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Full Length Article

Characteristics and Bioactivity of a Chlorogenic Acid-Producing Endophytic Bacterium Isolated from *Lonicera japonicae*

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Abstract

In present study, endophytes were isolated from medicinal plant extract of *Lonicera japonicae* for presence of chlorogenic acid (CGA) by HPLC and LC-MS. An endophytic baterium RB2, identified as *Bacillus subtilis*, was confirmed to produce chlorogenic acid and isomer neochlorogenic acid. Cinnamic acid and *p*-coumaric acid, two intermediates of CGA synthetic pathway, were detected in RB2 extract. The results suggested that RB2 may produce CGA in similar synthetic pathway to its host plant. The RB2 extract also exhibited certain antimicrobial activity with strongest effect against *Pseudomonas aeruginosa* and *Candida albicans*, and antioxidant activity with certain DPPH scavenging and FRAP value. RB2 extract displayed stability at low pH and high temperature, as for chlorogenic acid. The RB2 can be hopeful as the potential alternative source of CGA in future. © 2019 Friends Science Publishers

Keywords: Antimicrobial;·Antioxidant; Chlorogenic acid;·Endophyte;·Isolation

Introduction

Chlorogenic acid (CGA, 3-caffeoylquinic acid) is a common plant secondary metabolite composed of caffeic acid and quinic acid and plays an important role in protecting plant cells against environmental stress (Upadhyay and Rao, 2013). Previous studies showed that CGA has diverse physiological functions such as antimicrobials (Lou *et al*., 2011), anti-tumor, antivirus (Wang *et al*., 2009), antioxidant, scavenging free radical (Liang *et al*., 2016), preventing cardiovascular disease, neuroprotective effect (Hao *et al*., 2015), detoxicity (Wang *et al.*, 2018) and so on. CGA is produced in plant through phenylpropanoid pathway which leads to the synthesis of other important metabolites such as lignin, flavonoid and coumarin (Singh *et al.*, 2010).

Currently, the only source of CGA is extract from natural plants such as bark of *Eucommia ulmoides Oliver* or flower of *Lonicera japonicae* (Takamura *et al*., 2007). The pharmaceutical or commercial application of CGA is limited because of scarce plant sources, long growth period and low yield (Mills *et al*., 2013). It is necessary to find alternative source of CGA to overcome such defects. Endophytes, the micro-organisms that colonize inside plant tissues, had been proved to produce the same or similar metabolites with host plants (Yu *et al*., 2010) and to involve in many essential activities such as promoting plant growth, nitrogen fixation and withstanding environmental stress (Eljounaidi *et al*., 2016). Hence, endophyte may be served as potential alternative source of some plant natural metabolites.

In present study, endophytes were isolated from a Chinese traditional herb *L. japonicae* a potential source of CGA. Thus, chlorogenic acid-producing endophyte was screened and confirmed by HPLC and LC-MS. In addition, the isomers, derivatives, and intermediate metabolites of CGA were detected as well. Furthermore, antimicrobial and antioxidant activities of endophyte were invested. The characteristics of CGA-producing endophyte then were discussed preliminarily.

Materials and Methods

Sources of the Plants

L. japonicae plant samples were collected from Chengdu, Sichuan Province, China. The roots, stems, leaves and flowers of healthy plants were separately collected, followed by immediately processing in laboratory.

Isolation and Culture of Endophytes

Plant samples were surface sterilized through a three-step procedure: soaking in 70% ethanol for 3 min, soaking in 5.2% NaOCl 5 min and finally washing in sterilized water. Samples were then cut into 1 to 2 cm segments and inserted into potato dextrose agar (PDA) medium plates and beef extract peptone medium plates respectively.

Some rinsed sterile water and surface sterilized intact samples were used as negative controls. All plates were incubated at 28℃ (for PDA medium) or 37℃ (for beef extract peptone medium) respectively for 3 days. The emerging endophytic fungi and bacteria were numbered and stored.

Fermentation and Extraction of Endophytes

All endophytes were inoculated into 100 mL corresponding liquid medium, and fermented for 3 days at 37℃ or 28℃ on 150 rpm. After 30 min ultrasonically disrupting, cultural solution then were extracted by adding 75% ethanol, adjusting pH to 2–3 and adding ethyl acetate. The solution was vacuum concentrated to dry and dissolved in 2 mL methanol (chromatographically pure, Fischer) as samples for HPLC and LC-MS screening. In addition, 10 g flowers of *L. japonicae* were extracted as positive control.

Screening of CGA-producing Endophyte

The solution of endophytes and *L. japonicae* were filtered by 0.22 μm membrane for HPLC and LC-MS. All standards including CGA, isomers and derivatives, intermediate metabolites (Sigma-Aldrich, USA) were dissolved in methanol to 0.1 mg/mL.

HPLC was performed by Waters C18 reversed phase column (5 μm, 4.6×150 mm, Waters, Milford, MA, USA). Mobile phase was methanol: ultrapure water (15:85 volume) with flow rate of 0.5 mL/min. Detection was made at wavelength of 324.8 nm. The samples of the same retention time and absorption wavelength as CGA standard were selected for LC-MS analysis.

LC-MS analysis was carried out using a Waters Quattro Premier XE mass spectrometer (Waters, USA). Chromatographic separation was completed on a Waters Acquity BEH C18 column (50×2.1 mm, 1.7 μm). Methyl cyanide (A) and 1% acetic acid (B) was used as mobile phase at a flow rate of 0.25 mL/ min. A gradient elution was performed as follows: 90% A for 3 min, 65% A for 10 min and 90% A for 2 min. Samples were ionised with electrospray ionization (ESI) in negative mode. Multiple-reaction monitoring (MRM) was used to monitor ion transition. Source and desolvation temperature were 110℃ and 400℃. Capillary voltage was 2.8 kV. Cone and estracter voltage were 20 V and 5 V, respectively. Desolvation gas and cone gas were 700 L/h and 40 L/h. The collision energy was 15 eV.

Identification of CGA-producing Endophyte

An endophytic bacterium RB2 confirmed to produce CGA was identified by microscopic morphologic characteristics and 16S rDNA sequence analysis. The standard procedures were performed as previously described (Weisburg *et al*., 1991). The genome DNA of RB2 was extracted with genome kit (Takara, Japan). The PCR amplified products were inserted into pMD19-T vector (Takara, Japan) and then sequenced (Invitrogen, China). Multiple alignments and phylogenetic tree construction were performed with Clustal W and Neighbour-Joining. The 16S rDNA sequence of RB2 was submitted to GenBank nucleotide sequence data libraries.

Antimicrobial Activity

The antimicrobial activity of endophytic RB2 was assessed with a variety pathogenic fungi and bacteria as test organism including *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Salmonella typhimuriu* ATCC 13311, *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922, *Candida albicans* ATCC 10231, *Fusarium oxysporum* ATCC 16608, *Phytophthora infestans* ATCC MYA1113, *Mucor racemosus* ATCC42647 and *Rhizoctonia solani* ATCC 28628. All microorganisms were provided by China General Microbiological Culture Collection Center (CGMCC, Beijing China). Antimicrobial activity was carried out using the standard disc diffusion assay method. Paper disks eluted with methanol and standard chlorogenic acid were used as negative and positive controls, respectively. Antimicrobial activity was evaluated by measuring diameter of the inhibition zone (mm) produced by RB2 extract. The various concentrations (10–100 mg/mL) of extract of RB2 were used to identify the minimum inhibitory concentrations (MIC) value against each test organism. Besides, the effects of temperature (20–50℃) and pH (4–10) on antimicrobial activity of RB2 were studied.

Antioxidant Activity

The antioxidant activity of endophytic RB2 was assessed with FRAP assay and DPPH radical scavenging. The FRAP assay was performed basing on method of Benzie and Strain (1996) with some modifications. FRAP reagent was freshly prepared by mixing 100 mL of acetate buffer (0.3 *M*, pH 3.6), 10 mL of TPTZ solution (10 m*M* TPTZ in 40 m*M* HCl) and 10 mL of $FeCl_3$ (20 m*M*). The reaction mixture, containing 1.0 mL extract and 5.0 mL FRAP reagent, was measured at 593 nm after incubating at 37℃ for 10 min. $FeSO_4$ solutions were used to make calibration curve. Chlorogenic acid solution was used as positive control. The antioxidant power (FRAP value) of sample was calculated from calibration curve of $FeSO_4$ solution.

DPPH radical scavenging assay was performed using the method described previously with some modifications (Wang *et al*., 2008). Extract (0.2 mL) was added into 2.8 mL 0.1 m*M* DPPH (dissolved in methanol). Mixture was incubated for 30 min in the dark. Then absorbance of mixture was measured at 517 nm. Solution mixed with 0.2 mL methanol and 2.8 mL DPPH were used as controls and chlorogenic acid solution was used as standard for

comparison. The DPPH scavenging effect and half maximal inhibitory concentrations (IC50) were then calculated. The effects of temperature (20–50°C) and pH (4–10) on DPPH scavenging activity of RB2 extract were studied.

Results

Confirmation of CGA-producing Strain

In present study, 56 endophytes were isolated from CGA-producing plant *L. japonicae*, 34 from roots, 12 from stems, 7 from leaves and 3 from flowers. Morphology results revealed that 36 of endophytes were bacterial and 20 fungi. The results of HPLC showed that RB2, a bacterium isolated from roots of plant had the same retention time (8.373 min) as CGA standard and extract of *L. japonicae* (Fig. 1a, c and e). Meanwhile, the three samples had the same absorption peak in characteristic absorption wavelength (324.8 nm and 216.9 nm) (Fig. 1b, d and f).

Furthermore, LC-MS showed that RB2 extract and CGA standard had the same retention time (2.7 min) (Fig. 2a and b) and the same CGA parent ion m/z value (353.22) and quinic acid daughter ion m/z value (191) in mass spectra (Fig. 2c and d). The results above suggested that endophytic bacterium RB2 can produce CGA like host plant *L. japonicae* does.

Detection of Isomers and Intermediates

On the other hand, RB2 extract was analyzed by LC-MS with standard CGA isomers, CGA derivatives and intermediate metabolites. The retention time of isomers was as follows: neochlorogenic acid (1.82 min), chlorogenic acid (2.71 min) and cryptochlorogenic acid (3.02 min). While derivatives retention time were as follows: 1,3-dicaffeoylquinic acid (5.02 min), isochlorogenic acid B (8.43 min), 1,5-dicaffeoylquinic acid (8.63 min), isochlorogenic acid A (9.29 min), isochlorogenic acid C (10.49 min). The results showed that retention time of RB2 was identical to the CGA standard and CGA isomer neochlogenic acid (Fig. 3). Subsequently, RB2 had the same retention time with two CGA synthesis intermediates cinnamic acid (0.96 min, Fig. 4a and b) and *p*-coumaric acid (1.00 min, Fig. 4c and d) respectively.

Identification of the Selected Strain

Based on 16S rDNA sequence of RB2, phylogenetic tree exhibited that RB2 and *Bacillus subtilis* formed a distinct phylogenetic cluster while RB2 and other *Bacillus* strains are only distantly related (Fig. 5). Thus, endophytic RB2 was identified as a strain of *Bacillus subtilis*. The 16S rDNA sequence of RB2 was submitted to GenBank (NCBI) and deposited under the accession number AB218615. RB2 was preserved in China General Microbiological Culture Collection Center as strain number CGMCC 1.13618. In previous studies, a CGA-producing endophytic fungus from *Eucommia ulmoide Oliver* has been reported (Chen *et al*., 2010). Our study found CGA-producing endophytic bacterium.

Antimicrobial Activity

Antimicrobial effects of extracts were presented in Table 1. RB2 extract exhibited certain antibacteria and antifungi activity. The antimicrobial effect on bacteria, with bigger inhibition diameter zone and less MIC value, was better than of fungi. Extract produced optimum antibacterial effect against *Pseudomonas aeruginosa* with 17.6 mm inhibition diameter zone and 1.2 mg mL^{-1} MIC value against *Enterococcus faecalis.* Whereas, extract produced optimum antifungi effect against *C. albicans* with 8.3 mm inhibition diameter zone, and 4.5 mg mL^{-1} MIC value against *Fusarium oxysporum*. Thus, the effects of temperature and pH on antimicrobial activity against *Pseudomonas aeruginosa* and *C. albicans* were studied. The results showed that antimicrobial effect was reduced at high and low temperature (Fig. 6a). Optimum activity against *Pseudomonas aeruginosa* was observed at 30–35°C and 35–40°C against *C. albicans*. On the other hand, antimicrobial effect was better at low pH value. Optimum activity against *Pseudomonas aeruginosa* and *C. albicans* was founded at pH 5, whereas high pH value (8–10) leaded to weak effect (Fig. 6b).

Antioxidant Activity

The antioxidant activity of RB2 extract was tested using the DPPH and FRAP assays. The extract of RB2 exhibited certain antioxidant activity with 30.5% DPPH radicals scavenging effect, 10.2 μg/mL IC50 values and 0.976 μmol/L of FRAP value which were lower than that of chlorogenic acid standard. Furthermore, effects of temperature and pH on DPPH scavenging were studied. The results showed that antioxidant activity of sample was influenced by temperature and pH value. Optimum antioxidant activity of extract was observed at 35°C and pH 5 (Fig. 7a and b). DPPH scavenging of chlorogenic acid was decreased sharply at high temperature (decreased 45% at 50°C) and high pH (decreased 55% at pH 10). However, DPPH scavenging of extract decreased 23% at 50°C (Fig. 7a) and 34% at pH 10 (Fig. 7b).

Discussion

Endophytes of *L. japonicae* were specifically rich in roots and correspond with the hypothesis that endophytes may originate from rhizosphere bacteria (Strobel, 2003). CGA is typically abundant in buds and leaves of *L. japonica* but absent in roots and stems (Cai *et al.*, 2013). However, the

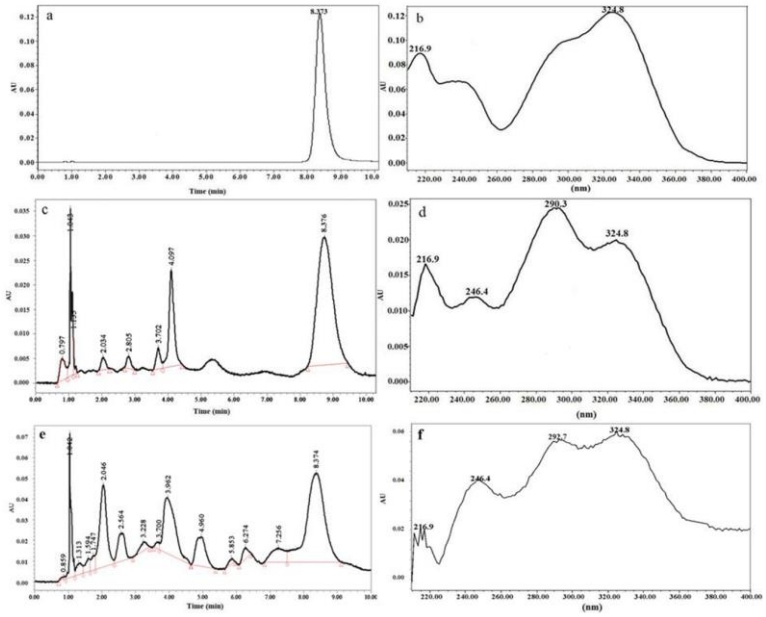

Fig. 1: HPLC chromatogram: retention time and absorption spectrum of authentic chlorogenic acid (**a**, **b**), extract of RB2 (**c**, **d**) and extract of *L. japonicae* (**e**, **f**)

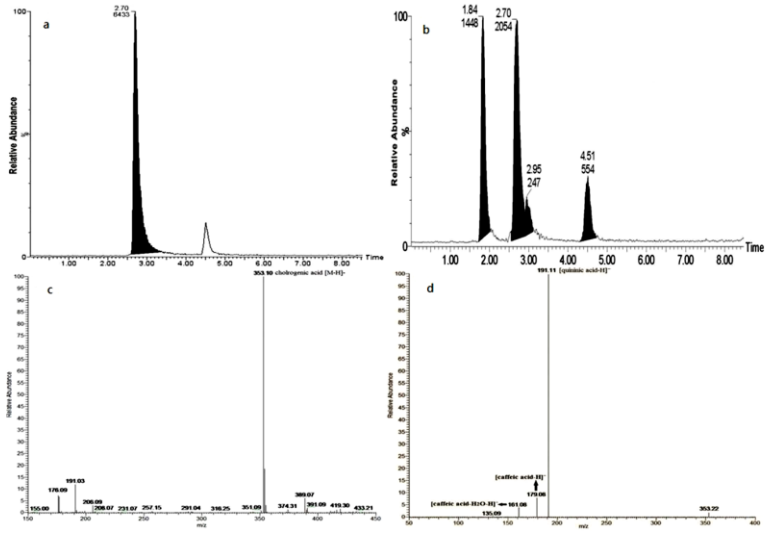

Fig. 2: MRM chromatogram of authentic chlorogenic acid (**a**) and RB2 extract (**b**); Mass spectra at the retention time 2.70 min (**c**, **d**)

distribution of endophytes was opposite to CGA in plant. This is possibly because CGA produced by plants and endophytes transferred to flower and leaf cells to defend against invasion of pathogen.

CGA is synthesized from phenylalanine through phenylpropanoid pathway in plants which is usually accompanied by several isomers and derivatives. In present study, RB2 produced CGA and only one isomer neochlogenic acid. Furthermore, RB2 fermentation broth determined the presence of cinnamic acid and *p*-coumaric acid as intermediates of phenylpropanoid pathway. It is speculated that endophytic bacterium RB2 may have similar pathway with host plant to produce CGA. As a result of long-term symbiosis with plant, endophytes possibly had relevant genes through gene transfer from host plant, thereby forming the same or similar pathway and products (Moore *et al*., 2002).

As an antimicrobial agent, chlorogenic acid increased the outer and plasma membrane permeability and released cytoplasm macromolecules, which led to cell death (Lou *et al.*, 2011). The antimicrobial property of chlorogenic acid was observed in *E. coli* (Kabir *et al*.,

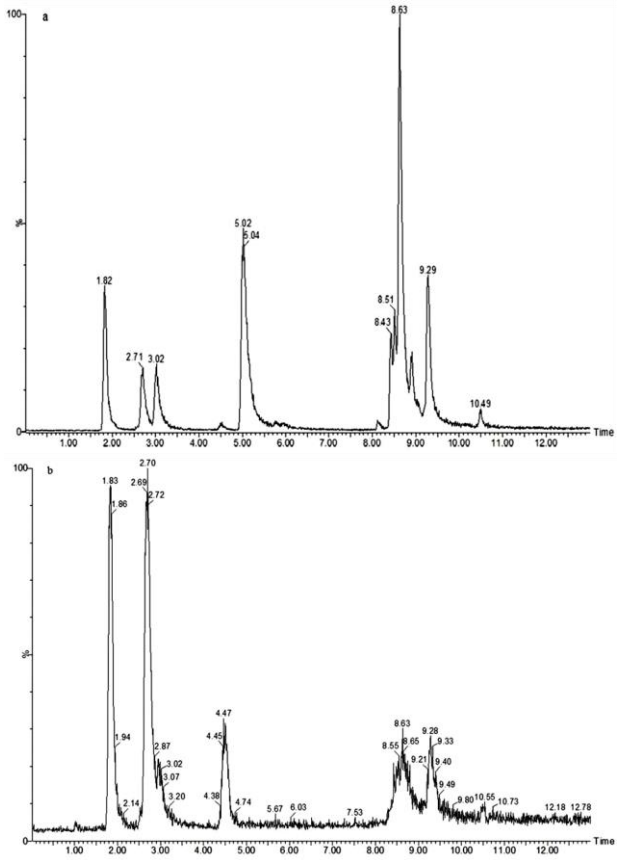

Fig. 3: MRM chromatogram of authentic chlorogenic acid and its isomers and derivatives (**a**) and RB2 extract (**b**)

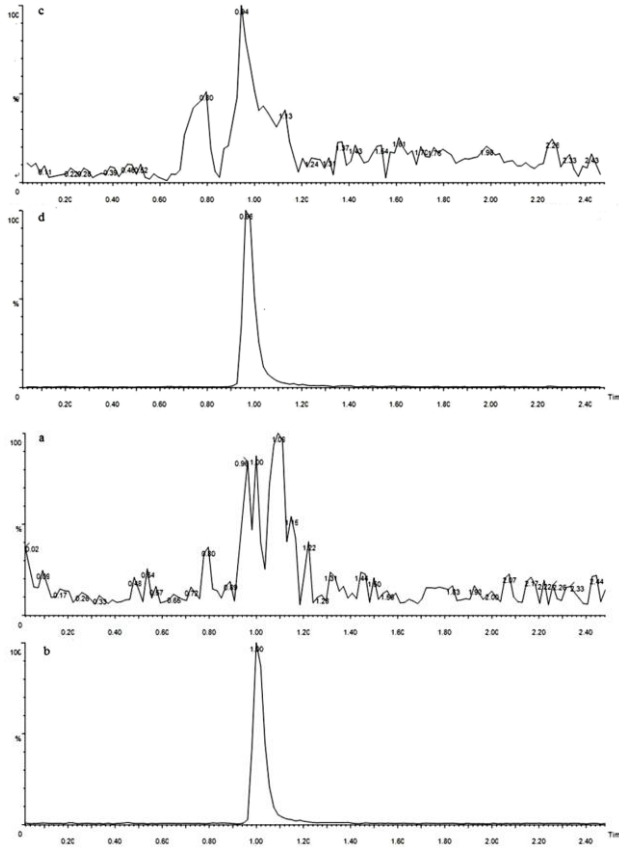

Fig. 4: MRM chromatogram of RB2 extract (**a**, **c**) and chlorogenic acid synthetic intermediate metabolites *p*-coumaric acid (b) and cinnamic acid (d)

2014), *Staphylococcus aureus* (Li *et al*., 2014) and *Stenotrophomonas maltophilia* (Karunanidhi *et al.*, 2012). Chlorogenic acid from different plant sources had different antibacterial spectrum. In previous studies, chlorogenic acid from artichoke or kale exhibited strongest antibacterial activity to *B. subtilis* and *Aspergillus niger* (Zhu *et al.*, 2004), or *Moraxella catarrhalis* (Ayaz *et al*., 2008) respectively. Comparatively, in present study, extract of RB2 exhibited strongest antibacterial activity to *Pseudomonas aeruginosa* and *C. albicans*. Chlorogenic acid also showed thermal stability and acidic stability (Friedman and Jürgens, 2010). Thus, antibacterial property of chlorogenic acid is related to its structural pH and salt concentration (Canillac and Mourey, 2004). Previous study reported optimum pH 4 against *E. coli* (Kabir *et al*., 2014). While in present study, optimum temperature and pH were 35–40°C and pH 5, respectively. High pH and low temperature reduced greatly the effect of extract, while low pH and high temperature reduced effect with small range. Our study accorded with the conclusion of Friedman (Friedman and Jürgens, 2000). The different optimum pH may be the result of different test bacteria.

AS a polyphenol, cholorogenic acid exhibited antioxidant property with FRAP reaction, scavenging DPPH and hydroxyl radical (Urbaniak *et al*., 2017). In some studies, the antioxidant properties of plant extracts, such as *L. japonica* (Wu, 2007) and *Eriobotrya japonica* (Jung *et al.*, 1999), have been shown to come from chlorogenic acid. In our study, extract of RB2 displayed certain antioxidant with more DPPH scavenging than of kale (Ayaz *et al*., 2008) and Chinese hawthorn (Zheng *et al.*, 2018), less than *Eriobotrya japonica* (Jung *et al*., 1999). Interestingly, the antioxidant properties of the extracts were also affected by temperature and pH, which has not been reported in previous studies. There was a large decrease of DPPH scavenging of chlorogenic acid at high temperature and high pH, while DPPH scavenging of RB2 extract decreased not significantly under the same conditions. These may be due to the antioxidant property of derivatives or isomers of chlorogenic acid in RB2 extract (Urbaniak *et al*., 2017).

Conclusion

In present study, an endophytic *Bacillus subtilis* RB2, isolated from root of *L. japonica*, was confirmed to produce CGA like the host plant does. This study highlighted the

Table 1: Antimicrobial activity of extract of endophytic bacterium RB2

Test organism	Inhibition diameter zone (mm)	MIC (mg /mL)
Bacteria		
Enterococcus faecalis	9.4	1.2
Escherichia coli	16.4	1.5
Pseudomonas aeruginosa	17.6	3.0
Salmonella typhimuriu	12.4	2.5
Staphylococcus aureus	8.5	3.4
Fungi		
Candida albicans	8.3	6.0
Fusarium oxysporum	6.3	4.5
Mucor racemosus	7.2	7.3
Phytophthora infestans	7.5	5.0
Rhizoctonia solani	6.5	6.0

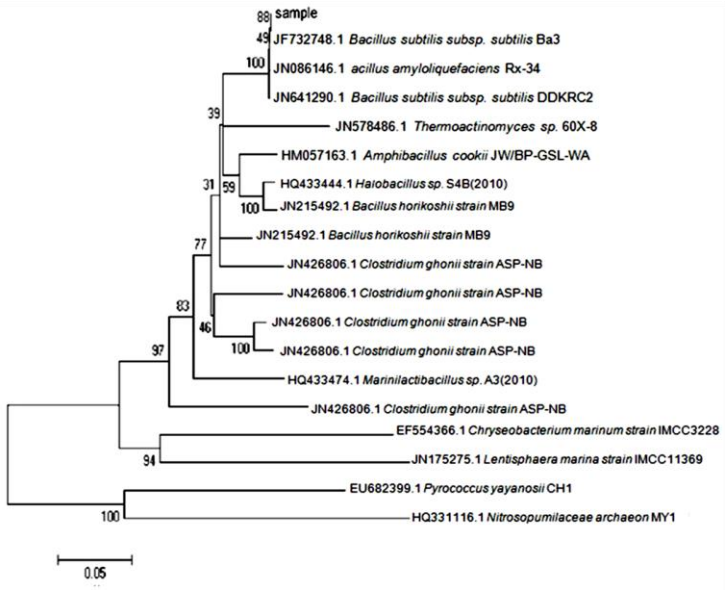

Fig. 5: Phylogenetic tree of the endophytic bacterium RB2

antimicrobial and antioxidant properties of RB2 extract. Therefore, RB2 was expected to be an alternative source of CGA. Further works on genes of CGA synthesis pathway in RB2 and optimizing fermentation condition to produce CGA were required.

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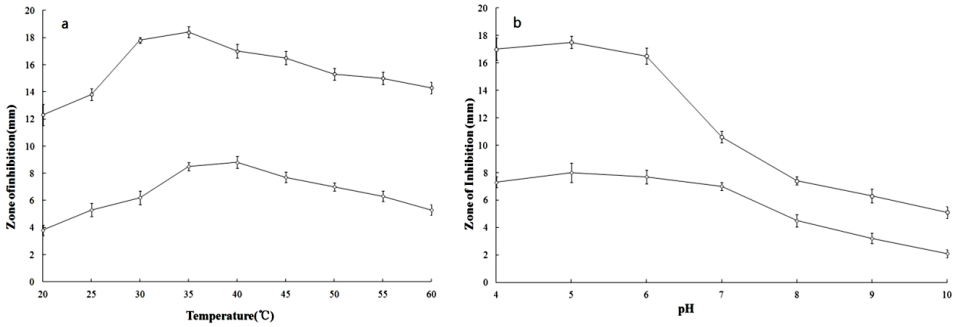

Fig. 6: Effects of temperature (**a**) and pH (**b**) on antimicrobial activity of RB2 extract

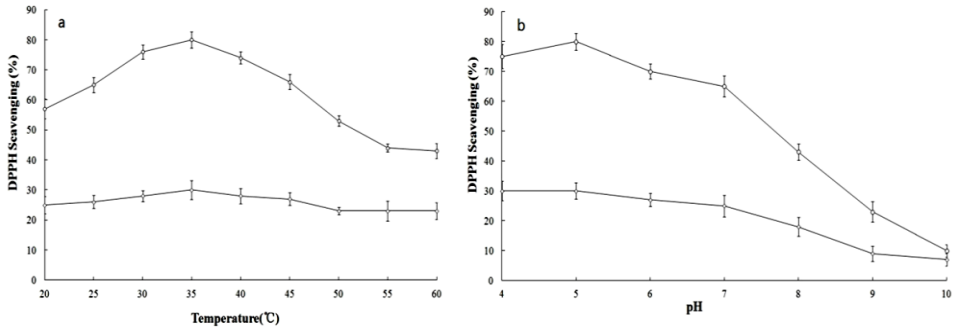

Fig. 7: Effects of temperature (**a**) and pH (**b**) on antioxidant activity of RB2 extract

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