Chapter 1

Isolation and Characterization of Cypermethrin Degrading Bacteria Screened from Contaminated Soil

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1. Introduction

A current environmental concern is the contamination of aquatic ecosystem due to pesticide discharges from manufacturing plant, agricultural runoff, leaching, accidental spills and other sources [1, 2]. Synthetic pyrethroid insecticides were introduced into widespread use for the control of insect pests and disease vectors more than three decades ago. In addition to their value in controlling agricultural pests, pyrethroids are at the forefront of efforts to combat malaria and other mosquito-borne diseases [3] and are also common ingredients of household insecticide and companion animal ectoparasite control products [4]. Cypermethrin is a type of synthetic pyrethroids (SPs), a class of pesticides widely used for insect control in both agricultural and urban settings around the world [5].

The use of SPs in China has increased sharply since many organophosphate products, such as methamidophos and parathion, are being phased out for agricultural use. With such extensive application, many adverse effects, such as pest resistance, residues in foods, and environmental contamination are public safety concerns [6, 7]. Although SPs are widely considered safe for humans, numerous studies have shown that exposure to very high concentrations of SPs might cause human health problems [8]. Such effects include bioaccumulation toxicity; immune suppression, endocrine disruption; modify electrical activity in various parts of the nervous system, neurotoxicity, lymph node and splenic damage, and carcinogenesis [9-11]. In addition, bees, fish, crabs, tadpoles, arthropods, and other non-target organisms are extremely sensitive to the toxic effects of SPs [12-15].

Cypermethrin is more effective against pests including moth pests of cotton, fruits and vegetable crops. Extensive and improper use of this kind chemicals leads to greater health risk to plants, animals and human population which had been reviewed time to time by several researchers [16]. One of the major problems asides from toxicity and carcinogenicity of pesticides is their long persistence in nature that amplifies the toxicity and health risk problems in the area of contamination [17].

Therefore, it is necessary to develop a rapid and efficient disposal process to eliminate or minimize the concentrations of SPs in the environment. A variety of physical and chemical methods are available to treat the soils contaminated with hazardous materials but many of these physical and chemical treatments do not actually destroy the hazardous compounds but are bound in a modified matrix or transferred from one phase to another [18, 19], hence biological transforming is essential. The biological treatment of chemically contaminated soil involves the transformation of complex or simple chemical compounds into non-hazardous forms [20]. For biodegradation, ideally the target pesticide will be able to serve as the sole carbon source and energy for microorganisms, including the synthesis of appropriate enzymes if need able. The specificity of enzymes active against xenobiotic compounds differs from one microorganism to another.

In the light of this fact, biodegradation, especially microbial degrading, has proven to be a suitable method for insecticide elimination. Previous studies indicated that microbes play important roles in degrading and detoxifying SPs residues in the environment. Thus far, many reports have described the biodegradation of cypermethrin by various bacteria, including *Ochrobactrum lupini*, *Pseudomonas* aeruginosa, *Streptomyces aureus*, and *Serratia* spp.[21-23], but there is few research describing biodegradation of pesticides by *Rhodobacter sphaeroides*. Among the different genera of pesticide-degrading bacteria, the photosynthetic (PSB) genus *Rhodobacter* has a special status in the ecosystem, since its metabolic functions are extraordinarily versatile, including degradation of various organic compounds, nitrogen fixation, hydrogen production [24], as a biofertilizer for promoting plant growth and increasing grain yield [25], and 5-aminolevulinic acid production which has multiple functions including a relatively strong herbicidal effect in clover [26]; therefore, microbes belonging to this genus are ideal choice for degrading pesticide residues.

The research aim was to identify the potential microbial strain able to utilize cypermethrin from the contaminated soil. In this study, the pesticide degrading potential of a bacterial culture is examined with the hope of isolation and characterization of cypermethrin degrading potentials in the contaminated soil. In addition, the optimum dose and the suitable conditions for cypermethrin degradation using laboratory scale were also evaluated. The results of the present study suggest that the use of potential microorganisms in the treatment system can successfully overcome many of the disadvantages associated with the conventional method used for the degradation of inhibitory compound.

2. Materials and methods

2.1. Chemicals and media

Standard analytical grade sample of 100 μg/mL cypermethrin (99.8% purity) was purchased from the Agro-Environmental Protection Institute, Ministry of Agriculture (Tianjin, China). Acetonitrile, methanol and hexane were of chromatographic grade while other chemicals were of analytical grade. Cypermethrin dissolved in acetone solution was added to desirable concentration in medium as the sole carbon source. Mineral Salts Medium (MSM) (g/L): 1.0 NH_4NO_3, 1.0 NaCl, 1.5 K_2HPO_4, 0.5 KH_2PO_4, 0.2 $MgSO_4$ $7H_2O$, pH 7.0. For solid plate, 1.5% (w/v) agar was added. Medium were sterilized by autoclaving at 121℃ for 30 min before use.

2.2. Enrichment, isolation and screening of bacterial strains

An activated sludge sample was collected from the wastewater treatment pool of a pesticide plant located in Changsha (Hunan, China), which had produced cypermetrin over 5 years. Wastewater sludge enrichment was performed by placing 10 g activated sludge in a 250 mL-Erlenmeyer flask containing 100 mL sterilized MSM media with an initial cypermethrin concentration at 20 mg/L, and incubated in a light incubator (PRX-450D, China) at 37℃ and 7500 lux; the flasks were shaken 3–5 times per day. After 10 days or so, the medium turned red-brown, a 5 mL aliquot of the culture was inoculated into 100 mL of fresh MSM medium containing 50 mg/L cypermetrin, and the new mixture was incubated for another 10 days under the same conditions. The medium was gradually acclimated to increasing concentrations of cypermetrin ranging from 50 to 200 mg/L at intervals of a week. After about 10 transfers, a mixed microbial population was diluted in series, and then streaked on MSM agar medium plate containing 100 mg/L cypermethrin. The dilution series was repeated at least 5 times, until single colony was achieved. The abilities of isolates to degrade cypermethrin were determined by gas chromatography (GC) according to Yin et al and Chen et al [23, 27]. The relatively higher degradation ability colonies were selected for further degrading studies. These organisms were stored long-term on porous beads in a cryopreservative fluid at -20℃ and short-term on agar plates at 4℃

2.3. Characterization and identification of the cypermethrin degrading isolates

A cypermethrin degrading isolate designated as $S_{10\text{-}1}$ showed the highest degradation rate was selected for further study. The purified $S_{10\text{-}1}$ was identified on the basis of its morphological characteristics and results of biochemical tests and 16S rRNA gene sequence analysis. The isolate $S_{10\text{-}1}$ was grown on MSM agar plates containing 50 mg/L cypermethrin at 37℃ and 7500 lux for 7 days, its cell morphology, method of reproduction, and the structure of its inner photosynthesis membrane and flagella were observed by transmission electron microscope (JEM-6360, JEOL) and/or scanning electron microscope (JSM-6360LV, JEOL).

The isolate $S_{10\text{-}1}$ was further confirmed by 16S rRNA gene sequence. The DNA was extracted and purified using the Qiagen genomic DNA buffer set. PCR amplification was performed as described by Mirnejad et al [28]. The 16S rRNA sequencing was performed by Beijing Liuhe

Huada Genomic Company (Beijing, China). The sequences with the highest 16S rDNA partial sequence similarity were selected and compared by CLUSTAL W. Phylogenetic and molecular evolutionary analyses were conducted by MEGA 4.0 software with the Kimura 2-paremeter model and the neighbor joining algorithm [29]. Confidence estimates of branching order were determined by bootstrap resampling analysis with 1000 replicates.

2.4. Inoculum preparation

Unless otherwise stated, the inoculants for this experiment were bacteria cultured in a 130 mL serum bottle containing 120 mL of PSB medium in a light incubator at 35℃ and 7500 lux. At the exponential phase (about 2–3 days), the cell pellets were harvested via centrifugation (5000×g, 10 min), washed 3 times with 50 mL of KH_2PO_4-K_2HPO_4 (0.15 mol/L, pH 7.0), and then suspended in the same phosphate buffer as the inoculants. In order to avoid the effects of hydrolysis and photolysis, each treatment was set in triplicate with non-inoculated samples as control under the same conditions and analyzed in the same manner. Samples for residual pesticide concentration analysis were collected from the cultures at regular intervals.

2.5. Optimal conditions for degrading cypermethrin by $S_{10\text{-}1}$

To determine the optimal conditions for degrading cypermethrin by $S_{10\text{-}1}$, single-factor test was designed in this study under different conditions. To confirm the effects of temperature on degradation, the media were placed in illuminating incubators at 10, 20, 25, 30, 35, and 40℃, respectively. To determine the effect of cypermethrin concentration on degradation, MSM media were added with cypermethrin ranging in concentration from 100 mg/L to 800 mg/L. The media were prepared at pH values from 4.0 to 11.0 buffers for the measurement of the effects of pH on degradation. All experiments were conducted in triplicate. The non-inoculated controls throughout the studied were implemented at the same condition in order to exclude the abiotic degradation affection.

2.6. Extraction of cypermethrin for residue analysis

The extraction and quantification of cypermethrin residue in the media was modified slightly from method described in Yin et al [27] and Liu et al [30]. At different time intervals, triplicate populations were sampled for cypermethrin concentration analysis. Cypermethrin was extracted three times from the media with 100 mL of hexane. The hexane extracts from the same samples were combined, dried with anhydrous sodium sulfate, and concentrated by exposure to nitrogen gas to near dryness on a rotary evaporator at room temperature, and then dissolved in 5 mL of hexane for GC detection. Before detection the residues were purified using hexane pre-poured Florisil® columns (Agilent SAMPLIQ Florisil®, USA) and 0.22 μm membranes (Millipore, USA), and were then recovered in 5 mL of hexane; finally, the residues were analyzed by performing GC. Preliminary experiments showed that the recovery of cypermethrin in the above extraction and analysis procedures was >90%.

Residue analyses of cypermethrin degradation were performed using an Agilent 6890N GC system (Agilent Technologies, USA) equipped with an electron capture detector (μ-ECD); an

HP-5 5% phenyl methyl siloxane capillary column (30 m × 320 μm × 0.25 μm; Agilent Technologies, USA) was used for separation, with helium as the carrier gas (flow rate, 1 mL/min). Other GC parameters included an inlet temperature of 250℃ and a detector temperature of 300℃; initially, the oven temperature was 150℃ for 2.0 min, was ramped to 280℃ at 15℃/min, and then maintained at 280℃ for 5.0 min. The injection volume was 1.0 μL. Samples were introduced in split-less mode. Concentrations were determined by analyzing peak area with an authentic cypermethrin standard.

2.7. Detection of cypermethrin metabolites

Metabolites were isolated from the culture filtrates of the organism grown in cypermethrin (100 mg/L) by extraction with acetonitrile, before and after acidification to pH 2 with 2 M HCl, and the residue obtained was dissolved in hexane [22]. The metabolites were identified and analyzed using the GC/MS system (Agilent 7890A/5975, Agilent Technologies, USA) equipped with electron ionization (EI). EI (70 eV) was performed with a trap current of 100 mA and a source temperature of 200℃. Full scan spectra were acquired at m/z 45–500 at 2 sec per scan. The metabolites were confirmed by standard MS, data collection and processing were performed using Agilent MSD ChemStation software containing the Agilent chemical library.

3. Results and discussion

3.1. Isolation and characterization of cypermethrin degrading bacterium

After repeated enrichment and purification processes, we obtained approximately 20 strains of organisms with different colony morphologies from the activated sludge samples. But the degradation experiments showed the isolate $S_{10\text{-}1}$ possessed the relatively higher degradation, capacity of degrading cypermethrin (100 mg/L) by 90.4% after incubating 7 days at pH 7.0 and temperature 35℃ (Fig. 3a). And $S_{10\text{-}1}$ utilized cypermethrin as its sole carbon and energy source in MSM. Thus strain $S_{10\text{-}1}$ was selected for further detail investigation.

$S_{10\text{-}1}$ is a gram-negative, anaerobic bacterium. The morphology of the $S_{10\text{-}1}$ colonies, cultured for 10 days on MSM agar plate, were reddish-brown, smooth, circular, wet, nontransparent, glistening, and with entire margins (Figure 1a). The physiological and biochemical characteristics of $S_{10\text{-}1}$ are shown in Table 1. SEM observations showed that the cells are ovoid to rod shaped (Figure 1b), sometimes even longer, measuring about 0.5–0.9 μm in width and 1.2–2.0 μm in length, and are motile by means of polar flagella (Figure 1c). Internal photosynthetic membranes appear as lamellae underlying and parallel to the cytoplasmic membrane (Figure 1d). The culture suspension was reddish-brown in color. In vivo absorption maxima of intact cells (Figure 1e) were recorded at 378, 455, 480, 510, 592, 806, and 865 nm, indicating the presence of bacteriochlorophyll a and carotenoids of the spheroidene series [31]. These morphological and biochemical properties are identical to the genus *Rhodobacter* [31].

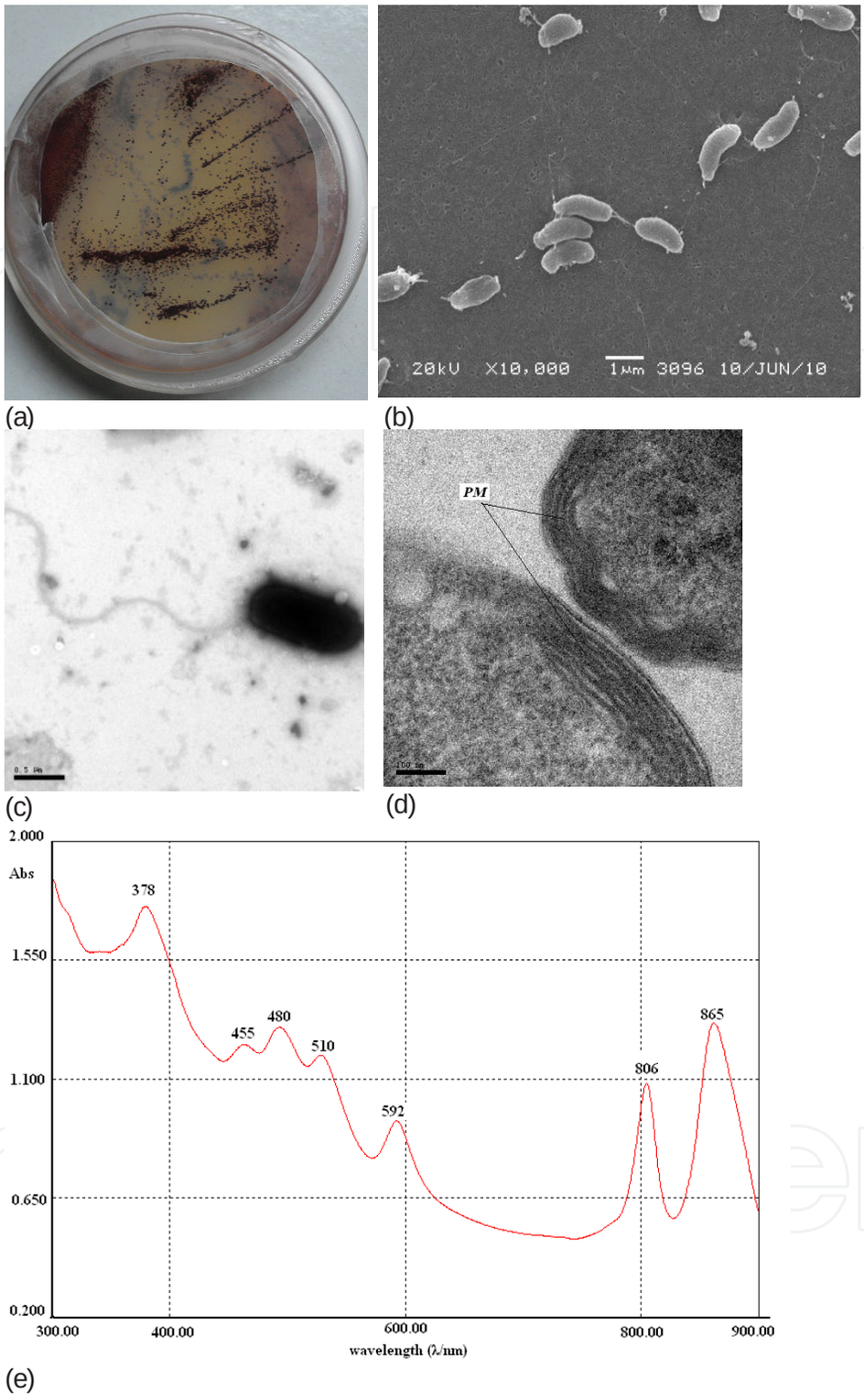

Figure 1. The characterization of strain $S_{10\text{-}1}$. (a) The morphology of the $S_{10\text{-}1}$ colonies, cultured for 10 days on MSM agar plate; (b) Scanning electron micrograph of strain $S_{10\text{-}1}$ (10,000×); (c) Electron micrograph of negatively stained $S_{10\text{-}1}$ cells showing polar flagella (40,000×); (d) Transmission electron micrograph of $S_{10\text{-}1}$: a cross-section showing the photosynthetic membrane (*PM*) lying parallel to the cytoplasmic membrane (200,000×); (e) Absorption spectra of living $S_{10\text{-}1}$ cells.

Items	Results	Items	Results	Items	Results
Gram stain	-[a]	3% NaCl	-	Aerobic dark growth	+
Motility	+[b]	M. R reaction	-	Succinate utilization	+
Hydrogen sulfide	+	Citrate utilization	+	Mannitol utilization	-
V–P reaction	-	Acid from carbohydrates	-	Glycerol utilization	+
Gelatin liquefaction	+	Indole production	-	Pyruvate utilization	+
Catalase	+	Urease	-	Benzoate utilization	-
Oxidase	+	Pigment production	+	Ammonia utilization	+
Strach hydrolysis	+	Nitrate reduction	+	Tartrate	-

Note: [a] Negative/Substrate not utilized; [b] Positive/Substrate utilized.

Abbreviation: VP-Vogues Proskauer; MR-Methyl Red.

Table 1. Physiological and biochemical characteristics of S_{10-1}

3.2. Phylogenetic analysis and identification of S_{10-1}

A 1380-bp 16S rRNA fragment was amplified from the genomic DNA of S_{10-1} and sequenced (Genebank Accession NO. HM193898). Phylogenetic analysis of 16S rRNA revealed that S_{10-1} belonged to the genus *Rhodobacter sphaeroides* (Figure 2). S_{10-1} was temporarily identified as *R. sphaeroides* according to its morphology, colony and cultural properties, physiological and biochemical characteristics, absorption spectra (living cells), internal photosynthetic membrane, and phylogenetic analysis.

Microbial belong to the genus *Rhodobacter*, which are known to play a major role in the treatment of organic wastewater, since they can utilize a broad range of organic compounds as carbon and energy sources; moreover, they are ubiquitous in fresh water, soil, wastewater, and activated sludge. Thus they have been selected for the treatment of many types of wastes [32-34], while *R. sphaeroides* appears to be a new bacterium that may participate in efficient degradation of cypermethrin. To our knowledge, there is not any information concerning the ability of *R. sphaeroides* to degrade cypermethrin and other SPs. However, reports showed that *R. sphaeroides* could effectively degrade pesticides including 2,4-d, quinalphos, monocrotophos, captan and carbendazim [35].

3.3. Effect of temperature on cypermethrin degradation in MSM

Cypermethrin was degraded by S_{10-1} during incubation temperatures ranging from 10℃ to 40℃. The cypermethrin residues were detected after 7 days' treatment. In cultures incubated at 10℃ and 20℃, the results show that the degradation rate were 15.3% and 23.8%. However, in cultures incubated at higher temperature, i.e. 25℃, 30℃ and 35℃, the degradation rate reached 70.4%, 87.4% and 90.4% within 7 days, respectively, but the degradation rate was only

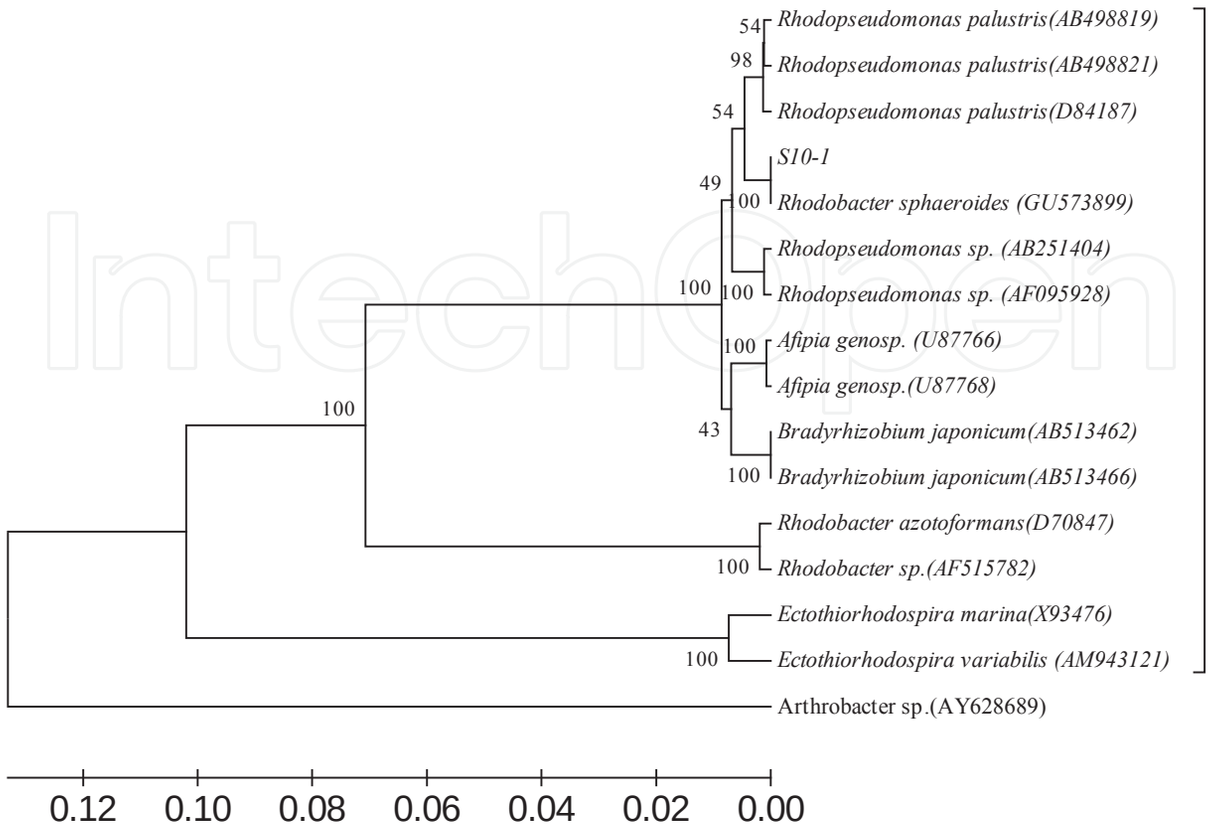

Figure 2. Phylogenetic tree constructed by the neighbor-joining method based on 16S rDNA sequences of $S_{10\text{-}1}$ and related strains. Bootstrap values are given at branching points. The sequence of *Arthrobacter* spp. (AY628689) was selected as an out group. The tree was constructed using the neighbor-joining method. Bootstrap values at nodes were calculated using 1,000 replicates (only values >70% are indicated). The GeneBank accession numbers for 16S rRNA gene sequences are shown in parentheses.

61% when incubated at temperature 40℃ for 7 days. The best temperature for degradation was 35℃ (Figure 3a). Similar results were reported by Lin et al [36] who reported temperature significantly influenced cypermethrin degradation by *Streptomyces* sp. strain HU-S-01. Our results also reveal that cypermethrin degradation occurred at 30–35℃ indicating strain $S_{10\text{-}1}$ preferred relatively high temperature condition. These results were consistent with previous findings of Chen et al [21]. It is possible that some key enzyme(s) responsible for cypermethrin degradation have their optimum enzymatic activity over such range of temperature. In non-inoculated controls at different temperatures, abiotic degradation was negligible throughout the studies.

3.4. Effect of initial concentration on cypermethrin degradation in MSM

Cypermethrin degradation at different initial concentrations by strain $S_{10\text{-}1}$ was investigated. The cypermethrin degradation rates were found to be 90.4%, 60.3%, 38.4%, 32.3%, and 28.7% at concentrations of 100, 200, 400, 600, and 800 mg/L, respectively (Figure 3b). At low cypermethrin concentration ranging from 100 to 200 mg/L, the degradation rate reached above 60%

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within 7 days. However at high concentration (400 to 800 mg/L), only about 30% was degraded within 7 days. It might be because of the fact that microbial degradation starts slowly and requires an acclimation period before rapid degradation occurs at high concentration. Similar results were reported by Lin et al [36] who reported that initial concentration of carfofuran was significantly efficiently degraded by *Pichia anomala* strain HQ-C-01 in contaminated soils. In non-inoculated controls at different initial concentrations, abiotic degradation was negligible throughout the studies.

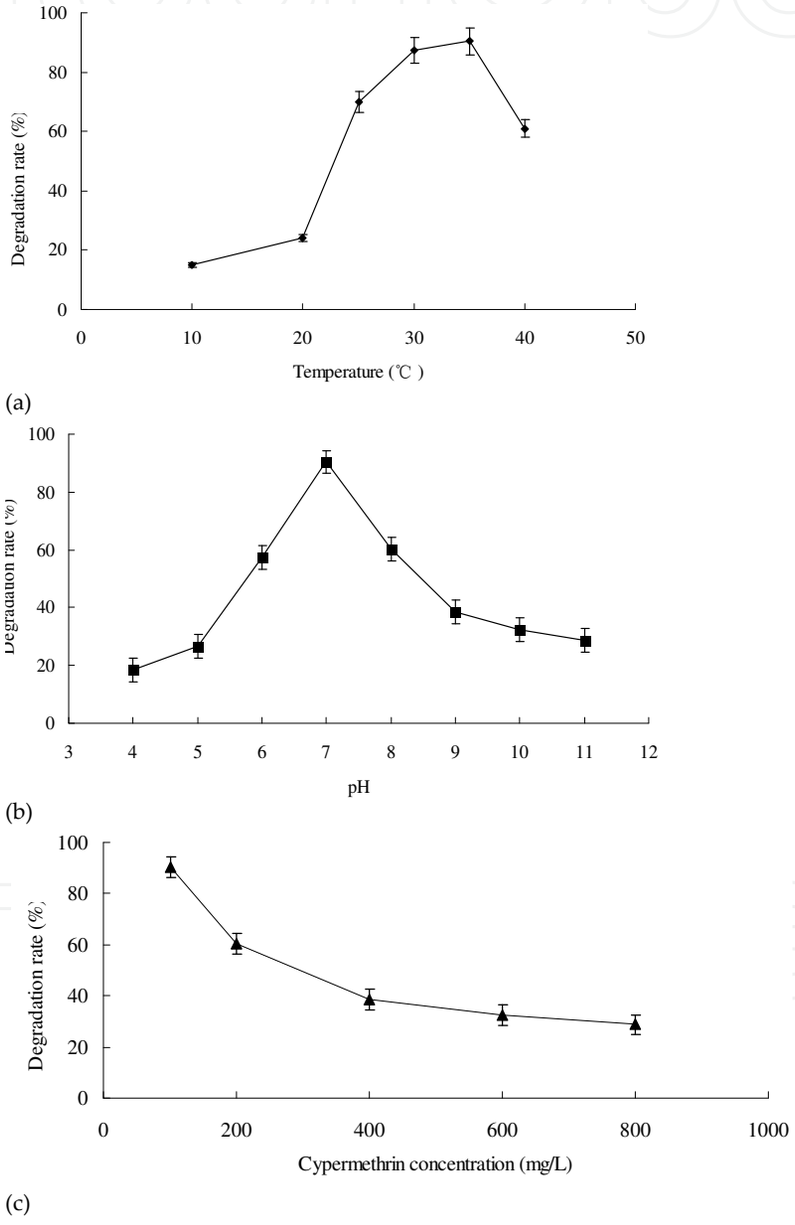

Figure 3. Optimal conditions for degrading cypermethrin by $S_{10\text{-}1}$. (a) Effect of temperature on the degradation of cypermethrin by $S_{10\text{-}1}$; (b) Effect of the initial cypermethrin concentration on the degradation by $S_{10\text{-}1}$; (c) (d) Effect of pH on the degradation of cypermethrin by $S_{10\text{-}1}$. Error bars represent standard deviation (SD) from the mean. Error bars smaller than symbols are not depicted.

3.5. Effect of pH on cypermethrin degradation in MSM

The pH is also an important factor, which significantly effects the degrading ability of bacteria capable of degrading toxicities [37, 38]. To determine the effect of pH on degradation, MSM medium prepared with different pH buffers, fortified with 100 mg/L cypermethrin, and incubated at 35℃ and 7500 lux. Eight different pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0) were tested in the optimization experiment. The result showed that the degradation rate were 18.5%, 26.7%, 57.5%, 90.4%, 60.3%, 38.4%, 32.3%, 28.7%, respectively (Figure 3c). The optimal initial pH value for degradation was between 6.0 and 8.0. Results revealed that $S_{10\text{-}1}$ was able to degrade cypermethrin over a wide range of pH. Similar results were reported by Zhang et al [36] who reported that initial pHs were significantly efficiently degraded by two *Serratia* spp., and rapid degradation of cypermethrin at high pH while it was relatively low at acidic pH. In non-inoculated controls at different pH conditions, abiotic degradation was negligible throughout the studies.

3.6. Identification of cypermethrin degradation metabolites

The degradation metabolites of cypermethrin by strain $S_{10\text{-}1}$ were extracted and identified by GC/MS using Agilent MSD ChemStation software containing the Agilent chemical library. GC/MS analysis of the metabolites showed the presence of 4 products. These compounds corresponded with cyclopropanemethanol (Figure 4a), 5-methoxy-2-nitrobenzoic acid (Figure 4b), 3,5-dimethoxybenzamide (Figure 4c), and 5-aminoisophthalic acid (Figure 4d). The retention times of these compounds were 13.609, 14.874, 16.980, and 17.323 min, respectively.

Previous studies had reported about the biodegradation pathway of SPs [21, 39, 40]. In the molecular structure of SPs there is an ester bond which is not as firm as other chemical bonds. Literature indicated that the first step in the microbial degradation and detoxification of SPs is the hydrolysis of its carboxyl ester linkage [23, 36, 41]. However, the chemical bond broken of cypermethrin metabolites are not detected as that described in a previous study. It is evident from our GC/MS results that $S_{10\text{-}1}$ degraded cypermethrin by reductive dechlorination, oxidation or/and hydrolysis to transform to other 4 metabolites. The cypermethrin degradation pathway appeared to be different to the initial steps of SPs degradation by *Ochrobactrum lupine*, *Pseudomonas aeruginosa*, *Pseudomonas aeruginosa* and *Achromobacter* sp. [21, 22, 42, 43]. Moreover, no 3-phenoxybenzoic acid (3-PBA) was detected in the metabolites by GC-MS after 7 days of treatment, while 3-PBA was generally regarded as the major metabolite after hydrolysis of SPs in soil and water [21, 36, 42-44]. Owing to its antimicrobial activities [23, 45] and transient GC/MS detectable peak [21, 45], biodegradation of 3-PBA was rarely reported. Chen et al reported that fenvalerate was degraded by hydrolysis of the carboxylester linkage to yield 3-PBA, and then the intermediate was further utilized for bacterial growth by strain ZS-S-01, finally resulted in complete mineralization [42]. So, we speculated that carboxylesterases and oxidoreductases involved in degradation of cypermethrin by strain $S_{10\text{-}1}$, that needed to be testified by further experiments.

On the other hand, *R. sphaeroides* are metabolically flexible and under different situations they can grow chemoheterotrophically, chemoautotrophically, photoheterotrophically, and photoautotrophically [46]. Because of this multiplicity of growth modes there has been

considerable interest in studying of degrading toxic compounds [35, 47]. The structural components of this metabolically diverse organism and their modes of integrated regulation are encoded by a genome of ~4.5 Mb in size [46]. Moreover, its large inventory of transport and chemotaxis genes also implies that *Rhodobacter* is adept at sensing and acquiring diverse compounds from its environment [48-50].

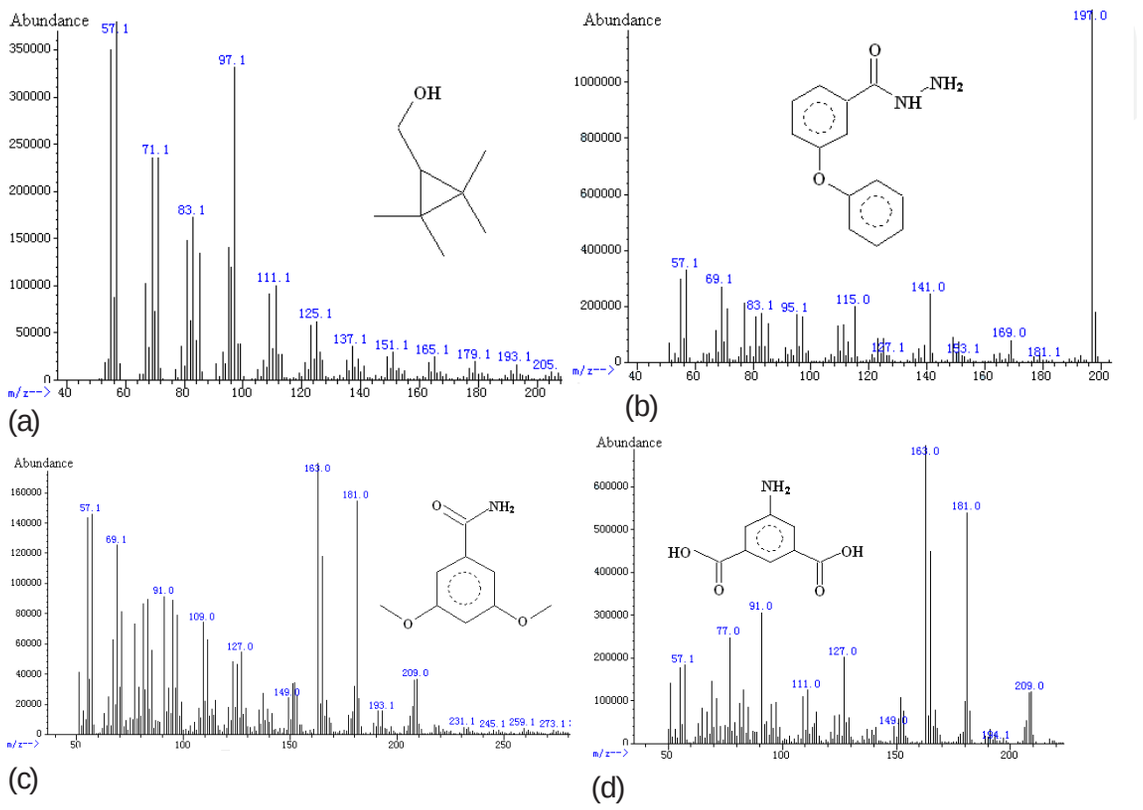

Figure 4. GC/MS spectra of four main metabolites produced during cypermethrin degradation by strain $S_{10\text{-}1}$. (a) cyclopropanemethanol; (b) 5-methoxy-2-nitrobenzoic acid; (c) 3,5-dimethoxybenzamide; (d) 5-aminoisophthalic acid.

4. Conclusion

R. sphaeroides strain $S_{10\text{-}1}$ was isolated from an activated sludge sample collected from the wastewater treatment pool of a pesticide plant. It can utilize cypermethrin as sole source of carbon, nitrogen and energy. The optimal temperature and pH for biodegradation of cypermethrin by strain $S_{10\text{-}1}$ were 35℃and pH 7.0, and the degradation rate reached 90.4% within 7 days under the optimal conditions. Four metabolic compounds were detected, hinting that there are complex redox reactions are involved in the cypermethrin degradation process.

In conclusion, our results indicated that strain $S_{10\text{-}1}$ could be a good choice for the bioremediation of cypermethrin contaminated water and soil. However, further studies such as its

interactions with environment, toxicological aspects, degradation enzymes, biochemical and genetic aspects are still needed before the application in actual field-scale bioremediation.

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