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Characterization of the propanil biodegradation pathway in *Sphingomonas* sp. Y57 and cloning of the propanil hydrolase gene *prpH*

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ABSTRACT

In our previous study, the isoproturon-degrading strain *Sphingomonas* sp. Y57 was isolated from the wastewater treatment system of an herbicide factory. Interestingly, this strain also showed the ability to degrade propanil (3,4-dichloropropionamilide). The present work reveals that Y57 degrades propanil via the following pathway: propanil was initially hydrolyzed to 3,4-dichloroaniline (3,4-DCA) and then converted to 4,5-dichlorocatechol, which was then subjected to aromatic ring cleavage and further processing. N-acylation and N-deacylation of 3,4-DCA also occurred, and among N-acylation products, 3,4-dichloropropionanilide was found for the first time. The gene encoding the propanil hydrolase responsible for transforming propanil into 3,4-DCA was cloned from Y57 and was designated as *prpH*. PrpH was expressed in *Escherichia coli* BL21 and purified using Ni-nitrilotriacetic acid affinity chromatography. PrpH displayed the highest activity against propanil at 40 °C and at pH 7.0. The effect of metal ions on the propanil-degrading activity of PrpH was also determined. To our knowledge, this is the first report of a strain that can degrade both propanil and 3,4-DCA and the first identification of a gene encoding a propanil hydrolase in bacteria.

1. Introduction

The intensive use of pesticides in agriculture is a matter of worldwide concern. These substances constitute a substantial source of contamination of non-target systems due to their overuse and application techniques. Contamination of different environmental niches via spray drift, volatilization, run-off and/or leaching has been widely reported in the literature [1–4]. Many pesticides persist in edaphic and aquatic environments either unaltered or only partially degraded. Most of these compounds, as well as their metabolites, represent human health and ecotoxicity threats and should therefore be removed from contaminated environments.

Propanil, a highly selective post-emergent contact herbicide, is one of the most extensively used herbicides for rice production worldwide and is ranked within the top 20 pesticides used in agriculture in the United States [5]. Propanil and its major metabolite 3,4-DCA are biologically active pollutants that cause acute toxicity in a wide range of aquatic species [5–8]. Propanil is also an important cause of death from acute pesticide poisoning, of which methemoglobinemia is an important manifestation [5]. In soil, biodegradation of propanil generates 3,4-DCA, which is also a product of the microbial transformation of other herbicides, such as diuron and linuron [8–11]. Compared with propanil, 3,4-DCA shows lower toxicity against fish and mammals [8,12]. However, 3,4-DCA can be converted by microbial peroxidases to 3,3′,4,4′-tetrachloroazobenzene (TCAB) and other azo products. There is great concern about TCAB because it is a carcinogen and a potential genotoxin [8].

Microbes play an important role in removing agrochemical residues and their metabolites from contaminated environments [13,14]. In previous studies, several bacteria and fungi capable of hydrolyzing propanil to 3,4-DCA were isolated from different environments [8,15–19]. Strains capable of degrading 3,4-DCA have also been isolated and characterized [10,20–25]. However, to the best of our knowledge, a single strain that can hydrolyze propanil to 3,4-DCA and further degrade 3,4-DCA has never been reported. Furthermore, while several hydrolases that transform propanil to 3,4-DCA have been purified from bacteria or fungi [26,27], no propanil hydrolase gene has been identified.

In our previous study, an herbicide-degrading strain *Sphingomonas* sp. Y57 [28], which can degrade isoproturon, diuron and propanil, was isolated from the wastewater treatment system of an herbicide factory. In this work, it was demonstrated that Y57 first hydrolyzed propanil to 3,4-DCA and subsequently degraded 3,4-DCA. The degradation pathway of propanil in Y57 was studied. *prpH*,

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Table 1
Strains and plasmids used in this study.

Strains and plasmids	Characteristic(s)	Source or reference
Strains		
Sphingomonas sp.Y57	Wild type; Propanil and 3,4-DCA degrader	Lab stock
E.coli DH5α	Host strain for cloning vectors	Lab stock
E.coli BL21 (DE3)	Expression host	Lab stock
Plasmids		
pUC118 *Bam*H I/BAP	*Bam*H I digested DNA fragment cloning vector	TaKaRa
pUC118-T1T2	pUC118 Derivative carrying the *Sau*3A I digested DNA fragment containing *prpH*.	This study
pT-*prpH*	pMD18-T Derivative carrying the *prpH*	This study
pET-29a(+)	Expression vector	Novagen
pET-*prpH*	pET-29a(+) Derivative carrying the gene *prpH*	This study

encoding a propanil hydrolase responsible for converting propanil to 3,4-DCA, was cloned from Y57 and was expressed in *Escherichia coli*; the target protein was then purified and characterized.

2. Materials and methods

2.1. Chemicals and media

Propanil (97%) and 3,4-DCA (98%) were purchased from Nanjing Trust Chem. Co., Ltd. and Alfa Aesar A Johnson Matthey Co., respectively. Methanol used for liquid chromatography was purchased from Jiangsu Hanbon Science & Technology Co., Ltd. All other chemicals used in this study were of analytical grade. Minimal salt medium (MSM), prepared at a pH of 7.0, contained ($g\,L^{-1}$) NaCl 1.0, NH_4NO_3 1.0, K_2HPO_4 1.5, KH_2PO_4 0.5, $MgSO_4{\cdot}7H_2O$ 0.2. Concentrated stock solutions of propanil and 3,4-DCA were prepared in methanol at a concentration of $10\,g\,L^{-1}$. Luria-Bertani (LB) medium, prepared at a pH of 7.0, contained ($g\,L^{-1}$) tryptone 10.0, yeast extract 5.0 and NaCl 5.0. To make selective media, ampicillin and kanamycin were added at concentrations of 100 and $50\,mg\,L^{-1}$, respectively.

2.2. Strains, plasmids and DNA-manipulation techniques

Strains and plasmids used in this study are listed in Table 1. Oligonucleotide synthesis and DNA sequencing reactions were performed by Invitrogen Biotechnology Co., Ltd. DNA was gel-purified using the AxyPrep DNA Gel Extraction Kit (Axygen). All enzymes were used as specified by the supplier (TaKaRa Biotechnology (Dalian) Co., Ltd.).

2.3. Degradation of propanil and 3,4-DCA by Y57

In order to analyze biodegradation of propanil and 3,4-DCA, one loop of strain Y57 was inoculated into LB medium and incubated overnight at 30 °C. After harvest by centrifugation (4000 × *g*, 5 min), the cell pellet was washed twice with sterilized MSM, and suspended in MSM to give an OD_{600} of 1.0. Propanil and 3,4-DCA degradation tests were performed by adding propanil or 3,4-DCA to the cell suspension at a concentration of $30\,mg\,L^{-1}$. The cell suspension was shaken in a rotary shaker at 180 rpm at 30 °C, and samples of the suspension were taken at regular intervals. All samples were immediately extracted with an equal volume of dichloromethane and analyzed by high performance liquid chromatography (HPLC) as described below.

2.4. Degradation of 4,5-dichlorocatechol by Y57

4,5-Dichlorocatechol (4,5-DCCAT) degradation tests were performed in MSM. Y57 cells were washed in MSM (as above) and diluted 1:100 into 100 mL MSM containing $50\,mg\,L^{-1}$ 4,5-DCCAT as the sole carbon source. The cell suspension was shaken at 180 rpm on a rotary shaker at 30 °C, and samples of the suspension were taken at regular intervals. The optical densities (OD_{600}) of the samples were measured with a Shimadzu UV-2450 UV–vis spectrophotometer (Shimadzu Corporation, Japan). All samples were immediately extracted with an equal volume of dichloromethane and analyzed by HPLC.

2.5. Identification of propanil and 3,4-DCA degradation products

Samples (10 mL) were taken at regular intervals during propanil and 3,4-DCA degradation by Y57 as described above. Cell suspensions lacking propanil and 3,4-DCA were used as negative controls. Propanil and 3,4-DCA (in MSM) controls were included as well. All samples were extracted with an equal volume of dichloromethane; the organic layer was dried and re-dissolved in methanol. To identify the metabolites, samples were analyzed by HPLC and gas chromatography–mass spectrometry (GC–MS).

For the HPLC analysis, the separation column (internal diameter, 4.6 mm; length, 25 cm) was filled with Kromasil 100-5 C18. The mobile phase was methanol:water (80:20, v:v), and the flow rate was $0.8\,mL\,min^{-1}$. The detection wavelength was 246 nm.

GC–MS analyzes were performed on a Thermo Trace DSQ mass spectrometer. Helium was used as the carrier gas at a flow rate of $1.2\,mL\,min^{-1}$. Gas chromatography was conducted using a RTX-5MS column (15 m × 0.25 mm × 0.25 mm, Restek Corp., US). The column temperature profile was programmed as follows: hold at 50 °C for 1.5 min; increase to 150 °C at 40 °C min^{-1} and hold for 1 min; increase to 200 °C at 10 °C min^{-1} and hold for 6 min; finally, increase to 260 °C at 50 °C min^{-1} and hold at 260 °C for 6 min. The injector temperature was set at 220 °C with a split ratio of 20:1. The interface temperature and ion source temperature were both set to 250 °C. The column outlet was inserted directly into the electron ionization source block, operating at 70 eV.

2.6. Cloning and sequence analysis of a propanil hydrolase gene

The genomic DNA of Y57 was randomly digested with *Sau*3A I. 4–6 kb length DNA fragments were recovered by gel purification, ligated into *Bam*H I/BAP-treated pUC118 and transformed into *E. coli* DH5α cells. Transformants were spread onto LB agar containing $100\,mg\,mL^{-1}$ ampicillin and incubated at 37 °C overnight. Colonies were transferred onto LB agar plates supplemented with $100\,mg\,L^{-1}$ ampicillin and $400\,mg\,L^{-1}$ propanil. The solubility of 3,4-DCA is higher than that of propanil in water, so a clear transparent halo forms around colonies that can hydrolyze propanil to 3,4-DCA. Such colonies were selected and further tested by HPLC.

Nucleotide and amino acid sequence analyzes were performed using BioEdit software. BlastN and BlastP were used for the nucleotide and protein sequence searches (www.ncbi.nlm.nih.gov/Blast). Enzyme MWs and PIs were predicted using the ExPASy Proteomic Server (http://www.expasy.org/).

2.7. Expression and purification of PrpH

To express PrpH in *E. coli* using the pET29a expression system (Novagen), *prpH* was amplified from Y57 genomic DNA. Forward and reverse primers, *prpH*-F (5′-CATATGTCCAACACCGGCTTCTA-CACGC-3′, forward) and *prpH*-R (5′-CTCGAGGCCCAGCAGC-ACGGCCGCCATC-3′, reverse) incorporated *Nde* I and *Xho* I sites (underlined), respectively, into the amplified product. The PCR product was cloned into the pMD18-T vector (TaKaRa) and sequenced. The *prpH* DNA fragment was then digested by *Nde* I and *Xho* I, gel-purified and ligated into the corresponding site of pET29a, generating pET-*prpH*, which was transformed into *E. coli* BL21 (DE3). Transformants were subcultured into 50 mL LB medium and were allowed to grow until reaching a culture density of 0.5 (OD_{600}). Then, *prpH* expression was induced by adding isopropyl-β-d-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, and after a 12 h incubation at 18 °C, cells were harvested by centrifugation, washed twice and suspended in binding buffer (20 mM Na_3PO_3, 500 mM NaCl, 20 mM imidazole; pH 7.8). The cell suspension was disrupted by sonication. Cell debris was removed by centrifugation. The supernatant was loaded onto a His-Bind resin (Novagen). After elution of the nontarget proteins with elution buffer (20 mM Na_3PO_3, 500 mM NaCl; pH 6.0) containing 50 mM imidazole, the target protein was eluted with elution buffer containing 200 mM imidazole. The enzyme was dialyzed against 20 mM Tris–HCl (pH 8.0) containing 200 mM NaCl for 24 h and was concentrated with self-indicating silica gel. The purified enzyme was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.8. Enzyme assays

Enzymatic activities toward propanil were determined in 20 mM Tris–HCl (pH 8.0) unless described otherwise. Then, 5 μl of propanil stock solution was added to 0.5 mL of Tris–HCl buffer containing 25 μl enzyme solution. The mixture was incubated for 3 h at the temperatures given. Reactions were stopped by adding 1.5 mL dichloromethane, and the substrate was extracted and analyzed by HPLC. To study the effect of incubation temperature, samples were incubated at temperatures ranging from 20 °C to 60 °C. The effect of pH was studied at 40 °C in Na_2HPO_4-citric acid buffer. Metal ions were added to final concentrations of 1 mM and 10 mM to study their effects on PrpH activity. Three replicates were used for each sample. One activity unit in this study was defined as the amount of enzyme required to catalyze the formation or hydrolysis of 1 μmol product or substrate per min.

2.9. GenBank accession number

The GenBank accession numbers of gene *prpH* and the 16S rRNA gene sequence of strain *Sphingomonas* sp. Y57 are JN024622 and DQ092868, respectively.

3. Results and discussion

3.1. Propanil degradation in MSM by Sphingomonas sp. Y57

As shown in Fig. 1C, during the degradation of propanil, one metabolite with the retention time of 5.53 min was detected. This product had nearly the same retention time as authentic 3,4-DCA (Fig. 1B and C) and was identified as 3,4-DCA by GC–MS (data not shown). The kinetics of degradation of propanil and production of 3,4-DCA were then concurrently investigated. Over 98% of propanil (30 mg L^{-1}) was degraded by cell suspensions of strain Y57 within 16 h (Fig. 2A). During the first 8 h, 3,4-DCA was accumulated. After this point, 3,4-DCA concentration began to decrease, indicating

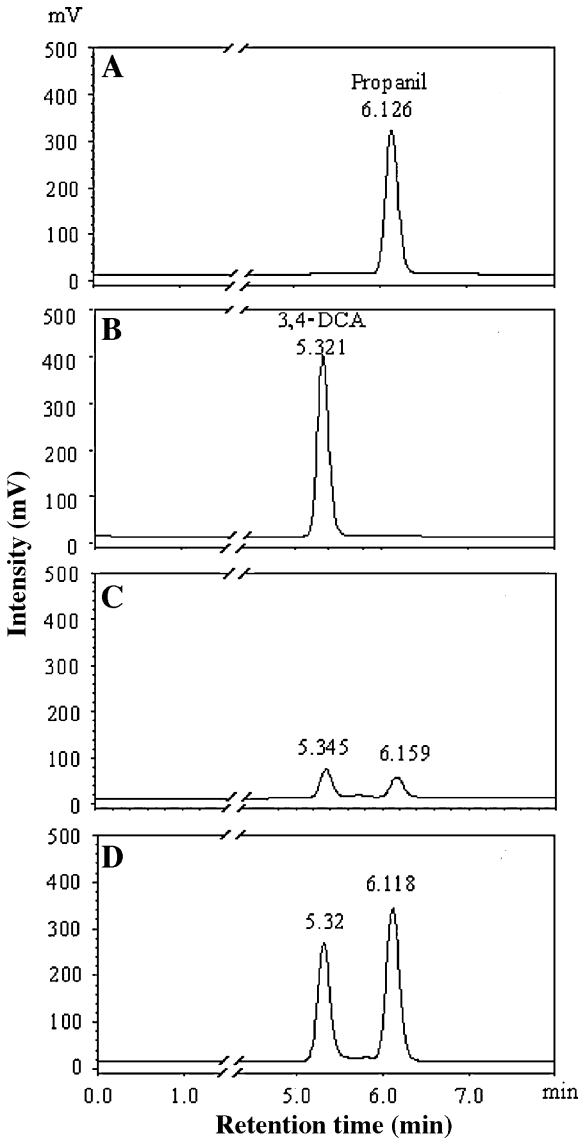


Fig. 1. Identification of propanil and its putative degradation intermediates by comparing the HPLC retention times with standard chemicals. (A) propanil standard. (B) 3,4-DCA standard. (C) Propanil degraded by Y57. (D) Propanil hydrolyzed by PrpH.

that Y57 could not only transform propanil into 3,4-DCA but could also degrade 3,4-DCA. This was further demonstrated by 3,4-DCA degradation test (Fig. 2B). Many propanil-degrading strains have been reported [8,15–19], but none are known to degrade propanil beyond 3,4-DCA.

3.2. Identification of the metabolites during 3,4-DCA degradation by Y57

As shown in Fig. 3, four metabolites appeared during the degradation of 3,4-DCA. The predicted chemical structures, retention times and characteristic ions of the mass spectra are listed in Table 2. The detection of three 3,4-DCA N-acylation products indicated that 3,4-DCA underwent N-acylation and N-deacylation events during the degradation. Among these N-acylation products, 3,4-dichloroformylanilide and 3,4-dichloroacetanilide were reported during the degradation of 3,4-DCA in previous studies [25,29–31], but 3,4-dichloropropionanilide (propanil) was here detected for the first time. Metabolite B was identified as 4,5-dichlorocatechol (Fig. 3a and c and Table 2), indicating that 3,4-DCA was converted to 4,5-dichlorocatechol. As shown in Fig. 2C, Y57

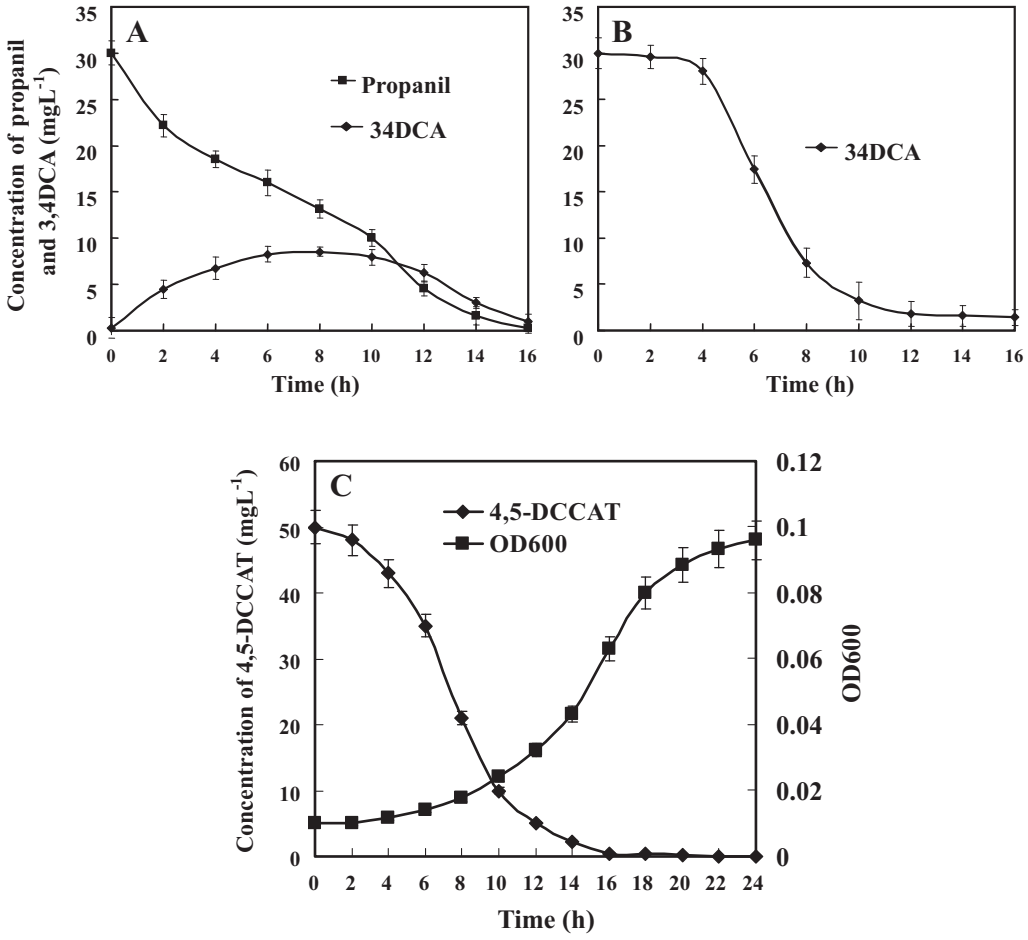
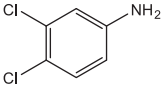
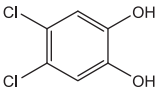
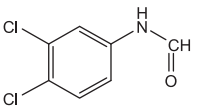
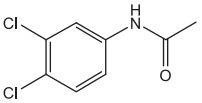
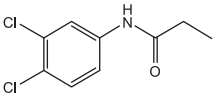


Fig. 2. (A) Degradation dynamics of propanil in MSM by Y57. (■) The degradation of propanil; (♦) the accumulation of 3,4-DCA. (B) Degradation dynamics of 3,4-DCA in MSM by Y57. (C) Degradation dynamics of 4,5-DCCAT in MSM by Y57. (♦) The degradation of 4,5-DCCAT; (■) the growth of Y57.

Table 2
3,4-DCA and its metabolites identified by GC–MS.

Megtabolite	RT (min)	Characteristic ions in GC–MS (*m*/*z*)	Chemical structural formula in NIST library	Name
A	4.57	160.91, 133.91, 125.96, 98.90, 89.95, 80.44, 72.92, 62.93,		3,4-Dichloroaniline (3,4-DCA)
B	6.39	177.89, 159.94, 148.98, 131.91, 114.91, 96.98, 84.98, 70.89, 61.95		4,5-Dichlorocatechol
C	7.06	188.93, 177.85, 160.95, 134.06, 124.72, 113.09, 108.80, 98.98, 89.97, 71.00, 62.95, 56.93		3,4-Dichloroformylanilide
D	7.47	202.93, 160.91, 144.89, 132.87, 125.97, 108.89, 98.89, 89.97, 72.90, 62.92		3,4-Dichloroacetanilide
E	8.15	216.96, 160.90, 144.89, 132.94, 125.97, 108.91, 100.01, 89.95, 73.94, 62.93,		3,4-Dichloropropionanilide (propanil)

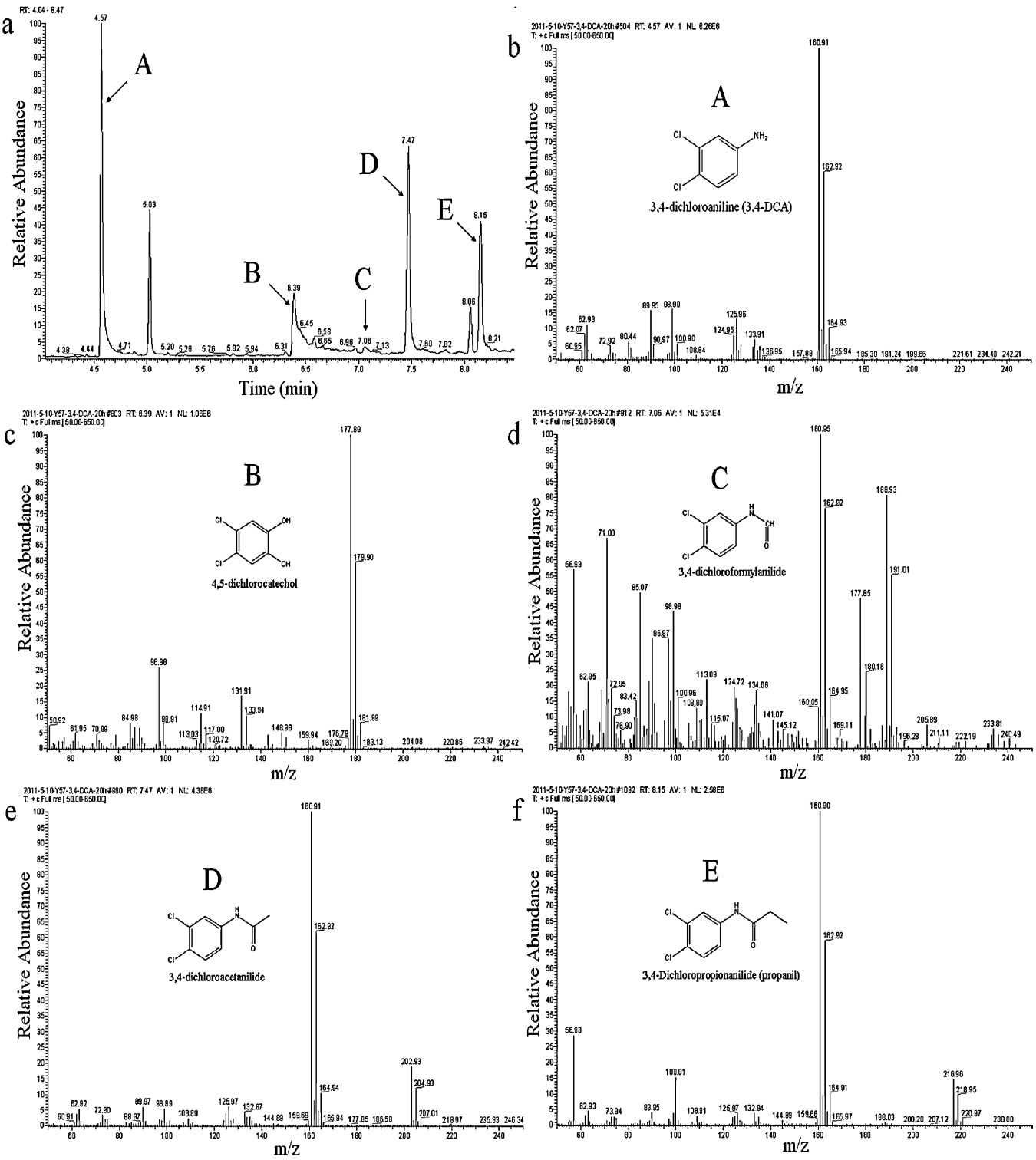


Fig. 3. GC–MS mass spectrum analysis of the 3,4-DCA intermediates transformed by Y57: (a) the GC profiles for 3,4-DCA and its intermediates A, B, C, D, E. The retention times of each compounds was 4.57, 6.39, 7.06, 7.47 and 8.15, respectively. (b–f) The characteristic ions of compounds A–E in GC–MS. They were identified as 3,4-DCA, 4,5-dichlorocatechol, 3,4-dichloroformylanilide, 3,4-dichloroacetanilide and 3,4-dichloropropionanilide (propanil), respectively.

could degrade 4,5-dichlorocatechol and also utilize it as its sole carbon source for growth. Based on the above results, we proposed a degradation pathway of propanil in Y57 (Fig. 4).

Y57 was originally isolated, using isoproturon as the sole carbon source, from the wastewater treatment system of an herbicide factory that produced several kinds of herbicides including isoproturon, diuron and propanil. Notably, these herbicides are all aniline derivatives. Y57 has the ability to degrade a series of aniline derivatives such as 4-IA (4-isopropylaniline), 3-CA (3-chloroaniline), 4-CA (4-chloroaniline) and 3,4-DCA (data not shown). Clearly its acquisition of the ability to degrade aniline derivatives, whether by horizontal gene transfer or mutation, gave strain Y57 advantages over other strains for survival the wastewater treatment system.

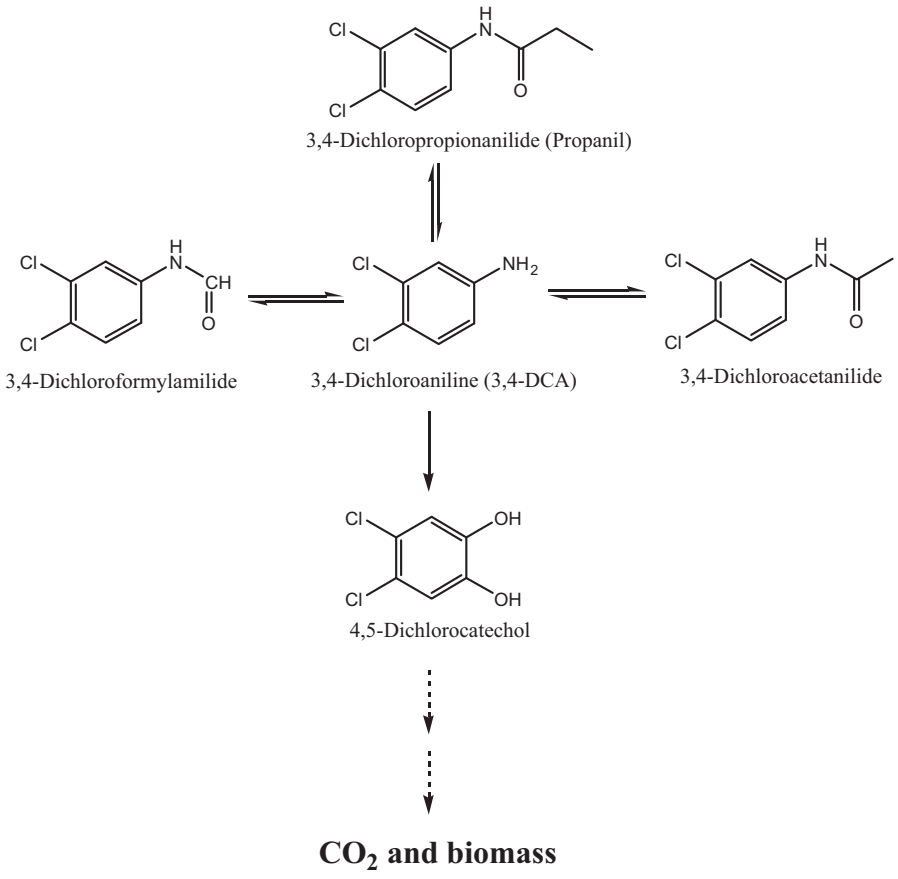


Fig. 4. Proposed pathway for degradation of propanil and 3,4-DCA in *Sphingomonas* sp.Y57.

3.3. Cloning and sequence analysis of the propanil hydrolase gene

Although propanil is known to be hydrolyzed to 3,4-DCA by microbes [8], and several propanil hydrolases responsible for transforming propanil to 3,4-DCA have been purified from bacteria or fungi [26,27], there are no reports of the cloning of a propanil hydrolase gene. To clone the propanil hydrolase gene from Y57, a gene library was constructed from Y57 genomic DNA.

Three positive clones were obtained from approximately 6000 transformants. Their propanil-hydrolyzing abilities were verified by HPLC. One positive clone, 3250 bp in length, was selected for further study. Putative ORFs were then subcloned into pMD18-T and transformed into *E. coli* DH5α to determine their ability to hydrolyze propanil. The ORF encoding the propanil hydrolase was designated as *prpH*.

Sequence analysis indicated that the *prpH* ORF was 1110 bp long and that it encoded a protein of 369 amino acids. Proteomic predictions (ExPASy) showed that the molecular weight of PrpH is 40146.6 Da and the theoretical pI of PrpH is 5.20. The ORF started with ATG and ended with TAG. A putative ribosomal binding site (AGGAAG) was located 10 bp upstream of the start codon. The G + C content was 69.28%.

The *prpH* nucleotide sequence and predicted protein sequence were compared with those in the GenBank database by an online alignment search. Y57 *prpH* shares a high level of similarity with a series of putative histone deacetylases (HDACs) at both the nucleotide and protein sequence level. PrpH showed the highest protein sequence identity (72%) with a putative histone deacetylase from *Sphingomonas wittichii* RW1 (GenBank Accession Number YP_001263732.1) and 46% identity with a verified bacterial histone deacetylase-like amidohydrolase (HDAH) from *Alcaligenes* strain FB188 (DSM11172) [32,33]. We speculated that PrpH was a bacterial histone deacetylase homologue that had the capability to remove the N-propyl group from propanil. *S. wittichii* RW1 cannot convert propanil to 3,4-DCA (data not shown), indicating that PrpH is different from the histone deacetylase RW1, although it may have obtained this function through gene mutation during long-term exposure to propanil.

3.4. Expression, purification and characterization of PrpH

PrpH was expressed in *E. coli* BL21 (DE3) and purified using Ni-nitrilotriacetic acid affinity chromatography. The purified enzyme gave a single band on SDS-PAGE (Fig. 5A). The molecular mass of the denatured enzyme was approximately 40 kDa, in agreement with the molecular mass deduced from the amino acid sequence (40146.6 Da) (GenBank accession number JN024622).

The effects of temperature, pH and metal ions on the propanil-degrading activity of PrpH were assayed with the purified enzyme (Fig. 1D). As shown in Fig. 5B and C, the optimal temperatures and pH for PrpH were 40 °C and 7.0, respectively. The results shown in Fig. 5D indicated that Li^+, Mg^{2+}, Ga^{2+} and Mn^{2+} did not remarkably influence enzyme activity at concentrations of 1 mM or 10 mM but that Ag^+, Cd^{2+}, Zn^{2+}, Cu^{2+} and Hg^{2+} were strong inhibitors of PrpH. Approximately 40% to 60% of the PrpH activity was inhibited by Ni^{2+} and Co^{2+} at the tested concentrations. At a low concentration (1 mM), Cr^{3+}, Fe^{2+}, Fe^{3+} and Al^{3+} slightly influenced the enzyme activity, but at a high concentration (10 mM), they all strongly inhibited enzyme activity. Notably, Zn^{2+} was a strong inhibitor of PrpH at a concentration of 1 mM, which is in agreement with

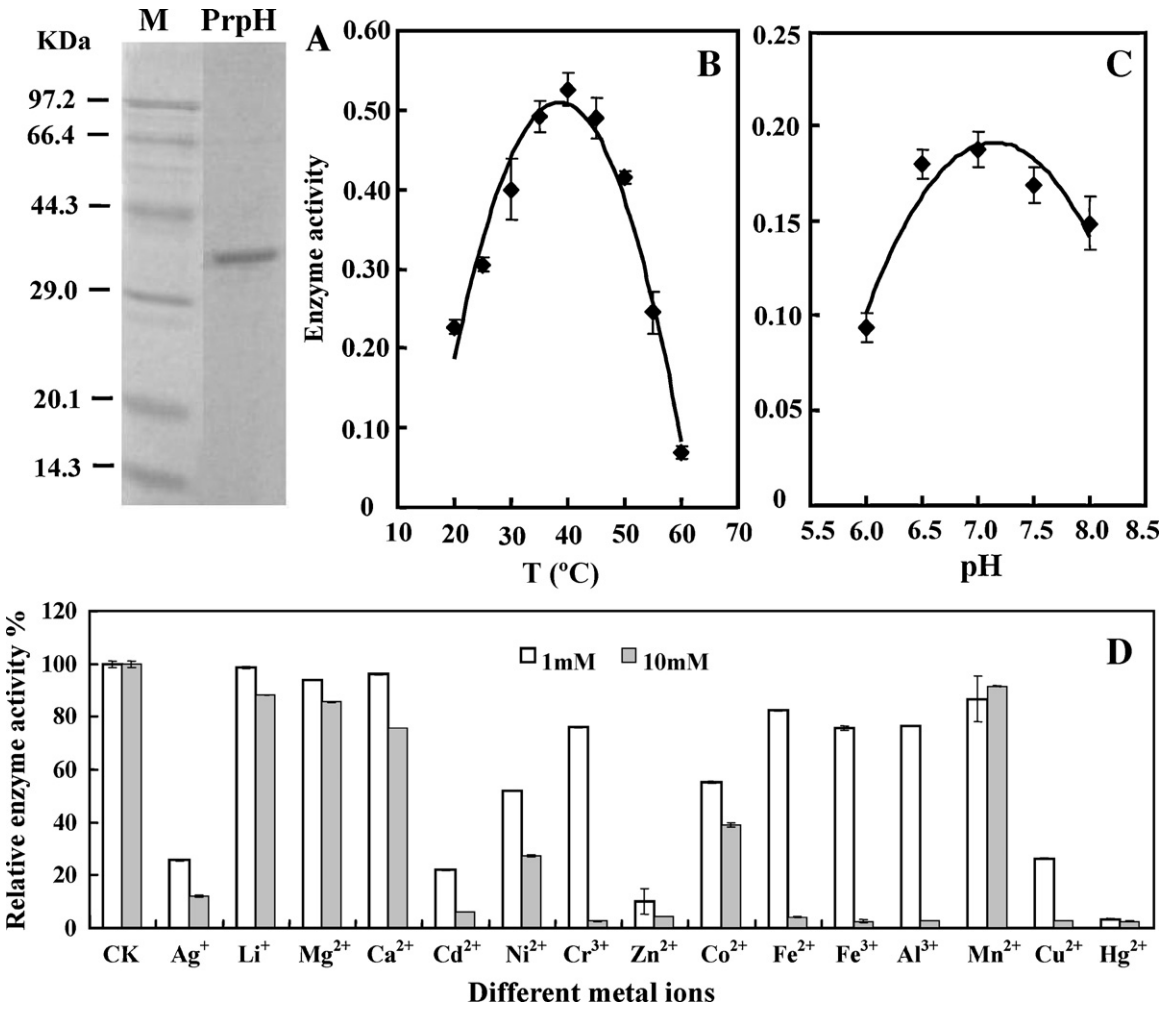


Fig. 5. Characterization of the purified recombinant PrpH produced from the recombinant *E. coli* BL21(DE3) strain harboring pET29a-prpH: (A) SDS-PAGE analysis of the purified recombinant PrpH, (lane M) low weight protein marker; (lane PrpH) purified recombinant PrpH. (B) Effect of temperature on enzyme activity of PrpH. (C) Effect of pH on enzyme activity of PrpH. (D) Effect of different metal ions on enzyme activity of PrpH.

published propanil degradation tests using Y57 cells [28]. However, HDAH from *Alcaligenes* strain FB188 contains a Zn^{2+} ion in the active site, which contributes significantly to catalytic activity, and Zn^{2+} was able to increase the catalytic activity of HDAH (2.2-fold) at a concentration of 1 mM [32]. This activity may indicate that PrpH is different from HDAH. Additionally, besides propanil, stain Y57 was also able to degrade other types of herbicides, such as isoproturon and diuron, but PrpH could not degrade any other herbicides except propanil (data not shown), indicating the existence of other herbicide-degrading enzymes in strain Y57.

4. Conclusion

The isoproturon-degrading strain Y57 degrades both propanil and 3,4-DCA. It first hydrolyzes propanil to 3,4-DCA, which it converts to 4,5-dichlorocatechol before metabolizing it further. N-acylation and N-deacylation of 3,4-DCA also occurred in strain Y57, and N-propionylation was found for the first time. The gene encoding a propanil hydrolase responsible for transforming propanil to 3,4-DCA was cloned from strain Y57 and was designated as *prpH*. Its protein product was expressed in *E. coli*, purified and characterized. To the best of our knowledge, strain *Sphingomonas* sp. Y57 is the first bacterium reported to degrade both propanil and 3,4-DCA, and the gene *prpH* is the first propanil hydrolase gene to be identified in bacteria.

Acknowledgements

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