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Original Research Paper

Isolation and Characterization of the Carbendazim-Degrading Strain Djl-5B

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ABSTRACT

Carbendazim (MBC) is one of the most widely used agricultural fungicides worldwide, but this chemical adversely affects animal health and ecosystem function. As such, effective methods for remediating MBC-contaminated soils must be developed. In this study, a novel MBC-degrading bacterium, namely, djl-5B, was isolated from MBC-treated soil. Based on its phenotype features as well as physiological, biochemical, and phylogenetic characteristics, the strain was identified as *Pseudomonas* sp. The optimal degradation condition for strain djl-5B was determined to be at 30°C and pH 7.5 with an inoculum size of 7%. Inoculation of strain djl-5B to MBC-contaminated soil significantly decreases the degradation half-life of MBC from 14.15 d to 3.06 d in non-sterile soil and from 24.75 d to 3.30 d in sterile soil. Results of high-pressure liquid chromatography-mass spectrometry analysis revealed that the degradation product of strain djl-5B contains 2-aminobenzimidazole, 2-hydroxybenzimidazole and benzimidazole. The newly isolated strain effectively degrades MBC and thus exhibits potential for environmental rehabilitation. This study provides basic information and technical support for future utilization of strain djl-5B as an ecofriendly bioremediation agent.

INTRODUCTION

Carbendazim (methyl-1H-benzimidazol-2-ylcarbamate or MBC) is one of the most active compounds of benzimidazole (BZ) fungicides that exhibit protective and curative activities against a wide range of fungal diseases in fruits and vegetables, particularly grapes (Boudina et al. 2003, Rodríguez-Concepción et al. 2002), MBC is also an active substance and the main degradation product of benomyl or thiophanate-methyl (Mazellier et al. 2002). A large portion of MBC, which has a stable and long residual period (with a half-life up to 12 months) (Austin et al. 1976), infiltrates the soil and environment because of its extensive application in agricultural production; hence, MBC is a serious threat to human and animal security as a result of its natural migration in the food chain (Cuppen et al. 2000, McCarroll et al. 2002). MBC is a suspected mutagen, carcinogen, and endocrine disruptor in mammals (Selmanoglu et al. 2001, Farag et al. 2011).

With increasing awareness on environmental protection and serious effects of pesticide residues, research on the degradation of MBC residues has gained increased attention. MBC is degraded in natural soils mainly through microbial metabolism, in addition to chemical and physical processes (Kannaiyan et al. 1975). Nevertheless, systematic research on the screening of degradation bacteria and their characteristics to restore the soil and remediate MBC-contaminated soils is rare. Thus, this paper describes the isolation and degradation characterization of *Pseudomonas* sp. strain djl-5B, which can utilize MBC as the sole carbon source. Metabolites in the culture filtrate of the isolated strain djl-5B are analysed. This study provides a proof-of-concept demonstration of the potential of the strain in the bioremediation of MBC-contaminated soils.

MATERIALS AND METHODS

Materials

Experimental soils: Soil samples for isolation were vegetable greenhouse soils collected in May 2013 from Yangling (Shaanxi Province, China) with a 5-year history of repeated MBC application. Soil samples for degradation were the topsoil of a flower nursery without MBC treatment.

Culture medium: LB liquid medium (pH = 7.0) contained 10.0 g of NaCl, 10.0 g of peptone, 5.0 g of yeast powder, and 1 L of distilled water. LB solid medium constituted all ingredients of LB liquid medium added with 1%-2% agar. Basic salt medium (pH = 7) comprised 1.0 g of NaCl, 1.5 g of K_2HPO_4, 0.5 g of KH_2PO_4, 2.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4.7H_2O$, and 1 L of distilled water. Screening liquid medium consisted of all the ingredients of the basic salt medium added with BMC. Screening solid medium contained the ingredients of the screening liquid medium with additional 1%-2%agar. All the media were sterilized in an

autoclave at 121°C for 30 min.

Main reagents and instruments: The main reagents included MBC, 2-aminobenzimidazole, (2-AB), 2-hydroxyphenyl benzimidazole (2-HB), BZ, and O-phenylendiamine (OP) standard substance (J & K Chemical Technology Co., Ltd.). The main instruments included LTQ-XL Liquid Chromatography Mass Spectrometer (American Thermoelectric Company), Hitachi S-4800 Field Emission Scanning Electron Microscope (Japanese Hitachi Company) and ZHWY-1102 Constant Temperature Shaker (Shanghai Zhicheng Analysis Instrument Manufacturing Co., Ltd.).

Methods

Soil enrichment and isolation of MBC-degrading bacteria: Briefly, 10 g of newly collected soil samples were added to 100 mL of the screening liquid medium containing 50 mg/L MBC at 30°C. After shaking the culture for 5 h at 150 r/min, the suspended matter was drained and inoculated (5%) into 100 mL of the screening liquid medium. MBC concentration was increased by 50 mg/L each time until the concentration reached 300 mg/L. The final screening culture medium underwent a gradient dilution (10^{-1}-10^{-11}) with three repetitions for each gradient. The gradient was smeared on the solid screening medium containing 200 mg/L MBC at 30°C. Strains with different morphologies and a large colony were isolated and purified repeatedly until pure bacterial strains were obtained. The pure strains were stored through slant preservation.

Strain identification: The screened strains were subjected to basic phenotypic, physiological and biochemical identifications, and their microstructures were observed using field-emission scanning electron microscopy. The molecular identification of 16S rDNA was performed by Beijing AUGCT Biological Technology Co., Ltd., and homology comparison was conducted between the 16S rDNA sequences of the screened strains and those in Genbank with BLAST. ClustalX version 1.83 and 4.0 MEGA software were adopted to create a phylogenetic tree with the neighbor-joining method.

Growth of the strain and its ability to degrade MBC: Under pH 7.0 and 30°C, 5 mL of the strain seed fluid was inoculated into 100 mL of the degradation medium with 8 mg/L MBC content (solubility of MBC in water). The fluid was cultured at 150 r/min for 72 h. Regular sampling was conducted every 12 h to determine MBC content and growth in the system. Increase in the amount of MBC was measured using dilution-plating method.

Determination of the degradation characteristics of the strain: Briefly, 1%, 3%, 5%, 7%, 9%, and 11% cultured cells were inoculated into 100 mL of the degradation medium with 8 mg/L MBC and stored at 150 r/min for 72 h under pH 7.0 to determine the influence of the amount of inoculant on the activity of the degradation bacteria. Briefly, 5 mL of the seed fluid was also inoculated into 100 mL of the degradation medium containing 8 mg/L MBC, and maintained at 150 r/min for 72 h at 15°C, 20°C, 25°C, 30°C, 35°C, 40°C and 45°C to test the effect of temperature on the activity of the degradation bacteria. Under the optimal temperature for strain degradation, 5 mL of the strain seed fluid was inoculated into 100 mL of the degradation medium with 8 mg/L MBC and cultured at 150 r/min for 72 h under pH values of 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 to assess the effect of pH on the activity of the degradation bacteria. Furthermore, 10, 50, 100, 200, and 300 mg/L MBC samples were added as the sole carbon source into the degradation culture medium. Strain djl-5B was inoculated under the optimum degradation conditions and sampled every 12 h during the total test time of 72 h to determine the influence of different initial concentrations of MBC on the degradation bacteria.

Determination of the degradation ability of the strain for MBC: Soil samples used for the degradation experiment were obtained and divided into four groups: group A: sterilized soil sample without bacterial agents; group B: fresh and bacteria-free soil sample; group C: sterilized soil samples with bacterial agents; and group D: fresh soil sample with bacterial agents. The amount of MBC added in the soil samples was 10 mg/kg, and the djl-5B degradation bacteria agent was 150 CFU/g. The MBC and bacterial agent were evenly distributed on the soils and cultured at 25°C. Sterile water was provided to maintain the moisture content of the soils at approximately 10%. The treated soils were sampled and determined every 48 h to investigate the degradation conditions and ability of MBC-degrading bacteria for 10 d.

Identification of degradation products: The samples were pretreated using the method proposed by Wang (Wang et al. 2010). Briefly, 8 mg/L MBC was added to the degradation medium at pH 7.0. The MBC-degrading bacterial seed liquid (5%) was inoculated and shaken at 150 r/min for 72 h at 30°C. Subsequently, 5 mL of the obtained mixture was placed in the graduated test tube with acetone of the same volume and sealed with a stopper. After shaking the mixture for 2 min, the solid NaCl solution was saturated and then placed in boiling water to separate water and acetone phases. The acetone phase (5 mL) was separated by drying the solution at room temperature with nitrogen gas. HPLC-MS was adopted for the detection.

Determination conditions of HPLC-MS: Waters XTerra C_{18} (4.6 nm × 150 mm, 5 μm) was selected as the chromato-

graphic column. The ratio of methanol to water (with aqueous phase containing 0.1% formic acid and 10 mmol/L ammonium formate) was set at 1:1 (V/V) for the mobile phase. Subsequently, 10% to 80% methanol was treated with a gradient elution for 0-5 min, 80%-100% of methanol for 5-8 min, 100% methanol for 8-18 min, 100%-10% methanol for 18-20 min, and 100% water for 20-25 min. The other conditions included a detection wavelength of 280 nm, flow velocity of 500 µL/min, a sampling volume of 5 µL, a column temperature equal to room temperature, and a detector, namely, LTQ-XL mass spectrometer equipped with electrospray ion source (ESI, positive ion mode).

RESULTS AND ANALYSIS

Separation and identification of MBC-degrading bacteria: The Gram-negative MBC-degrading strain djl-5B was isolated from the MBC-contaminated soil. The morphological observation results (Fig. 1) show that strain djl-5B exhibits a globular shape without flagella and grows relatively quickly in the culture media. The lengths of the observed bacterial colonies are between 0.4 µm and 0.6 µm and between 0.5 µm and 0.8 µm. Orange colony with neat edges appears within 2-3 d and grows into a circular shape with an opaque and smooth surface and a length between 0.4 and 0.8 mm. The results of the physiological and biochemical test results indicate that methyl red and indole test are positive when strain djl-5B comes in contact with the enzymes. Moreover, V-P and 3-keto lactose are negative in starch hydrolysis. Subsequently, a phylogenetic tree was created based on the results of 16S rDNA, and bacteria were initially determined as *Pseudomonas* sp. djl-5B (Genbank serial No.: KT230830) (Fig. 2).

Degradation characteristics of MBC-degrading bacteria: The growth curve and MBC-degradation dynamics of strain djl-5B were determined under pH 7.0 and 30°C (Fig. 3). The degradation curve shows that MBC degrades quickly in the sample with strain djl-5B 36 h before the experiment. The degradation rate abruptly decreases after 48 h and reaches 99.1% after 72 h. Based on the growth curve, the strains grow relatively slow within 24 h of the experiment, which may be due to the relatively high MBC content in the system, thereby inhibiting the growth of the strain to a certain extent. Nonetheless, the growth rate of the strain increases and reaches the logarithmic phase after 48 h with decreasing MBC in the system through time.

The amount of strain inoculant also affects the degradation of MBC (Fig. 4). The degradation rate of MBC increases with increasing inoculation amount of strain djl-5B in the system. When the amount of the inoculant reaches 7%, the rate of degradation decreases and tends to stabilize. Based on comprehensive evaluation, the most effective dosage of the djl-5B degradation strain is 7%.

As shown in Fig. 5, temperature affects the MBC-degradation activity of the bacteria within seven setting temperatures. Degradation activity is the highest at 30°C with a deg-

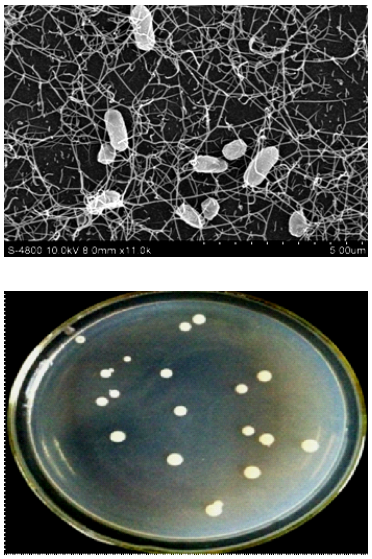

Fig. 1: Morphological observation of strain djl-5B. (A: Micrograph of strain djl-5B obtained through a transmission electron microscope; and B: bacterial colony of strain djl-5B).

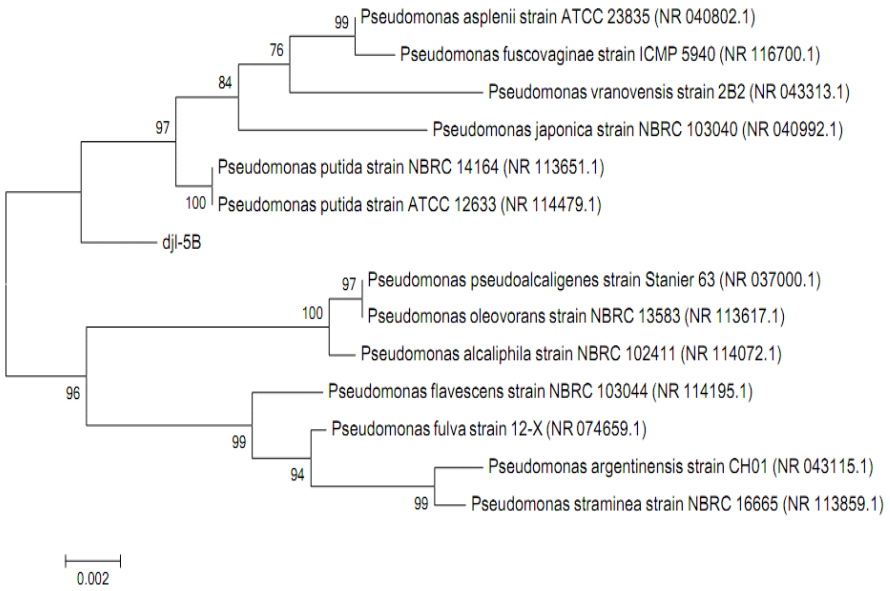

Fig. 2: Phylogenetic tree of strain djl-5B.

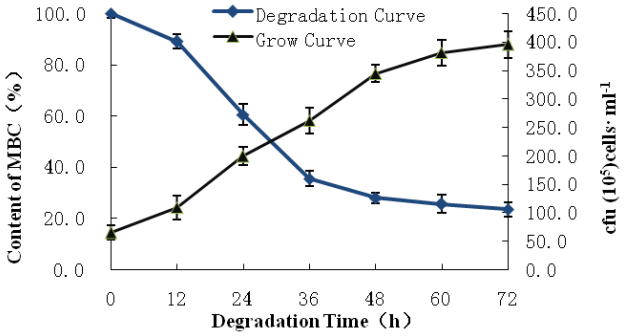

Fig. 3: Growth of strain djl-5B and degradation of MBC in the sample with strain djl-5B.

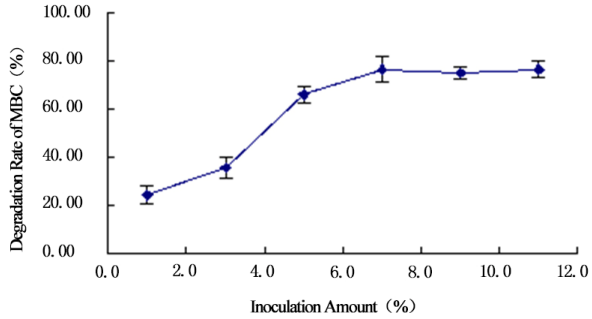

Fig. 4: Effect of the amount of inoculant on the degradation activity of strain djl-5B.

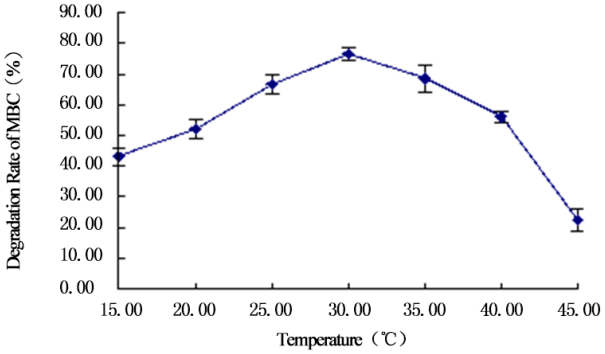

Fig. 5: Effect of temperature on the degradation activity of strain djl-5B.

radation rate of 76.50%. At 20°C-40°C, the degradation rate of strain djl-5B is higher than 50%, demonstrating the good adaptability of the strain to temperature changes.

Fig. 6 indicates that pH also affects MBC-degradation activity. Degradation activity is optimal at 30°C and pH 7.5, with a degradation rate of 86.33%. Between pH 6.5 and 8.5, the degeneration rate of strain djl-5B is higher than 50%, indicating that the strain can be applied in weak acidic and slightly alkaline soils.

Under pH 7.0 and 30°C, 7% of strain djl-5B was inoculated to investigate its degradation ability in reference to

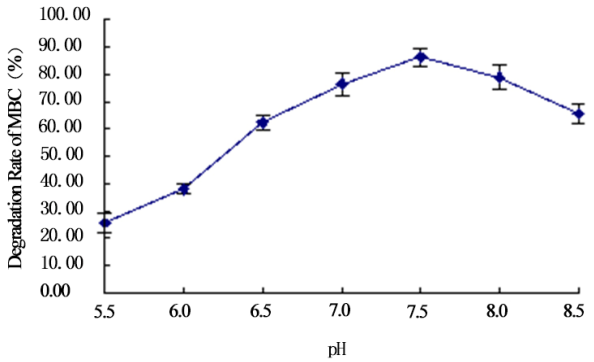

Fig. 6: Effect of pH on the degradation activity of strain djl-5B.

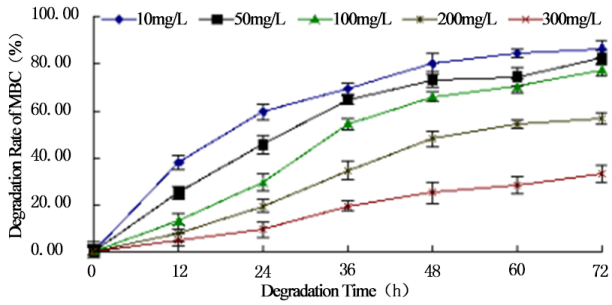

Fig. 7: Effect of initial MBC concentration on MBC degradation by strain djl-5B.

MBC with different initial concentrations (Fig. 7). The degradation rates of strain djl-5B corresponding to 10 and 50 mg/L MBC are 86.50% and 82.55%, respectively, within 72 hours, whereas that corresponding to 300 mg/L MBC is 33.46%. The MBC-degradation rate of strain djl-5B decreases evidently at the beginning with increasing MBC concentration. The decrease in MBC degradation rate may be due to the large amount of MBC that inhibits the growth of strain djl-5B. Generally, strain djl-5B exhibits degradation effect on MBC at five different concentrations.

MBC degradation in the soil by the degradation strain: The MBC-degradation dynamics of strain djl-5B is presented in Table 1. The half-life of MBC in sterile soils (A) without djl-5B degradation bacteria reaches 24.75 days after 10 days of testing, whereas that of MBC in non-sterile soils (B) occurs as long as 14.15 days. By contrast, MBC degradation decreases to 3.30 and 3.06 days in sterile soils (C) and non-sterile soils (D) with djl-5B degradation bacteria, respectively. The results indicate that hydrolysis and photolysis of MBC in the soil is very slow, and biodegradation plays a significant role in the entire degradation process. In addition, indigenous microorganisms in the soil, increase the degradation rate because their half-lives are still more than 10 days. Exogenous djl-5B degradation bacteria can significantly improve MBC

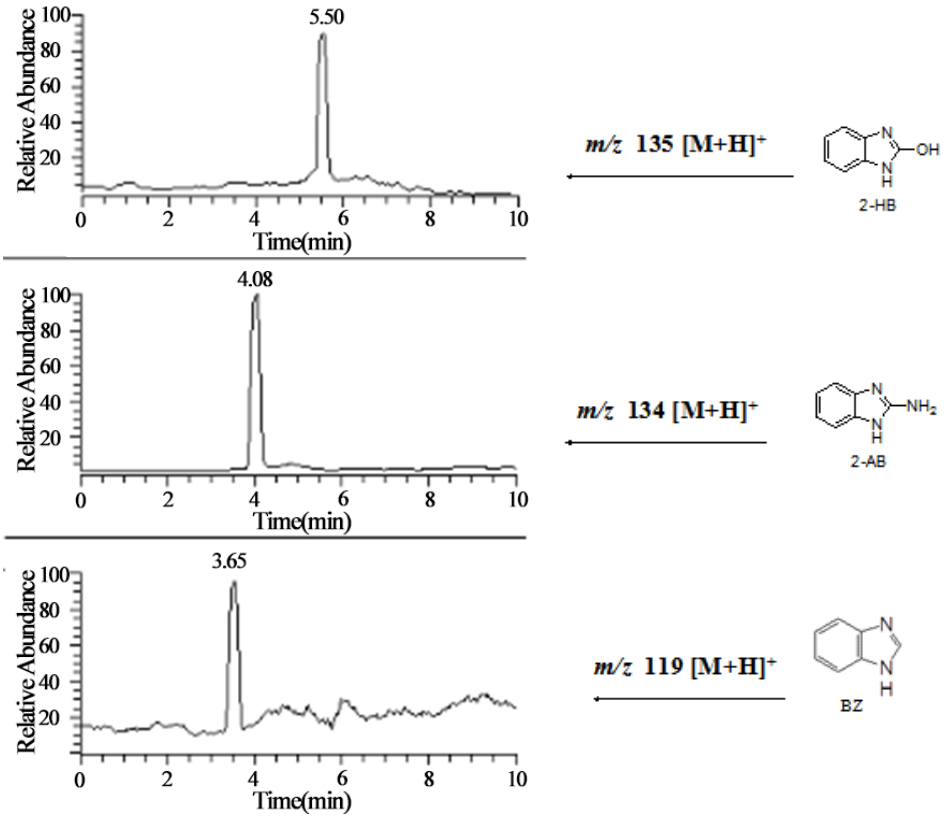

Fig. 8: Determination of MBC degradation products of the degradation bacteria.

degradation with their half-life within 4 days.

Determination of MBC degradation products of the degradation bacteria: The LC-MS technique was adopted to analyse the major products of MBC degraded by strain djl-5B, which mainly include 2-amino benzene (2-AB), 2-hydroxy benzimidazole (2-HB), and BZ (Fig. 8). As reported in the literature (Fang et al. 2010), OP is not detected in the degradation products. Based on the results and the findings in the literature (Fang et al. 2010, Pandey 2010), strain djl-5B converts MBC into 2-amino benzene first, which is further converted into 2-hydroxy benzene and BZ.

DISCUSSION

MBC-degrading bacteria mainly include *Nocardioides* sp. (Pandey et al. 2010) and *Rhodococcus* sp. (Wang et al. 2010, Holtman et al. 1997, Xu et al. 2006). *Pseudomonas* sp. djl-5B strain with stable degradation characteristics was isolated and identified in the experiment as a new kind of MBC-degrading bacteria, which can significantly enrich the germplasm resources of MBC-degrading strains.

Environmental conditions significantly affect the growth of microorganisms and degradation of pesticides. Changes in temperature, pH and moisture content directly affect the degradation of pesticides by microorganisms. As a result, investigating the degradation of MBC by microorganisms within a certain range of temperature and pH can reflect the potential of using microorganisms in pollution restoration. Strain djl-5B exhibits evident degradation effect on MBC. Moreover, the MBC degradation rates at 20°C-40°C and pH 6.5-8.5 are more than 50%, demonstrating that this bacteria can be potentially used in environment restoration with relatively good environmental adaptability and strong degradation stability.

Microorganisms play an important role in decomposing pesticides in an ecological system. The significant potential of these microorganisms to degrade or convert foreign compounds have been crucial research topics in the field of environment protection. Most microorganisms that can effectively degrade pesticides originate from soils. The biodegradation rate of MBC in the soil by strain djl-5B, which was selected and isolated in the experiment, is apparently higher than the hydrolysis and photolysis rate of MBC, as well as the degradation rate by indigenous microorganisms in the soils. Thus, the absolute advantage of these microorganisms in degradation is demonstrated. The results provide a theo-

Table 1: Degradation and dynamic half-life equation of MBC in soil with strain djl-5B.

Sample	Degradation equation	Coefficient of correlation (R^2)	Degradation half-times (d)
A	$C_t=99.996e^{-0.028t}$	0.9795	24.75
B	$C_t=99.552e^{-0.049t}$	0.8959	14.15
C	$C_t=113.02e^{-0.2101t}$	0.9787	3.30
D	$C_t=111.4e^{-0.2265t}$	0.9880	3.06

A: Sterile soils without strain djl-5B; B: non-sterile soils without strain djl-5B; C: sterile soils with strain djl-5B; D: non-sterile soils with strain djl-5B.

retical basis for the application of strain djl-5B in the restoration of filed soils.

CONCLUSION

MBC significantly contributes to the resistance of plant to diseases in the past five decades. However, increasing concerns about the non-target toxicity of MBC result in the demand for new methods to safely and efficiently eliminate MBC contamination in the soil. In this study, a novel high-efficiency MBC degradation bacterial strain was isolated from MBC-treated soil and identified as *Pseudomonas* sp. djl-5B. The optimal degradation condition for strain djl-5B includes a temperature of 30°C, pH 7.5 and an inoculum size of 7%. The degradation results demonstrate that the inoculation of strain djl-5B to MBC-contaminated soil significantly reduces the degradation half-life of MBC. In addition, the degradation process of MBC by strain djl-5B is demonstrated through HPLC-MS analysis. In this process, MBC is first converted to 2-AB, which is then decomposed into 2-HB and BZ. These results demonstrate that strain djl-5B exhibits strong potential for remedying MBC contamination in the soil because of its highly efficient and eco-friendly degradation process. Thus, this study also provides basic information and technical support for future utilization of strain djl-5B as a bioremediation agent. Nevertheless, further studies on the hydrolysis mechanism of MBC by strain djl-5B are still necessary.

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