Microbial Degradation of Benzimidazole Fungicide Carbendazim by Bacillus velezensis HY-3479

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Abstract

Carbendazim (Methyl benzimidazol-2-ylcarbamate: MBC) is a fungicide of the benzimidazole group which is widely used in the cultivation of pepper, ginseng, and many other crops. To remove the remnant carbendazim, many rhizobacteria are used as biodegradation agents. A bacterial strain of *Bacillus velezensis* HY-3479 was found to be capable of degrading MBC in M9 minimal medium added with 250 mg/L carbendazim. The strain had a significantly higher degradation rate compared to the control strain *Bacillus subtilis* KACC 15590 in HPLC analysis and HY-3479 had the best degradation rate of 76.99% at 48 hours. In gene expression analysis, upregulation of carbendazim degrading genes (*mhel*, *hdx*) was observed in the strain. HY-3479 was able to use MBC as the sole source of carbon and nitrogen but the addition of 12.5 mM NH_4NO_3 significantly raised the degradation rate. HPLC analysis showed that the degradation rate increased to 87.19% when added with NH_4NO_3. Relative gene expression of *mhel* and *hdx* also increased higher for samples with NH_4NO_3 supplement. The enzyme activity of carbendazim degrading enzyme and the 2-aminobenzimidazole degrading enzyme was found to be highly present in the HY-3479 strain. The biodegrading activity of strain HY-3479 may be developed as useful means for bioremediation and used as a potential microbial agent in sustainable agriculture.

Introduction

Carbendazim, which is also known as Methyl benzimidazol-2-ylcarbamate (MBC), is a benzimidazole fungicide widely used in the cultivation of fruits and vegetables and is especially used in Asian countries. China and Korea are known for using MBC-similar pesticides in the cultivation of pepper, ginseng, and many other crops. Carbendazim has refractory and stable nature, which makes it difficult to degrade. But to efficiently raise these vegetables, pesticides including carbendazim are necessarily spread, and this causes the overuse of chemical pesticides. This leads to environmental problems and health problems for humans and animals. According to the agricultural report in Scientific American, pesticides are killing the organisms such as earthworms, beetles, and ground-nesting bees. This greatly affects reducing soil biodiversity (Gunstone et al. 2021). Soil remaining carbendazim causes high toxicity in soil which can affect plant metabolism and can disrupt the microbial communities (Xiao et al. 2013). It is also known to cause abnormality in the liver, testes, and endocrine system even if exposed at a low dose (Aire 2005; Kiigemagi et al. 1991). Applying beneficial rhizobacteria may be one of the solutions to efficiently remove carbendazim-contaminated soil.

The local farmlands in Asian countries tend to use chemical pesticides to maximize crop production and the usefulness of environmental microbes in the agricultural sector continues to rise. This study was initiated to see if functional microorganisms with various beneficial characteristics can be applied to bioremediation and maintaining sustainable agriculture. There are many kinds of microorganisms used for plants and agriculture. These include plant growth-promoting rhizobacteria (PGPR), antifungal bacteria, and bioremediating microbes. To efficiently remove harmful pesticides from contaminated soil, bioremediation through microbial agents is becoming necessary work.

Bioremediation is a process in which biological reactions are taken to remove waste materials including pesticides and toxic chemicals and to break them down into smaller reusable units. The term bioremediation became popular as the demand for environmental protection keeps growing. Microorganisms take a great part in this bioremediation (Zhang et al. 2020). Many microbes are found to have biodegradation activity in various types of environments and soil. The majority of these degrading microorganisms belong to the genera *Acinetobacter, Bacillus, Flavobacterium, Gloeophyllum, Microbacterium, Paenibacillus, Pseudomonas, Ralstonia, Rhodococcus*, and *Xanthofacter* (Fang et al. 2010; Watanabe 2001; Zhang et al. 2020). These rhizobacteria and fungi may act individually or by microbial consortiums. Factors affecting microbial bioremediation include biological factors, temperature, pH, moisture content, nutrient availability, O_2 concentration, and many more (Zhang et al. 2020). Some advantages of microbial bioremediation are that it is a natural process and a cost-effective process. It is also an eco-friendly and sustainable way of degradation since it does not use any harmful chemicals (Dell'Anno et al. 2012). Recent trends in bioremediation involve integrated system biology and metabolic engineering (Dangi et al. 2019). System biology optimizes bioremediation by understanding gene expression, degrading enzymes, sequence information, and pathways. These studies fall in the “omics” techniques (genomics, metabolomics, proteomics, and transcriptomics). Some researchers also undergo metabolic engineering which involves genetic engineering of degrading genes into new microbial hosts. This leads to improvements in yield and productivity of the secondary metabolites produced by microorganisms.

The objective of this study was to investigate the biodegradation activities of the soil-isolated antifungal strain *Bacillus velezensis* HY-3479. An additional function which is carbendazim-degrading ability was pursued (Song et al. 2022). The metabolite study was performed using HPLC analysis, gene expression analysis was performed with the RT-qPCR of formerly discovered genes, and enzymatic tests of MBC and its metabolite, 2-Aminobenzimidazole, using hydrolyzing system (Pandey et al. 2010; Long et al. 2021).

Materials and Methods

Materials

For the growth of the HY-3479 strain, LB (Luria-Bertani) medium was purchased from BD Difco and was used for various experimental purposes. Carbendazim (MBC), 2-Aminobenzimidazole (2-AB), and 2-Hydroxybenzimidazole (2-HB) standards for HPLC were purchased from Sigma-Aldrich (USA). 250 mg/L of carbendazim was added to M9 minimal medium and stored at 4°C until usage. The strain of *Bacillus subtilis* subsp. *stercoris* (KACC 15590) was provided by Korean Rural Development Administration (KRDA). A strain from *Bacillus subtilis* was selected as a comparative strain based on research that supports the biodegrading ability of *B. subtilis* against MBC (Salunkhe et al. 2014; Singh et al. 2019).

The MBC degradation rate of HY-3479 and metabolite concentration change analyzed by HPLC

The method applied to HPLC analysis was adopted from the study of Lee et al. (2020) with modifications. After the bacterial sample was grown overnight in LB broth medium, it was centrifuged at a speed of 12000 rpm for 10 min at 4°C. The supernatant was collected and it was mixed with acetonitrile in a 1:1 volume ratio (v/v) and vortexed for 20 min. After vortexing, each sample was separately filtered with a 0.2 µm Whatman syringe filter (Whatman, Germany). Pretreated samples were then collected in an HPLC 2 ml vial and kept in a -20°C freezer until analysis. HPLC analysis was performed on HPLC-PDA Perkin Elmer Flexar SQ 300 MS model equipped with YMC-Triart C18 Column (150 mm x 4.6 mm x 5 µm). HPLC conditions were as follows: mobile phase used was HPLC grade acetonitrile and water in 7:3 ratios, gradient mode was isocratic, injection volume was 20 µl, the flow rate was 0.7 ml/min, the column oven temperature was 40°C, and detector wavelength was set at 281 nm (Lee et al. 2020; Wang et al. 2010). More detailed conditions are listed in Supplementary Table S1.

MBC-degrading gene analysis

RNA extractions of the HY-3479 strain were performed using the Takara Minibest Universal RNA Extraction Kit (Takara, Japan). The cDNA was synthesized during the real-time quantitative PCR (RT-qPCR) procedure and RNA concentration was set to 100 ng/µl. The instrument used was the StepOne Plus Real-Time PCR system (Applied Biosystems, USA). Onestep TB Green RT-PCR kit including ROX dye (Takara, Japan) was used and the 16S rRNA gene was used as a reference gene (Kirk et al. 2014). Carbendazim-degrading genes from Long *et al.* were selected for gene analysis of strain HY-3479 (Long et al. 2021). Two genes selected were *mhel* (MBC hydrolyzing esterase I) and *hdx* (hydroxylase). MheI enzyme is responsible for the first step of degradation which is MBC to 2-AB and hydroxylase is responsible for the second degradation step which is 2-AB to 2-HB (Pandey et al. 2010). Primers used for the carbendazim degrading genes were created using Primer3 software (ver 0.4.0) and primer information is listed in Supplementary Table S2. The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) was used to determine the relative quantification of gene expression in each sample including the comparative strain. Results of RT-qPCR were calculated and expressed in RQ values (Relative quantification values) (Lee et al. 2008).

Degradation ability promotion by inducing agent NH_4NO_3

Degradation activity promotion was tested by adding NH_4NO_3 supplements in HPLC analysis and RT-qPCR experiments (Zhang et al. 2013). For HPLC analysis, M9 minimal medium was added with 12.5 mM NH_4NO_3 together with 250 mg/L MBC (Zhang et al. 2013). The rest of the procedure was repeated and compared with the samples without the NH_4NO_3 supplements. For RT-qPCR, RNAs were extracted in a 12h-interval of the bacterial samples grown in a medium supplied with NH_4NO_3. The rest of the procedure was repeated and the PCR results and cycle threshold (Ct) were compared with the samples without the NH_4NO_3 supplements. Data were computed to analyze whether NH_4NO_3 addition gave promoting or inhibiting of degradation ability.

MheI and hydroxylase enzyme activity tests

Enzymatic activity tests of strain HY-3479 were conducted with the method from Lei et al. (2017). Bacterial samples were grown overnight and equilibrated to OD_{600} = 0.7 and centrifuged at 12000 rpm for 10 min at 4°C. The supernatant was collected and kept in a -20°C freezer before use. Carbendazim was used as a substrate for the detection of mheI enzyme activity and 2-aminobenzimidazole was used as a substrate for the detection of hydroxylase enzyme activity. In each 14 ml conical tube, 5 ml of the reaction mixture was composed of 0.1 M citric acid buffer adjusted to pH 7.0, 0.2 M sodium phosphate dibasic, and 1.25 mg (250 µg/ml) of MBC or 2-AB (Lei et al. 2017; Jing-Liang et al. 2006). 200 µl of supernatants were added to each tube and it was incubated at 37°C for 60 min. After incubation, the reaction was terminated by warming in a 70°C water bath for 5 min, and 5 ml of ethyl acetate was pipetted for extraction. Anhydrous sodium sulfate was added gradually to dry the samples. UV-Vis Spectrophotometer DU730 (Beckman-Coulter, USA) was used to measure absorbance at 287 nm for determining the concentration difference of MBC and 2-AB after the hydrolysis activity of the samples.

Statistical analysis

All experiments including HPLC, qPCR, and enzyme tests were conducted at least 3 times. Statistical analysis was performed with GraphPad Prism program version 8.0.2. Data were analyzed with multiple unpaired t-tests and statistical significances were calculated using the Holm-Sidak method with P = 0.05.

Sequence accession numbers

The NCBI Genbank accession number of *Bacillus velezensis* HY-3479 16S rRNA sequence is ON653038. The Genbank accession number of mheI and hydroxylase are GQ454794.1 and OCC19062.1, respectively.

Results

The MBC degradation rate of HY-3479

To determine the optimum concentration of MBC, the HY-3479 strain was inoculated in M9 minimal medium with various concentrations of carbendazim pesticide ranging from 125 mg/L to 1 g/L. The strain was able to survive well in the condition of 250 mg/L MBC medium. With this concentration, bacterial growth of the HY-3479 strain peaked at 24 hours. Gradual growth was observed from 12 hours to 24 hours and a decrease in cell numbers afterward (Fig. 1). A total of 3 samples were analyzed using HPLC analysis according to appropriate conditions. Samples include negative control DW, the comparative strain *Bacillus subtilis* KACC 15590, and test strain HY-3479. The comparative strain (KACC 15590) sample showed barely any difference from the original pesticide sample with concentrations ranging around 220 to 240 mg/L of remaining MBC. For the HY-3479 strain, degradation was rapidly occurring until 12 hours of exposure and the concentration remained similar until 48 hours. A clear difference in degrading action was observed in HY-3479 compared to the KACC 15590 strain and negative control. The lowest concentration was 57.53 mg/L equivalent to a degradation rate of 76.99% (Fig. 2).

Metabolite concentration change analyzed by HPLC

Intermediate metabolites experiments were conducted simultaneously with the MBC degradation rate test. Two standard materials, 2-AB and 2-HB, were used for comparison with MBC pretreated sample peaks. Before the retention time (RT) of MBC, small collapsed peaks were appearing in around RT of 1.6 to 2.3 minutes. 2-HB is predicted to be appearing in RT of 1.7 minutes and 2-AB is predicted to be appearing in RT of 2.1. Collapsed peaks seemed to be a mixture of secondary metabolites including 2-AB and 2-HB. Peak areas were calculated and converted to concentration(mg/L) for RT at 1.7 for 2-HB and 2.1 for 2-AB. Concentration changes after 48 hours are shown in Fig. 3. The amounts of metabolites had clear differences between the strains. Compared to the KACC 15590, the HY-3479 strain had a significant increase for both metabolites produced. It showed that HY-3479 with NH_4NO_3 supplement had the highest production of 2-AB and 2-HB. It was especially higher for 2-AB with 56.91 mg/L metabolite production.

Carbendazim-degrading gene expression analysis of HY-3479

The gene expression level of carbendazim degrading gene *mheI* (MBC-hydrolyzing esterase I) was investigated and shown in Fig. 4a. *mheI* expression for the HY-3479 strain exhibited significant change compared to the strain KACC 15590. HY-3479 strain showed a drastic increase in expression level with a mean RQ value of 709.19. The hydroxylase *hdx* gene, which is involved in secondary degradation, showed a different pattern with *mheI* (Fig. 4b). For the HY-3479 strain, an increase in expression level was observed. It was 4.39 times relatively higher than the comparative strain KACC 15590. HY-3479 strain had significant differences in gene expression for *mheI* and *hdx* genes.

Degradation ability promotion by inducing agent NH_4NO_3

During HPLC analysis, NH_4NO_3-added samples were compared with the non-promoter group. NH_4NO_3 supplement group showed a slower degradation rate after 12 h for the HY-3479 strain (Fig. 2). Gradual differentiations of MBC were observed and eventually higher degradation rate at 48 h. The final concentration of MBC in the HY-3479 strain was 32.03 mg/L and the degradation rate was 87.19%. Amounts of intermediate metabolites had a slightly higher outcome for 2-HB but a significant increment for 2-AB (Fig. 3). The addition of NH_4NO_3 gave promoting effects in the action of carbendazim-degrading bacteria.

During gene expression analysis, RNAs extracted with NH_4NO_3 supplement were used in RT-qPCR. There were significant differences in gene expression levels after applying NH_4NO_3 RNA samples. HY-3479 strain had a great promotion of gene expression level for both degrading genes (*mheI, hdx*). The RQ value of *mheI* significantly increased from 709.19 to 2491.39 when NH_4NO_3 was added while the gene

expression level of *hdx* had a moderate difference (Fig. 4a). The RQ value of *hdx* increased from 4.39 to 6.03 in HY-3479 strain (Fig. 4b).

Mhel and hydroxylase enzyme activity tests of HY-3479

Enzyme activities of HY-3479 were tested for MBC degrading enzymes and 2-AB degrading enzymes using MBC and 2-AB as a substrate in the hydrolyzing system. DW group had no variance of MBC and 2-AB at 287 nm absorbance measurement. Comparative strain *B. subtilis* KACC 15590 had a slight change in MBC concentration which was not significant at all and its MBC enzyme degradation rate was 1.51%. HY-3479 strain had a significant change in MBC composition. The calculated MBC enzyme degradation rate for HY-3479 was 75.46% (Fig. 5a). 2-AB degradation was more eminent in all strains. Comparative strain KACC 15590 had a 35.22% of 2-AB enzymatic degradation while the HY-3479 strain had an excellent degrading ability with a 2-AB degradation rate of 82.92% (Fig. 5b).

Discussion

The strain *Bacillus velezensis* HY-3479 was isolated from our previous study (Song et al. 2022) and this strain had an impressive ability to degrade the widely-used fungicide carbendazim (MBC). The HY-3479 endured 250 mg/L of MBC in M9 minimal medium and degraded a significant amount of MBC pesticide after 48 h of exposure. *B. velezensis* was reported as a potential biocontrol agent in the studies of Kim et al. (2021), Myo et al. (2019), and Pour et al. (2021). Demands for eco-friendly microbial agents in agriculture are arising, and this study contributes to the agronomy field with a novel strain with the exceptional performance of MBC biodegradation.

To begin with, HPLC analysis showed that the MBC was degraded by the HY-3479 strain with significant differences in concentration after treatments of the microbial strain. In addition, the HPLC chromatogram showed not only a decrease in the final concentration of MBC but also showed the presence of intermediate metabolites produced during the degradation process (Supplementary Fig. S1). These results suggest that HY-3479 has a notable degrading ability and could be utilized as a bioremediation agent. It has a similar result to the study of Lee and co-workers (Lee et al. 2020). *Rhodococcus* sp. 3 − 2 and 6 − 2 successfully degraded carbendazim in minimal salt medium (MSM) at about a degradation rate of 96%. *Bacillus* species from the study of Salunkhe et al. (2014) also displayed biodegradation of MBC pesticides. This paper urged that 4 strains of *B. subtilis* are capable of degrading MBC up to 95.2% in 3 mg/L of MBC present in grape samples.

In the mRNA expression analysis, the two degrading enzyme genes, *mhel*, and *hdx*, involved in the biodegradation of carbendazim were highly expressed in the HY-3479 strain compared to strain KACC 15590. HY-3479 showed an exceeding expression level which was around 700 RQ values. Moreover, *hdx* expression was analyzed next and the microbial strain altered the transcriptions of genes at a certain amount. The *hdx* gene of the HY-3479 strain had a significant difference in expression level but at a much lower level of increments. This might suggest that the HY-3479 strain has more capability in the first degradation step and less contribution to the second degradation step. Similarly, this trend of

degrading enzyme genes up-regulation was monitored in isolated strains of *Bacillus* spp. (Bhatt et al. 2019a). In this study, pesticide-degrading genes (*est* & *aldh*) were highly expressed by *B. thuringiensis* SG4 and *Bacillus* sp. Sulfo3. There were some more genes found to be involved in the further degradation of MBC in the report of Long *et al*., but our study was focused on investigating the gene expression of *mheI* (MBC-hydrolyzing esterase I) and *hdx* (hydroxylase) (Long et al. 2021; Fang et al. 2016).

The degrading ability of strain HY-3479 was pursued through HPLC analysis and gene expression analysis. Moreover, the researchers assumed that the degradation activities could be improved by the addition of promoting substances. Two chemicals selected were $Cd(NO_3)_2$ and NH_4NO_3 (Arya and Sharma 2016). These potential promoters of carbendazim degradation were selected based on the studies of Xiao et al. (2013) and Zhang et al. (2013). Unfortunately, $Cd(NO_3)_2$ supplement did not have any effects on the results of HPLC and RT-qPCR (data not shown). However, the NH_4NO_3 supplement was effective in both HPLC analysis and gene expression analysis. Clear differences were observed in the HPLC analysis of NH_4NO_3 supplement samples. Final concentrations of remnant MBC were significantly decreased compared to the non-promoter group in the HY-3479 strain. The action of the degradation enzyme seemed to be accelerated by providing a nitrogen source needed in the degradation process. The study of Zhang et al. (2013) reported that the addition of NH_4NO_3 greatly increased the degradation rate of carbendazim by *Rhodococcus erythropolis* djl-11. More importantly, the dji-11 strain had a significant reduction of degradation in the absence of NH_4NO_3. The escalation of degradation rate was obvious in HPLC analysis, and gene expression of HY-3479 had vivid increments in the *mheI* gene and a small increase for the *hdx* gene. These results confirm that the HY-3479 strain has more interaction in the primary degradation step and slightly lower interaction in the degradation of 2-AB to 2-HB. In a further study, consortium biodegradation might also be applied to boost the degradation of MBC (Chen et al. 2012; Bhatt et al. 2019b).

Some notable changes were also examined in the enzyme actions of strain HY-3479, which was analyzed by optical density change of the enzyme-substrate assays. Comparative strain KACC 15590 barely changed the MBC substrate in the hydrolyzing system. HY-3479 caused a significant difference in MBC substrate amount and a higher degradation rate than the KACC 15590. Interestingly, RT-qPCR data of *mheI* matches with the results of enzyme-substrate assays. In addition, 2-AB substrate assays showed that KACC 15590 and HY-3479 strains both have a certain degradation rate of 2-AB but a much greater amount for HY-3479. The study of Jing-Liang et al. (2006) discovered that *Rhodococcus qingshengii* djl-6 could utilize carbendazim substrate as a sole carbon and nitrogen source which is similar to our results. To countenance more about this idea, the report of Lei et al. (2017) showed that the MheI enzyme activity of *Microbacterium* sp. was eminently present.

Enzyme action was also compared with other species with *mheI* and *hdx* from NCBI gene and protein databases (Tripathi et al. 2018). Microorganisms that are known for having the *mheI* gene were *Microbacterium*, *Mycobacterium*, *Nocardioides*, and *Rhodococcus* genera (Jing-Liang et al. 2006; Lei et al. 2017; Pandey et al. 2010). The metagenomics study by Fang et al. (2018) showed the presence of *mheI*

in *Mycobacterium* and the presence of *hdx* in *Dechloromonas* and *Pseudomonas*. Considering only a few strains with *mhel* and *hdx* genes, strain HY-3479 could be a promising bacterial agent that takes part in carbendazim degradation.

Conclusion

This study focuses on examining the outstanding microbial bacterium that can effectively degrade MBC pesticides and be practically used in maintaining sustainable agriculture. *Bacillus velezensis* HY-3479 was able to survive in the M9 minimal medium added with 250ppm MBC pesticide. HPLC analysis showed that the degradation rate after 48 hours was 76.99% for HY-3479 and 87.18% for HY-3479 with NH_4NO_3 supplement. Intermediate metabolites from MBC degradation were produced in a meaningful amount. Lastly, mRNA expression level analysis was performed with 2 MBC degrading genes which are *mhel* (MBC hydrolyzing enzyme I) and *hdx* (hydroxylase). All gene expressions were significantly higher than that of the comparative strain *Bacillus subtilis* KACC 15590. Throughout these experiments, observed results support a clear MBC degradation in strain HY-3479. This microbial strain could degrade MBC into 2-AB and 2-AB into 2-HB via a hydrolytic mechanism of degrading enzymes. Strain HY-3479 has the possibility to be utilized as a potential bioremediation agent in the near future. However, further study of metagenomics and transcriptomics might be needed to determine the exact mechanisms of HY-3479 action on MBC and substances that are involved in the degradation pathway of carbendazim.

Declarations

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Competing Interest The authors have no relevant financial or non-financial interests to disclose, and that they all agree with the submission of the manuscript.

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Ethics approval Not applicable

Consent to participate Not applicable

Consent to publish Not applicable

Data availability Data used in the present study are available from the corresponding author on reasonable request.

Code availability Not applicable

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Figures

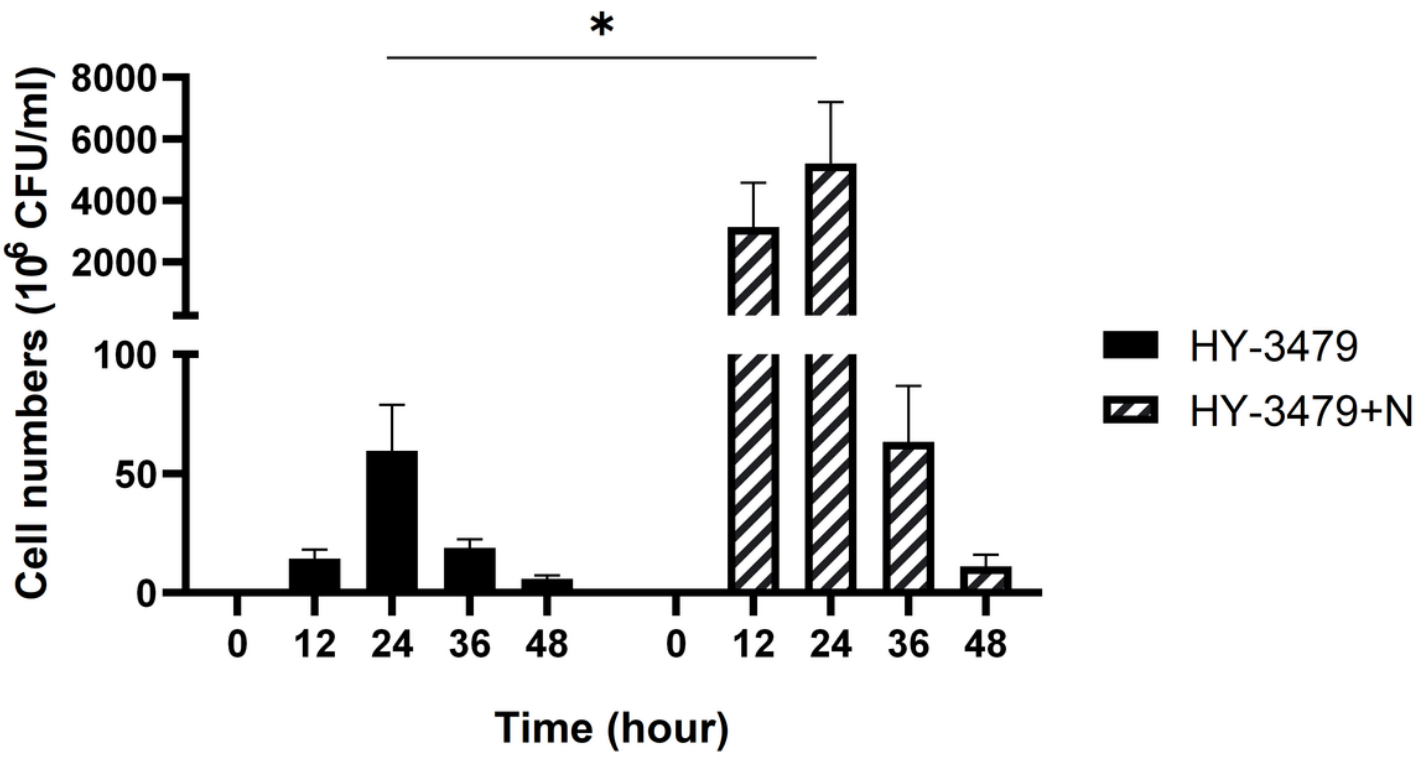

Figure 1

Cell numbers of degrading bacteria (HY-3479) while 48 hours of degradation action occurs. Cell numbers are expressed in 10^6 CFU/ml for 12-hour intervals. HY-3479+N represents HY-3479 with NH_4NO_3 supplements. Experiments were repeated 3 times and asterisks above the columns indicate the significance of the values ($P < 0.05$)

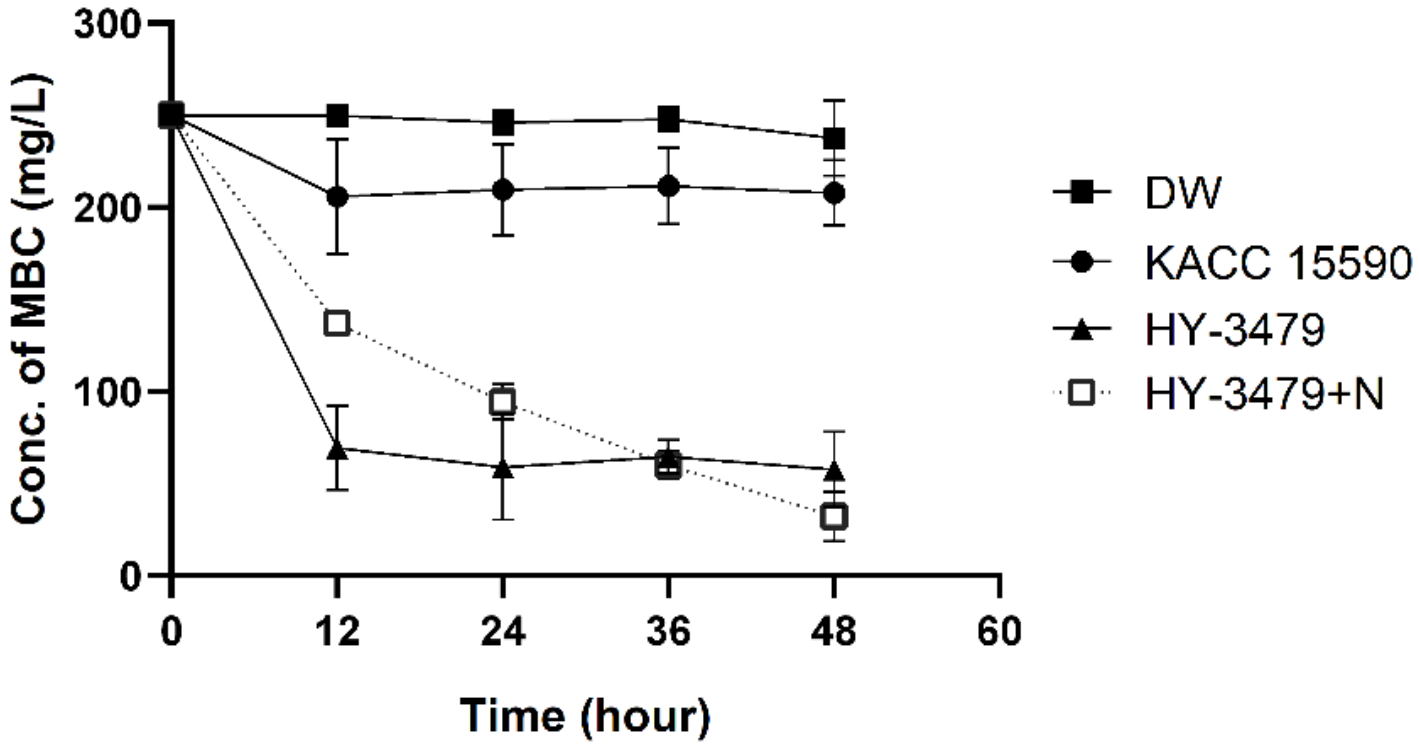

Figure 2

Carbendazim degradation rate of DW, comparative strain KACC 15590, and HY-3479 analyzed by HPLC. Comparisons of degradation rate of HY-3479 without any supplement (HY-3479) and degradation rate of HY-3479 with NH_4NO_3 supplements (HY-3479+N) can be seen. HPLC experiments were repeated 3 times with similar results (n = 3)

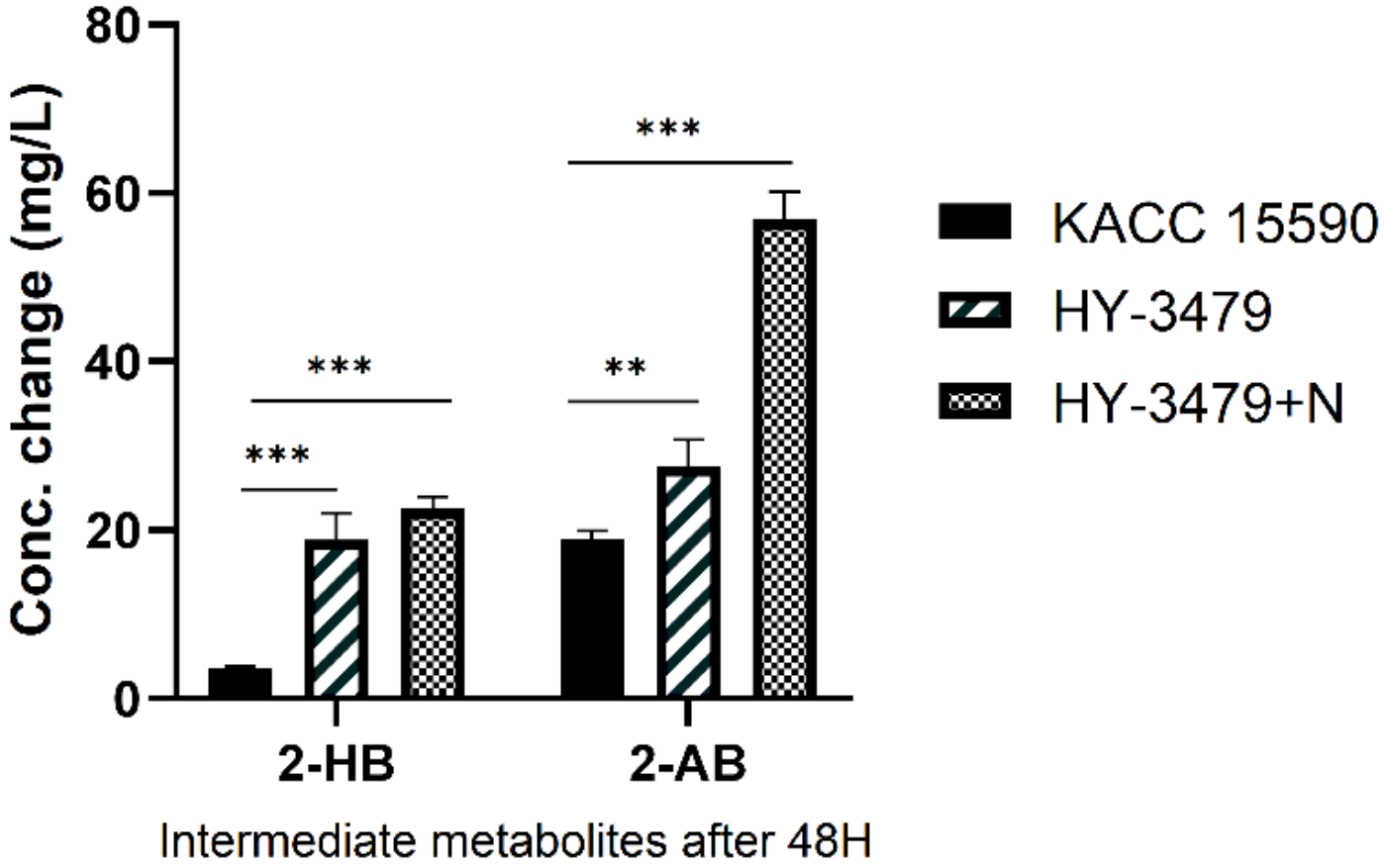

Figure 3

Average amount of intermediate metabolites (2-HB & 2-AB) produced during bacterial degradation of carbendazim. 2-HB and 2-AB concentrations were calculated from the peak area of each HPLC chromatogram. Experiments were repeated 3 times and asterisks above the columns indicate the significance of the values ($P < 0.05$)

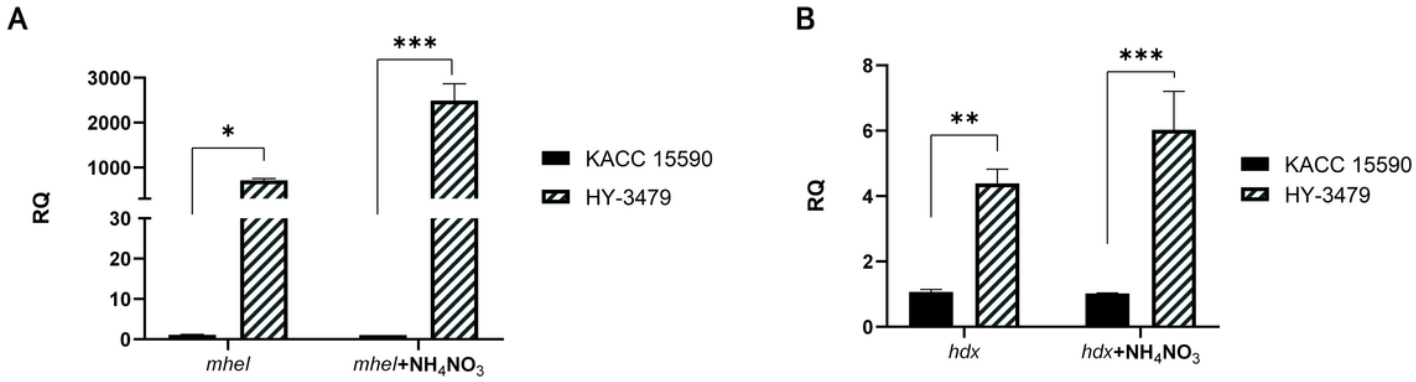

Figure 4

mRNA expression level of *mhel* gene (A) and *hdx* gene (B) in strain HY-3479. Presence of carbendazim degrading genes were analyzed between HY-3479 and KACC 15590. A comparison of the HY-3479 sample and HY-3479 with NH_4NO_3 supplements is shown. Data are presented in relative quantification (RQ) values. Error bars indicate the SD value of the mean (n = 3). Asterisks above the columns indicate the significance of the values (P < 0.05)

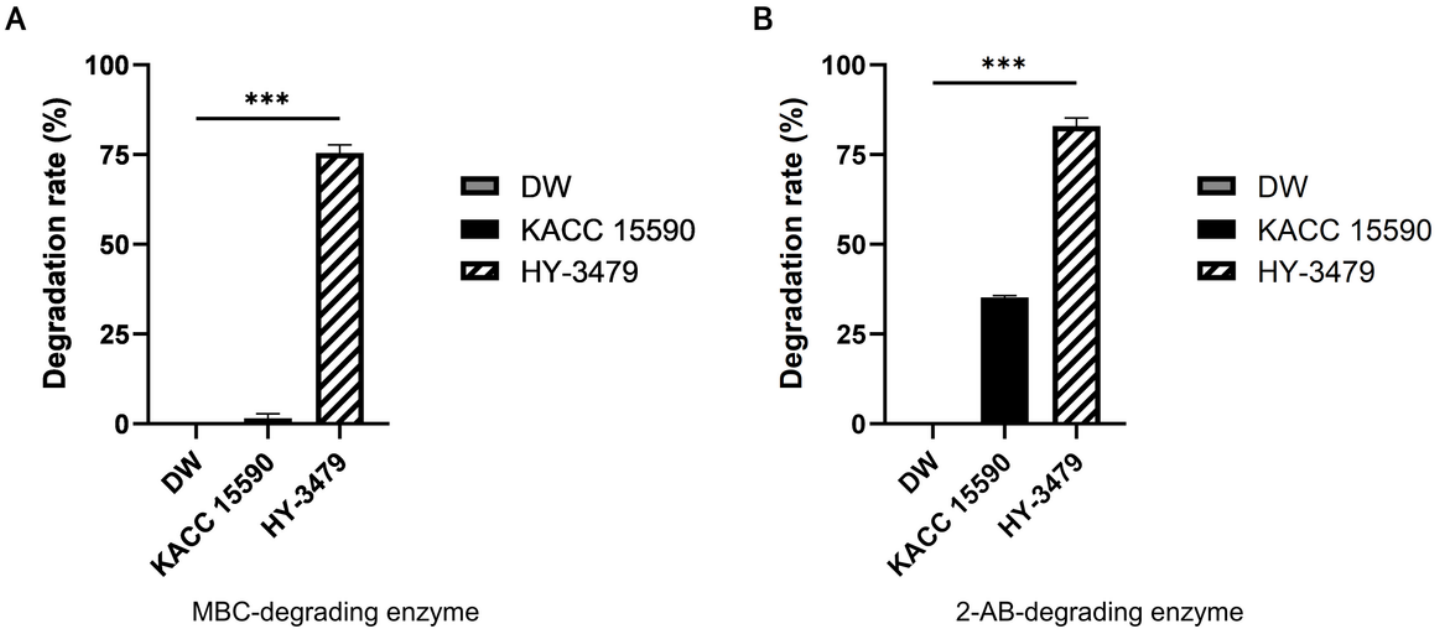

Figure 5

Spectrophotometric analysis of MBC-degrading enzyme (A), and 2-aminobenzimidazole(2-AB)-degrading enzyme test (B) in the hydrolyzing system (OD = 287 nm). Degradation rates were calculated by the absorbance difference during the inoculation of bacterial samples. Experiments were repeated 3 times and asterisks above the columns indicate the significance of the values (P < 0.05)

Supplementary Files

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