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ORIGINAL PAPER

Isolation and characterization of an abamectin-degrading *Burkholderia cepacia*-like GB-01 strain

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Abstract Abamectin is widely used in agriculture as an insecticide and in veterinary as an anti-parasitic agent, and has caused great environmental pollution by posing potential risk to non-target soil invertebrates and nearby aquatic systems. A bacterium designated GB-01, which was capable of degrading abamectin, was isolated from soil by enrichment culture method. On the basis of morphological, physiological and biochemical characteristics, combined with phylogenetic analysis of 16S rRNA gene, the bacterium GB-01 was identified as *Burkholderia cepacia*-like species. The bacterium GB-01 was able to utilize abamectin as its sole carbon source for growth, and could degrade more than 90% of abamectin at initial concentrations of 50 and 100 mg l^{-1} in mineral salt medium in 30 and 36 h, respectively. The longer degradation cycle was observed with abamectin concentrations higher than 100 mg l^{-1}. Optimal growth temperatures and pH values with highest degradation rate were 30–35°C and 7–8, respectively. Two new degradation products were identified and characterized by high performance liquid chromatography-tandem mass spectrometry (HPLC–MS/MS) based mass spectral data and a plausible partial degradation pathway of abamectin was proposed. This is the first report in which an abamectin-degrading *Burkholderia* species isolated from soil was identified and characterized.

Keywords Biodegradation · Abamectin · *Burkholderia cepacia* · Metabolites · HPLC–MS/MS

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Introduction

Abamectin, also called avermectin B_1 (a natural macrocyclic lactone), is a fermentation product from a strain of *Streptomyces avermitilis* discovered by the collaboration of The Kitasato Institute and Merck Sharp & Dohme in 1979 (Burg et al. 1979). Abamectin is a mixture of two homologues, avermectin B_{1a} and avermectin B_{1b} (Fig. 1) in a molar ratio of at least 4:1 (Pesticide manual 1997), and possesses potent anthelmintic and insecticidal activities. It is extensively used against a broad spectrum of endoparasites and ectoparasites in animals, and against different phytophagous pests of field crops, ornamentals,

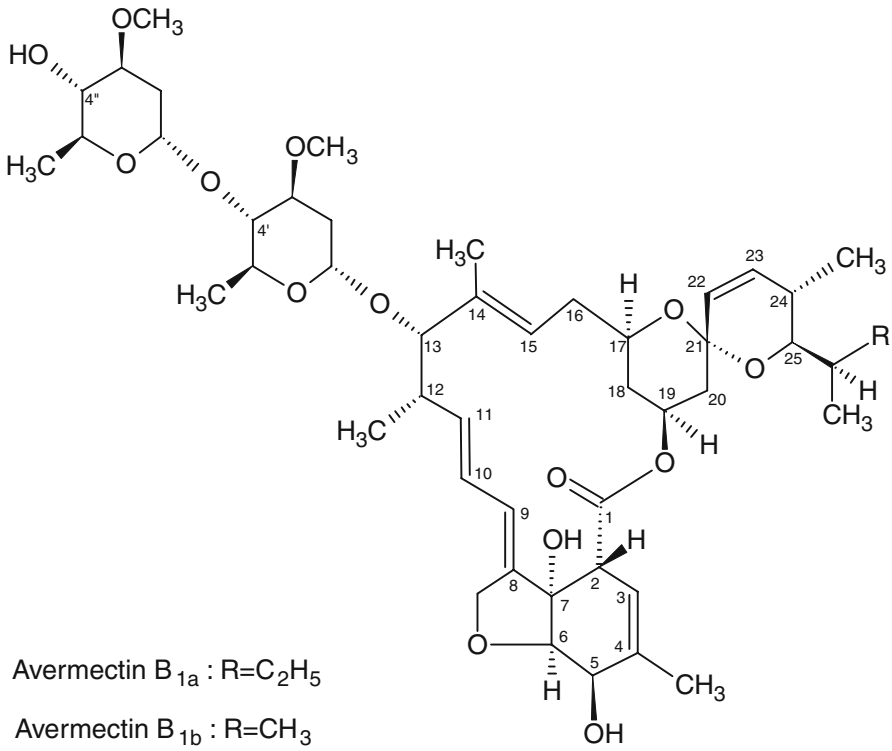

Fig. 1 Chemical structure of the two major components of abamectin

vegetables, and fruits and also used in controlling fire ants (Lofgren and Williams 1982; Kolar et al. 2006; Kamel et al. 2007).

The primary target of avermectins is the nervous system of parasites. They interact with the GluCl (glutamate-gated chloride) channels and GABA (gamma-amino butyric acid)-gated chloride channels in arthropods and nematodes causing strong chloride influx, which results in disrupted neural signal transmission (Martin et al. 2002). The mode of action of avermectins is not, however specific to parasitic arthropods and nematodes, and consequently may affect other organisms in the environment (McKellar 1997).

Abamectin undergoes little metabolism within the target organism and in most of the cases up to 98% of the dose given to the animal is hence excreted as parent compound, primarily in the feces with less than 2% in urine (Gruber et al. 1990; Lumaret and Errouissi 2002). The physical and chemical properties of avermectins indicate that, once they enter the environment, they can persist for extended periods of time at concentrations high enough to exert toxic impacts. A number of reports indicated the persistence of abamectin for 14–70 days in soil, soil-feces and feces (Kozuh Erzen et al. 2005). Recent studies on photo degradation kinetics of abamectin revealed that natural light does not produce any chemical transformation on this molecule, and it possesses limited possibilities of photosensitized degradation induced by environmental light (Escalada et al. 2008).

Abamectin shows effects on reproduction, biological function and survival of non-target terrestrial organisms that have important roles in the food web (Strong 1992; Shipp et al. 2000; Zhu et al. 2006; Jensen et al. 2007; Diao et al. 2007; Kolar et al. 2008). Moreover, abamectin can runoff from the sites of application, so the possibility exist that agricultural use of pesticide or administration to animals could result in its indirect introduction to nearby water bodies, leading to the exposure of aquatic organisms. The compound is expected to have adverse effects on the aquatic environment due to its high toxicity, even at very low concentrations (Halley et al. 1993; Tisler and Kozuh Erzen 2006).

However the risks posed by abamectin depend on its persistence in the environment. Bioremediation is considered to be a cost effective and major tool for the removal of contaminants in the environment.

Successful application of bioremediation technologies has been reported earlier for many compounds. Till date, only one bacterial strain capable of degrading abamectin, isolated from the sludge of waste water treatment plant of abamectin manufacturing factory, has been reported (Li et al. 2008). However, there are no reports on successful isolation of abamectin-degrading bacteria from soil. The present study reports a new abamectin-degrading bacterium, and its characteristics of utilization and degradation of abamectin as sole carbon source. The understanding of the physiological characteristics and optimum growth conditions of this strain may contribute to its successful application for bioremediation in soil. In addition, the biodegradation products of the abamectin were also identified, and a plausible partial degradation pathway was proposed. To our knowledge this is the first report of aerobic degradation of abamectin by a *Burkholderia* species isolated from soil.

Materials and methods

Chemicals and media

Abamectin (purity 97.1%, analytical standards, Riedel-de-Häen) was purchased from Sigma–Aldrich (New York, USA). HPLC gradient grade methanol was purchased from Sigma–Aldrich (St. Louis, USA). All other reagents used in this study were of highest analytical-reagent grade.

Mineral salt medium (MM) (1.0 g l^{-1} NaCl, 1.0 g l^{-1} NH_4NO_3, 1.5 g l^{-1} K_2HPO_4, 0.5 g l^{-1} KH_2PO_4, 0.1 g l^{-1} $MgSO_4{\cdot}7H_2O$ and pH 7.0) was used for microbial enrichment steps, isolation procedures and degradation experiments. The Luria–Bertani medium (LB), was used for cell growth. Solid media were prepared by adding 1.5% (w/v) agar. All of the media were sterilized at 121°C for 25 min before use.

Strain isolation and characterization

The soil samples were collected randomly from surface layer (0–10 cm) of a citrus orchard field situated in Jiangxi province of China, which has been exposed to the usage of abamectin for many years. This soil has the characteristics of 154 g kg^{-1} sand, 272 g kg^{-1} silt, 574 g kg^{-1} clay, 1.6 g kg^{-1} Total Nitrogen, 0.6 g kg^{-1} Total Phosphorus, 15.0 g kg^{-1} Total Potassium and 18.2 g kg^{-1} Organic matter, while the litter layer was absent. Soil samples were homogenized well, and an aliquot of 10 g non-sieved soil was mixed in 100 ml of MM medium using waring blender. About 10 ml of this mixture was transferred into the flask containing 90 ml MM medium supplemented with 100 mg l^{-1} abamectin to serve as sole carbon source for the microbial community. The enrichment culture was placed on a rotary shaker (150 rpm) in the dark at 30°C. About 5 ml of enrichment culture was taken and transferred into fresh, sterile MM medium, containing 100 mg l^{-1} abamectin. In total, five reinoculation steps of the enriched culture were conducted to ensure the maintenance of the degradation ability of bacteria and to enrich the key organisms for abamectin biodegradation. Dilutions of the sequential enrichment were plated onto MM agar plates containing 100 mg kg^{-1} abamectin, and bacterial colonies grown on plates were tested for their abamectin-degrading capabilities. One strain designated GB-01, which possessed the highest abamectin-degrading ability utilizing the abamectin as sole carbon source for growth, was purified and selected for the further investigation.

The colony morphology and microscopic characters of strain GB-01 were examined under a light microscope, as well as under a transmission electron microscope (Hitachy TEM system, Japan). Biochemical tests for the identification were carried out according to the Bergey's Manual of Determinative Bacteriology (Holt et al. 1994). Further, the identification of strain GB-01 was confirmed by API 20 NE kit (bioMerieux).

The genomic DNA of enrichment pure strain GB-01 was extracted by the method of high-salt-concentration precipitation (Miller et al. 1988). The partial 16S rRNA gene fragment was amplified using the universal primer set, 27f (5′-AGA GTT TGA TCC TGG CTC AG-3′) as forward and the 1492r (5′-TAC GGT TAC CTT GTT ACG ACT T-3′) as reverse primer (Lane 1991). The PCR products were purified by using BioDev's Gel Extraction System B (100), and ligated into the linear vector pMD18-T (TaKaRa Biotechnology, Dalian, China) and then transformed into competent *E. coli* DH5α cells. An automatic sequencer (Applied Biosystem, model No. 3730) was used to determine the 16S rRNA gene

sequence from cloned inserts. The amplified partial 16S rRNA gene sequence was deposited in GenBank under the accession number FJ793552.

Alignment with different 16S rRNA gene sequences from GenBank was performed using Clustal X 1.8.3 (Thompson et al. 1997) with default settings. Phylogenesis was analyzed with MEGA version 4.0 software (Tamura et al. 2007) and distance was calculated using the Kimura 2 parameter distance model. Phylogenetic tree was built using the neighbor-joining method. Each dataset was bootstrapped 1000 times.

HPLC analysis

Abamectin depletion was measured using high performance liquid chromatography (HPLC). Aliquots (10 ml) of growing cultures were taken and partitioned 3 times each with 10, 5, and 5 ml dichloromethane. Extracts were pooled, dried over anhydrous sodium sulfate and evaporated using a vacuum rotary evaporator at room temperature. The final residue was dissolved in 5 ml methanol, thoroughly mixed in an ultrasonic bath for 5 min, and 20 µl of the resulting solution was injected into a reverse-phase high-performance liquid chromatography column for HPLC analysis (HPLC, 600 Controller, Rheodyne 7725i Manual injector and 2487 Dual λ Absorbance Detector; Waters Co., Milford, MA). The separation column for the HPLC (4.6 mm $\times$ 250 mm, 5 µm) was filled with Kromasil 100^{-5} C18. The mobile phase contained methanol:water (80:20, v/v) and the flow rate was 1.0 ml min^{-1}. The wave length of UV absorbance detector was fixed at 245 nm.

Biodegradation assay of abamectin by strain GB-01

Unless otherwise stated, the inoculum for all the experiments was prepared by growing strain GB-01 in 100 ml of LB supplemented with 5 mg l^{-1} abamectin overnight at 30°C and 150 rpm on a rotary shaker till the late exponential growth phase. Cells were harvested by centrifugation at 6,000*g* for 5 min at room temperature. Cell pellets were washed twice with 25 ml of sterilized 0.5 M phosphate buffer (pH 7.5), mixed in sterilized MM medium, and adjusted to approximately 1.5 optical densities at 600 nm (OD_{600}). A ultra-violet spectrophotometer (UV-2401PC, Shimadzu, Japan) was used to determine the OD_{600} value through out the studies. For all degradation experiments, the cells were inoculated at the level of 2% (v/v) into 250 ml flask containing 100 ml of MM medium supplemented with abamectin as sole carbon source. The control experiments, without inoculation of microorganism were carried out under the same conditions. All of the degradation experiments were performed in triplicates.

In order to find out the optimum temperature for the growth and degradation ability of strain GB-01, reactors containing MM medium supplemented with 50 mg l^{-1} abamectin as sole carbon source and pH 7.0 were prepared. All the reactors were inoculated with 2 ml inoculum of strain GB-01, and incubated at 4, 20, 25, 30, 35, 40 and 45°C, respectively.

MM media with various initial pH values (4, 5, 6, 7, 8, 9 and 10) containing 50 mg l^{-1} abamectin were prepared to conclude the optimum pH for the growth of strain GB-01, and removal of abamectin.

The effect of initial concentration of abamectin on the degradation potential of strain GB-01 was also determined in batch reactors under aerobic conditions. Different initial concentrations of abamectin (10, 25, 50, 100, 150 and 200 mg l^{-1}) were prepared, and incubated with strain GB-01 at determined optimum temperature and pH for 48 h on a rotary shaker operating at 150 rpm in dark. Residual abamectin was determined at the interval of every 6 h.

Abamectin degradation time-course and growth of the strain GB-01 were monitored concomitantly in the 100 ml MM medium containing 50 mg l^{-1} abamectin. The culture was allowed to incubate for 0, 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 h at determined optimum temperature and pH, on a rotary shaker operated at 150 rpm in dark. Samples were collected periodically at defined intervals and assayed for OD_{600} value to get the cell growth curve, while the concentration of residual abamectin was analyzed by HPLC.

Mass spectrometry

To find out the degradation pathway, the metabolites were extracted from growing culture of GB-01 on abamectin (100 mg l^{-1}) in MM medium using dichloromethane before and after acidification to pH 2 adding 1 M HCl. Extracts were dried over

anhydrous sodium sulfate, and evaporated using a vacuum rotary evaporator at room temperature. The residue was dissolved in 2 ml methanol, thoroughly mixed in an ultrasonic bath for 5 min and subjected to HPLC–MS/MS analysis. The HPLC system conduced as described above, and linked to a Mass Spectrometer equipped with electron spray ionization (ESI) source. A Finnigan TSQ Quantum Ultra AM triple quadrupole Mass Spectrometer (Thermo Electron Corporation, USA) was used to detect unknown biodegradation products. Mass Spectrometer was operated in the electron spray ionization mode with a negative polarity {(−)-ESI–MS}. Parameters were optimized by continuous infusion of standards. Mobile phase was directly introduced into the ion source without splitting. Full-scan (−)-ESI–MS mass spectra were obtained by scanning from 200 to 1000 *m/z* (mass over charge ratio). The structures of the unknown metabolites were elucidated by applying multiple-stage fragmentation studies using tandem mass spectrometry (MS/MS). Culture media extracts of the reference control samples (non-inoculated but supplemented with abamectin) were also prepared and analyzed in the same way.

Results

Isolation and characterization of the abamectin-degrading strain GB-01

After acclimation and selective enrichment, a total of seven bacterial strains with the ability to degrade abamectin were obtained. Among these, the strain GB-01 was selected for further investigations due to its comparatively higher degradation rate. It was able to use abamectin as the sole carbon source for growth under aerobic conditions. The strain formed smooth colony with an average diameter of approximately 0.6–1 mm. Cells of strain GB-01 were straight rods, having rounded ends, with dimensions of 0.4–0.7 μm in width and 1.2–1.7 μm in length, and occasionally formed pairs or short chains. The isolate was a gram negative bacterium. Oxidase, nitrate reduction and beta-glactocidase activity were found positive, while arginine dihydrolase, beta-glucosidase (aesculin hydrolysis), protease (gelatin liquefaction), tryptophanase and urease activity were negative. The organism could assimilate the L-arabinose, D-glucose, D-mannose, D-mannitol, *N*-acetylglucosamine, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, and phenylacetic acid, while assimilation of D-maltose was not observed.

Comparative analysis of partial 16S rRNA gene sequence of strain GB-01 illustrated very high similarity with the species of genus *Burkholderia*, and more than 99% gene homology was observed with *Burkholderia cepecia* complex (Bcc) species. Phylogenetic analysis of 16S rRNA gene also clustered the strain GB-01 within the clade of *Burkholderia cepecia* complex (Bcc) species in phylogenetic tree (Fig. 2). On the basis of morphological, physiological and biochemical properties, combined with 16S rRNA gene sequence analysis, the strain GB-01 was identified as *Burkholderia cepacia*-like species.

Biodegradation of abamectin by strain GB-01 in cell culture

The bacterium grew on abamectin as sole carbon source and degraded more than 90% of abamectin at initial concentration of 50 mg l^{-1} in 30 h. During the incubation period, the concentration of abamectin declined very slowly in first 6 h, indicating an initial lag phase for the growth of strain GB-01. The highest degradation rates were observed between 9 and 24 h of incubation. When the abamectin residue was nearly undetectable after 30 h, the final OD_{600} increase reached to its maximum values (Fig. 3). It was clear that growth of strain GB-01 was accompanied by the degradation of abamectin, as no significant growth was observed in the reactors without supplementation of abamectin as sole carbon source in MM medium.

Degradation of abamectin and the growth of strain GB-01 occurred over a wide range of temperatures. The strain grew well from 25 to 35°C, and there was no significant difference of cell growth and degradation ability at temperatures 30 and 35°C. However, the greatest growth of the strain GB-01 and abamectin degradation rate were observed at 30°C (Fig. 4).

The strain GB-01 could degrade abamectin over a wide range of pH values (from 6.0 to 9.0). There was no significant difference of abamectin degradation ability and cell growth at pH 7 and 8. However, the maximum cell growth and abamectin degradation rate were recorded at pH 7 (Fig. 5).

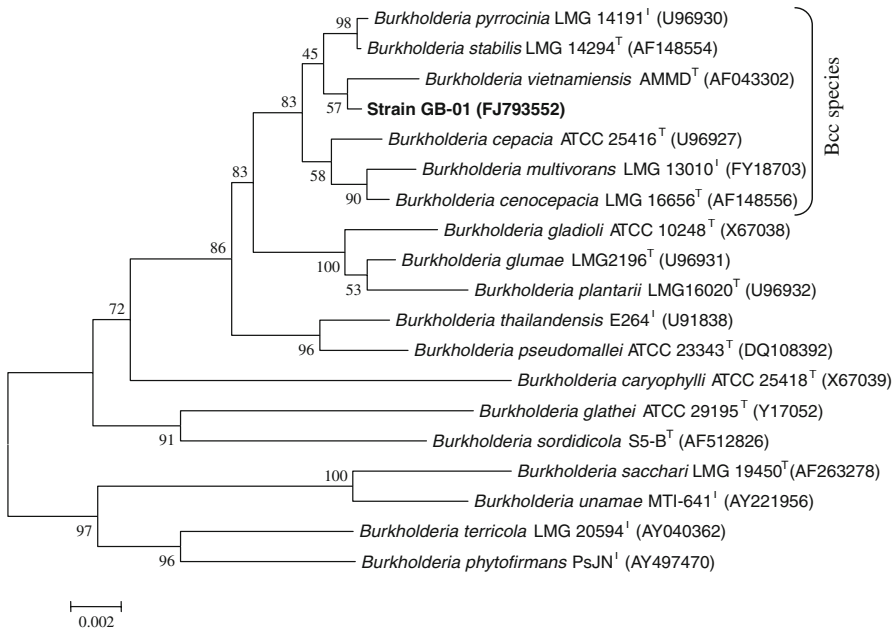

Fig. 2 Phylogenetic tree constructed by the neighbor-joining method based on 16S rRNA gene sequences of strain GB-01 and related *Burkholderia* type strains. GenBank accession numbers are given in *parentheses*. *Numbers* at the nodes indicate the percentages of bootstrap samplings, derived from 1,000 replicates, supporting the internal branches. The *scale bar* corresponds to 0.002 estimated nucleotide substitutions per sequence position

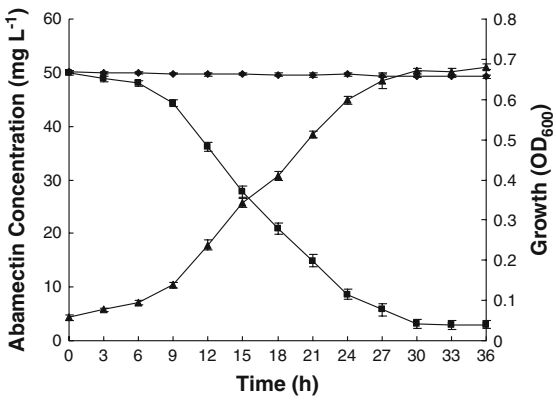

Fig. 3 Utilization of abamectin as sole carbon source for growth by strain GB-01 (temperature 30°C, pH 7, 150 rpm, and abamectin 50 mg l^{-1}). Symbols: *filled diamond*, abamectin concentration in non-inoculated culture; *filled square*, abamectin concentration in growing culture; *filled triangle*, growth of strain GB-01. *Error bars* represent the standard error of three replicates

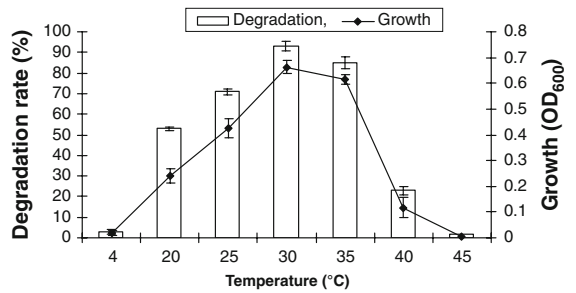

Fig. 4 Effect of temperature on abamectin degradation by strain GB-01 (pH 7, 150 rpm, 30 h incubation, abamectin 50 mg l^{-1}). *Error bars* represent the standard error of three replicates

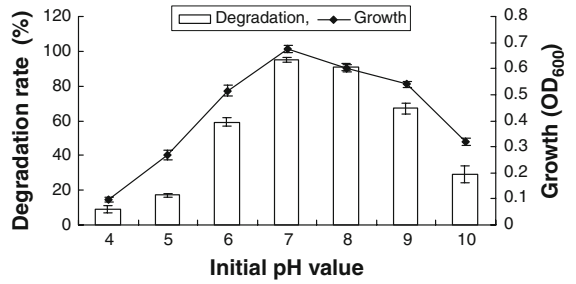

Fig. 5 Effect of initial pH on degradation of abamectin by strain GB-01 (temperature 30°C, 150 rpm, 30 h incubation, abamectin 50 mg l^{-1}). *Error bars* represent the standard error of three replicates

In MM medium (pH 7, 30°C and 150 rpm), strain GB-01 was capable of degrading more than 90% of abamectin at an initial concentration of 100 mg l^{-1}. When the initial concentration of abamectin was 50 and 100 mg l^{-1}, maximum degradation was achieved by strain GB-01 within 30 and 36 h, respectively. However, initial concentrations of 150 and 200 mg l^{-1} resulted in a bit longer lag phase, and

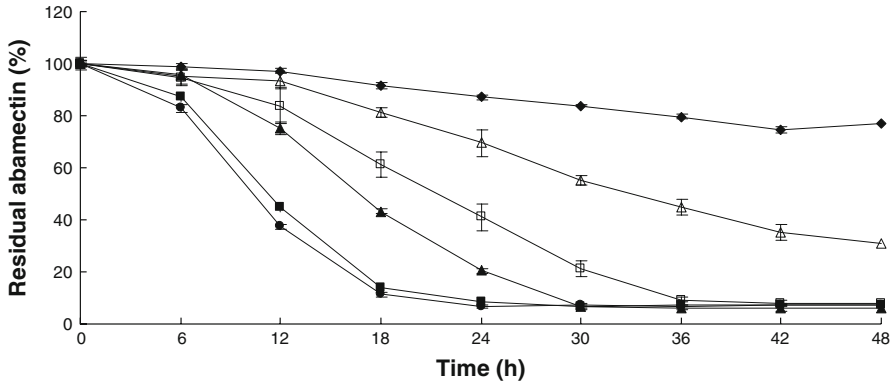

Fig. 6 Effect of initial concentration of abamectin on degradation ability of strain GB-01 (temperature 30°C, pH 7, 150 rpm). Symbols: *filled circle*, 10 mg l^{-1}; *filled square*, 25 mg l^{-1}; *filled triangle*, 50 mg l^{-1}; *open square*, 100 mg l^{-1}; *open triangle*, 150 mg l^{-1}; *filled diamond*, 200 mg l^{-1}. *Error bars* represent the standard error of three replicates

strain GB-01 could degrade maximum 70% and 23% of abamectin in 48 h, respectively (Fig. 6).

Degradation products and pathway of abamectin

HPLC–MS analysis revealed the presence of two major biodegradation products with retention times 8.94 and 10.42 min respectively besides avermectin B_{1b} at 9.34 min and avermectin B_{1a} at 9.80 min (Fig. 7a). In full-scan HPLC-(−)-ESI–MS spectra, de-protonated molecules i.e. $[M–H]^-$ ions, were mainly produced and mass signals of the proposed metabolite I (*m/z* 393.01, $[M–H]^-$) and metabolite II (*m/z* 339.03, $[M–H]^-$) corresponding to their retention times are shown (Fig. 7b). The MS/MS analysis of the proposed metabolites gave us an idea about the fragmentation pattern of their $[M–H]^-$ precursor ions. Metabolite I (*m/z* 393, $[M–H]^-$) produced the characteristic fragment ions of *m/z* 349, *m/z* 325, *m/z* 239, and *m/z* 183, while metabolite II (*m/z* 339, $[M–H]^-$) produced the characteristic fragment ions of *m/z* 295, *m/z* 239, *m/z* 197 and *m/z* 183. Structures of these metabolites were suggested on the basis of their MS/MS product ions spectra and fragmentation patterns (Fig. 8). With reference to the tentative identification and structural elucidation of these metabolites, we have proposed a plausible partial biodegradation pathway of abamectin by strain GB-01 (Fig. 9).

Discussion

A novel bacterium capable of degrading abamectin was isolated from soil by enrichment technique and named as strain GB-01. Polyphasic taxonomic data analysis revealed that strain GB-01 has very close relationship with the members of *Burkholderia cepecia* complex (Bcc) species of genus *Burkholderia*. So, based on the phenotypic characteristics and 16S rRNA gene similarity criterion (Stackebrandt and Goebel 1994), this bacterium was identified as *Burkholderia cepacia*-like strain GB-01.

Burkholderia cepecia is not a single species, rather it is a complex of related species or genomovars referred to as *Burkholderia cepacia* complex (Bcc) (Coenye and Vandamme 2003; Coenye et al. 2003; Payne et al. 2005). It is a group of highly versatile bacteria capable of a variety of complex interactions such as the degradation of aromatic pollutants (Baldwin et al. 2005; Mahenthiralingam and Vandamme 2005; O'Sullivan and Mahenthiralingam 2005), and protection and growth promotion of plants (Parke and Gurian-Sherman 2001). In general the species of Bcc are phenotypically nearly indistinguishable, as all of the Bcc species demonstrate considerable phenotypic variability, even with an extended panel of biochemical tests (Vandamme et al. 1997; Henry et al. 2001; Vanlaere et al. 2008a, b). Genetic methods have proven very useful for Bcc species identification. Phylogenetic comparison of the full-length 16S rRNA gene sequence of Bcc bacteria can distinguish all the formally named species (Coenye and Vandamme 2003). However, partial 16S rRNA gene sequencing is not sufficiently discriminatory to resolve all the species, as the gene is >98% identical for the members of Bcc (Mahenthiralingam et al. 2008). In short, identification of Bcc species is a complicated challenge, requiring a combination of multiple molecular diagnostic procedures, and is mostly restricted to reference centers. Based on all these facts, we did not propose a formal name for this strain at this time, as more

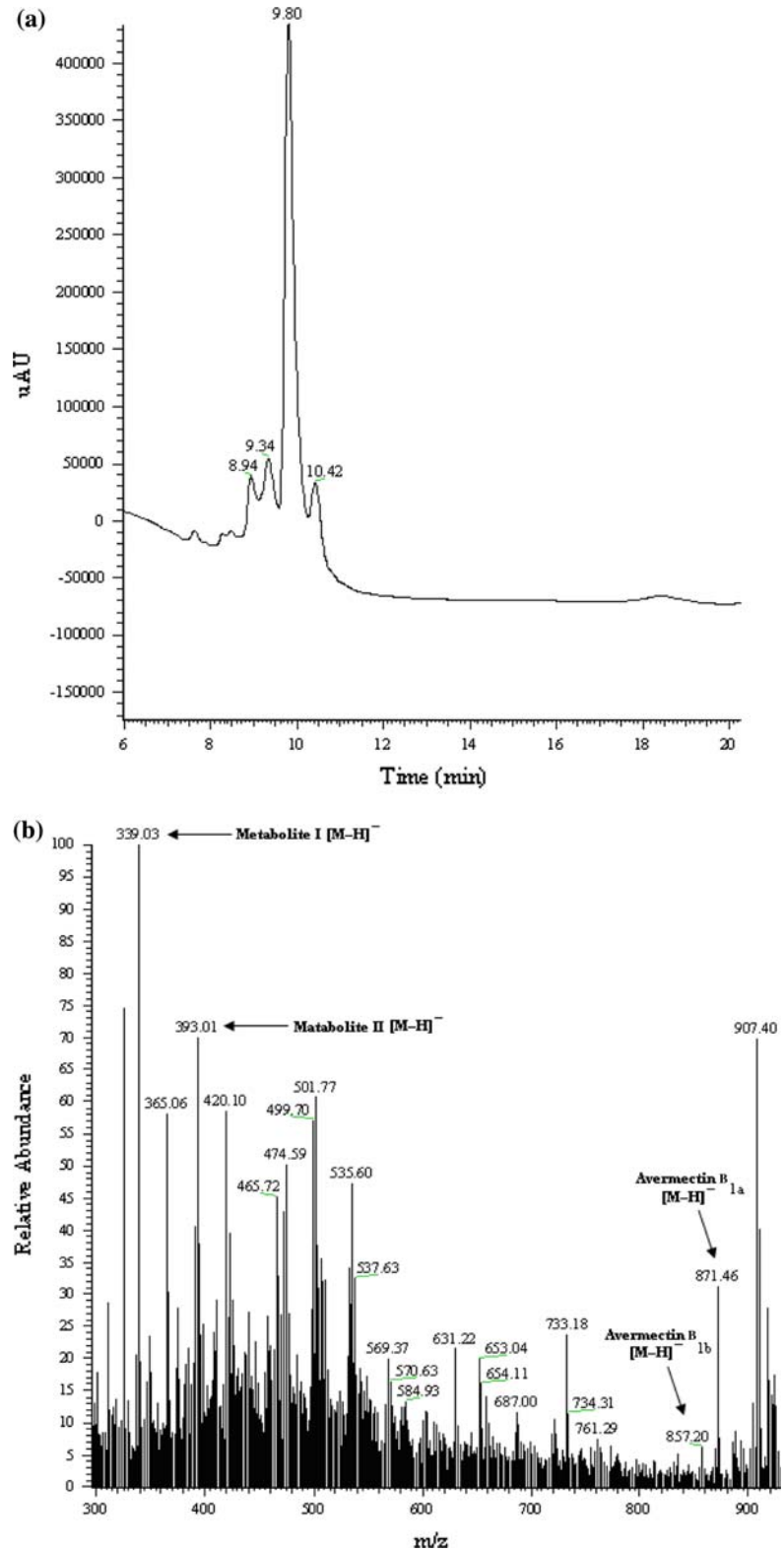

Fig. 7 **a** HPLC–ESI–MS chromatogram of the abamectin biodegradation by strain GB-01. **b** HPLC-(−)-ESI–MS spectrum of the intermediates of abamectin biodegradation by strain GB-01

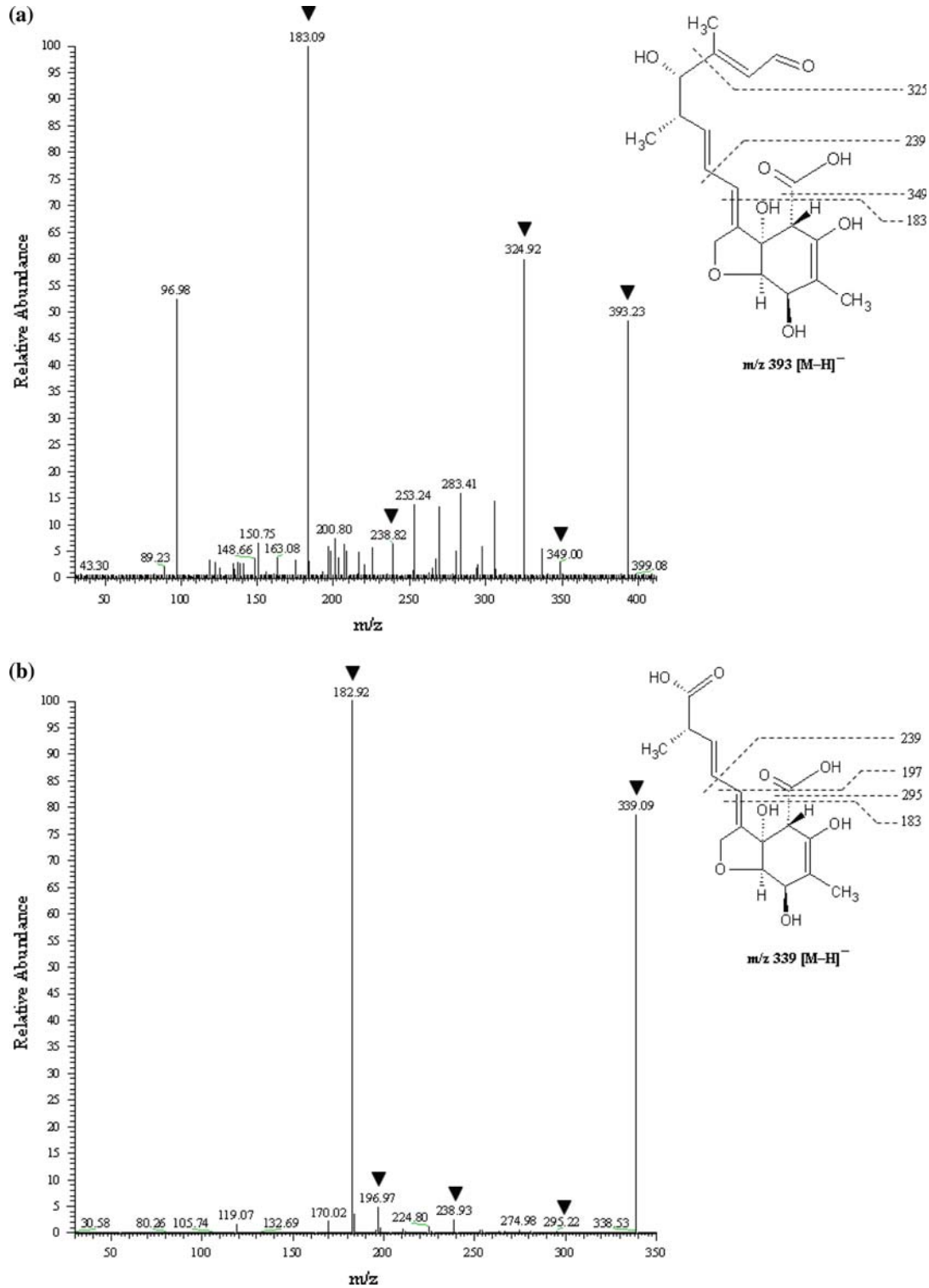

Fig. 8 (−)-ESI–MS/MS product ion spectra of deprotonated presumed metabolites along with suggested structures. **a** Metabolite I, *m/z* 393 $[M–H]^-$, **b** metabolite II, *m/z* 339 $[M–H]^-$

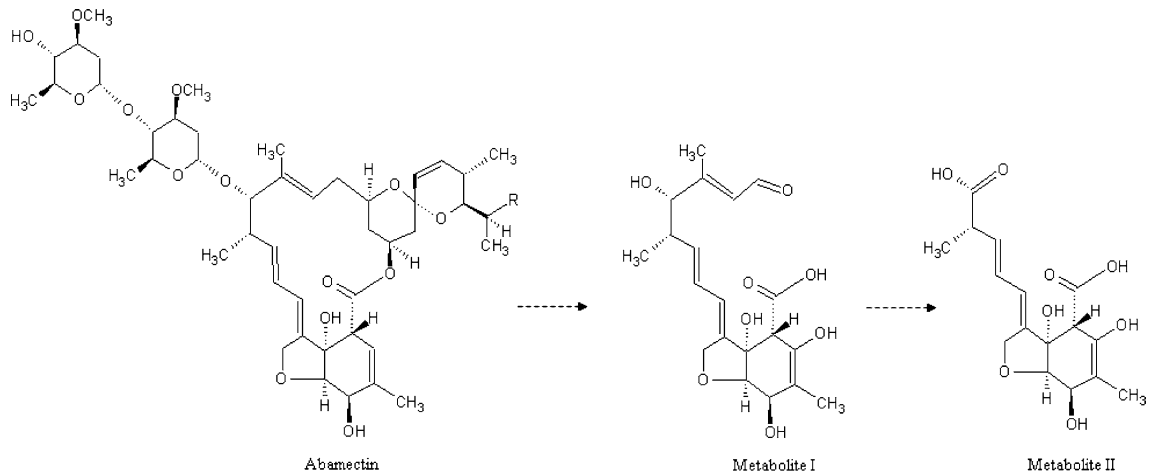

Fig. 9 Proposed partial degradation pathway of abamectin by strain GB-01 based on presumed metabolites

detailed taxonomic work is required to avoid misidentification.

Bcc seems to have the ability to metabolize virtually anything available to it (Balashova et al. 1999; Inguva and Shreve 1999; Bhushan et al. 2000; Leahy et al. 2003; Chaillan et al. 2004; Olaniran et al. 2004; Tillmann et al. 2005). Some *Burkholderia* strains such as JS150 (ex *Pseudomonas* sp. JS150) are known to have multiple oxygenase pathways for the dissimilation of aromatic compounds, which may further expand the range of substrates that can be co-oxidised (Haigler et al. 1992; Johnson and Olsen 1997). The strain GB-01 grew well in the mineral salt media supplemented with abamectin, and could utilize more than 90% of available abamectin as the sole carbon source. Abamectin degradation rate was very rapid at temperatures ranging from 30 to 35°C and pH 7–8. These results indicated the bioremediation potential of the strain GB-01 to be used in the field conditions.

The fate of avermectins in soil (Bull et al. 1984), plants (Maynard et al. 1989a) and animals (Maynard et al. 1989b) have been reported, however the mechanism of bacterial degradation is still unclear. Tandem mass spectrometry is a powerful technique for the structural characterization of unknowns, and has been especially useful when linked with a 'soft' ionization technique such as elctrospray (Gianelli et al. 2000). In the HPLC-(−)-ESI–MS analysis, two major biodegradation products were observed. Metabolite I (*m/z* 393, $[M–H]^-$) seemed to be produced due to the removal of two oleandrose moieties, addition of –OH at C(3), oxidation of C(16)–C(17) to –CHO, and the breakage of C–O bond between C(1)–C(19) resulting in the opening of entire lactone ring. Regarding the removal of oleandrose moieties, similar biodegradation mechanism has been reported by Li et al. (2008). However, they reported the removal of only one oleandrosyl moiety, while our strain GB-01 seemed to remove the both alpha-L-oleandrose moieties of abamectin. Moreover, Gianelli et al. (2000) also observed the fragmentation of avermectins at C(17) during the mass spectrometry, indicating that C(17) may be a point of substitution in avermectins. Metabolite II (*m/z* 339, $[M–H]^-$) may be produced from metabolite I due to the oxidation of C(13)–C(14) bond. Above mentioned metabolites have not been reported earlier, and we suggest their structures on the basis of their product ion MS/MS spectra and fragmentation patterns, as reported by many researchers for different chemicals (Eichhorn et al. 2005; Haghedooren et al. 2006; Muller et al. 2007; Medana et al. 2008). In MS/MS spectrum of metabolite I, the fragment ion *m/z* 325 may be the result of an allylic cleavage between C(13)–C(14). The fragment ions *m/z* 349 from metabolite I and *m/z* 295 from metabolite II may be produced due to the loss of CO_2 (−44) i.e. $393 - 349 = 44$ and $339 - 295 = 44$ from both of the parent ions respectively, indicating the presence of –COOH in both of the metabolites. The fragment ion *m/z* 239 may be produced due to allylic cleavage between C(9)–C(10) in both of the metabolites respectively. Similarly the fragment ion *m/z* 183 may be generated due to an allylic cleavage between

C(8)–C(9) and removal of –COOH from both of the metabolites respectively. The fragment ion *m/z* 197 from metabolite II may be produced due to the allylic cleavage between C(9)–C(10) and removal of–COOH. Although the complete structural elucidation of these two unknown metabolites can not be achieved without further analysis e.g. HPLC-NMR, the structures presented here are the best possible structures deduced by MS/MS fragmentation pathways.

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