e-ISSN:2320-3528
p-ISSN:2347-2286

Research & Reviews: Journal of Microbiology and Biotechnology

Aerobic Degradation of Buprofezin via Novel Degradation Intermediates by *Rhodococcus* sp. strain RX-3

Ruixue Li, Chun Dai, Guangli Wang*, Shaoxian Wu, Yubao Gong, Yuanyuan Jiang, Zhijia Wang, Naiyue Sun
College of Life Sciences, Huaibei Normal University, 235000, Huaibei, PR China

Research Article

Received date: 30/11/2015
Accepted date: 19/02/2016
Published date: 29/02/2016

***For Correspondence**

Guangli Wang, College of Life Sciences, Huaibei Normal University, 235000, Huaibei, PR China, Tel: +86 561 3803024

E-mail: wanf-3344@163.com

Keywords: Buprofezin; Degradation pathway; Biodegradation; *Rhodococcus* sp.

ABSTRACT

Buprofezin is a commonly used chemical with satisfactory efficacy against sucking insect pests, but its disposal causes serious environmental problems. In this study, a bacterial strain RX-3 isolated by continuous enrichment from buprofezin-treated soil was tested for biodegradation of buprofezin. The isolates were most similar to *Rhodococcus* sp. based on their morphological, physiological and biochemical characteristics, as well as phylogenetic placement inferred from 16S rRNA gene sequence. Strain RX-3 was found capable of utilizing buprofezin as the sole source of carbon for growth over a wide range of temperature (25-45°C) and pH (5.0-9.0) conditions. It could almost completely degrade 60 mg/L of buprofezin within 80 h, and in the presence of metals such as Ba^{2+}, Zn^{2+} and Cu^{2+}. In addition, six newly identified metabolites formed during buprofezin degradation were detected and identified by gas chromatography-mass spectrometry (GC-MS), from which we proposed a novel degradation pathway. Our results suggest that *Rhodococcus* sp. RX-3 could be a potential bioremediation agent of buprofezin-contaminated environments.

INTRODUCTION

Buprofezin is a new insecticide growth regulator that disrupts the development of immature forms by interference with chitin synthesis [1-3]. This compound affects important insect pests, including whiteflies, planthoppers, leafhoppers, and scales [4]. It is a stable compound with a long half-life in the natural environment, i.e. 26-220 days in aerobic soils [4], and approximately 36-104 days under flooded field conditions [5]. Hence, its residues can persist at application sites and in common livestock like tea, rice, potatoes, citrus fruits, cotton, and vegetables, and thus could easily induce cumulative effects which are harmful to human health [4].

The intensive use of buprofezin in agriculture, along with improper storage and its adverse effects makes the compound highly hazardous [6], and its treatment and disposal have emerged as important environmental concerns. Although the pesticide can be degraded through chemical and physical processes, microbe-mediated metabolism still remains to be the main mechanism responsible for its degradation in the environment [6,7]. However, until now, only three bacterial strains capable of degrading buprofezin have been documented [4,5,8], and the degradative pathway has not yet been fully uncovered [4,5]. In this report, we described the isolation and characterization of a *Rhodococcus* sp. strain capable of using buprofezin as the sole carbon source for growth. Characteristics of microbial degradation were also evaluated and a novel buprofezin-degrading pathway was proposed.

MATERIALS AND METHODS

Culture medium and chemicals

Bacteria were enriched and tested for degrading activity in basal salts medium (MM) [5] containing K_2HPO_4 (1.5 g/L), KH_2PO_4 (0.5 g/L), NH_4NO_3 (1.0 g/L), $MgSO_4 \cdot 7H_2O$ (0.2 g/L) and NaCl (1.0 g/L). MM was supplemented with buprofezin (60 mg/L; Sigma-Aldrich) to enrich the buprofezin-degrading bacteria, here referred to as MM-1. Luria-Bertani medium (10.0 g/L tryptone, 5.0 g/L yeast extract, and 5.0 g/L NaCl, pH 7.0) was used for preparation of buprofezin-degrading strain seed in liquid [5]. Solidified media was prepared with 1.5% (w/v) agar.

Enrichment, screening and identification of buprofezin-degrading bacteria

A total of 10 g of soil samples were collected from a buprofezin-manufacturing factory, in Jiangsu (China) and were then added to 100 mL MM-1 medium in a 500-mL Erlenmeyer flask. Inocula were incubated at 30°C with shaking at 160 rpm. Every week, 5 mL of culture were harvested by centrifugation at 5000 Xg for 5 min at 4°C before being transferred into a new enrichment medium. After three transfers, the enrichment cultures assumed to contain bacteria capable of degrading buprofezin were further diluted and cultivated in LB agar containing 100 mg/L buprofezin. Colonies grown on these plates were isolated and further purified before being tested for their buprofezin-degrading capabilities following the method described by Chen et al. [5]. Out of the 4 isolates which tested positive, only the isolate RX-3 was considered for other tests and further characterized due to its high degrading activity. The model isolate was grown in LB plates for 48 h at 30°C to characterize the colony, morphology and biochemical activities following the Bergey's Manual of Determinative Bacteriology [9].

Identity of RX-3 was also verified by phylogeny by amplifying and sequencing the 16S rRNA gene. In passing, total genomic DNA was extracted using the high salt precipitation protocol from 10 mL of cells grown in LB media and incubated at 30°C overnight [10]. The target gene was amplified using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGCTACCTTGTTACGACTT-3') [11]. PCR cycles include initial denaturation step at 95°C for 10 min, followed by 30 cycles of 30 s denaturation step at 94°C, 30 s annealing step at 56°C, and 30 s elongation step at 72°C and a final extension step at 72°C for 10 min using TProfessional standard thermocycler (Biometra, Germany) [12]. Amplified products were verified for the correct size by agarose (1%) gel electrophoresis. Then, the target DNA fragment was purified with a SanPrep PCR purification kit (Sangon Biotech) and then sent to Sangon Biotech Co., Ltd. (Shanghai, China) for single pass Sanger sequencing also using the same PCR primers. Similar and related sequences were compared to and downloaded from NCBI GenBank database using the BLASTn method (http://www.ncbi.nlm.nih.gov/blast). Multiple sequence alignment was performed using Clustal X 1.8.3 with default settings [13]. Phylogenetic analysis was carried out in Mega 6.0 [14] using the Kimura 2-parameter distance model. A phylogenetic tree was built using the Neighbor Joining method with bootstrap support test repeated 1000 times. The 16S rRNA gene sequence of RX-3 was sequenced (1411 bp) and deposited in the GenBank database under accession number KR906529.

Growth and degradation experiments

To prepare the bacteria for tests, strain RX-3 was pre-cultured in 10 mL of LB medium at 30°C with shaking at 160 rpm for 24 h until late-exponential growth. Then, cells were harvested by centrifugation at 5000 Xg for 5 min at 4°C, washed twice with MM and re-suspended in fresh MM (OD600=1.0). For all experiments, unless otherwise stated, the cells were incubated at 1% (v/v) ratio with the medium and further incubated in an incubator shaker (160 rpm, 30°C). All of the treatments were performed in triplicate.

Screening of the bacterial isolate for buprofezin degrading activity was carried out in 250 mL Erlenmeyer flasks containing 100 mL of MM-1. Then, 10 mL of samples were collected from each flask every 16 h and buprofezin was extracted with an equal volume of dichloromethane [5]. The concentration of buprofezin was determined by high performance liquid chromatography (HPLC) and the biomass was monitored by measuring OD600 as described below. To investigate the effect of the initial pH and temperature on buprofezin biodegradation, incubation temperatures (25°C, 30°C, 35°C, 40°C, 45°C) and initial medium pH (5.0, 6.0, 7.0, 8.0, 9.0) were studied.

Effects of metal ions on buprofezin degradation

Effects of metal ion on the biodegrading activity of the isolated bacteria was also determined by adding 60 mg/L buprofezin to MM in combination with the different concentrations of metal ions ranging from 0.1 mM to 10 mM, referred hereafter as MM-2. The resuspended bacterial cultures were incubated in 10 mL of MM-2 with different concentrations (0.1 mM, 1 mM and 10 mM) of metal ions (Cu^{2+}, Ca^{2+}, Ba^{2+}, Li^{+}, and Zn^{2+}). After 24 hours, the buprofezin was extracted and concentrations were determined as described previously [15].

Chemical analysis

Biomass concentration of RX-3 was monitored by measuring optical cell density at wavelength 600 nm (OD600) using UNIC 4802 (Shanghai China). Concentrations of buprofezin however were analyzed by HPLC following the procedures described by Chen et al. [5]. In brief, buprofezin was first extracted with an equal volume of dichloromethane followed by the addition of dehydrated anhydrous sodium sulfate and then concentrated by revolving evaporation. After which, the residual was dissolved in an equal volume of methanol. The concentration of buprofezin was measured by HPLC (Agilent 1200) with photodiode array detector. It was equipped with YWG-C18 reversed phase column (250 mm length 4.6 mm internal diameter) and 2487 Dual λ-Absorbance Detector. The mobile phase was in methanol: water (85:15, v/v), with the flow rate of 0.6 mL/min. The target compound was detected at 245 nm.

The metabolites expressed during the assays were also determined using the previously described methods with some modifications [4]. These were prepared by incubating cells in 10 mL liquid MM-1 for 5 days and extracted for GC-MS analysis. This time however, the GC-MS was equipped with electron ionization (EI) mode of 70 eV and with a mas range of 30-600 Da. Analysis was done using the column with 30 m length × 250 µm inside diameter ×0.25 µm film thickness. Temperature profile included initial temperature of 110°C for 1 min, then increased to 240°C at 20°C/min and held for 3 min. Helium was used as the carrier gas at the flow of 1.2 mL/min.

e-ISSN:2320-3528
p-ISSN:2347-2286

RESULTS AND DISCUSSION

Strain isolation and identification

A total of four bacterial strains were obtained after enrichment and were further tested for their degrading capability under different conditions. Strain RX-3 was remarkable because of its relatively high capability to degrade buprofezin, and was then selected for further investigations.

Morphological and biochemical tests indicated that strain RX-3 is an aerobic, non-motile, Gram-positive bacterium. Colonies of RX-3 on LB agar were smooth, moist, yellowish, circular, and with regular edges. The most similar sequence of the RX-3 16S rRNA gene was identified to be *Rhodococcus baikonurensis* GTC 1041T (accession no. AB071951) as revealed by BLASTn search against the NCBI GenBank with 99.77% similarity. Phylogenetic analysis also verified this by clustering the sequences, with bootstrap support of 1000 times. Based on the information, including the phenotypic and phylogenetic characteristics, the isolate RX-3 was preliminary identified as a putative *Rhodococcus* sp. **(Figure 1).**

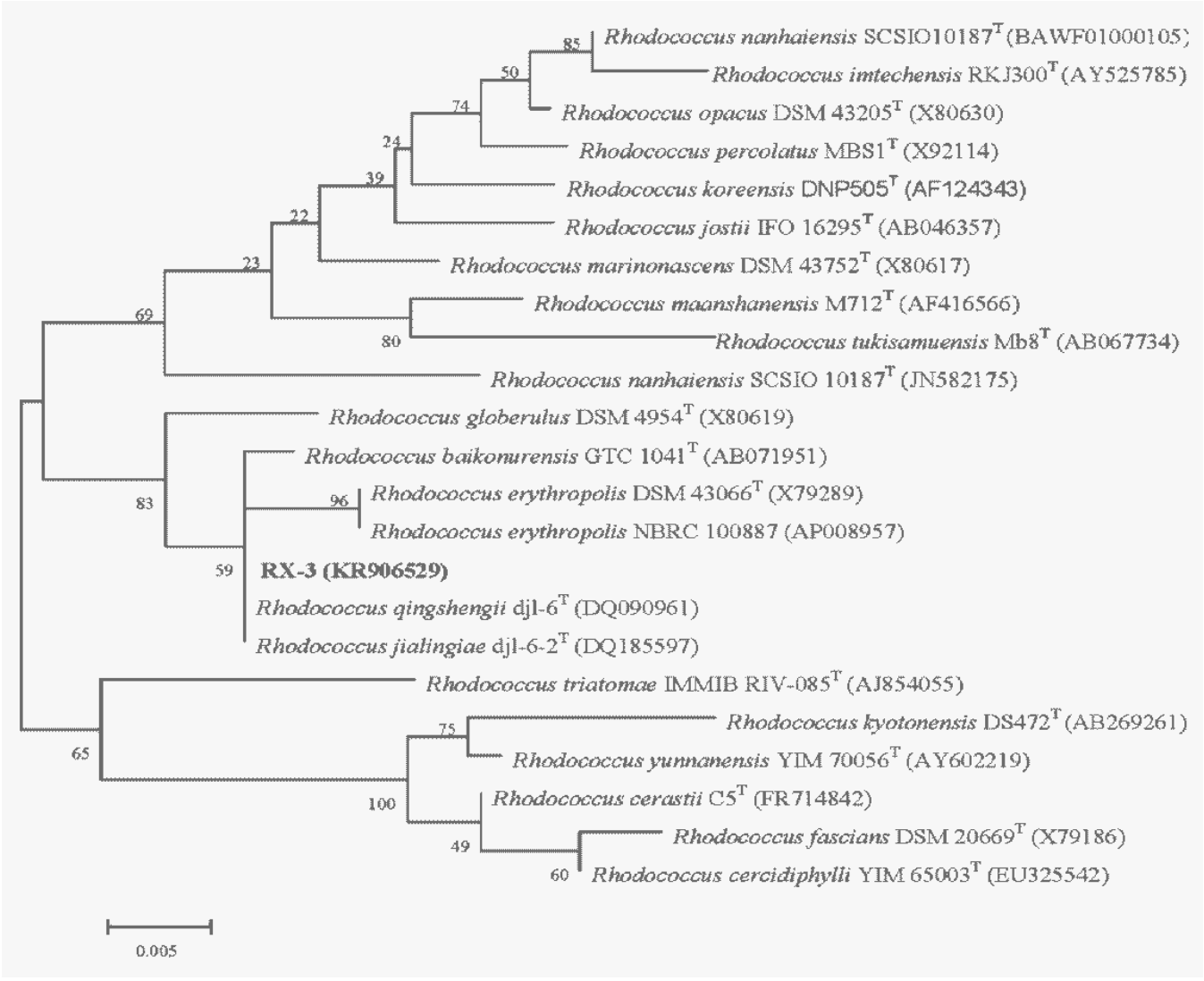

Figure 1. Phylogenetic tree generated from full length 16S rRNA gene sequence including that of strain RX-3 and 20 other reference strains from NCBI GenBank constructed using the neighbor joining method and the MEGA 6.0 program.

Members of this genus are saprophytic and commonly found in soil and water, and many isolates are engaged in the biodegradation of many toxic organic pollutants such as 17α-methyltestosterone [16], quinoline [17], 4-nitroaniline [18], 4-nitrophenol [19], estrogen [20] and methyl tert-butyl ether [21]. *Rhodococcus* sp. RX-3 rapidly degraded buprofezin, indicating that the strain has great potential application in removing the buprofezin residues from environment and agricultural products.

Biodegradation of buprofezin by RX-3 in liquid culture

This study revealed that RX-3 could efficiently degrade buprofezin by making it as the sole carbon source. It was markedly different from the other three isolates. **Figure 2** shows that 98.01% of the buprofezin was degraded in MM-1 within 80 h by strain RX-3. Biomass also increased with time of incubation suggesting cell growth and reproduction. The efficiency of buprofezin degradation by strain RX-3 was related to the pH and temperature conditions which were optimum at 30°C and 7.0, respectively (Date

e-ISSN:2320-3528
p-ISSN:2347-2286

not shown). This showed that buprofezin is an efficient carbon source for the strain RX-3. Present results were similar to Li et al. [6] who reported that approximately 92% of the buprofezin was degraded by strain YL-1 after 48 h.

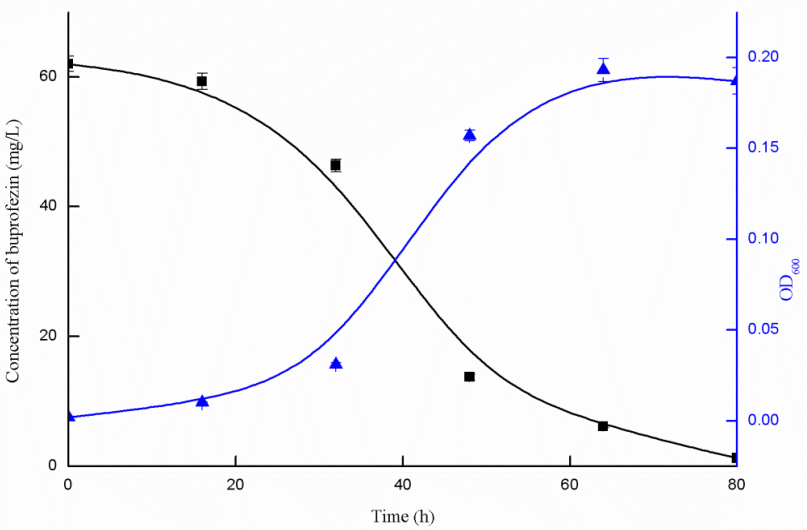

Figure 2. Buprofezin degradation dynamics and growth curve of *Rhodococcus* sp. strain RX-3. Closed; squares: buprofezin concentration; closed triangles: growth of strain RX-3. Error bars represent the standard deviations based on triplicate experiments.Essi assim sunt volupta diti nest

Effects of metal ions on buprofezin biodegradation

Metal ions which have inhibitive effects on the biodegradation activity and growth of microorganisms are often found at high concentrations in industrial wastewater [22]. Therefore, it is an excellent condition and source to isolate candidate bacteria that could perform bio-treatment of buprofezin and metals co-contaminated environments [15]. As shown in **Figure 3**, rate of degradation increased with increased of Cu^{2+} and Ba^{2+} concentrations. It is interesting to note that buprofezin was significantly degraded at low concentrations of Li^{+} and Ca^{2+}, but the activity decreased once the concentration rose above 1.0 mM. These suggest that RX-3 is possibly capable of bioremediation on both buprofezin and metals co-contaminated environments.

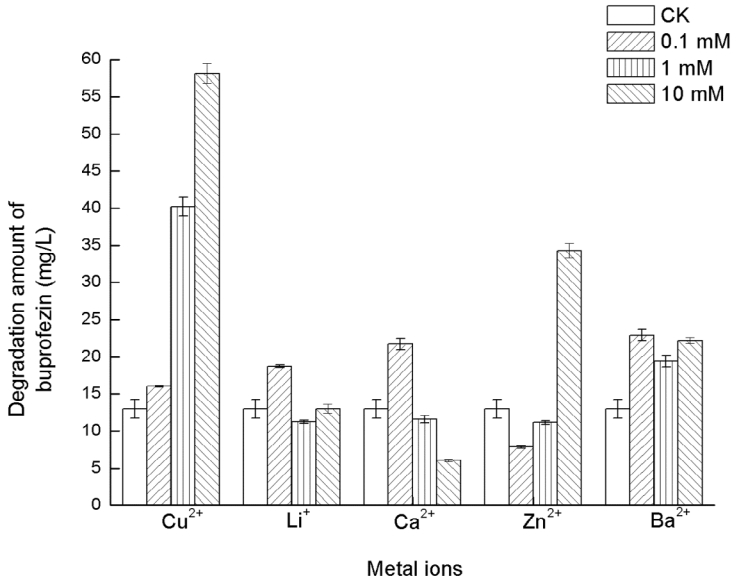

Figure 3. Effect of added metal ions on the degradation of buprofezin by strain RX-3 within 24 h. The initial concentration of buprofezin was 60 mg/L. CK was MM-1 culture free of metal ions.

Identification of the metabolites during buprofezin biodegradation

The metabolites produced during buprofezin degradation by strain RX-3 was identified by GC-MS. Compound A, which has already been reported in other species was found and identified as buprofezin based on the molecular weight (305), retention time (9.874) and the characteristic fragment ion peaks (290, 249, 216, 190, 131, 115, and 105; **Figure 4**, **Figure 5a**). Further, our results showed that nine metabolite compounds were present in the degradation process **(Figure 4 and Table 1)**, which includes Compound B (retention time of 9.526; **Figure 5b**), Compound C (retention time of 8.136; **Figure 5c**), Compound D (retention time of 7.189; **Figure 5d**), Compound E (retention time of 7.059; **Figure 5e**), Compound F (retention time of 5.920; **Figure 5f**), Compound G: (retention time of 5.295; **Figure 5g**), Compound H (retention time of 4.660; **Figure 5h**), Compound I (retention time of 4.313; **Figure 5i**), and Compound J (retention time of 3.409; **Figure 5j**).

e-ISSN:2320-3528
p-ISSN:2347-2286

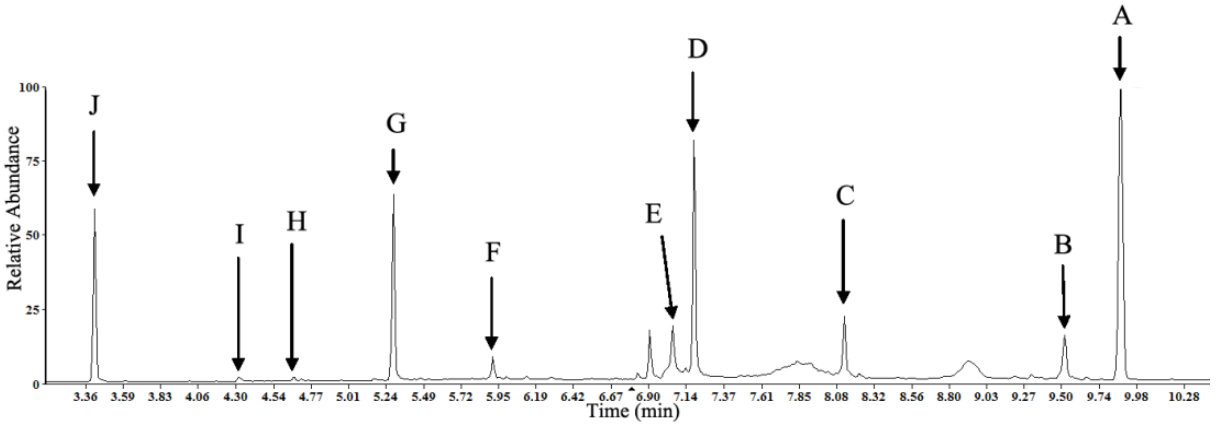

Figure 4. The GC-MS chromatograms of buprofezin and the metabolites appeared during buprofezin degradation by *Rhodococcus* sp. strain RX-3.Compound A: Buprofezin. Compound B: (((N' - (tert-butyl) – N - isopropylcarbamimidoyl) thio) methyl) (phenyl) carbamic acid. Compound C: 2 - (ethylimino) – 3 – isopropyl – 5 – phenyl - 1, 3, 5 – thiadiazinan – 4 - one. Compound D: 2 - (tert-butylimino) – 3 – isopropyl - 1, 3, 5 – thiadiazinan – 4 - one. Compound E: 2 – imino – 3 – isopropyl – 5– phenyl - 1, 3, 5 – thiadiazinan – 4 - one. Compound F: 2 – imino – 3 – methy l – 5 – phenyl - 1, 3, 5 – thiadiazinan – 4 - one. Compound G: formamidomethyl N – tert – butylmethanimidothioate. Compound H: (N - phenylformamido) methyl methanimidothioate. Compound I: N - (mercaptomethyl) – N - phenylformamide. Compound J: formamidomethyl N – isopropylmethanimidothioate.

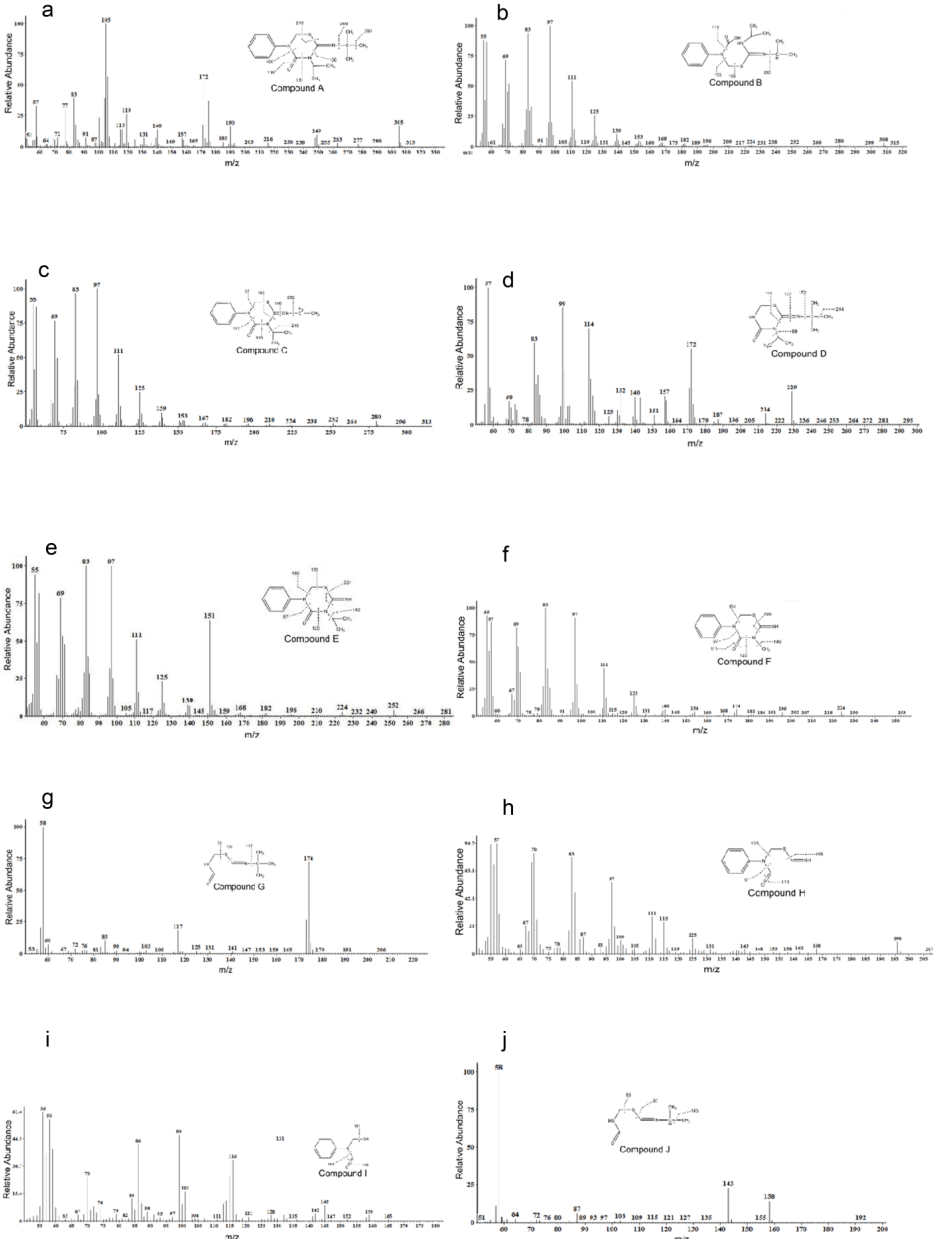

Figure 5. The characteristic fragment ions of buprofezin and buprofezin metabolites analyzed by GC-MS.

e-ISSN:2320-3528
p-ISSN:2347-2286

Table 1: A summary of corresponding chemical names, retention times (Rt) and characteristic ions identified by GC-MS.

Compound	Chemical name	Rt (min)	Characteristic ions in GC-MS (m/z)
A	Buprofezin	9.874	305, 290, 249, 216, 190, 131, 115, 105
B	(((N'-(tert-butyl)-N-isopropylcarbamimidoyl) thio) methyl)(phenyl)carbamic acid	9.526	308, 280, 196, 168, 153, 139,125, 111, 97, 83
C	2-(ethylimino)-3-isopropyl-5 -phenyl-1,3,5-thiadiazinan-4-one	8.136	280, 252, 210, 196, 182, 153, 139, 125, 111, 97, 83
D	2-(tert-butylimino)-3-isopropyl -1,3,5-thiadiazinan-4-one	7.189	229, 214, 172, 157, 114, 99, 83, 69
E	2-imino-3-isopropyl-5-phenyl -1,3,5-thiadiazinan-4-one	7.059	252, 224, 182, 151, 139, 125, 111, 97, 83
F	2-imino-3-methyl-5-phenyl -1,3,5-thiadiazinan-4-one	5.920	224, 196, 174, 154, 140, 125, 111, 97, 83
G	formamidomethyl N-tert -butylmethanimidothioate	5.295	174, 141, 117, 103, 85, 58
H	(N-phenylformamido)methyl methanimidothioate	4.660	196, 168, 143, 125, 111, 97, 83
I	N-(mercaptomethyl)-N-phenylformamide	4.313	165, 159, 145, 131, 116, 101, 99, 86, 70
J	formamidomethyl N- isopropylmethanimidothioate	3.409	158, 143, 87, 58

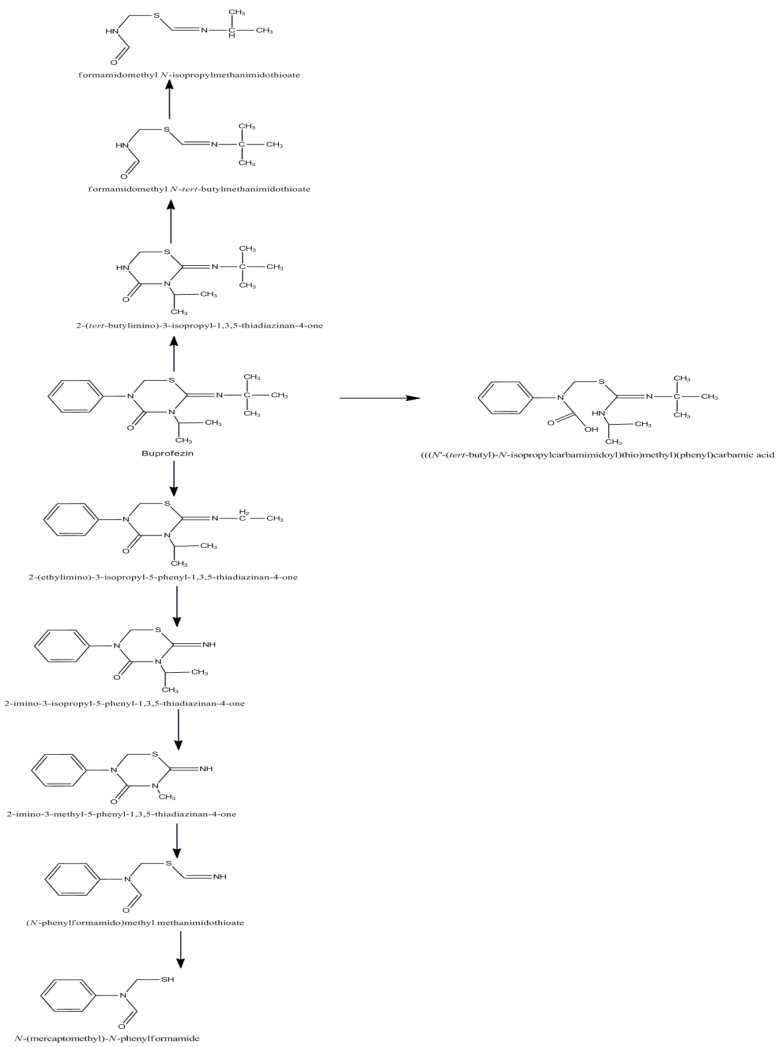

Figure 6. Proposed metabolic pathways for the degradation of buprofezin by *Rhodococcus* sp. strain RX-3. Compounds B, C, F, H, I, and J were first to be reported in this study to be possibly involved in the degradation of buprofezin.

The pathway of buprofezin metabolism in strain RX-3 was studied by metabolite identification and the proposed pathway is shown in **Figure 6**. During the buprofezin degradation process, a total of 9 metabolic compounds were produced. Except for compounds D, E and G, which were previously identified by Li et al. [4] and Chen et al. [5], the other compounds were newly identified in this degradation process. We proposed three possible metabolic pathways for buprofezin degradation **(Figure6).** In pathway A, the heterocyclic ring of buprofezin was opened by hydrolysis to form a new compound identified to be Compound B. In pathway B, the first step involved in the degradation of buprofezin was the sequential loss of N-tert-butyl and N-isopropyl to form compounds identified as C, E and F. Compound C was already reported previously [5], while Compounds C and F were identified to be new. The second step in this transformation pathway B is believed to result from a hydrolysis, followed by a redox reaction to form a novel structure N-(mercaptomethyl)–N-phenylformamide. Pathway C involved the removal first of the benzenic ring from buprofezin to generate compound D, similar to previous reports [4], and later transformed into formamidomethyl N–tert-butylmethanimidothioate also identified by Li et al. [4]. This process is followed by its conversion into a new structure formamidomethyl N-isopropylmethanimidothioate.

Thus, with regards to its observed relatively high degrading activities and ability to metabolize buprofezin, strain RX-3 is a good candidate to further study the mechanisms and its potential application in removing buprofezin from contaminated-soils.

CONCLUSIONS

A novel *Rhodococcus* sp. strain RX-3 utilized buprofezin as the sole carbon source was isolated and identified from buprofezin-treated soil. Strain RX-3 could degrade 98.01% of buprofezin in MM-1 within 80 h. Nine metabolites from buprofezin degradation were identified, and six new metabolites were first reported. The detailed study on the degradation of buprofezin by a single bacterial strain suggests that *Rhodococcus* sp. strain RX-3 could be a promising candidate for the remediation of environments contaminated with buprofezin.

ACKNOWLEDGEMENTS

This work was supported by grants from the Chinese National Natural Science Foundation (31100083), Foundation for Young Talents in College of Anhui Province, Natural Science Foundation of the Educational Commission of Anhui Province (KJ2015A049), Provincial Natural Science Foundation of Anhui (1508085MC49), Scholar Backbone Supporting Plan of Huaibei Normal University, and Construction Project from College Scientific Research Innovation Team of Anhui Province--Ecological Restoration and Utilization of Coal Mining Subsidence Area.

REFERENCES

1. Das C, et al. Effect of pH on the persistence behavior of the insecticide buprofezin in water under laboratory conditions. B. Environ Contam. Tox. 2004; 72: 307-311.

1. Izawa Y, et al. Inhibition of chitin biosynthesis by buprofezin analogs in relation to their activity controlling Nilaparvata lugens Stål. Pestic Biochem Phys. 1985; 24: 343-347.

2. Prabhaker N and Toscano N-C. Toxicity of the insect growth regulators, buprofezin and pyriproxyfen, to the glassy-winged sharpshooter, Homalodisca coagulata Say (Homoptera: Cicadellidae). Crop Prot. 26: 495-502.

3. Li C, et al. Biodegradation of buprofezin by *Rhodococcus* sp. strain YL-1 isolated from rice field soil. J. Agr Food Chem. 2012; 60: 2531-2537.

4. Chen K, et al. 2011. Isolation of a buprofezin co-metabolizing strain of *Pseudomonas* sp. DFS35-4 and identification of the buprofezin transformation pathway. Biodegradation. 2011; 22: 1135-1142.

5. Errami M, et al. Electrochemical degradation of buprofezin insecticide in aqueous solutions by anodic oxidation at boron-doped diamond electrode. Res Chem Intermediat. 2013; 39: 505-516.

6. Burrows H, et al.Reaction pathways and mechanisms of photodegradation of pesticides. J. Photoch Photobio. B.2002; 67: 71-108.

7. Li C, et al. Isolation, identification and characterization of a buprofezin-degrading bacterium BF 3. J. Environ Sci-China. 2011; 31: 965-970.

8. Gibson T and Gordon R. Bergey's manual of determinative bacteriology. By RE Buchanan and NE Gibbons, Williams and Wilkins Co. Baltimore. 1947; Md. 529-550.

9. Miller S, et al. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 1988; 16: 1215.

10. Sun Q, et al. Aerobic biodegradation characteristics and metabolic products of quinoline by a Pseudomonas strain. Bioresource Technol. 2009; 100: 5030-5036.

11. Patel V, et al. 2012. Phenanthrene degradation by Pseudoxanthomonas sp. DMVP2 isolated from hydrocarbon contaminated sediment of Amlakhadi canal, Gujarat, India. J. Hazard Mater. 201: 43-51.

12. Li R, et al. Biochemical degradation pathway of dimethoate by *Paracoccus* sp. Lgjj-3 isolated from treatment wastewater. Int Biodeter Biodegr. 2010; 64: 51-57.

13. Tamura K, et al. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 2013; 30: 2725-2729.

14. Wu Z, et al. Isolation of a heavy metal-resistant 4-Chloronitrobenzene degrader *Cupriavidus* sp. D4 and cloning of its cnb genes. Int Biodeter Biodegr.2011; 65: 871-876.

15. Homklin S, et al. 2012. Degradation of 17α-methyltestosterone by *Rhodococcus* sp. and *Nocardioides* sp. isolated from a masculinizing pond of Nile tilapia fry. J. Hazard Mater. 2012; 221: 35-44.

16. Zhu S-n, et al. Degradation of quinoline by *Rhodococcus* sp. QL2 isolated from activated sludge. J. Hazard Mater. 2008; 160: 289-294.

17. Khan F, et al. Aerobic degradation of 4-nitroaniline (4-NA) via novel degradation intermediates by *Rhodococcus* sp. strain FK48. J. Hazard Mater. 2013; 254: 72-78.

18. Yamamoto K and Nishimura M, et al. 2011. Identification and characterization of another 4-nitrophenol degradation gene cluster, nps, in *Rhodococcus* sp. strain PN1. J. Biosci Bioeng. 111(6): 687-694.

19. Kurisu F, et al. Degradation of natural estrogen and identification of the metabolites produced by soil isolates of *Rhodococcus* sp. and *Sphingomonas* sp. J. Biosci Bioeng. 2010; 109: 576-582.

20. Lee E-H and Cho K-S. Effect of substrate interaction on the degradation of methyl tert-butyl ether, benzene, toluene, ethylbenzene, and xylene by *Rhodococcus* sp. J. Hazard Mater. 2009; 167: 669-674.

21. Kuo CW and Genthner B. Effect of added heavy metal ions on biotransformation and biodegradation of 2-chlorophenol and 3-chlorobenzoate in anaerobic bacterial consortia. Appl Environ Microb. 1996; 62: 2317-2323.