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ORIGINAL PAPER

Isolation and characterization of *Pseudomonas* sp. CBW capable of degrading carbendazim

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Abstract With the intensive application of carbendazim in greenhouse production of vegetables and the production of medicinal herbs, there is an increasing need to find a way to remediate carbendazim-contaminated soil. A bacterial stain capable of utilizing carbendazim as the sole source of carbon and energy was isolated from soil. The isolate was designated CBW and identified as a member of *Pseudomonas* sp. based on its colony morphology, 16S rRNA gene sequencing and Biolog analysis. About 87.1 and 99.1% of carbendazim at concentrations of 1.0 and 10.0 mg l^{-1} in mineral salts medium were removed by the isolate CBW after incubation for 3 days, respectively. The optimal pH value for the isolate CBW to degrade carbendazim was 7.0. The degradation rate of carbendazim by the isolate CBW was found to increase slightly with temperature. According to the metabolites detected and identified in the present study, it was proposed that carbendazim was first converted to 2-aminobenzimidazole, which was then transformed to 2-hydroxybenzimidazole, 1,2-diaminobenzene, catechol, and finally to carbon dioxide. The results indicate that the isolate CBW is a new bacterial resource for biodegrading carbendazim and might be used for bioremediation of sites heavily contaminated by carbendazim and its derivatives.

Keywords Carbendazim · Degradation · Metabolism · *Pseudomonas* sp. · Bioremediation

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Introduction

Carbendazim, a representative benzimidazolic compound, is a systemic fungicide and used to control a broad range of diseases on arable crops, fruits, vegetables, ornamentals, and medicinal herbs. It is also the major metabolic product of some other systemic fungicides, such as benomyl and thiophanate-methyl (Sandahl et al. 2000; Boudina et al. 2003). It is widely used in greenhouse production of vegetables and the production of medicinal herb *Rhizoma Atractylodis Macrocephalae*. To protect *Rhizoma Atractylodis Macrocephalae* from the infection of *Fusarium oxysporum Sch1* and *Sclerotium rolfsii Sacc*, 50% carbendazim wettable power is usually recommended to be applied into soil 3–4 times over a growing season as often as every 10–15 days if needed (Pan et al. 2006). The residual characteristics of carbendazim in the soil environment have been previously investigated. The half-life of carbendazim in soil varies from about 3 days to 12 months, depending on the soil type (Torstensson and Wessén 1984; Sangita et al. 1995; Jin et al. 2005). Repeated applications of this fungicide may result in a high residual concentration in the soil. Residual

carbendazim in soil could be uptaken by plants through the roots, seeds or leaves and, afterwards, is transferred to the whole plant (de la Huebra et al. 2000). As a result, carbendazim residue may be incorporated into and transferred along the food chain (Das et al. 2003; Burrows and Edwards 2004; Reisinger et al. 2006).

Various toxic effects have been reported after carbendazim exposure. These include down-regulation of humoral immunity (Singhal et al. 2003), sloughing of germ cells (Nakai et al. 2002), spermatogenic failure (Yu et al. 2009), and reproductive toxicity (Rajeswary et al. 2007). Morinaga et al. (2004) found that carbendazim is a potential endocrine disruptor in humans. Additionally, the intensive application of carbendazim should be assessed as a high environmental risk in soil ecosystem. Residual carbendazim in soil may reduce microbial function, and alter microbiota composition (Wang et al. 2009).

Due to the intensive application of carbendazim and its adverse effects, there is an increasing need to find a way to enhance carbendazim degradation in soil. However, only a few strains capable of degrading carbendazim have been documented (Holtman and Kobayashi 1997; Zhang et al. 2005; Xu et al. 2006). In the present study, a new bacterial strain CBW capable of utilizing carbendazim as the sole carbon and energy sources was isolated. Degradation characteristics of carbendazim by the isolated strain CBW were examined.

Materials and methods

Chemicals

Carbendazim (98.5%) was purchased from Dr. Ehrenstorfer GmbH, Augsburg, Germany. 2-Aminobenzimidazole (98%) and 2-hydroxybenzimidazole (98%) were purchased from Alfa Aesar-A Johnson Matthey Company, Ward Hill, MA, USA. All other chemicals and solvents were of analytical grade.

Isolation of microorganism

Four soil samples were taken from the fields used for the production of earthworms, vegetables, corn, and rice in Huajiachi campus, Zhejiang University, Hangzhou, China. To isolate microbes capable of degrading carbendazim, 5 g of the soil sample was placed in a 100 ml Erlenmeyer conical flask containing 50 ml of mineral salts medium (MSM) ($MgSO_4 \cdot 7H_2O$, 0.4 g; $FeSO_4$, 0.02 g; K_2HPO_4, 0.2 g; $(NH_4)_2SO_4$, 0.2 g; $CaSO_4$, 0.08 g; distilled water, 1000 ml; pH 7.0) supplemented with carbendazim of 20 mg l^{-1}. The mixture was incubated in the dark on an orbital shaker at 30°C and 150 rpm. After incubation for 4 days, 3.0 ml of the supernatant was transferred into fresh mineral salts medium containing carbendazim at the concentration of 20 mg l^{-1} and incubated for 4 days under the same conditions as described above. The culture was successively acclimated 3 times in mineral salts medium with carbendazim as the sole source of carbon and energy. Subsequently, the bacterial culture was cultivated onto mineral salt agar medium plates and incubated at 30°C for 5 days. A bacterial strain capable of utilizing carbendazim as the sole source of carbon and energy was isolated from the soil sample taken from the field used for the production of earthworms and labeled as CBW.

Growth of microorganism

To examine the growth of CBW, the mineral salts medium of pH 7.0 was supplemented with 10 mg l^{-1} of carbendazim as the sole source of carbon and energy. Each flask was inoculated with suspension of CBW to reach a biomass level of $OD_{610} = 0.2$. All flasks were incubated in a rotary shaker at 150 rpm and 30°C in the dark. The OD_{610} values were measured at 0, 1, 2, 3, 4, and 5 days, respectively. All treatments were performed in triplicate, the control experiment without carbendazim was performed under the same conditions.

Identification of microorganism

Identification of the isolate CBW was performed on the basis of the colony morphology, 16S rRNA gene sequencing and Biolog analysis. The colony morphology of the isolate CBW was monitored on agar plates containing 10 mg l^{-1} of carbendazim and LB medium without carbendazim after incubation for 2 days at 30°C, and the cell morphology was observed by a transmission electron microscope (Hitachi H-7650, Hitachi High-Technologies Corp., Tokyo, Japan) after incubation for 24 h in liquid LB medium at 30°C. Sequence analysis of the 16S rRNA gene was accomplished by Invitrogen Biotechnology

Limited Company, Shanghai, China. Biolog analysis was performed on MicroStation System with Microbial Identification Software 4.20.05 according to the manufacture's instructions (GN2 Microtiter Plate, Biolog Inc., Hayward, CA, USA).

Inoculum preparation

The isolated bacterial strain CBW was cultured in 250 ml Erlenmeyer flasks containing 100 ml LB medium (beef extract, 10 g; peptone, 5 g; sodium chloride, 5 g; distilled water, 1000 ml; pH 7.0) supplemented with carbendazim of 20 mg l^{-1}. At the exponential phase, the microorganisms were harvested by centrifugation (6500×*g*, 5 min). The bacterial sediment was washed twice with 30 ml of 0.1 mol l^{-1} NaH_2PO_4–Na_2HPO_4 buffer, and then suspended in the same buffer and used to inoculate the degradation cultures.

Degradation of carbendazim by the isolate CBW in mineral salts medium

To assess the ability of the isolate CBW to degrade carbendazim, the mineral salts medium was supplemented with carbendazim as the sole source of carbon and energy. Each flask was inoculated with suspension of the isolate CBW to reach a biomass level of $OD_{610} = 0.05$. All flasks were incubated in a shaker at 30°C and 150 rpm in the dark. At regular time intervals, the whole culture was sampled for determination of carbendazim. Each treatment was replicated 3 times, and the control experiment without inoculation of the isolate CBW was carried out under the same conditions.

To examine the effect of carbendazim concentration on its degradation, the mineral salts medium was fortified with carbendazim at levels of 1.0, 10.0, and 100.0 mg l^{-1}, respectively. The medium was prepared with pH 5.0, 7.0, and 9.0 buffer (0.1 mol l^{-1} NaH_2PO_4–Na_2HPO_4) for the measurement of pH effect on the degradation. To investigate the effect of temperature on the degradation, the medium was incubated at 20, 30, and 40°C, respectively.

Determination of carbendazim in the cultures by HPLC

The culture was entirely transferred to a separatory funnel and extracted 3 times with 50 ml CH_2Cl_2 for each. After dehydration through anhydrous sodium sulfate, the organic phases were collected in a 250-ml flat bottom flask and concentrated to almost dryness with a slight N_2 stream. Methanol was added to dissolve carbendazim and the volume was made up to 10 ml for the determination by HPLC. HPLC analysis of carbendazim was conducted with Agilent technologies 1200 equipped with diode array detector (DAD). The chromatographic separation was achieved on an Eclipse XDB-C18 column (150 mm × 4.6 mm i.d., 5 μm) at room temperature. And 10 μl samples were detected by measuring the absorbance at 281 nm with an elution of methanol and water mixture (45:55, v/v) at a flow rate of 0.8 ml min^{-1}.

Extraction and identification of carbendazim metabolites

To obtain enough quantities of the possible metabolites for separation and identification with HPLC–MS–MS, 0.3 mg of carbendazim was added into 30 ml of mineral salts medium inoculated with the isolate CBW. After incubation for 0–36 h under conditions of 30°C and 150 rpm, the mixture was extracted 3 times with 50 ml of ethyl acetate. The organic phase was collected in a 250 ml flat bottom flask through anhydrous sodium sulfate, and concentrated to almost dryness with a slight N_2 stream. Methanol was added to dissolve carbendazim and the possible metabolites and the volume was made up to 10 ml and then analyzed by HPLC–MS–MS. Separation was achieved on Eclipse XDB-C18 column (150 mm × 4.6 mm i.d., 5 μm) with elution of methanol/water (50/50, v/v) or methanol/water (60/40, v/v) containing 0.1% acetic acid at a flow rate of 0.3 ml min^{-1}. The separated metabolites were ionized by electrospray with positive polarity and scanned with a mass range (50–250 m/z).

Results

Characterization of the isolate CBW

A bacterial strain CBW capable of utilizing carbendazim as the sole source of carbon and energy was isolated from the soil sample. The growth of the isolate CBW in MSM supplemented with

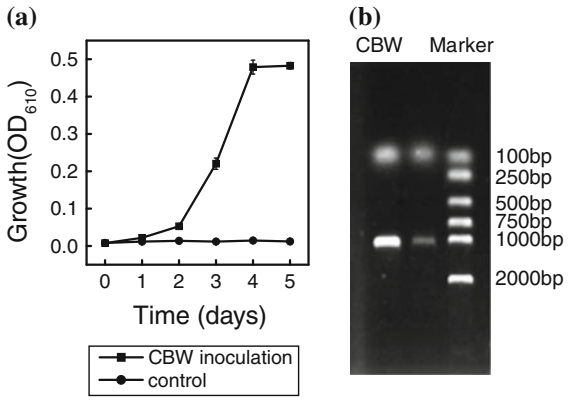

Fig. 1 Growth of the isolate CBW in mineral salts medium with carbendazim as the sole source of carbon and energy under conditions of 30°C and pH 7.0 (**a**) and agarose gel electrophoresis of 16S rRNA gene amplified from the isolate CBW (**b**)

carbendazim of 10 mg l^{-1} as the sole source of carbon and energy under conditions of 30°C and pH 7.0 in the dark is shown in Fig. 1a.

The isolate CBW is a gram-negative, aerobic, and rod shaped bacterium. The colony morphology of the isolate CBW on plain agar plate was oyster white, smooth, and eminentia. To identify the isolate CBW, both Biolog identification and 16S rRNA gene sequencing were conducted. The results of Biolog identification system indicated that the isolate is similar to *Pseudomonas* sp. with a similarity of 0.536 and a distance of 5.81. An approximately 1.0 kb 16S rRNA gene fragment was amplified from the total DNA of the isolate CBW (Fig. 1b) and sequenced (NCBI GenBank Accession No. GQ454926). After alignment with other 16S rRNA gene sequences in GenBank, it had a high degree of similarity (99%) to other members of *Pseudomonas* sp. Therefore, the isolated strain CBW is denoted here as *Pseudomonas* sp., and it has been deposited in the China Center for Type Culture Collection (CCTCC) under the CCTCC number M208046.

Effect of substrate concentration on carbendazim degradation by the isolate CBW

Effect of carbendazim concentration on its degradation by the isolate CBW was examined in MSM containing carbendazim at concentrations of 1.0, 10.0, and 100.0 mg l^{-1} under conditions of 30°C and pH 7.0 (Fig. 2). In all controls without the isolate CBW, the hydrolysis percentages of carbendazim at three concentrations were less than 11.7%. Compared to the controls without inoculation, degradation of carbendazim was enhanced obviously by the addition of the isolate CBW. Carbendazim was degraded more quickly within the first 3 days and then much more slowly during the following period of time. About 87.1 and 99.1% of carbendazim at concentrations of 1.0 and 10.0 mg l^{-1} were removed from the inoculated MSM, respectively, after incubation for 3 days. However, exposure of the isolate CBW to carbendazim at the level of 100.0 mg l^{-1} might inhibit its ability to degrade carbendazim, and about only 9.9% of carbendazim at the concentration of 100.0 mg l^{-1} was removed from the inoculated MSM.

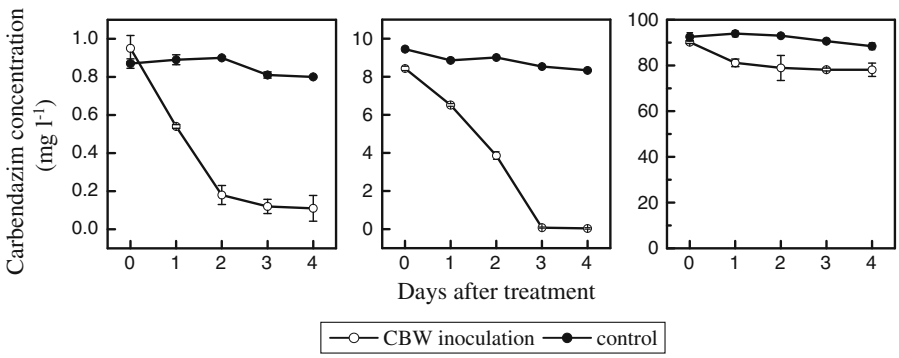

Fig. 2 Degradation of carbendazim at concentrations of 1.0, 10.0, and 100.0 mg l^{-1} by the isolate CBW in mineral salts medium under conditions of 30°C and pH 7.0

Effect of pH on carbendazim degradation by the isolate CBW

The effect of pH on degradation of carbendazim of 10.0 mg l^{-1} was carried out at 30°C (Fig. 3a). In all controls without the isolate CBW, the hydrolysis percentages of carbendazim at pH 5.0, 7.0, and 9.0 were less than 6.1%. After 4 days of incubation in the dark, 54.7, 94.9, and 83.0% of carbendazim were removed at pH 5.0, 7.0, and 9.0, respectively. The corresponding degradation rates at pH 5.0, 7.0, and 9.0 were calculated to be 1.36, 2.20, and 1.94 mg day^{-1} l^{-1}, respectively.

Effect of temperature on carbendazim degradation by the isolate CBW

Carbendazim degradation by the isolate CBW with the increase of temperature from 20 to 40°C is shown in Fig. 3b. In all controls without the isolate CBW, the hydrolysis percentages of carbendazim at 20, 30, and 40°C were less than 20.7%. It could be observed that carbendazim was degraded almost completely after incubation for 3 days, the corresponding degradation rate at 20, 30, and 40°C was 2.6, 2.7, and 2.8 mg day^{-1} l^{-1}, respectively. This indicated that the degradation rate was slightly increased with temperature.

Identification of carbendazim metabolites

After incubation for 12 h, two metabolites of carbendazim (10.0 mg l^{-1}) were identified with mass ion at m/z of 135.45 and 192.48 by MS. With the reference information of standard compounds and reported carbendazim degradation pathway, these compounds were proposed to be 2-hydroxybenzimidazole (2-HB) ($C_7H_6N_2O$, Fig. 4c) and carbendazim itself (Fig. 4a), respectively. The compounds corresponding to the other peaks were unidentified and were probably not metabolites of carbendazim.

To examine the possibility of accumulation of the metabolite 2-HB throughout the course of carbendazim degradation, the variation of its amount together with carbendazim was determined. The results showed that the level of 2-HB increased with carbendazim degradation during the period of 0-12 h and reached maximum during 12–16 h. Afterwards, 2-HB was degraded smoothly with a similar rate to that of carbendazim and removed almost completely at 36 h. This indicated that 2-HB will not accumulate in the medium in the course of carbendazim degradation.

Another chemical 2-aminobenzimidazole (2-AB) was found as a metabolite of carbendazim biodegradation in a previous study (Xu et al. 2006). However, it could not be detected in the course of carbendazim

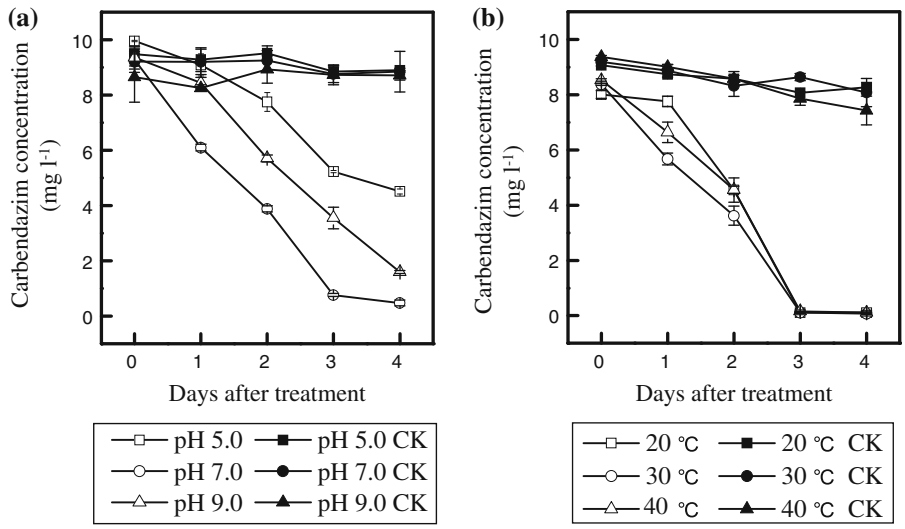

Fig. 3 Effect of pH (**a**) and temperature (**b**) on degradation of carbendazim at the concentration of 10.0 mg l^{-1} by the isolate CBW in mineral salts medium

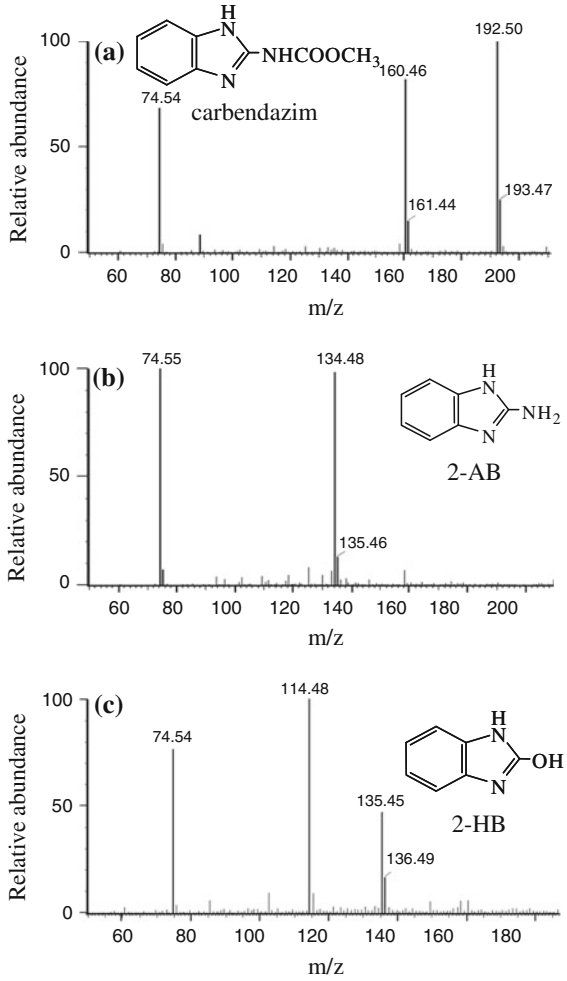

Fig. 4 Mass spectra of the metabolites of carbendazim transformed by the isolate CBW

degradation at the concentration of 10.0 mg l^{-1} in MSM by the isolate CBW. To further explore the possibility of 2-AB as a metabolite, 0.3 ml of carbendazim (1000 mg l^{-1}) was added three successive times into 30 ml of MSM inoculated with the isolate CBW every 12 h. After incubation for 36 h, the mixture was extracted with ethyl acetate and analyzed by HPLC–MS–MS. The metabolite 2-AB was found, and the corresponding MS spectrum is given in Fig. 4b.

To confirm the possible order of 2-HB and 2-AB in the course of carbendazim degradation, degradation of 2-AB at the concentration of 10.0 mg l^{-1} in 30 ml of the isolate CBW inoculated MSM under conditions of 30°C and pH 7.0 was carried out. About 46% of 2-AB was degraded after incubation for 36 h. Meanwhile, 2-HB (II, 10.747 min) was detected as a metabolite of 2-AB after HPLC separation with a mobile phase of methanol and water mixture (60:40, v/v) containing 0.1% of acetic acid at a flow rate of 0.3 ml min^{-1}, and confirmed with MS (Fig. 4c).

The metabolite 2-HB can also be degraded by the isolate CBW. After incubation under conditions of 30°C and pH 7.0 for 24 and 48 h, 46.7 and 98.9% of 2-HB at the concentration of 10 mg l^{-1} in the isolate CBW inoculated MSM were removed, respectively. To identify the possible metabolites of 2-HB, acetone, dichlorom, and ethyl acetate were selected as solvent for their extraction, respectively. However, no metabolite could be found in the course of 2-HB degradation by the isolate CBW.

Although no metabolite was extracted and identified from 2-HB degradation, it is reasonable to speculate 1, 2-diaminobenzene and catechol as possible metabolites based on the structures of 2-HB and carbendazim itself. To approve this assumption, degradation of 1, 2-diaminobenzene (1.0 mg l^{-1}) and catechol (0.5 mg l^{-1}) in the isolate CBW inoculated MSM was performed under conditions of 30°C and pH 7.0, respectively. After incubation for 24 h, 1, 2-diaminobenzene was degraded almost completely. About 30% of catechol was degraded within 12 h.

Degradation pathway of carbendazim

The present results indicated that carbendazim can be degraded rapidly by the isolate CBW. 2-AB and 2-HB were detected and indentified as major metabolites, 1, 2-diaminobenzene and catechol are suspected to be transitional metabolites. Therefore, the proposed pathway for carbendazim degradation by the isolate CBW was that carbendazim was converted into 2-AB in the first step, and to 2-HB in the second stage, and then to 1, 2-diaminobenzene and catechol, and finally to carbon dioxide (Fig. 5).

Discussion

Up to now, only a few strains capable of degrading carbendazim have been reported, such as *Rhodococcus erythropolis* (Holtman and Kobayashi 1997), *Ralstonia* sp. 1-1 (Zhang et al. 2005), *Rhodococcus* sp. djl-6

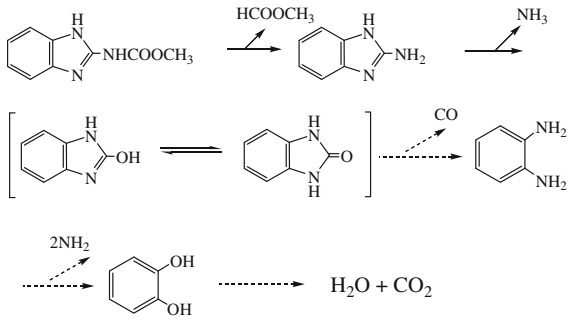

Fig. 5 The proposed degradation pathway of carbendazim by the isolate CBW. The reaction indicated by dotted line showed the compounds were uncertained

(Xu et al. 2006), *Rhodococcus qingshengii* sp. djl-6^T (Xu et al. 2007), *Burkholderia cepacia*, *Aeromonas hydrophila*, *Sphingomonas paucimobilis* (Kalwasinska et al. 2008a, b), and *Bacillus pumilus* NY97-1 (Zhang et al. 2009). In the present study, a new bacterial strain CBW capable of utilizing carbendazim as the sole carbon and energy sources was isolated and identified as *Pseudomonas* sp. Fuchs and de Vries (1978a, b) also reported that four *Pseudomonas* spp. strains capable of degrading carbendazim were isolated. Kalwasinska et al. (2008a, b) showed that *Pseudomonas luteola* and *Pseudomonas fluorescens* were the most efficient organisms for the degradation of carbendazim. The results obtained indicated that the degradation rate of carbendazim by the isolate CBW was enhanced with increasing concentrations (1–10 mg l^{-1}) and temperature (20–40°C), respectively. Similar to our results, Zhang et al. (2009) reported that the degradation rate of carbendazim by *Bacillus pumilus* NY97-1 was measured to be 42.44–90.07% with its increasing concentrations ranging from 10 to 300 mg l^{-1} and temperature. However, a different result was found that the degradation rate of carbendazim was not significantly different at different temperatures (15, 25, 30, and 37°C), except that the degradation ability at 15°C decreased slightly (Pattanasupong et al. 2004a). In our study, neutral condition was favorable for the degradation of carbendazim by CBW, whereas higher or lower pH inhibited the carbendazim degradation. A similar result was reported by Zhang et al. (2009), who found that the lowest degradation percentage of carbendazim by *Bacillus pumilus* NY97-1 was detected at pH 4. Pattanasupong et al. (2004b) also found that the degradation ability of the immobilized consortium for carbendazim markedly decreased at pH 4 and 5.

In the present study, carbendazim was converted to 2-AB and then rapidly to 2-HB by CBW. The two metabolites could be further degraded by CBW. Although no further metabolites were detected, the potential metabolites 1,2-diaminobenzene and catechol could be degraded rapidly, suggesting that 2-HB is further converted by ring-cleavage through degradation of 1,2-diaminobenzene and catechol, and probably even to carbon dioxide. Fuchs and de Vries (1978a, b) reported that carbendazim could also be degraded to 2-AB by *Pseudomonas* spp., but no further transformation of 2-AB was found, which may be due to 2-AB was toxic to the microorganism and inhibited its growth at a relatively low concentration. As a second metabolite of carbendazim, benzimidazole was detected in the course of carbendazim transformation by *Rhodococcus erythropolis* CB11 (Holtman and Kobayashi 1997). However, *Rhodococcus erythropolis* CB11 could not utilize benzimidazole as the sole carbon source. Both benzimidazole and 2-AB were found as metabolites of carbendazim degradation by *Rhodococcus* sp. djl-6 (Xu et al. 2006). In this study, the degradation of carbendazim by CBW might be a mineralization process. Nevertheless, Zhang et al. (2005) reported that carbendazim is a poor microbial energy source and not the most suitable substrate for *Ralstonia* sp. 1-1, indicating that the degradation pathway may be a co-metabolic process.

The results presented here indicated that carbendazim can be degraded and detoxified rapidly by CBW without supplementation of other carbon or nitrogen sources. Such characteristics are desirable in microorganisms to be used for bioremediation purpose. Some studies showed that carbendazim in soil was decomposed mainly by microorganisms (Helweg 1977; Yarden et al. 1990). Therefore, the isolate CBW might play a possible bioremediation role for soils contaminated by carbendazim. It is well known that the successful remediation of a soil contaminated with a specific compound relies not only on the use of an effective microbial strain, but also on many abiotic and biotic factors. To use CBW in remediation of carbendazim-contaminated soil, physical–chemical and biological factors controlling the survival and activity of CBW in soil should be further investigated.

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