Nematicidal activity of volatile organic compounds produced by Bacillus altitudinis AMCC 1040 against Meloidogyne incognita

Bo Zhou (zhoubo2798@163.com)
Shandong Agricultural University
Lin Ye
Ningxia University
Jian-Yu Wang
Shandong Agricultural University
Xiao-Fang Liu
Shandong Agricultural University
Qi Guan
Shandong Agricultural University
Nong-Xiao Dou
Shandong Agricultural University
Jian Li
Jimei University
Qian Zhang
Shandong Institute of Pomology
Yan-Ming Gao
Ningxia University
Min Wang
Shandong Wanhao Fertilizer Co., LTD
Jian-She Li
Ningxia University

Research Article

Keywords: Root-knot nematodes, Biological control, Bacillus altitudinis, Volatile organic compounds

Posted Date: April 1st, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1500056/v1

Abstract

The application of nematicidal microorganisms and their virulence factors provides more opportunities to control root-knot nematodes. *Bacillus altitudinis* AMCC 1040, previously isolated from suppressive soils, showed significant nematicidal activity, and in this study, nematicidal substances produced by *Bacillus altitudinis* AMCC 1040 were investigated. The results of the basic properties of active substances showed that these compounds have good thermal stability and passage, are resistant to acidic environment and sensitive to alkaline conditions. Further analysis showed that it is a volatile component. Using HS-SPME-GC/MS, the volatile compounds produced by *Bacillus altitudinis* AMCC 1040 were identified and grouped into four major categories: ethers, alcohols, ketone, and organic acids, comprising a total of eight molecules. Six of them possess nematicidal activities, including 2,3-butanedione, acetic acid, 2-isopropoxy ethylamine, 3-methylbutyric acid, 2-methylbutyric acid and octanoic acid. Our results further our understanding of the effects of *Bacillus altitudinis* and its nematicidal metabolites on the management of *Meloidogyne incognita* and may help to find less toxic nematicides for root knot nematodes control.

1. Introduction

Plant parasitic nematodes (PPNs) are among the most important pests worldwide, with economic losses caused by PPNs estimated at $157 billion per year, and this figure may be a significant underestimate because many growers, especially in developing countries, are unaware of plant parasitic nematodes (Abad et al. 2008; Li et al. 2015). To date, more than 4,100 species of PPNs have been described, and among the species, root-knot nematodes (RKNs) belonging to the genus *Meloidogyne* are the most destructive, ranking first among the top 10 plant-parasitic nematodes (Jones et al. 2013; Coyne et al. 2018). Chemical nematicides have been the primary means of controlling RKNs for decades and are unlikely to change in the near future (Chen et al. 2019). However, most nematicides are gradually being banned or severely restricted due to their notorious toxicity for environmental and human health reasons (Collange et al. 2011). Therefore, it has become increasingly important to seek efficient and environmentally friendly alternatives to control RKNs (Li et al. 2015).

Biological control has shown good promise as an eco-friendly approach to reduce nematode damage (Cheng et al. 2017). In recent years, the use of microorganisms and their bioactive metabolites for plant diseases management has received more attention (Kaur et al. 2016). Bacteria are the most abundant organisms in the soil and as natural enemies of RKNs, several species, such as *Pasteuria*, *Pseudomonas* and *Bacillus* have been widely described, which affect nematodes through different mechanisms of action, such as: competition for nutrients, parasitism, and interference with nematode–plant-host recognition (Tian et al. 2007). However, as a whole, the production of virulence factors plays a crucial role in the expression of biocontrol activity of bacteria, and therefore, understanding those secondary metabolites of nematicides is essential for the development of new generations of pesticides (Marieta and Susan 2019). The different nematicidal compounds emitted by bacteria can be divided into two categories: enzymes and secondary metabolites (Castaneda-Alvarez and Aballay 2016). In addition,

many microbial secondary metabolites show high potential as commercial agents due to their safety and high nematicidal activity (Li et al. 2012).

In our two previous studies, we have evaluated the biocontrol potential of *Bacillus altitudinis* AMCC1040 in *in vitro* experiments, pot experiments, and field experiments (Wang et al. 2021a, b). The objectives of our study were: (1) to clarify the basic properties of the active substances; (2) to identify nematicidal VOCs by using headspace solid-phase micro-extraction coupled with gas chromatography–mass spectrum (HS-SPME–GC/MS); (3) to determine the nematicidal activity of VOCs by in vitro experiment using pure commercial compounds.

2. Materials And Methods

2.1 Cultures

The bacteria strain was isolated in October 2017 from a suppressive soil sample in Shandong province, China, and subsequently stored at the Shandong Agricultural Microbiology Collection Center (AMCC) under the registration number AMCC 1040 (Wang et al. 2021b).

Representative specimens of *Meloidogyne incognita* were used as targets for nematicidal activity bioassays. Egg masses of *Meloidogyne incognita* were collected from infected tomato roots after 30 days of incubation in a greenhouse at Shandong Agricultural University. Before use, they were surface sterilized with 1.5% NaClO solution, washed with distilled water, and then transferred to Baermann funnel at 25℃ to obtain second-stage juveniles (J2s) (Eloh et al. 2015).

2.2 Nematicidal activity bioassay

The bioassay of nematicidal has been described in our previous studies (Wang et al. 2021b). In summary, 100–150 *Meloidogyne incognita* J2s were mixed with in 0.4 mL of distilled H_2O with 0.1 mL of the culture supernatant in 24 microporous plates and kept in dark at 28°C. To prepare a cell-free culture supernatant, cultures were centrifuged at 12,000 rpm for 5 min at 4°C and then filtered through 0.22 μm Millipore filters. BPY medium (Beef extract 5.0 g, Peptone 10.0 g, Yeast extract 5.0 g, Glucose 10.0 g, NaCl 5.0 g, ddH_2O 1000 mL, pH 7.0) was used as a negative control. The nematodes were considered dead if their bodies were linear or insensitive to mechanical contact. The experiment was performed twice with three replicates per each treatment. The corrected mortality rates were calculated using the following formula: Mortality (%) = [(mortality percentage in treatment − mortality percentage in untreated control) / (100 − mortality percentage in untreated control)] × 100 (Bi et al. 2018).

2.3 Partial Characterization of Active Metabolites

A good understanding of the basic properties of nematicidal substances can be of great help in developing more effective analytical strategies. The active substances were characterized in terms of: dynamic nematicidal activity bioassay, thermal and pH stability, solubility, generation stability,

adsorption/ion exchange properties and volatility. All the experiments were performed twice with three replicates per treatment.

2.3.1 Dynamic nematicidal activity bioassay

Two experiments were designed to assess the dynamic nematicidal activity: (1) The virulence capacity varied with the time of incubation of *Bacillus altitudinis*. The incubation process was divided into two stages. To prepare the seed culture, the strains were activated on solid BPY medium at 37°C for about 18 h. Individual colonies were then selected and inoculated into a 250 mL erlenmeyer flask containing 50 mL of liquid BPY medium, followed by incubation at 37℃ for 12 h with constant shaking at 200 rpm. In the second stage, 100 mL of BPY medium was placed into a 500 mL Erlenmeyer flask and inoculated with 1 mL seed culture. The culture conditions were the same as described above, and samples were taken every two hours for bioassay of nematicidal activity until juvenile mortality were stable at 100%. (2) Minimum time to reach maximum juvenile mortality. In this section, mortality of J2 was counted every 10 min using 48 h of fermentation broth.

2.3.2 Heat, pH and generation stability

To determine thermal stability, the culture supernatant was heated at different temperatures (50, 60, 70, 80, 90, 100, 120℃) for 30 mins and subsequently tested for the nematicidal activity as its temperature dropped to ambient.

The culture supernatant was adjusted to pH 2 to 12 and subsequently incubated at 28°C for 30 min, then the pH was adjusted back to its original value (approximately 5.06) and tested for nematicidal activity.

In another experiment, a single colony was incubated continuously at monthly intervals for a total of 10 times, and each generation was tested for nematicidal activity to determine the stability of generation.

2.3.3 Solubility

To determine the solubility of the active metabolites, the culture filtrate was fully extracted with organic solvents including n-butanol, ethyl acetate, cyclohexane, chloroform. The organic solvents were mixed fermentation supernatant liquid (100 mL) in equal proportion, transferred to a separatory funnel, and left for 12 h. The organic and aqueous phase were separated, and the organic phase was combined after repeated extraction three times (300 mL in total). The residual aqueous phase was removed by adding an appropriate amount of anhydrous Na_2SO_4 to the organic phase, and the organic extracts were dried over anhydrous sodium sulfate and then centrifuged at 100 rpm, 40℃, -0.1 Mpa on a rotary vacuum evaporator. The resulting yellow residue was dissolved in 5 mL methanol and transferred to a 10 mL glass bottle then placed in a vacuum desiccator at -0.1 Mpa. After complete removal of methanol, the gummy mass were redissolved with 0.5% DMSO and subsequently tested for nematicidal activity using 0.5% DMSO as negative control (Sharma et al. 2014).

2.3.4 Adsorption/ion exchange properties

Four amberlite resins were used in the adsorption/ ion exchange experiments, including XAD 16, XAD 1180, FPC 3500, and FPC 23H. The resins were soaked in equal volume of methanol for 24 h according to the manufacturer's requirements and then loaded onto a column (the bed volume was about 100 mL, 30 cm × Φ 2 cm). The column was rinsed thoroughly with distilled water until the eluate was clear and free of methanol order. The column was then soaked in 5% NaOH for 6 h and rinsed with distilled water until neutral, finally, soaked in 5% HCl for 6 h, rinsed with distilled water until neutral (Wang et al. 2019). 200 mL culture supernatant was passed through the column at a flow rate of 0.5 BV/h, followed by elution with 200 mL of water. In the next sections, the column was eluted with 200 mL of 50% methanol and 100% methanol sequentially for the adsorbent resin and 200 mL of 0.1 mol/L and 0.5 mol/L HCl sequentially for the ion exchange resin with absolute desalting. The collection rate was 50 mL/tube, 16 fractions in total in each resin. The flow-through fluid of XAD 16, XAD 1180 were tested for the nematicidal activity directly and the methanol part was treated in the same way as described above. Regarding FPC 3500 and FPC 23H, the pH was adjusted back to the original value (4.71–5.06) and tested for nematicidal activity.

2.3.5 Volatility

Further freeze-drying experiments were used to investigate the volatility of substances. 100 mL of culture supernatant was completely pre-frozen at -80℃ for 12 h, and subsequently freeze-dried through lyophilization to obtain yellow viscous solid. The yellow viscous solid was redissolved with 100 mL ddH_2O and subsequently tested for nematicidal activity. In another experiment, 100 mL of culture supernatant was evaporated on a rotary vacuum evaporator at 100 rpm, 60℃, -0.1 Mpa, and the yellow solid in the distillation flask was re-dissolved with 100 mL ddH_2O, while the liquid in the condensation flask was collected and then tested nematicidal activity separately.

2.4 Analysis of VOCs by using HS-SPME-GC/MS

Bacillus altitudinis AMCC 1040 was cultured for 48 h. Culture supernatants were prepared as described above, and volatiles were collected and analyzed via HS-SPME-GC/MS (Gu et al. 2007). The fiber (50/30 μm DVB/CAR/PDMS) used for headspace solid-phase microextraction was first preconditioned at 250°C for 30 min (Murungi et al. 2018; Estupiñan-López et al. 2018). To adsorb the volatiles, the pre-cleaned fiber was inserted into a 50 mL headspace vials containing 25 mL of fermentation broth and soaked at 95℃ for 1 hr. Subsequently, the fiber was manually inserted into the injector port and desorbed at 250°C for 5 min (Zhai et al. 2018). A GC–MS QP 2010 Ultra (Shimadzu, Japan) gas chromatograph–mass spectrometer, equipped with Rtx-5MS (60m×0.32mm×0.25μm) column was used for chromatographic separation. The injector operated in splitless mode. The injector, interphase and ion detector temperatures was 230℃, 250℃, and 220℃, respectively. Grade 5.0 helium was used as the carrier gas at a flow rate of 3 mL/min. The column was held at 50℃ for 2 min, then ramped up to 120℃ at 2°C/min and finally to 200℃ at 4°C/min and held for 1 min. The EI ion source was acquired at 70 eV with an acquisition range of m/z 35 and 500. according to the mass spectrometry and database NIST 08, NIST 08s and mass spectra from the FNSC 1.3 library were compared to determine the identification of VOCs.

2.5 Nematicidal activity of VOCs produced by *Bacillus altitudinis* AMCC1040

The nematicidal activity of 2,3-butanedione, acetic acid, acetoin, 2-isopropoxy ethylamine, 2,3-butanediol, 3-methyl-butanoic acid, 2-methyl-butanoic acid and octanoic acid was evaluated for mortality of J2s. All reagents were analytical pure and purchased from Aladdin. Since octanoic acid is slightly soluble in water, a master solution of 0.2 µL/mL was prepared by adding 2 µL of octanoic acid (Aladdin, 99% purity) to 9998 µL distilled water. Stock solutions (1 µL/mL) of other compounds were prepared in sterile distilled water. The solutions should be prepared at the time of use and should not be left for too long after preparation. In vitro experiments were performed as described in 2.2. The working concentration range of each compound is from 0.01 µL/mL to 1 µL/mL to detect the $LC_{100/12h}$ of each compound.

2.6 Statistical Analysis

All statistical analyses were performed using analysis of variance (ANOVA) with the SAS statistical software. Pie charts and bar charts were made by GraphPad Prism 6.

3. Results

3.1 Partial Characterization of Active Metabolites

The results for the 120 min time point (Fig. 1a) revealed that the mortality rate of J2s caused by the culture supernatant was already close to 100% within 90 minutes, indicating that the substances produced by *Bacillus altitudinis* AMCC 1040 were effective in killing nematodes. Further experiments on the variation of virulence capacity with increasing *Bacillus* incubation time showed that the mortality of J2s increased linearly from 8 h to 12 h, which was opposite to the trend of the change of pH. Interestingly, the two curves were approximately symmetrical with a central point at 10 h, from which the pH decreased to about 5.0, while the mortality of J2s increased from 33.71–91.53% within two hours (Fig. 1b).

The nematicidal effect of the acid treatment did not change, killing more than 90% of J2s at the pH 2.0 to 6.0, but at pH 8.0, the alkali treatment started to decrease (56.66%) and almost disappeared at pH 12.0 (25.31%) (Fig. 2a). The next experiments showed that the substance was thermal stable since the boiled culture filtrate had no effect on the mortality of J2s at 120℃ (Fig. 2b). The results concerning the stability over generations showed that the activity remained above 90% after 10 months of continuous passages (Fig. 2c).

Organic solvent extraction experiments showed that all four solvents, including n-butanol, ethyl acetate, cyclohexane and chloroform, were ineffective in extracting the active substances, with the highest mortality rate of about 20% (Fig. 2d). The results related to the adsorption/ion exchange properties experiments revealed that the selected four resins were completely ineffective. The active fractions presented in the flow-through fluid and aqueous phase (Fig. 3).

Further lyophilization experiments showed that no activity was found in the yellow viscous solids produced, even when concentrated 10-fold. Also, the yellow solid obtained by rotary evaporation was also inactive; in contrast, the liquid in the condensation flask showed the same pH and nematicidal activity as the fermentation supernatant. Therefore, combined with the above experimental results, we speculate that the virulence factors produced by *Bacillus altitudinis* AMCC 1040 may be related to volatile organic acids.

3.2 Analysis of Volatile Organic Compounds

BPY medium is commonly used for bacterial culture and it has been shown to have little effect on nematode activity in our previous studies (Wang et al. 2021b). In the present study, *Bacillus altitudinis* AMCC 1040 were cultured in BPY medium at 37°C for 48 h at 200 rpm, and the VOCs were analyzed by SPME-GC-MS. Chromatograms of BPY medium and culture supernatant were overlaid to reveal compounds produced exclusively by the *Bacillus altitudinis* AMCC1040. As shown in Fig. 4 and Table 1, eight different VOCs were detected, covering a wide range of alcohols, ethers, ketones and acids, for a total of eight molecules. 2,3-butanedione, acetic acid, acetoin, 2-isopropoxy ethylamine, 2,3-butanediol, 3-methyl-butanoic acid, 2-methyl-butanoic acid and octanoic acid. As predicted above, organic acids provided the highest percentage, while alcohols were the next highest.

Table 1
Nematicidal activities of VOCs presented in fermentation broth

Compound	RT (min)	Relative (%)	Peak number	Group	$LC_{100/12h}$ μL/mL
2,3-Butanedione	2.736	96	1	ketone	0.5
Acetic acid	3.291	98	2	acid	0.05
Acetoin	3.837	98	3	ketone	/
2-Isopropoxy ethylamine	5.535	81	5	ether	0.8
2,3-Butanediol	5.829	96	6	alcohol	/
3-Methyl-butanoic acid	7.308	96	7	acid	0.3
2-Methyl-butanoic acid	7.701	96	8	acid	0.3
n-Octanoic acid	23.273	97	10	acid	0.03
/: Minimum killing concentration ≥ 1μL/mL					

3.3 Nematicidal activity of VOCs

All the VOCs were further examined for nematicidal by using commercial compounds. As shown in Table 1, different compounds showed different toxicity against nematodes. All four organic acids showed strong nematicidal activity, with octanoic acid showing the highest activity with the $LC_{100/12h}$ values of 0.03 μL/mL and acetic acid ranking second with the $LC_{100/12h}$ values of 0.05 μL/mL. In addition, the two

isomeric organic acids 2-methyl-butanoic acid and 3-methyl-butanoic acid had the same $LC_{100/12h}$ value of 0.3 μL/mL. Among the two ketones tested, the $LC_{100/12h}$ value of 2,3-butanedione was 0.5 μL/mL, however, no significant nematicidal activity of acetoin was found. In addition, 2-isopropoxy ethylamine also showed good nematicidal activity, in contrast, 2,3-Butanediol was not as active, with a minimum killing concentration of ≥ 1μL/mL.

4. Discussion

In 2006, Shivaji et al. first isolated *Bacillus altitudinis* from air samples at 24 km altitude and proposed it as new species (Shivaji et al. 2006). Subsequent studies demonstrated its antimicrobial activity, such as, *Streptomyces scabies*, *Pseudomonas aeruginosa*, and *Escherichia coli*, while, the nematicidal activity of *Bacillus altitudinis* against *M. incognita* has been previously reported (Li et al. 2019; Hwang et al. 2016; Ni et al. 2017). However, the nematicidal molecules released by *Bacillus altitudinis* have not been systematically studied. Therefore, the aims of this study was to analyze the nematicidal substance of *Bacillus altitudinis* AMCC 1040. Unlike previous studies that limited the active factors to volatile components, the substances we searched for in this study were completely unknown (Zhai et al. 2018; Rajer et al. 2017; Yu et al. 2015). After a series of processes, including dynamic nematicidal activity bioassays, thermal and pH stability and solubility analysis, we finally found that the active substances were volatile compounds and were retained in water. Here, we propose that it may be more effective to determine if it is volatile as a first step when analyzing unknown potential nematicidal compounds.

Microbial volatile organic compounds (MVOCs) are produced by a variety of microorganisms, usually in the form of complex mixtures of low molecular weight lipophilic compounds (Kanchiswamy et al. 2015). In recent decades, many researchers have been trying to identify molecules from MVOCs that could be used in agriculture (Terra et al. 2017). Previous studies have shown that various microorganisms can produce nematicidal volatile compounds, such as *Pseudomonas* spp., *Bacillus* spp., *Fusarium* spp., and *Daldinia* spp. (Gu et al. 2007; Liarzi et al. 2016; Freire et al. 2012). However, as mentioned above, little is known about the effects of volatile compounds released by *Bacillus altitudinis* on root-knot nematodes. In this study, six nematicidal volatile molecules with $LC_{100/12h}$ values ranging from 0.03 to 0.8 μL/mL were analyzed based on GC/MS and nematicidal activity bioassays. These six nematicidal volatile molecules were previously found in botanical and microbial materials. For example, Barros et al. demonstrated that 2,3-butanedione was present in the mixture of nematicidal VOCs produced by *Azadirachta indica* (Barros et al. 2014). In this study, we demonstrated that organic acids play a major role in the mixture of volatiles produced by *Bacillus altitudinis* AMCC 1040. Volatile fatty acids have long been known for their nematicidal activities (Zhang et al. 2012). For example, Seo et al. reported that 2-methylbutyric acid play an important role in nematicidal activity against *Bursaphelenchus xylophilusdisease* (Seo et al. 2014). Masahiko et al. found that the nematicidal activity of acetic and n-butyric acids were almost equal, and their mixtures showed an additive effect (Masahiko et al. 2009).

Despite the interest in MVOCs due to their high activity and low residues, their application is still limited due to their difficulty in crop plants in open agricultural systems with the rapid evaporation (Song and

Ryu 2013). Therefore, further research is needed to explore the performance of MVOCs in practical applications, which will facilitate the development of new low toxicity nematicides.

5. Conclusions

To clarify the nematicidal active substances produced by *Bacillus altitudinis* AMCC1040, we characterized the active substances in terms of dynamic nematicidal activity bioassay, thermal and pH stability, solubility, generation stability, adsorption/ion exchange properties and volatility, and finally found them to be volatile substances. In combination with HS-SPME-GC/MS analysis and *in vitro* experiments, we found that *Bacillus altitudinis* AMCC1040 produced six nematicidal compounds, namely 2,3-butanedione, acetic acid, 2-isopropoxy ethylamine, 3-methylbutyric acid, 2-methylbutyric acid and octanoic acid. This study provides a better understanding of the nematicidal mechanism of *Bacillus altitudinis* AMCC 1040, however, it is unclear whether these volatiles act independently or in combination to affect nematodes, which is a direction for our future research.

Declarations

Acknowledgments and FUNDING

This work was supported by the Key R&D project of Shandong Province (2020CXGC010803), Major Applied Agricultural Technology Innovation Projects of Shandong Province (SD2019ZZ009), Key R&D project of Ningxia Hui Autonomous Region (2021BBF02006) and Quancheng Scholars Program of Jinan, China (No.00262019026), Shandong Provincial Natural Science Foundation, China (ZR2021MC183) and Modern Agricultural Industry Technology System of Shandong Province, China (SDAIT-06-13).

Author Contributions

All authors contributed to the study conception and design. Material preparation and data collection and analysis were performed by all authors. The first draft of the manuscript was written by Ye Lin and Jian-Yu Wang, all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figures

Figure 1

The dynamic nematicidal activity of Bacillus altitudinis AMCC1040. (a) The dynamic change of J2s mortality with exposure time; (b) The dynamic change of J2s mortality and the pH value of the media with culture time.

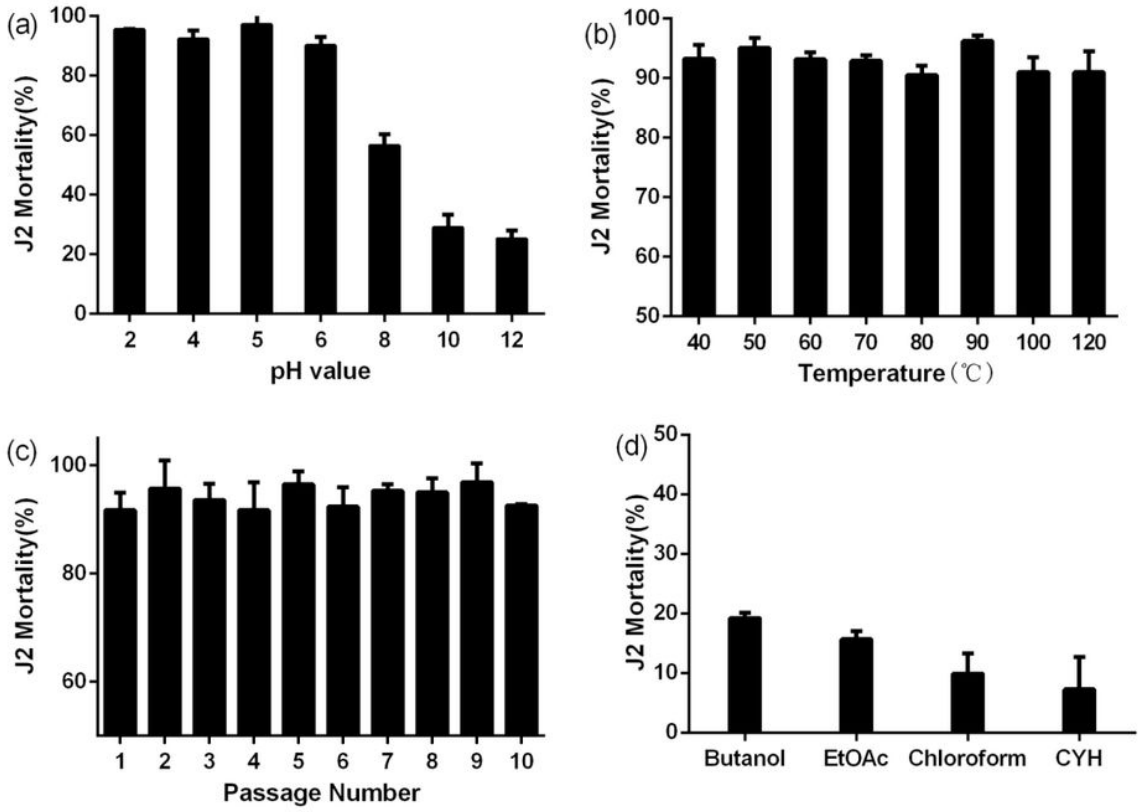

Figure 2

The stability and solubility of active metabolites. (a) pH stability; (b) Heat stability; (c) Generation stability; (d) Extraction effects of butanol, ethyl acetate (EtOAc), cyclohexane (CYH) and chloroform.

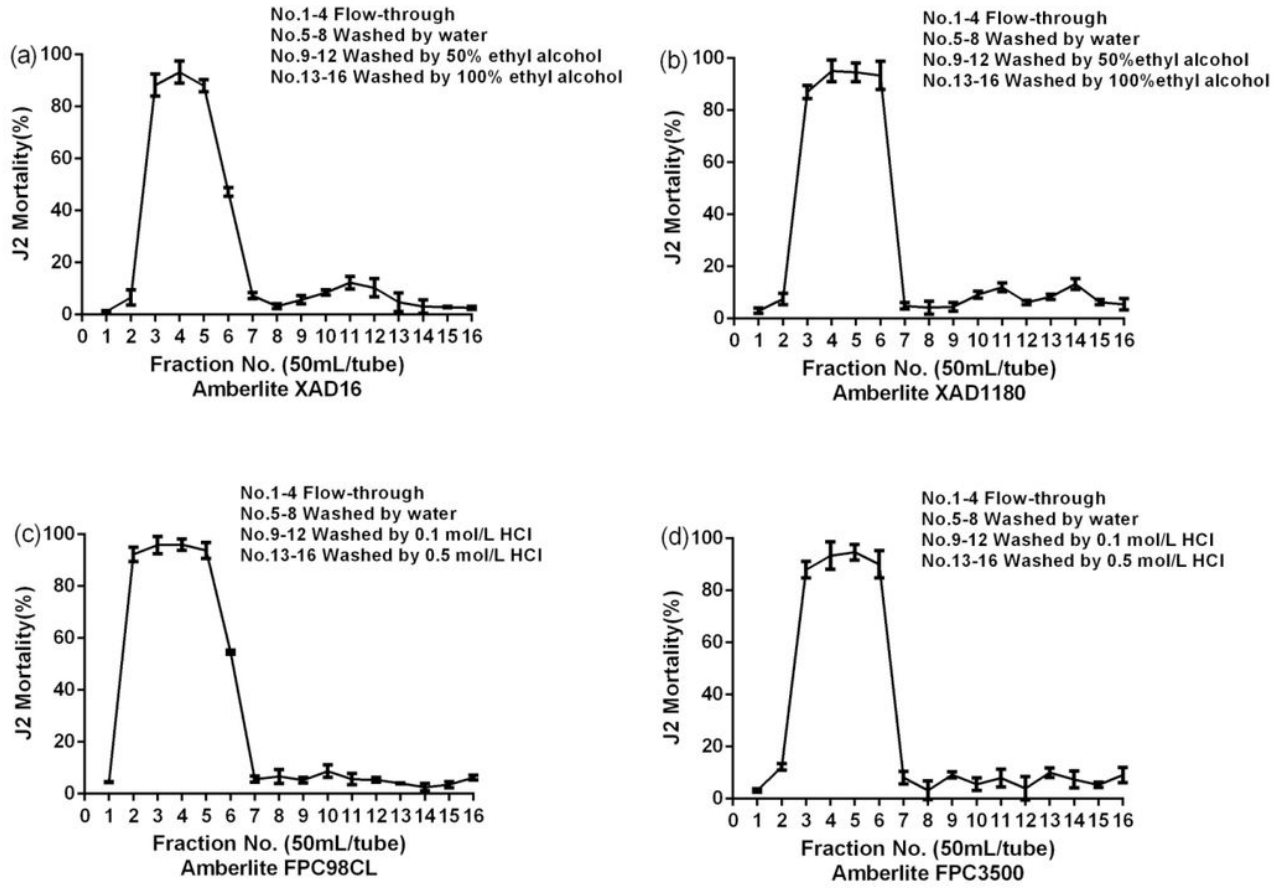

Figure 3

The adsorption/ ionexchange properties of active metabolites. (a) Amberlite XAD 16; (b) Amberlite XAD 1180; (c) Amberlite FPC 3500; (d) Amberlite FPC 23H.

Figure 4

The volatiles were extracted by HS-SPME and analyzed by GC-MS. *Bacillus altitudinis* AMCC1040 fermentation broth (A); BPY medium (B).