated from pesticide-polluted agriculture soils from Youngju, Gyeongbuk, Korea, using an enrichment culture in the MSM containing 200 μg/ml vinclozolin. Approximately 1 g of sample material was added to 20 ml of the liquid MSM containing 200 μg-vinclozolin ml^{-1} in 100-ml Erlenmeyer flasks and incubated with shaking (130 rpm) for 7 d at 30°C. Secondary enrichments were then established by inoculating 0.5 ml of the primary enrichments into 20 ml of a fresh liquid MSM containing 200 μg/ml vinclozolin and 3,5-DCA. Finally, pure cultures were obtained by repeated streaking on plates that contained the enrichment medium described above solidified with 1.8% agar. The cells were stored in 10% glycerol at -80°C. The strain designated T1-1, which grew on both the vinclozolin-MSM and the 3,5-DCA-MSM, was deposited in the Korea Collection of Type Cultures under Accession No. KCTC 10875 BP.

Identification of Strain T1-1

Strain T1-1 was characterized on the basis of its physiological and biochemical features, cellular fatty acid profiles, and phylogenetic position based on a 16S rDNA sequence analysis. The phenotypic profile of strain T1-1 was also investigated in duplicate using BIOLOG GP2 kits, following the manufacturer's instructions [21]. For the quantitative analysis of the cellular fatty acid composition, T1-1 was harvested, and the fatty acid methyl esters (FAMEs) were obtained from the washed cells by saponification, methylation, and extraction. The analysis by gas chromatography was controlled using MIS software (Microbial ID), and the peaks were automatically integrated and identified using the Microbial Identification software package [22].

For the sequence analysis, cells were harvested from a culture grown (at 30°C for 3 d) in an LB medium, and the isolation of the chromosomal DNA, PCR amplification, and direct sequencing of the purified products of strain T1-1 carried out as described in Yoon *et al.* [36].

In Vitro Toxicity Assays of Vinclozolin and 3,5-DCA

A toxicity evaluation with the human liver cancer cell line HepG2 was determined using an MTT reduction assay according to Saravanan *et al.* [24]. The HepG2 cancer cells were cultured in DMEM (Dulbecco's modified Eagle's medium; Gibco BRL, U.S.A.) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL, U.S.A.), 100 μg/ml penicillin, and 100 μg/ml streptomycin, and incubated at 37°C under 5% CO_2 in a humidified atmosphere. The medium was changed 2–3 times per week. After digestion with trypsin-EDTA, uniform amounts (~2×10^5) of HepG2 cells in the growth medium were inoculated into each well of a 96-well flat-bottom plate. After 24 h of incubation at 37°C in 5% CO_2, the growth medium was replaced with 100 ml of media containing different concentrations of vinclozolin and 3,5-DCA. After 48 h of incubation, 50 μM of a [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] (MTT) solution (5 mg/ml) was added to each well and incubated at 37°C for 4 h. The media were then carefully aspirated, and 150 μl of dimethyl sulfoxide (DMSO) was added to each well and pipetted up and down to dissolve the crystals. The cells were allowed to continue incubating at 37°C for 5 min, and then their absorbance was measured at 580 nm using a Microplate Reader (Asys Hitec, Expert96; Asys Co., Japan). The IC_{50} (concentration affecting 50% of the cells compared with the untreated control cells) values were graphically determined (plot not shown) according to the method of Dixon and Webb [7].

Biodegradation of Vinclozolin and 3,5-DCA

To quantify the substrate degradation, the vinclzolin or 3,5-DCA loss was measured over the duration of the experiment. The strains were precultured in 20 ml of the liquid MSM supplemented with 200 μg/ml vinclozolin at 30°C for 7 d. The precultures were then centrifuged at 15,000 rpm for 10 min at room temperature, and the cell pellets washed twice with a sterile 0.1 M phosphate buffer (pH 7.0). Next, the washed cells were adjusted to an OD of 0.3–0.5 at 550 nm using a 0.1 M phosphate buffer and inoculated into the MSM containing 200 μg/ml vinclozolin and 120 μg/ml 3,5-DCA, respectively, and incubated at 30°C in a rotary shaker (130 rpm) for 7 d. To prevent the pH effecting the chemical hydrolysis of vinclozolin, the initial pH value was adjusted to 5.0 [1]. The vinclozolin and 3,5-DCA concentrations and cell enumeration were measured at 24 h intervals during the incubation period. The total culture broth (vinclozolin, 3,5-DCA) was extracted with twice the volume of ethylacetate to prevent any extraction errors. After extraction with nitrogen gas, the vinclozolin and 3,5-DCA in the extract were analyzed using an HPLC (Sykam, Germany), consisting of a Solvent delivery system (S2100; Sykam, Germany) and UV-visible detector (S3210; Sykam, Germany) operated at 254 nm, and fitted with a

RP18-LiChrosorb column (Kanto Chemical Co., Japan). The mobile phase consisted of acetonitrile and water (7:3) at a flow rate of 1 ml/min. Aliquots of 20 ul were injected into the column, and the vinclozolin and its metabolites monitored using the UV detector at 254 nm. The retention time for 3,5-DCA and vinclozolin under these analytical conditions was 4.3 min and 5.3 min, respectively.

After treatment with T1-1, the toxicity of the culture broth was evaluated by yeast-based cell growth inhibition (spot test). The yeast, *Saccharomyces cerevisiae*, was cultured in a YPD (1% yeast extract, 1% polypeptone, 2% glucose) medium, and then the cells were collected and washed with a 0.1 M phosphate buffer (pH 7.0). The cell concentration was adjusted to 10^8 CFU/ml using distilled water. As the control experiment, 3,5-DCA was added to the MSM, extracted, and concentrated, as described, and then the extract added to 1 ml of the yeast suspension and cultured at 30°C. After 6 h, 10 µl of the treated yeast suspension was spotted on the YPD solid medium, and the yeast growth spot determined after 24 h of cultivation. In the case of T1-1 treatment, 3,5-DCA was added to the MSM, and then inoculated with T1-1. After 6 days, the culture broth was extracted and the growth-inhibition activity of the extract determined using the same methods.

RESULTS

Isolation and Identification of Strain T1-1

From 20 soil samples, three bacterial strains were isolated through repetitive enrichment cultures. Strains T1-1, V21, and V1 all showed vinclozolin-degradation activity, with degradation rates of 90% (20 µg/ml/d), 75% (18.13 µg/ml/d), and 67% (16.72 µg/ml/d), respectively. However, since T1-1 was capable of using both vinclozolin and 3,5-DCA as a carbon source, it was finally selected. Strain T1-1 was also found to utilize the following substrates as carbon and energy sources: catalase, dextrin, fructose, mannitol, sucrose, and alanine, but not oxidase, mannan, arabinose, lactose, or maltose.

The major cellular fatty acids were C16:0 (26.67% of total cellular fatty acids) and C18:1 (ω9c) (21.15% of total cellular fatty acids). The almost complete 16S rDNA gene sequence of strain T1-1 (1,333 nt) was determined directly following PCR amplification.

The phylogenetic analyses of the 16S rDNA gene sequences revealed a clear affiliation between strain T1-1 and members of the genus *Rhodococcus*. The closest sequence similarity was seen with *Rhodococcus baikonurensis* GTC 1041^T (99.77%) (Fig. 1). Thus, on the basis of the major fatty acid profiles, and phylogenetic and phenotypic data, strain T1-1 was assigned to the genus *Rhodococcus* as strain *Rhodococcus* sp. T1-1.

Effects of Vinclozolin and 3,5-DCA on Viability of Human Cell Lines

The inhibition of HepG2 cancer cell population growth by vinclozolin and 3,5-DCA is shown in Fig. 2. The HepG2 cell line was more sensitive to 3,5-DCA than vinclozolin

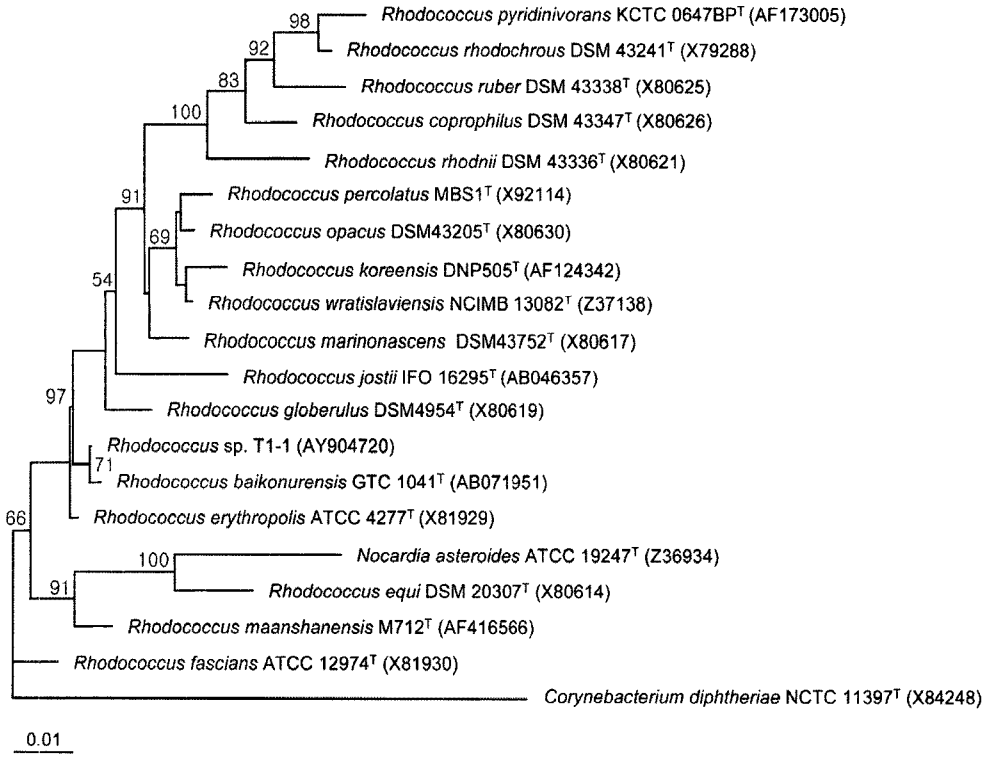

Fig. 1. Phylogenetic tree of strain T1-1 and related organisms, based on 16S rDNA sequences.
Distances were calculated using the neighbor-joining method. Numbers at branch points are bootstrap values (based on 1,000 samplings); only values greater than 50% are shown. GenBank accession numbers are given. *Corynebacterium diphtheriae* NCTC 11397T was used as the outgroup.

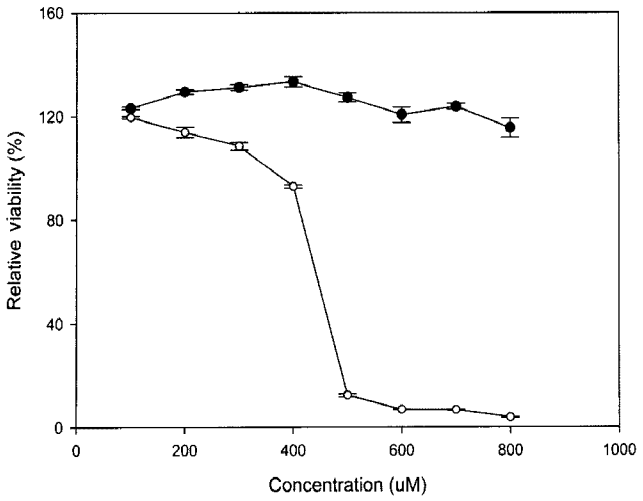

Fig. 2. Loss of viability for human cell line HepG2 after treatment with vinclozolin and 3,5-dichloroaniline.
Cell concentration: 1×10^5 cells/ml; 48 h CO_2 incubation at 37°C; MTT based; vinclozolin (●); 3,5-DCA (○).

(Fig. 2), as no significant decrease in viability was observed in the vinclozolin-exposed HepG2 after 48 h of treatment at various concentrations, whereas 3,5-DCA had a significant effect on the viability of the HepG2 cell lines. The cytotoxicity IC_{50} value for the 3,5-DCA treatment was 435 μM (Fig. 2).

Biodegradation of Vinclozolin and its Metabolites by *Rhodococcus* sp. T1-1

Rhodococcus sp. T1-1 was cultivated after being inoculated into a vinclozolin-MSM or 3,5-DCA-MSM (pH 5.0) at 30°C. Under batch culture conditions, the *Rhodococcus* sp. T1-1 cells were able to mineralize the vinclozolin and 3,5-DCA, as indicated by the decrease in the vinclozolin and 3,5-DCA concentrations and increase in the T1-1 biomass.

As such, the growth of *Rhodococcus* sp. T1-1 corresponded to the depletion of vinclozolin and 3,5-DCA after inoculation, and when vinclozolin or 3,5-DCA was omitted from the medium, no growth was observed and the pH remained at 5.0. Therefore, the results confirmed that the isolated *Rhodococcus* sp. T1-1 was able to use vinclozolin and/or 3,5-DCA as a sole carbon and energy source.

The degradation of vinclozolin was measured daily for 7 d, and Fig. 3A shows the disappearance of 200 μg/ml vinclozolin and the growth pattern of the T1-1 cells. In the vinclozolin-MSM, the cell density of *Rhodococcus* sp. T1-1 reached a maximum after six days, and then slightly decreased thereafter (Fig. 3A). The vinclozolin was rapidly degraded (Fig. 3A) from 200 to 20 μg/ml within 7 d (a decrease of 90%), whereas 3,5-DCA, a known product of vinclozolin degradation [1], was accumulated for six days from the start of cultivation, with an average of 2.03 μg/ml/d. Yet, these results also suggest that the degradation

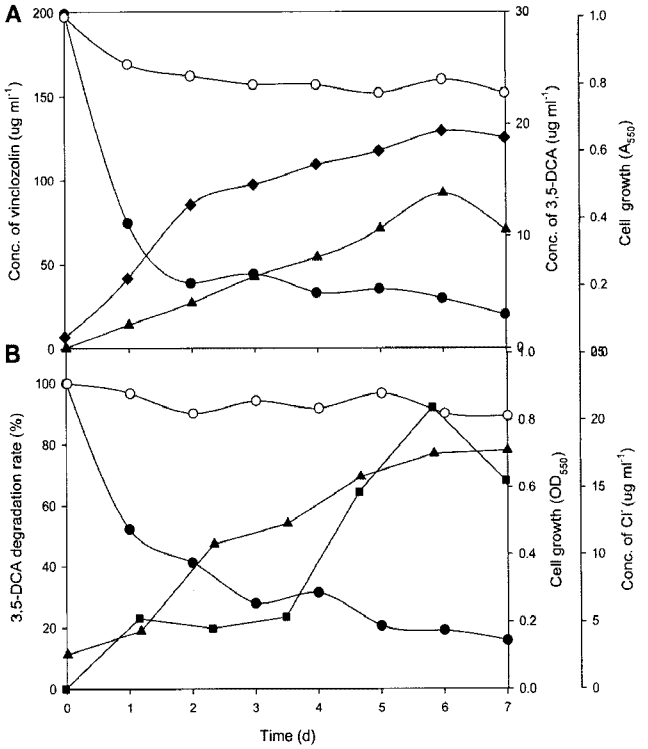

Fig. 3. Biodegradation of vinclozolin and 3,5-dichloroaniline by *Rhodococcus* sp. T1-1.
A. Degradation of vinclozolin (●), control vinclozolin (○), cell growth (◆), and production of 3,5-DCA (▲) in vinclozolin minimal salt medium over 7 d. **B.** Degradation of 3,5-DCA (●), control 3,5-DCA (○), cell growth (▲), and accumulated Cl^- (■).

of the metabolite 3,5-DCA after 6 d (Fig. 3A) was not due to chemical hydrolysis, but rather biodegradation by *Rhodococcus* sp. T1-1, as the concentration of 3,5-DCA decreased after 6 d, whereas the concentration of vinclozolin decreased to less than 21 μg/ml after 7 d (Fig. 3B). Therefore, the finding that strain T1-1 would seem to be able to degrade vinclozolin and its metabolite 3,5-DCA demonstrates the strain's versatility as regards pesticide biodegradation.

In the 3,5-DCA-MSM, the cells reached the stationary phase after about 3 d, and the 3,5-DCA was degraded from 120 to 19 μg/ml in 7 d (a decrease of 84.1%), and chloride accumulated from the degradation of 3,5-DCA (Fig. 3B). Consequently, these results suggest that 3,5-DCA was mineralized and degraded as a metabolite, confirming the efficacy of *Rhodococcus* sp. T1-1 for practical application in vinclozolin bioremediation. In addition, a GC/MS analysis detected phenol from the degraded 3,5-DCA (data not shown). As such, this would appear to be the first report of the degradation of 3,5-DCA by a bacterium that is also able to degrade vinclozolin. Upon further investigation, *Rhodococcus* sp. T1-1 was also found to degrade other dichloroaniline isomers, with the following order of degradation activity: 2,5-DCA>2,4-DCA>3,5-DCA>2,3-DCA>3,4-DCA (Fig. 4).

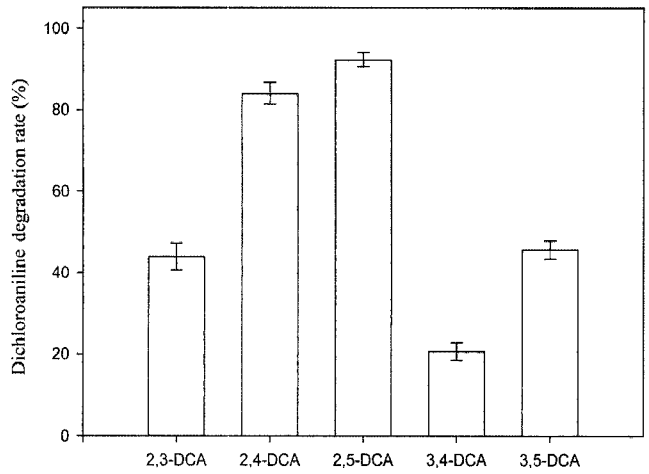

Fig. 4. Degradation ratios of dichloroaniline isomers by *Rhodococcus* sp. T1-1.

Biodegradation and Biodetoxification of Vinclozolin and 3,5-DCA by *Rhodococcus* sp. T1-1

The current authors previously reported on the toxic mechanism of 3,5-DCA using a unicellular higher eukaryote, *S. cerevisiae* [25]. Thus, since 3,5-DCA results in oxidative stress in a concentration-dependent manner in *S. cerevisiae*, treatment with 3,5-DCA followed by yeast viability measurements would seem to be a useful method for determining the toxicity of a culture broth containing 3,5-DCA. Moreover, the spot test used in this study is useful to evaluate the toxicity of a culture broth that includes residual vinclozolin and various possible toxic metabolites.

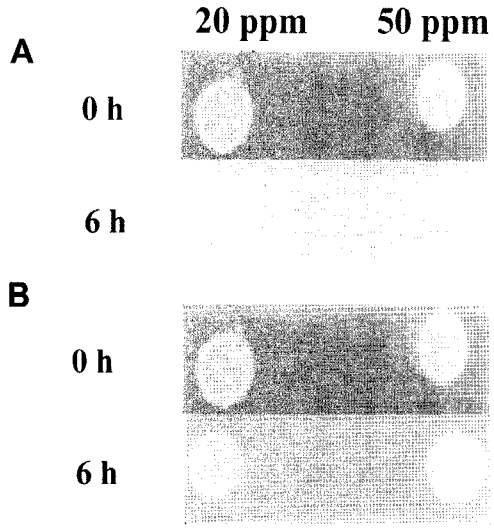

Fig. 5. Toxicity of culture broth containing 3,5-DCA based on yeast-based cell growth inhibition (spot test).
The yeast, *Saccharomyces cerevisiae*, was mixed with a culture broth extract containing toxic 3,5-DCA for 6 h, and then 10 μl of the treated yeast suspension was spotted on a YPD solid medium. The yeast growth spot was determined after 24 h of cultivation. The toxicity of 3,5-DCA was measured using a medium extract containing different concentrations of 3,5-DCA.

Thus, a spot test after 6 h of treatment with 50 ppm of 3,5-DCA revealed minor cell damage to the yeast, whereas a spot test with the T1-1-treated 3,5-DCA (50 ppm) did not show any cell growth inhibition (Fig. 5A). In addition, although severe cell growth inhibition was observed with the 50 ppm 3,5-DCA treatment, a spot test with the T1-1-treated 3,5-DCA (50 ppm) revealed similar cell growth to the non-treated control (Fig. 5B). Therefore, these results suggest that *Rhodococcus* sp. T1-1 has potential as a biocatalyst for vinclozolin and its metabolite bioremediation.

DISCUSSION

The principal toxic effects induced by vinclozolin and its metabolites are related to their anti-androgenic activity and ability to act as a competitive antagonist at the androgen receptor. Therefore, this paper reports on the biodegradation and detoxification of vinclozolin and its toxic metabolite 3,5-DCA. In several previous reports, vinclozolin has been utilized as a carbon source by a number of bacteria and fungi [13, 15].

For the biodegradation of vinclozolin, vinclozolin- and/or 3,5-DCA-degrading bacteria were isolated from pesticide-polluted agriculture soil. Among several isolated bacteria, strain T1-1 was selected based on being capable of using vinclozolin and 3,5-DCA as a source of carbon. As a result of major cellular fatty acid and 16S rDNA sequence analyses, strain T1-1 was identified as *Rhodococcus* sp. and named *Rhodococcus* sp. T1-1 [9].

Vinclozolin has previously been reported to influence sex differentiation in male rats owing to its anti-androgen potential, and also to foster the formation of kidney stones [10], thereby posing a potential risk to human beings and adaptation to oxidative stress [26]. Thus, a toxicity evaluation using the human liver cancer cell line HepG2 was conducted using an MTT reduction assay according to Saravanan *et al.* [24], where the cytotoxicity of vinclozolin (0–800 μM) and 3,5-DCA (0–800 μM), known as environmental hormones, was assessed [29].

The HepG2 cell line was found to be more sensitive to 3,5-DCA than vinclozolin (Fig. 2), which is similar to results previously reported by Valentovic *et al.* [30, 31]. The present results also suggested that 3,5-DCA, a degraded metabolite of vinclozolin, has more cytotoxic effects than vinclozolin. In another study on whether vinclozolin caused O_2-mediated radical damage in the HepG2 hepatoblastoma cell line, Sonia *et al.* [26] reported that prolonged *in vitro* treatment (24 h) with vinclozolin led to vinclozolin-induced oxidative stress, yet this action could be masked by an adaptive phenomenon. Thus, the detoxification of 3,5-DCA may be more important in the bioremediation of vinclozolin, an endocrine disrupting chemical.

Rhodococcus sp. T1-1 may be one of several bacteria with vinclozolin-degrading ability, where 3,5-DCA is the final product of the degradation or metabolic pathway of vinclozolin [1, 2]. In the present study, the degradation of vinclozolin was measured daily for 7 d. As shown in Fig. 3A, in the vinclozolin-MSM, the cell density of *Rhodococcus* sp. T1-1 reached a maximum after 6 days, and then decreased slightly thereafter, plus the vinclozolin was rapidly degraded from day 2. After 7 days, 90% of the vinclozolin was degraded, whereas 3,5-DCA, a known metabolite of vinclozolin degradation, was accumulated for 6 d from the start of cultivation. Experiments with an enrichment medium at pH 5.0 showed the microorganism to be capable of fungicide degradation; however, pH 5.5 was the most successful for culturing the vinclozolin-degrading microorganism and the biodegradation of the fungicide in pure cultures. In liquid cultures, the abiotic and biotic transformations of vinclozolin were pH-dependent, correlating with the recorded pH dependence of the chemical hydrolysis of fungicides in aqueous buffers [27, 32]. Yet, the present results suggested that the degradation of the metabolite 3,5-DCA after 6 d (Fig. 3A) was not due to chemical hydrolysis, but rather biodegradation by *Rhodococcus* sp. T1-1, as the concentration of 3,5-DCA decreased after 6 days, whereas the concentration of vinclozolin decreased after 7 days (Fig. 3B). Thus, the present finding that strain T1-1 was able to degrade vinclozolin and its metabolites demonstrates the strain's versatility for pesticide biodegradation, especially since the metabolites of the dicarboximide fungicide vinclozolin may be more severe endocrine disruptors and more resistant to bioremediation than vinclozolin [4, 17]. In Fig. 3B, highly active degradation of 3,5-DCA by strain T1-1 was induced after 3 days of incubation, and chloride accumulated as a result of the degradation of 3,5-DCA.

Therefore, the mineralization and degradation of 3,5-DCA, a metabolite of vinclozolin, by *Rhodococcus* sp. T1-1 confirms the strain's potential practical application for vinclozolin bioremediation. Although dicarbamate fungicide and 3,5-DCA isomer degradation by microorganisms has recently been reported, the 3,5-DCA degradation pathway has not been reported [5, 28, 33]. Accordingly, this would appear to be the first report of the degradation of 3,5-DCA by this bacterium, which is also able to degrade vinclozolin. Previous herbicide studies using bacteria have shown degradation products that have been implicated as detoxification products, as these types of compounds are generally more soluble in water and therefore more amenable to biodegradation and accelerated degradation by organisms.

Notwithstanding, in the present study, the degradation of the metabolite 3,5-DCA, produced from the degradation of vinclozolin, was considered more important, as 3,5-DCA is more toxic than vinclozolin. Thus, *Rhodococcus* sp. T1-1 can be effectively applied to degrade vinclozolin and its metabolite 3,5-DCA (the secondary pollution).

Acknowledgments

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