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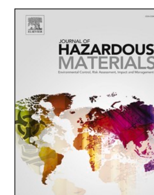

Enhancement of dicarboximide fungicide degradation by two bacterial cocultures of *Providencia stuartii* JD and *Brevundimonas naejangsanensis* J3

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ABSTRACT

Bioremediation is commonly conducted by microbial consortia rather than individual species in natural environments. Biodegradation of dicarboximide fungicides in brunisolic soil were significantly enhanced by two bacterial cocultures of *Providencia stuartii* JD and *Brevundimonas naejangsanensis* J3. The cocultures degraded 98.42 %, 95.44 %, and 96.81 % of 50 mg/L dimethachlon, iprodione, and procymidone in liquid culture within 6 d respectively, whose efficiency was 1.23 and 1.26, 1.25 and 1.23, and 1.24 and 1.24 times of strains JD and J3, respectively. The cocultures could effectively degrade dimethachlon, iprodione and procymidone to simple products. Moreover, the cocultures immobilized in a charcoal-alginate-chitosan carrier obviously surpassed free cocultures in terms of degradability, stability and reusability. In the field brunisolic soils treated by immobilized cocultures, 96.74 % of 20.25 kg a.i./ha dimethachlon, 95.02 % of 7.50 kg a.i./ha iprodione and 96.27 % of 7.50 kg a.i./ha procymidone were degraded after 7 d, respectively. Moreover, the lower half-lifes (1.53, 1.59 and 1.57 d) by immobilized cocultures were observed, as compared to free cocultures (3.60, 4.03 and 3.92 d) and natural dissipation (21.33, 20.51 and 20.09 d). This study highlights that strains JD and J3 have significant synergetic degradation advantages in rapid bioremediation of dicarboximide fungicide contamination sites.

1. Introduction

Three broad-spectrum dicarboximide fungicides dimethachlon [N-(3, 5-dichlorophenyl)-succinimide], iprodione [3-(3,5-dichlorophenyl) N-isopropyl-2, 4-dioxoimidazolidine-1-carboxamide] and procymidone [3-(3,5-Dichloropheny)-1, 5-di-methyl-3-azabicyclo [3.1.0] hexane-2,4-dione)] have been widely used worldwide for controlling various fungal infestations in plants in the last decades, such as *Botrytis cinerea*, *Botrytis allii*, *Sclerotinia sclerotiorum*, *Penicillium expansum*, *Monilinia fructigena*, *Rhizoctonia solani*, *Alternaria solani* and other many fungal pathogens (Osorio et al., 1994; Hamada et al., 2011; Grabke et al., 2014; Liu et al., 2016; Sun et al., 2018). However, a great many studies demonstrated that these fungicides are hazardous xenobiotics which present potential nephrotoxicity, hepatotoxicity and antiandrogen toxicity to non-target organisms and human beings (Kennedy et al., 2003; Health and Consumer Protection Directorate-General, 2002; Charles et al., 2005). For example, iprodione is even characterized as a probable carcinogen to human beings due to its moderately toxic to small animals (Morales et al., 2013). Moreover, their relatively long nature dissipation and low soil absorption result in moderately persistence and high mobility in soil and water systems (Carmona et al., 2001; Strömqvist and Jarvis, 2005; Wu et al., 2013; Allen et al., 2015). As a consequence of the widespread and inappropriate applications of these fungicides, their residues have frequently been detected in water, soil and farm products and posed risks to environment, wild lives and human beings (Sun et al., 2018; Allen et al., 2015; Zhang et al., 2017; Sun et al., 2019). Therefore, it is extremely important to eliminate dicarboximide fungicides from contaminated sites.

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In the natural environment, the removal of dicarboximide fungicides can be achieved through biological, chemical and photochemical degradation (Lassalle et al., 2014; Rifai et al., 2013). It is generally believed that microbial biodegradation is the main mechanism involved in the dissipation of organic contaminants (Haritash and Kaushik, 2009). Hence, biodegradation is extensively and preferentially used because it is an effectiveness, eco-friendliness, economy and expedience method compared with other methods (Haritash and Kaushik, 2009; Kuppusamy et al., 2016). In our previous studies, both *P. stuartii* JD and *B. naejangsanensis* J3 were firstly found to be capable of detoxifying dimethachlon, and *P. stuartii* JD or *B. naejangsanensis* J3 alone could completely mineralize dimethachlon (Zhang et al., 2019; Zhang et al., 2020). The metabolite 3,5-dichloroaniline generated from dimethachlon is highly nephrotoxic and carcinogenic(Lo et al., 1990). Several iprodione degradation microbes such as *Arthrobacter* sp. (Mercadier et al., 1997; Campos et al., 2016), *Pseudomonas fluorescens, Pseudomonas* sp. *and Pseudomonas paucimobilis* (Mercadier et al., 1997), *Achromobacter* sp. (Campos et al., 2016), *Microbacterium* sp. (Cao et al., 2017) and *Paenarthrobacter* sp. (Yang et al., 2018) have been reported. However, only *Microbacterium* sp. could mineralize 3,5-dichloroaniline which is the main metabolite of dicarboximide fungicides. Up to now, no pure microbes capable of degrading procymidone have been isolated. To make full use of microbial resources, the investigation of degradability of iprodione and procymidone by strains JD and J3 should be carried out.

There are many evidences that mixed microbial consortia exhibit good performance with regards to substrate tolerance and enhance the degradation of pollutants (Ghazali et al., 2004; Jia et al., 2019). Actually, bioremediation is commonly conducted by microbial consortia rather than individual species in natural environments and different strains or species fill different functional roles (Jia et al., 2016, 2019). All of aforementioned degradation microorganisms alone were used for the degradation of dimethachlon or iprodione, whether the cocultures of strains JD and J3 can degrade dimethachlon, iprodione and procymidone more efficiently and rapidly than single bacteria should be concerned. The success of bioremediation in contaminated sites depends on microbial survival, as well as the high cell densities, stability and reusability (Shi et al., 2018). Previous studies have shown that *P. stuartii* JD or *B. naejangsanensis* J3 immobilized in a charcoal-alginate carrier obviously improve degradability and adaptability of microbial cells (Zhang et al., 2019, [Zhang et al.,2020]2020), but their low mechanical stability were found in the field application. Alginate-chitosan beads were reported to have better mechanical strength than alginate or chitosan beads alone (Bedade et al., 2019). Thus, it is necessary to immobilize strains JD and J3 with a charcoal-alginate-chitosan carrier to improve their field adaptability.

The objective of this study was to evaluate the synergistic biodegradation of dicarboximide fungicides by the mixed consortium of strains JD and J3. The optimal degradation conditions for the cocultures were determined using an orthogonal design. Moreover, the metabolized pathway of dimethachlon, iprodione and procymidone by the cocultures were elucidated, respectively. The degradability, stability and reusability of immobilized cocultures immobilized in composite charcoal-alginate-chitosan beads were also described. Finally, the degradation of dimethachlon, iprodione and procymidone in the outdoor field brunisolic soil using immobilized cocultures were demonstrated, respectively. The obtained results would provide a better knowledge of the practical application of microbial consortia for the rapid bioremediation of sites contaminated by dicarboximide fungicides.

2. Materials and methods

2.1. Chemicals, medium and microorganisms

Dimethachlon (99.1 %) standard was purchased from Dr. Ehrenstorfer. Iprodione (99.0 %) and procymidone (99.0 %) standards were provided by Sigma Aldrich. All other chemicals were analytical grade or chromatographic grade. Bamboo charcoal (0.60 mm) was purchased from Shanghai Hainuo charcoal Co. Ltd., China. 40 % dimethachlon wettable powder (WP), 50 % iprodione WP, and 50 % procymidone WP was obtained from Jiangsu Heyi chemical Co. Ltd., China, FMC Corporation, and Sumitomo Chemical Corporation, respectively.

Minimal salt medium (MSM) (g/L) consisted of 0.2 $MgSO_4·H_2O$, 0.8 KH_2PO_4, 0.005 $FeSO_4·7H_2O$, 0.2 K_2HPO_4, 0.003 Na_2MoO_4, 0.1 $CaSO_4$, 1.0 $(NH_4)_2SO_4$ per liter of distilled water, pH 7.0. LB medium (g/L) contained10.0 tryptone, 3.0 yeast extract, 10.0 NaCl, pH 7.0. All media were sterilized at 121 °C for 30 min. *P. stuartii* JD and *B. naejangsanensis* J3 were isolated from Chinese Guiyang soils by our research group and conserved in our laboratory (Zhang et al., 2019, 2020). The 16S rDNA gene sequences of strains JD and J3 have been deposited in the Gen Bank database with accession number of MF 427701 and MF 427702, respectively. Strains JD and J3 are gram-negative bacterium, strain JD colony is approximately 2∼4 mm in diameter, milky white, and smooth after cultivation for 3 d on MSM plates at 30 ℃, and strain J3 colony is approximately 1.5∼4.0 mm in diameter, slightly glossy, primrose yellow, soft cells after cultivation for 3 d on MSM plates at 30 ℃.

2.2. Dicarboximide fungicide degradation experiments

Strains JD and J3 were grown in LB medium (30 ℃, 150 rpm) for 24 h, their cells were harvested by centrifugation (4 ℃, 8000 rpm, 15 min) and washed twice with fresh MSM. The pure and coculture suspensions of strains JD and J3 were used as the inocula respectively, and the ratio of JD and J3 in the cocluture consortium was 1:1(v/v). The pure and coculture suspensions were inoculated into 20 mL MSM containing 50 mg/L of different dicarboximide fungicides at 30 ℃ and 150 rpm, respectively. And the concentrations of the pure and coculture cells in 20 mL MSM were 6×10^8 cfu/mL. These specified concentrations were selected based on the results of pre experiments and previous studies (Zhang et al., 2019, 2020). No pure and coculture suspensions were added as the control. At 1 d intervals, the corrected degradation rates of different dicarboximide fungicides were determined.

To determine optimal conditions for degrading dicarboximide fungicides by the cocultures of strains JD and J3, an orthogonal design (L 9 [3^4]) was used to evaluate the effects of the following factors: medium pH (A, 6.5, 7.0, 7.5), temperatures (B, 25, 30, 35 ℃), coculture concentrations (C, 5.5, 6.0, 6.5×10^8 cfu/mL), fungicide concentrations (D, 50, 75, 100 mg/L). These specified factors and levels were based on previous studies (Zhang et al., 2019, 2020). The dependent variables were the degradation of different dicarboximide fungicides in MSM by the cocultures after 6 d of incubation, respectively. No cocultures was added as a control, the corrected degradation rates were calculated.

To determine the metabolites produced during dicarboximide fungicide degradation by the cocultures, the cell-free filtrates of 50 mg/L of different dicarboximide fungicides in MSM with inoculation of the cocultures under the optimal conditions were analyzed using GC/MS at different expected culture times, respectively.

2.3. Immobilization of the cocultures

The mixed cultures of strains JD and J3 were immobilized in a charcoal-alginate matrix as described by Zhang (Zhang et al., 2019). Sterile bamboo charcoal was added to the coculture suspension (6×10^{10} cfu/mL) at the ratio of 0.1:1 (w/v) and the mixture was homogenized by an orbital shaker for 6 h at 200 rpm. Subsequently, the mixture was mixed with 3% sterile sodium alginate solution (w/v) at the ratio of 0.1:1 (v/v) and then poured dropwise with a syringe in 2% sterile $CaCl_2$ solution (w/v) for 10 h gelation. The bamboo charcoal-sodium alginate (BS) beads were immersed in 1.0 % chitosan solution (w/v), stirred on a magnetic stirrer at 200 rpm for 1 h for coating, washed by deionized water to remove the excess chitosan, and then dried. Then the bamboo charcoal-sodium alginate-chitosan (BSC) beads were harvested. BS and

BSC beads entrapped approximately 5.01 × 10^9 cells (cfu) per gram. Subsequently, the biodegradability of different dicarboximide fungicides in MSM by BS beads (130 mg/mL MSM), BSC beads (130 mg/mL MSM), free cocultures (6.5 × 10^8 cfu/mL MSM) and immobilized materials without cocultures (130 mg/mL MSM) was evaluated, respectively.

In order to assess the storage stability of the freeze-dried and immobilized cocultures, they were stored at 4 ℃ and then withdrawn at intervals of 20 d and their degradation efficiencies were determined, respectively. The reusability of free and immobilized cocultures were investigated by determining the efficiency of repeated 10 batches of them degrading 50 mg/L dicarboximide fungicides, respectively. Between batches, immobilized cocultures were filtered off from the reaction medium and washed three times with sterilized water. Free cocultures were recycled by centrifugation after each batch (4 ℃, 8000 rpm, 15 min). For the stability and reusability test of each batch, the initial added concentrations of free and immobilized cocultures were 6.5 × 10^8 cfu/mL and 130 mg/mL, respectively. The cell growth concentration was determined after 5 d of degradation reaction in each batch.

2.4. Biodegradation of dicarboximide fungicides in agricultural field

The biodegradation of dicarboximide fungicides in brunisolic soils was carried out in agricultural field (N 26°27′41″, E 106°39′26″) which had not been sprayed with dimethachlon, iprodione and procymidone for more than 2 years. This soil had organic matter content of 4.37 %, pH of 6.65. Testing plots were sprayed with 40 % dimethachlon WP, 50 % iprodione WP, and 50 % procymidone WP at dose of 20.25, 7.50, and 7.50 kg a.i./ha (10-fold higher than the recommended dose), respectively. The area of each testing plot was set as 5 m^3, and three repeat plots were set for each treatment. After 12 h, the sprayed plots were remedied by 6.0 × 10^{10} cfu/mL of free cocultures and 90 kg/ha of immobilized cocultures, respectively. The control plots were treated with the same amount of water and immobilized materials without cocultures, respectively. At 1 d intervals, 2 kg of soil samples (0∼15 cm in deep) were collected randomly from each plot and the corrected degradation rates of different dicarboximide fungicides were determined. The dissipation of dicarboximide fungicides in brunisolic soil was calculated according to the exponential model equations:

$$C_t = C_0\, e^{-kt}$$

$$t_{1/2} = \ln 2/k,$$

where C_t is the residue at time (t), C_0 is the initial residue, k is the rate constant and $t_{1/2}$ is the half-life.

2.5. Analytical methods

The culture samples were centrifuged at 12,000 rpm for 5 min and the supernatant (10 mL) was extracted with equal volumes of acetonitrile by violent and ultrasonic shaker and then centrifuged at the same speed for 5 min. The extract (10 mL) was added 2.0 mL n-hexane after being dried by a gentle nitrogen stream, and then poured into the preactivated solid phase extraction column. The purified extract was diluted to 5 mL with methanol after nitrogen drying and filtered through 0.45 μm membrane for analysis. The dimethachlon, iprodione, and procymidone of soil samples (10.0 g) was respectively extracted with acetone (20 mL) and NaCl (2.0 g), and the remaining steps were the same as those of the culture samples. The dimethachlon, iprodione, and procymidone residues were analyzed by GC (Aglient 6890 N), respectively. Their separation was performed on a HP-5 capillary column (30 m × 0.32 mm × 0.25 μm). The temperature program started at 200 ℃ (held for 1 min) and then ramped to 280 ℃ at 10 ℃/min. Nitrogen (≥ 99.999 %) was employed as carrier gas (1.2 mL/min). The ECD detector and injector temperatures were set at 280 ℃ and 250 ℃, respectively. The injection volume was 1.0 μL. The dimethachlon, iprodione, and procymidone was quantified on the basis of a peak area from calibration curve, respectively.

The culture samples were extracted with equal volumes of acetonitrile, and then the supernatant was dehydrated, dried, and dissolved in methanol, and filtered through 0.45 μm membrane for analysis. The metabolic products of dimethachlon, iprodione, and procymidone during the degradation were identified by an electron ionization (EI) mode (70 eV) in GC/MS (Agilent 6890 N/5975), respectively. GC/MS was conducted using a ZB-5MS capillary column (30.0 m × 0.25 mm × 0.25 μm). The column temperatures were started at 50 ℃ (held for 2 min) and then increased to 320 ℃at 6 ℃/min (held for 2 min). The transfer line and EI source temperatures were set at 280 ℃ and 230 ℃, respectively. The flow rate of carrier gas (Helium, ≥99.999 %) was set at 1.0 mL/min. The injection volume was 1.0 μL.

3. Results and discussion

3.1. Degradation of dicarboximide fungicides by the cocultures

The degradability of the pure and cocultures of strains JD and J3 in MSM with dicarboximide fungicides were determined (Fig. 1). It is obvious that the degradation of dicarboximide fungicides by the pure and cocultures was very efficient. It is interesting to note that the strain JD or J3 also could efficiently utilize procymidone as a sole source of energy. To the best of our knowledge, it is the first report that procymidone has been degraded by pure microorganisms. The removal of dimethachlon, iprodione and procymidone was significantly accelerated ($P < 0.01$) when strains JD and J3 were grown in a coculture as compared to the pure culture. In pure culture, strains JD and J3 could remove 82.91 % and 80.83 %, 79.77 % and 81.32 %, and 80.78 % and 80.76 % of 50 mg/L dimethachlon, iprodione, and procymidone after 7 d of incubation, respectively (Fig. 1 A, B, C).

Combining strains JD and J3 resulted in rapid degradation of dicarboximide fungicides, and 85.56 %, 83.44 % and 84.68 % of 50 mg/L dimethachlon, iprodione and procymidone were metabolized within 5 d (Fig. 1). Dimethachlon, iprodione and procymidone were hardly detected in culturing broth of the cocultures of strains JD and J3 in 7 d, suggesting that the cocultures were a highly efficient approach for dicarboximide fungicide removal and advanced at least for 1 d as compared to the pure culture. Furthermore, the degradation rates in the coculture within 5–7 d of incubation followed the order dimethachlon > procymidone > iprodione, which might be caused by the complexity of the fungicide structure.

Some dicarboximide fungicide degradation microbes have been reported, such as *P. stuartii* (Zhang et al., 2019) and *B. naejangsanensis* (Zhang et al., 2020) for dimethachlon, *Arthrobacter* sp. (Mercadier et al., 1997; Campos et al., 2016), *Pseudomonas fluorescens, Pseudomonas* sp. *and Pseudomonas paucimobilis* (Mercadier et al., 1997), *Achromobacter* sp. (Campos et al., 2016), *Microbacterium* sp. (Cao et al., 2017) and *Paenarthrobacter* sp. (Yang et al., 2018) for iprodione. However, almost all of these microorganisms were only used for dimethachlon or iprodione degradation alone. Many evidences illustrated that the cooperative involvement of mixed microbial consortium enhanced the biodegradation of organic pollutants (Haritash and Kaushik, 2009; Sørensen et al., 2002; Sørensen et al., 2008). For instance, the cocultures of *B. naejangsanensis* and *Bacillus cohnii* had the capacity to mineralize natural rubber (Manasa and Veena, 2016), the degradation of cypermethrin was significantly accelerated in a coculture of *B. cereus* and *Streptomyces aureus* (Chen et al., 2012), some organophosphorus and organochlorine pesticides were also simultaneously degraded by a bacterial consortium which comprised of 10 organisms (Abraham et al., 2014).

In the present study, the cocultures of *P. stuartii* JD and *B. naejangsanensis* J3 significantly enhanced ($P < 0.01$) degradation of dimethachlon, iprodione and procymidone, demonstrating that strains JD and J3 consortia had the synergistically degradative capacities in the

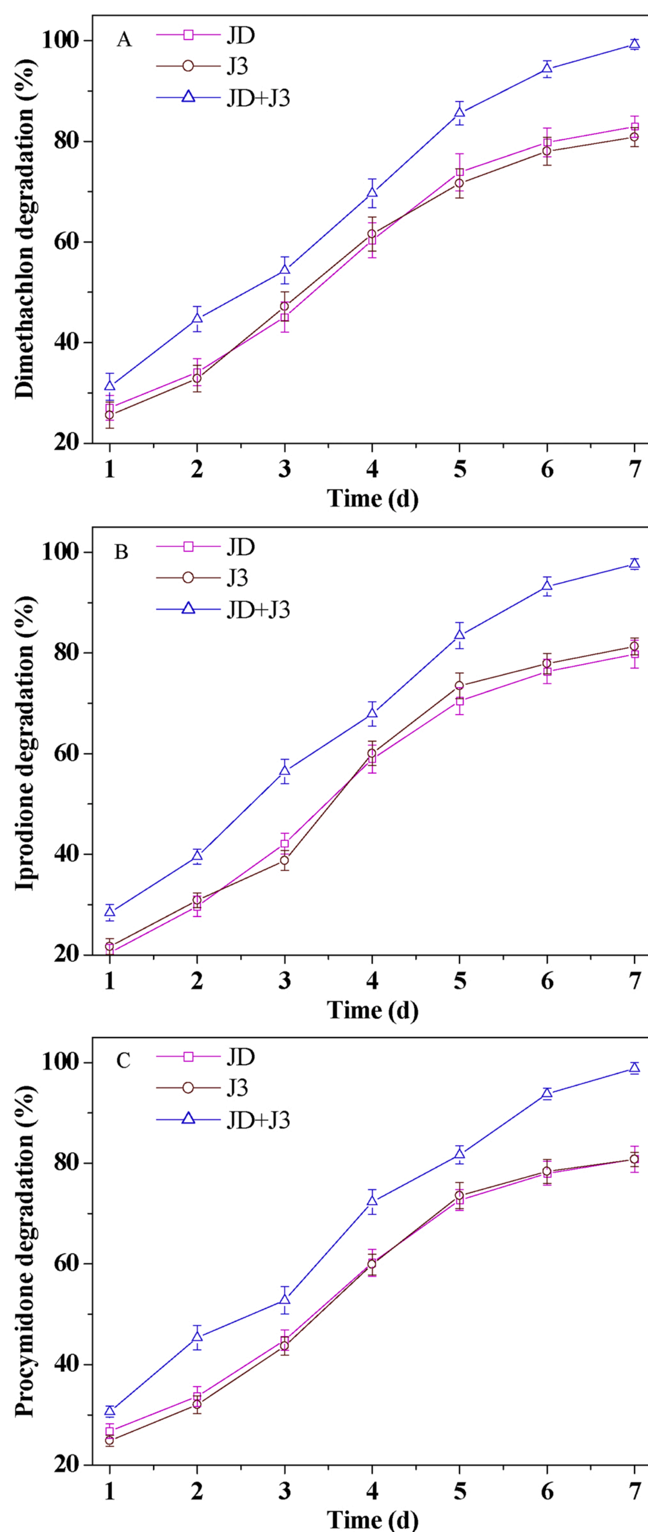

Fig. 1. Degradation of dimethachlon (A), iprodione (B) and procymidone (C) by the pure and cocultures of strains JD and J3, respectively. Vertical bars represent the standard errors of the means of three replicates, the same below.

degradation of dicarboximide fungicides. It might be because that the labor division and competition of mixed microbial consortia could theoretically reduce metabolic burden to substrate (Wang et al., 2012; Roell et al., 2019), thus the coexistence of strains JD and J3 promoted their utilizing abilities to fungicides. According to previous studies that free enzymes of strains JD and J3 could rapidly detoxify dimethachlon (Zhang et al., 2019, 2020), another possible reason was thus the cocultures increased the diversity of degradative enzymes for dicarboximide fungicides.

3.2. Optimization of degradation conditions for the cocultures

The efficiencies of removing dicarboximide fungicides were affected by culture parameters. The results of orthogonal test depicted in Table 1 indicate that the degradation of dimethachlon, iprodione and procymidone by the cocultures after 6 d of incubation was 98.42 %, 95.44 % and 96.81 %, whose efficiency was 1.23 and 1.26, 1.25 and 1.23, and 1.24 and 1.24 times of strains JD and J3, respectively. Further orthogonal analysis$k_i^A = \sum$the degradation at Ai, $R_i^A = \max\{k_i^A\} - \min\{k_i^A\}$ and $K_i^A = k_i^A/3$ were calculated. As seen from Table 1, the influence of factors on the dimethachlon, iprodione, and procymidone degradation in the order: D > A > B > C, D > B > A > C, and D > B > A > C according to the R values, respectively. These results demonstrated that the fungicide influenced degradation the most, followed by the temperature, pH and the cocultures. Furthermore, the K values reveal that the optimal condition for dicarboximide fungicide degradation was $A_2B_2C_3D_1$, which was pH 7.0, 30 °C, 6.5 × 10^8 cfu/mL of the cocultures, 50 mg/L of fungicides. It is in agreement with the degradation results of the pure culture of strains JD and J3 (Zhang et al., 2019, 2020).

3.3. Biotransformed pathways of dicarboximide fungicides

The transformed products of dimethachlon, iprodione and procymidone in the coculture medium extracts were identified by GC/MS. For transformed product analysis of dimethachlon, total five compounds including parent compound were detected by GC/MS on different culture times with the prominent protonated molecular ions at *m/z* 242.98, 161.99, 116.02, 94.11 and 140.03, respectively (Fig. S1). The metabolic pathway of dimethachlon in the coculture medium was in agreement with the pure culture medium alone (Zhang et al., 2019, 2020). Dimethachlon was firstly transformed into succinic acid and 3,5-dichloroanilin, then the latter was converted to phenol and further oxidized into muconic acid (Fig. 2).

For metabolic product analysis of iprodione, total six compounds including parent compound were detected by GC/MS on different culture times with the prominent protonated molecular ions at *m/z* 330.04, 244.99, 263.00, 161.99, 94.11 and 140.03, respectively (Fig. S2). On the basis of the molecular weight and the characteristic ion peaks, these compounds were identified as iprodione, N-(3,5-dichlorophenyl)-2,4-dioxoimidazolidine, 3,5-dichlorophenylurea acetic acid, 3,5-dichloroanilin, phenol and muconic acid, respectively. The possible metabolic pathways for iprodione were proposed based on the results of this study and previous reports (Mercadier et al., 1996; Mercadier et al., 1997; Campos et al., 2016; Yang et al., 2018) (Fig. 3). The amide bond of iprodione was initial hydrolyzed to N-(3,5-dichlorophenyl)-2,4-dioxoimidazolidine and a conjectural metabolite isopropylamine, and then the former was transformed to 3,5-dichlorophenylurea acetic acid, further metabolized to 3,5-dichloroanilin and probable glycine. Subsequently, 3,5-dichloroanilin was degraded to phenol and further oxidized into muconic acid.

For transformed product analysis of procymidone, total four compounds including parent compound were detected by GC/MS on different culture times with the prominent protonated molecular ions at *m/z* 283.40, 161.99, 94.11 and 140.03, respectively (Fig. S3). The structure of compounds were consistent with that of procymidone, 3,5-dichloroanilin, phenol and muconic acid, respectively. Based on these results and previous report (Vanni et al., 2000), we propose that the cocultures of strains JD and J3 could hydrolyze procymidone to a putative metabolite 2-(3,5-di-chlorophenylcarbamoil)-1,2-dimethyl-cyclopropane carboxylic acid and then further hydrolyzed into 3, 5-dichloroanilin and probable 1,2-dimethyl-cyclopropane dicarboxylic

Table 1
L 9 (3^4) orthogonal experimental results.

Test no.	pH (A)	Temp (B, ℃)	Cocultures (C, × 10^8 cfu/mL)	Fungicides (D, mg/L)	Dimethachlon degradation (%)	Iprodione degradation (%)	Procymidone degradation (%)
1	6.5	25	5.5	50	91.67	90.31	90.65
2	6.5	30	6.0	75	90.31	89.43	90.01
3	6.5	35	6.5	100	88.76	87.56	88.13
4	7.0	25	6.0	100	90.65	88.69	89.08
5	7.0	30	6.5	50	98.42	95.44	96.81
6	7.0	35	5.5	75	91.43	90.31	90.32
7	7.5	25	6.5	75	93.47	89.76	88.78
8	7.5	30	5.5	100	92.36	90.21	90.67
9	7.5	35	6.0	50	93.72	89.53	90.03
The affect order					D > A > B > C	D > B > A > C	D > B > A > C
Optimal level					$A_2B_2C_3D_1$		

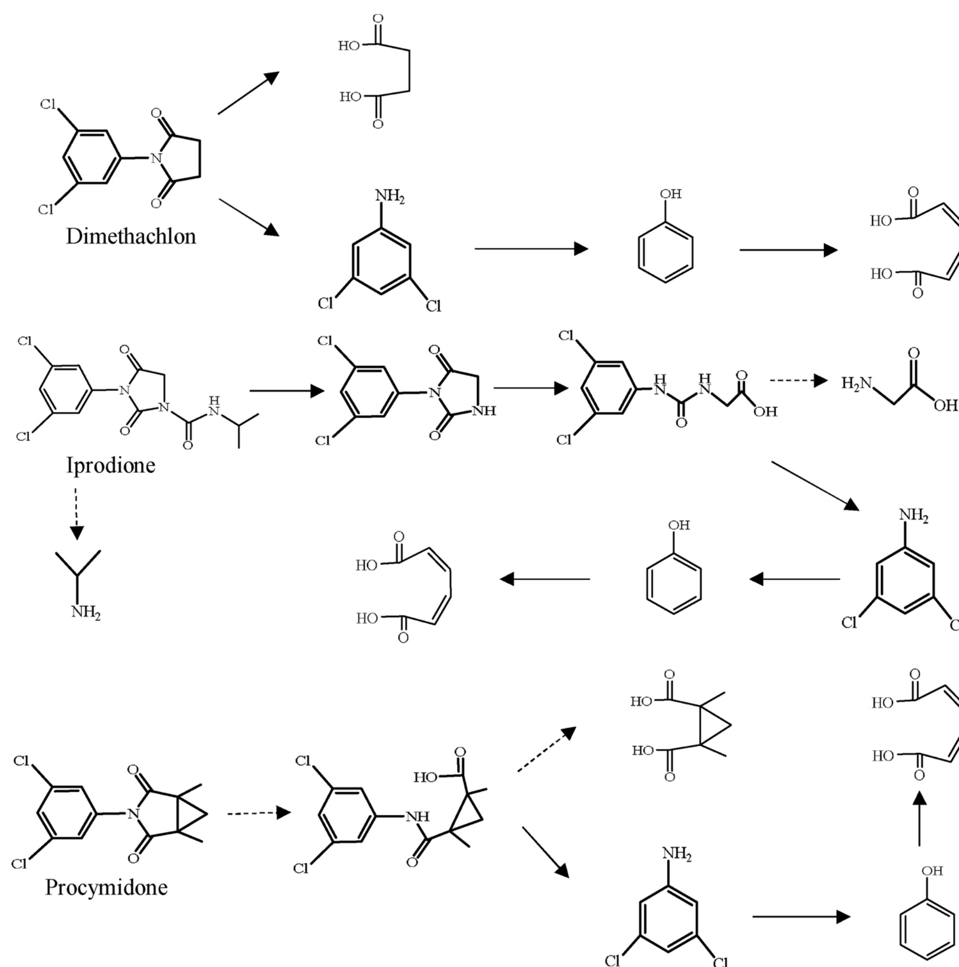

Fig. 2. Proposed metabolic pathways of dimethachlon, iprodione and procymidone by the cocultures.

acid. Perfectly, 3,5-dichloroanilin was effectively degraded by the same metabolic pathways of dimethachlon and iprodione.

The finally degradation product of iprodione by previously reported microbes was 3,5-dichloroanilin (Mercadier et al., 1996; Mercadier et al., 1997; Campos et al., 2016). However, the pure and cocultures of strains JD and J3 could effectively degrade 3,5-dichloroaniline to phenol and further oxidized into muconic acid. Therefore, this work provides a promising strategy for the rapidly and effectively bioremediation of dicarboximide fungicides.

3.4. *Dicarboximide fungicide removal by free and immobilized cocultures*

Free cocultures of strains JD and J3 were initially adsorbed and immobilized in bamboo charcoal-sodium alginate and then coated with chitosan, a comparative study was subsequently carried out for the degradation of dimethachlon, iprodione and procymidone using free, BS immobilized and BSC immobilized cocultures. As illustrated in Fig. 3, the degradations of dimethachlon, iprodione and procymidone in MSM after 5 d of inoculation were 85.67 %, 97.54 % and 100 %, 83.53 %,

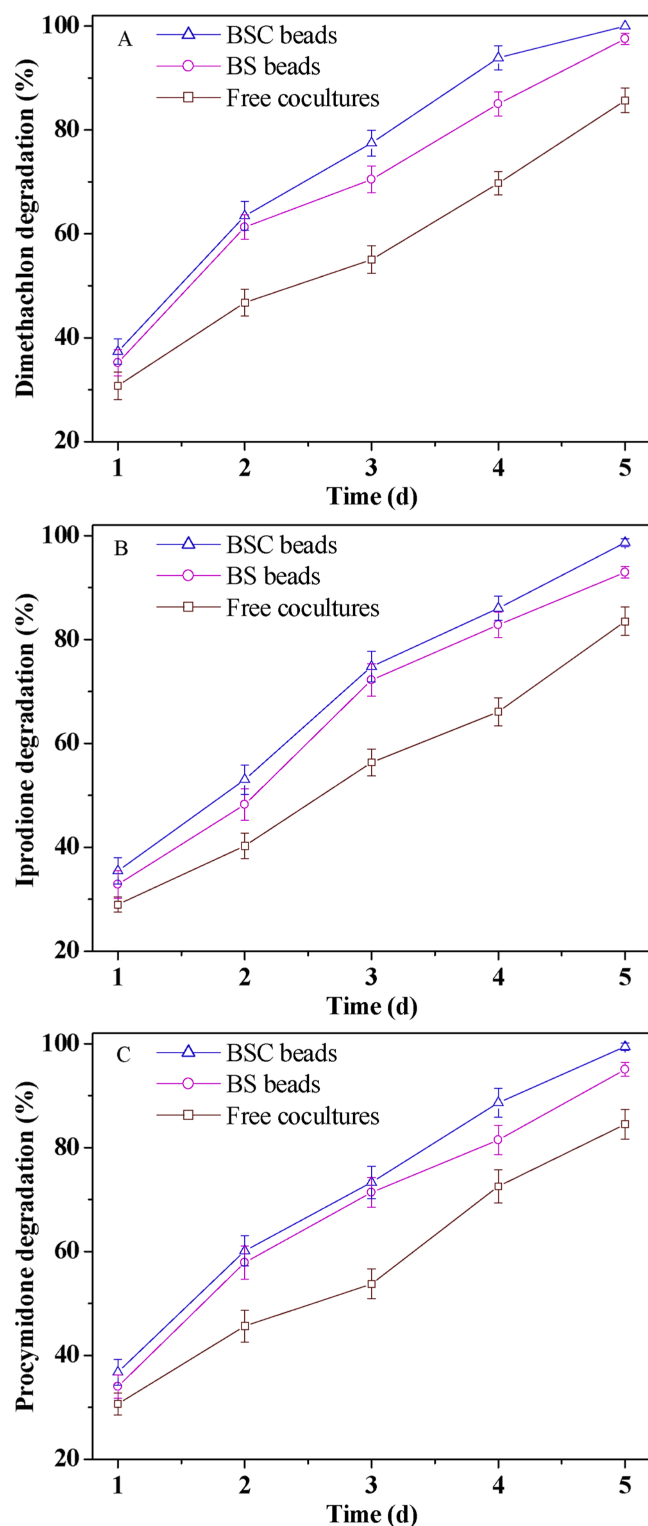

Fig. 3. Degradation of dimethachlon (A), iprodione (B) and procymidone (C) in MSM by free, BS immobilized and BSC immobilized cocultures, respectively.

93.94 % and 98.66 %, and 84.48 %, 95.06 % and 99.44 % for the free, BS immobilized and BSC immobilized cocultures, respectively. Obviously, immobilization enhanced the degradation efficiency of the cocultures for dicarboximide fungicides. Moreover, the coating of BS immobilized cocultures with chitosan significantly improved their biodegradability to dicarboximide fungicides. These good performances should result from two advantages of BSC immobilized beads: (i) the high porosities and large-effective surface areas of bamboo charcoal and the porous nature of chitosan are contributed to support the growth and physiological activity of the immobilized microorganisms (Zhang et al., 2019; Angelim et al., 2013; Chanratana et al., 2018). (ii) Chitosan is a polycationic polysaccharide, and it can spontaneously form polyelectrolyte complexes with polyanionic alginate (Wang et al., 2014), which promotes microbial utilization of fungicide metabolites.

The storage stability and cell growth of free and immobilized cocultures for dimethachlon, iprodione and procymidone degradation are depicted in Fig. 4. The storability and cell growth of the immobilized cocultures were much better than the free cocultures. After storage of

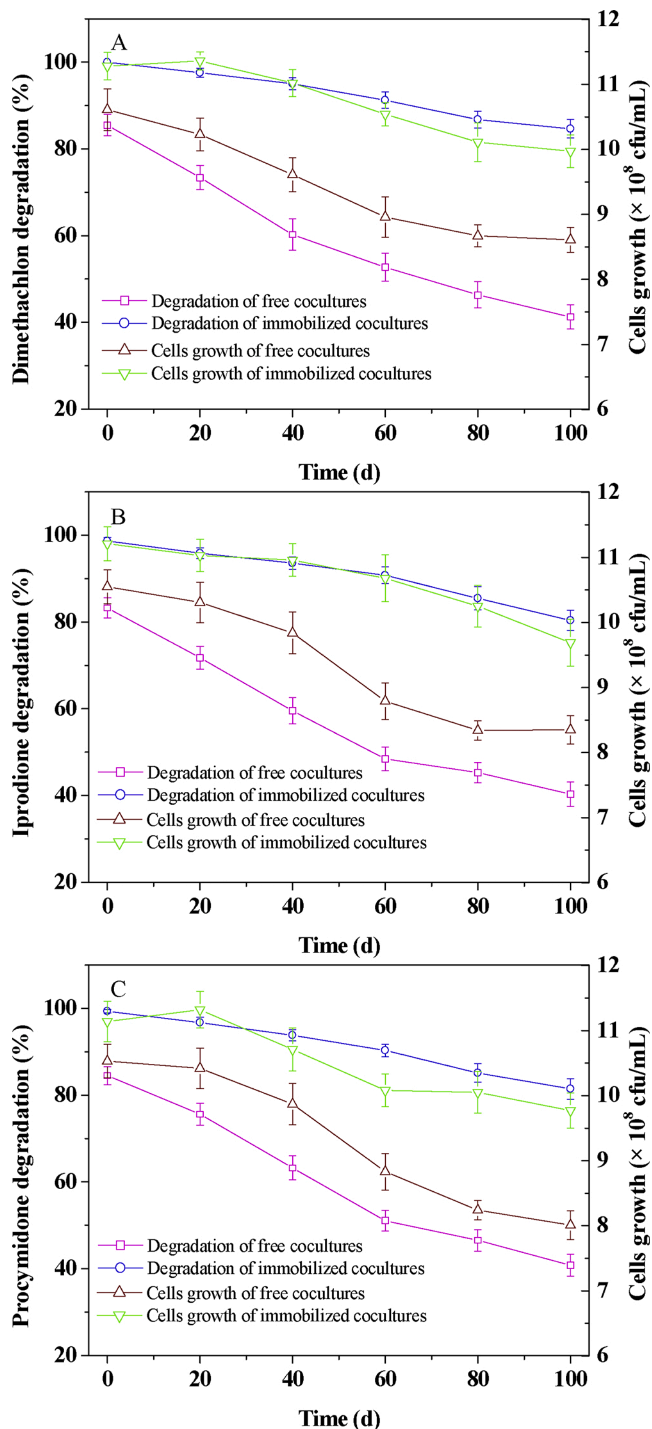

Fig. 4. Storage stability and cells growth of free and immobilized cocultures for dimethachlon (A), iprodione (B) and procymidone (C) degradation.

100 d, 41.25 % and 84.65 %, 40.32 % and 80.37 %, and 40.78 % and 81.46 % of dimethachlon, iprodione, and procymidone were degraded by free and immobilized cocultures, respectively (Fig. 4 A, B, C). The immobilized cocultures still kept approximately 81.42 %~84.65 % of their original degradability within 100 d of storage, while the free cocultures maintained only 48.24 %~48.44 %. As shown in Fig. 5, the immobilized cocultures had superior reusability and cell growth compared to the free cocultures during successive cycles of 5 d each. Only 6.89 %, 6.03 % and 6.55 % of dimethachlon, iprodione and procymidone were degraded by free cocultures within the seventh cycle, respectively (Fig. 5 A, B, C). While immobilized cocultures could retain 79.56 %, 78.32 % and 79.02 % of the degradation for dimethachlon, iprodione and procymidone after the tenth cycle, respectively (Fig. 5 A, B, C). Alginate-chitosan beads were reported to have good mechanical property (Bedade et al., 2019), and bamboo charcoal and chitosan were

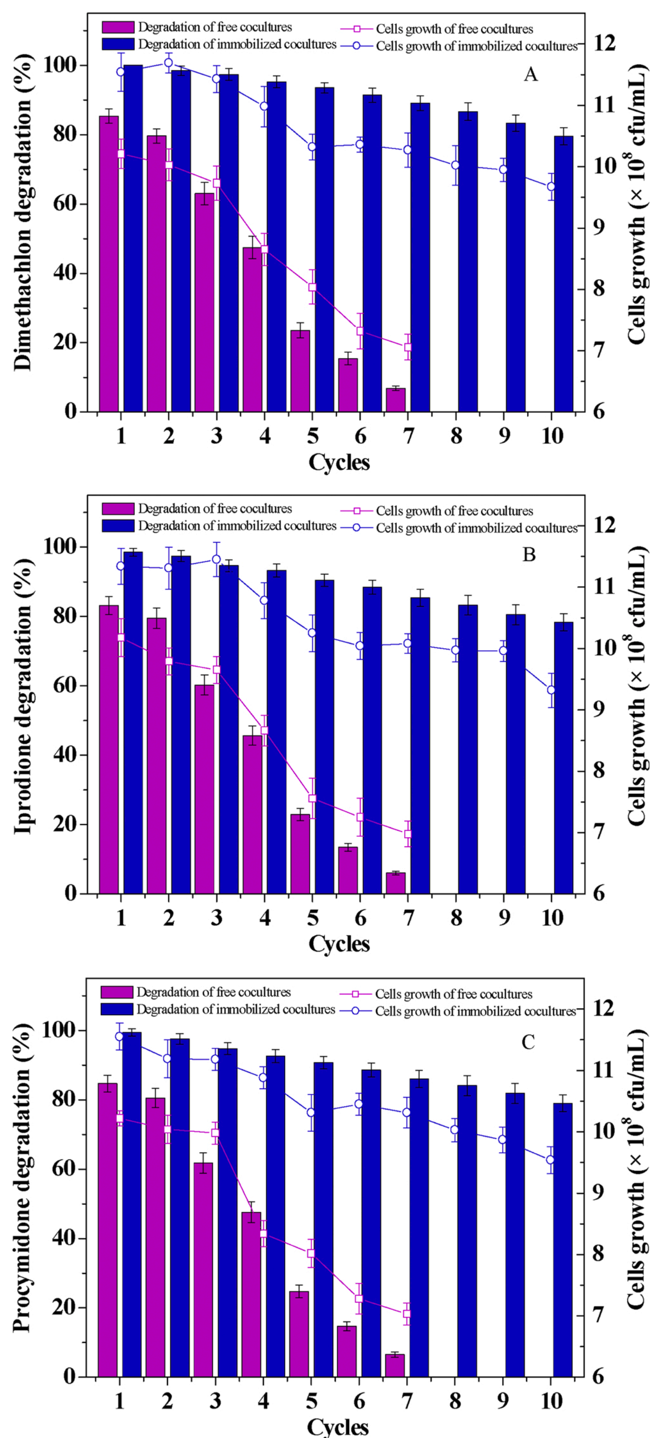

Fig. 5. Reusability and cells growth of free and immobilized cocultures for dimethachlon (A), iprodione (B) and procymidone (C) degradation.

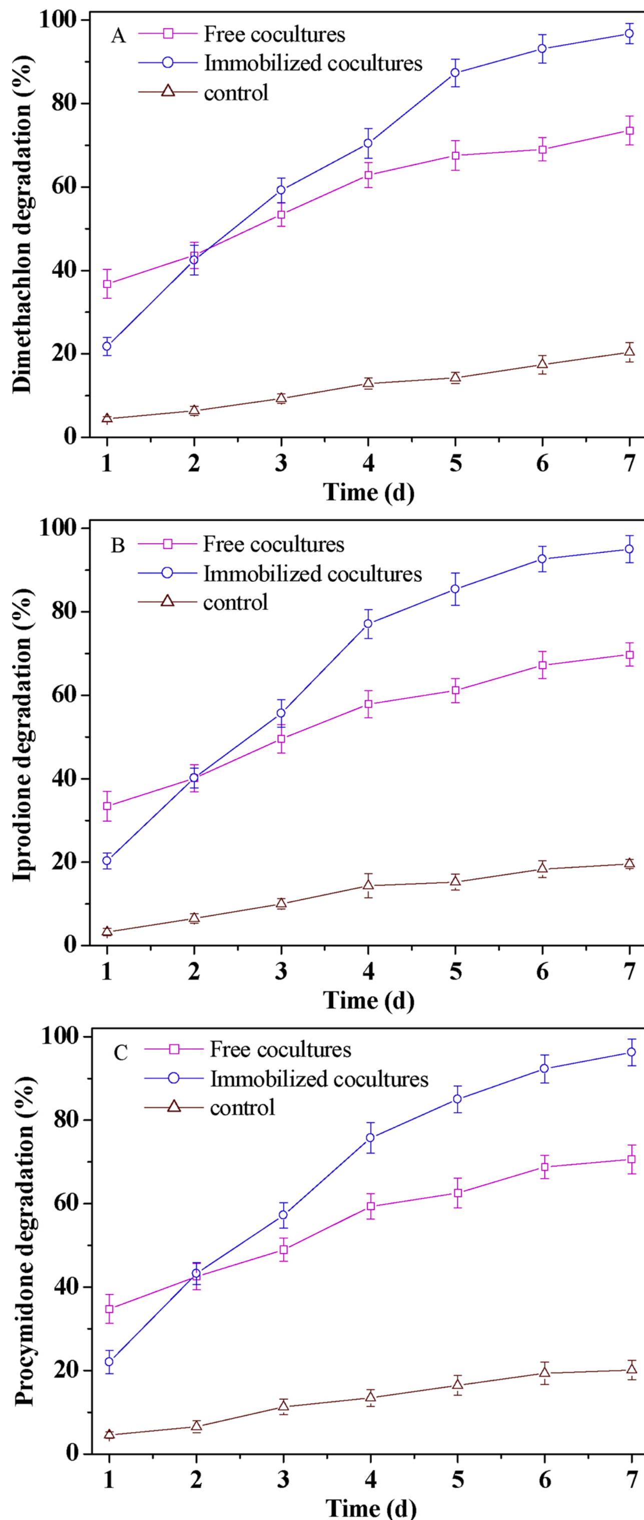

Fig. 6. Biodegradation of dimethachlon (A), iprodione (B) and procymidone (C) in field brunisolic soils.

Table 2
Kinetic parameters of dicarboximide fungicides biodegradation in brunisolic soils by free and immobilized cocultures.

Fungicides	Treatment	Initially residues (mg/kg)	Equation	R^2	$t_{1/2}$ (d)
Dimethachlon	Free cocultures	15.84	$C = 13.9260\ e^{-0.1927\ t}$	0.8854	3.60
	Immobilized cocultures	15.93	$C = 20.8379\ e^{-0.4539\ t}$	0.9716	1.53
	Control	15.88	$C = 15.8927\ e^{-0.0325\ t}$	0.9850	21.33
Iprodione	Free cocultures	6.57	$C = 5.8704\ e^{-0.1718\ t}$	0.9013	4.03
	Immobilized cocultures	6.63	$C = 8.5803\ e^{-0.4358\ t}$	0.9819	1.59
	Control	6.42	$C = 6.4328\ e^{-0.0338\ t}$	0.9773	20.51
Procymidone	Free cocultures	6.43	$C = 5.6972\ e^{-0.1767\ t}$	0.8946	3.92
	Immobilized cocultures	6.42	$C = 8.2522\ e^{-0.4416\ t}$	0.9812	1.57
	Control	6.51	$C = 6.4963\ e^{-0.0345\ t}$	0.9753	20.09

beneficial for the growth of microorganisms (Zhang et al., 2019; Angelim et al., 2013; Chanratana et al., 2018), these favorable conditions improved the stability and reusability of the immobilized cocultures.

3.5. Biodegradation of dicarboximide fungicides in filed soils

Fig. 5 exhibited the degradation of dicarboximide fungicides in filed brunisolic soils by free and immobilized cocultures. The degradation efficiencies of dimethachlon, iprodione, and procymidone by free cocultures, immobilized cocultures and control were 73.56 %, 96.74 % and 20.37 %, 69.78 %, 95.02 % and 19.59 %, and 70.65 %, 96.27 % and 21.12 % after 7 d, with their residual in soils decreased from initially 15.84, 15.93 and 15.88 mg/kg, 6.57, 6.63 and 6.42 mg/kg, and 6.43, 6.42 and 6.51 mg/kg to 4.19, 0.52 and 12.65 mg/kg, 1.99, 0.33 and 5.16 mg/kg, and 1.89, 0.24 and 5.14 mg/kg, respectively (Fig. 6 A, B, C). The results of the kinetic equation and half-life of dimethachlon, iprodione and procymidone residue dissipation in brunisolic soils were calculated in Table 2. The dissipation equation and half-life of dimethachlon, iprodione, and procymidone in soil for free cocultures, immobilized cocultures and control were $C = 13.9260\ e^{-0.1927\ t}$ ($R^2 = 0.8854$) and 3.60 d, $C = 20.8379\ e^{-0.4539\ t}$ ($R^2 = 0.9716$) and 1.53 d, and $C = 15.8927\ e^{-0.0325\ t}$ ($R = 0.9850$) and 21.33 d, and $C = 5.8704\ e^{-0.1718\ t}$ ($R^2 = 0.9013$) and 4.03 d, $C = 8.5803\ e^{-0.4358\ t}$ ($R^2 = 0.9819$) and 1.59 d, and $C = 6.4328\ e^{-0.0338\ t}$ ($R = 0.9773$) and 20.51 d, as well as $C = 5.6972\ e^{-0.1767\ t}$ ($R^2 = 0.8946$) and 3.92 d, $C = 8.2522\ e^{-0.4416\ t}$ ($R^2 = 0.9812$) and 1.57 d, and $C = 6.4963\ e^{-0.0345\ t}$ ($R = 0.9753$) and 20.09 d, respectively.

Previously, except for *P. stuartii* (Zhang et al., 2019) and *B. naejangsanensis* (Zhang et al., 2020), other dicarboximide fungicide degradation microbes have not been applied in situ sites (Mercadier et al., 1997;,Campos et al., 016; Cao et al., 2017; Yang et al., 2018). The findings here emphasized that the immobilized cocultures significantly accelerated the degradation of dicarboximide fungicides compared to the free cocultures and natural dissipation. Moreover, the free and immobilized cocultures of strains JD and J3 were more rapidly and efficiently than the pure culture for the remediation of dicarboximide fungicide contamination sites based on previous studies (Zhang et al., 2019; Zhang et al., 2020). The synergetic and efficient degradation superiorities of *P. stuartii* JD and *B. naejangsanensis* J3 significantly extend their applications of environmental bioremediation, providing a practical and cost-effective approach to remediate sites contaminated by various dicarboximide fungicides. To ensure the effectiveness of the large-scale application in the bioremediation of dicarboximide fungicide contamination sites in situ, however, the simultaneous degradability of mixed dicarboximide fungicides by these strains are need further investigation.

4. Conclusions

The cocultures of *P. stuartii* JD and *B. naejangsanensis* J3 significantly enhanced the degradation of dicarboximide fungicides. The optimal degradation conditions of the cocultures were determined. The cocultures could effectively degrade dimethachlon, iprodione and procymidone to simple products. The cocultures were successfully immobilized in a charcoal-alginate-chitosan carrier and manifested good the stability and usability. Effectively, the immobilized cocultures exhibited higher and faster degradation for dimethachlon, iprodione and procymidone in field brunisolic soils. The synergetic degradation advantages of strains JD and J3 significantly extend their applications for the rapid bioremediation of sites contaminated by dicarboximide fungicides.

Credit author statement

Xiaomao Wu, Yanyou Wu and Cheng Zhang designed research. Cheng Zhang, Jiaohong and Huaming An performed research. Cheng Zhang and Tao Zhang analyzed data. Cheng Zhang and Xiaomao Wu wrote the paper. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2020.123888.

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