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Table: 2, Figs : 5

Screening and identification of harmful bacteria associated with the accumulation of cinnamic acid in Wuyi Rock tea soil

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ABSTRACT

Continuous cropping soil samples of Wuyi rock tea for 8, 14, 20, 26, and 32 years were collected to identify the harmful strains of bacteria, which could accumulate cinnamic acid in rhizosphere soil. The *Priestia megaterium* BW16 bacteria was isolated and identified by morphological identification and phylogenetic analysis. The cinnamic acid production study showed that it transformed the L-phenylalanine into cinnamic acid and resulted in its accumulation in high concentration (4 g/L) within 7 d when supplemented with 1 % L-phenylalanine as sole carbon source. The optimum conditions for L-phenylalanine ammonia-lyase enzyme activity were found to be incubation temperature 35 °C, pH 7.0, and time allowed for enzyme reactivity was 20 min. The results showed that *P. megaterium* BW16 bacteria is responsible for the transformation of phenylalanine into cinnamic acid and may be a harmful bacteria for soil, which differs from some previous studies. It is helpful to further screen the antagonistic microorganism's application for tea soil and improve the soil ecological system.

Key words: Cinnamic acid, continuous cropping, 16S rRNA gene sequencing, harmful bacteria, planting years, rhizosphere soil, tea soil.

INTRODUCTION

Wuyi rock tea is one of the Chinese ten famous teas (17). Its export was 170 billion yuan in 2020 i.e., 22 % of the total agricultural income in Wuyishan City. However, in recent years, with the increasing planting area and planting years of Wuyi rock tea, long term perennial root cropping of tea has restricted the development of tea industry (9,14,15). The phenolic acids cause above phenomenon and cinnamic acid derivative and benzoic acids (*p*-hydroxybenzoic acid and 3,4 Dihydroxybenzoic acid) are allelopathic (1,28,32). Cinnamic acid and its derivatives are the model substances in many allelopathy studies and commonly accumulates in tea garden soil, which causes soil nutrients imbalance, salt accumulation, soil acidification and changes in the soil microbial community structure (13,29). Generally, the secondary metabolites (phenols and fatty acids) are produced by the acetic acid and shikimic acid pathways in plants (20). These secondary metabolites are easily transformed to metal chelates in soil and are absorbed by plant roots and caused the allelopathic effects (21). However, in addition to plant physiological metabolism, the microbial metabolism also causes cinnamic acid accumulation in soil. Earlier studies showed that *Escherichia coli* (25), *Rhodosporidium toruloides* (31), *Streptomyces lividans* (26) and *Pseudostellariae heterophylla* (34) high abundance in soil could convert L-phenylalanine into cinnamic acid through phenylalanine pathway, i.e. microorganisms are also the vital factor for the accumulation of cinnamic acid in crop soil. Thus, balancing the

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microbial community structure in soil is the key to improve the tea quality and yield. However, metagenomics research has focused on the harmful microorganisms and few studies have been done on the microorganisms that causes the accumulation of cinnamic acid in tea soil (13). Whether the existence of these harmful microorganisms will further deteriorate the tea soil, needs to be studied.

In this study, samples of Rougui tea soils with 8, 14, 20, 26 and 32 planting years were collected. Our previous metagenomics research and KEGG pathway analysis showed that the 3-dominant microorganisms (*Fusarium oxysporum, Priestia megaterium* and *Streptomyces lividansso*) increases the cinnamic acid in tea soil (9). Their pathway analysis showed the potential to increase the cinnamic acid. We screened and identified these microorganisms, then detected their transformation ability and related enzyme activities by adding L-phenylalanine as carbon source. Our studies showed that Bl17 was the main group of these microorganisms that accumulated a large amount of cinnamic acid in the soil samples of 20 and 32 years. This study aimed to screen and identify the key bacteria strain responsible for accumulation of cinnamic acid in tea rhizosphere soil.

MATERIALS AND METHODS

Sample Collection

Five soil samples (8, 14, 20, 26 and 32 years) were collected from Rougui tea plantation in Xing village, Wuyishan City, Fujian Province (latitude: 27°32′ N, longitude: 117°54′ E, altitude, 450-550 m, annual mean temperature:16-18°C and annual rainfall, 2000 mm during August 2021. Five-spot-sampling method (23-35 cm depth and 15-25 cm radius) was used to collect the rhizosphere soil. Part of samples were packed in sterile polybags and stored at 4 ^{o}C in refrigerator until use. Remaining sample was air-dried, crushed and sieved through 25-mesh sieve and was sub-sampled by the coning and quartering method.

Soil Physico-chemical properties

Soil pH, organic matter, available N and P were determined according to methods described in Jia *et al.* (9).

Spread-plate method for cinnamic acid-producing Bacteria Isolation

According to the previous metagenome analysis (9), the Luria-Bertani medium (1 L distilled water with 1 % tryptone, 0.5 % yeast extract, 1 % NaCl, 15 g agar) was used to screen the harmful bacteria. Normal saline (0.90 % NaCl) was used to dilute the soil sample, all samples were individually diluted 10 times with 10 mL saline solution, then the sample was diluted into 10^{-4} concentrations by gradient dilution. Then, the diluted soil suspension of each sample (0.1 ml) was spread on sterile Luria-Bertani medium plates, and nutrient agar plates aseptically in an airtight workhouse. The plates were incubated at 37 °C for 24 h and all colonies were picked up and transferred to the conical flask containing 100 mL LB medium and incubated at 37 °C for 24 h. Then, the cells were harvested by centrifugation in cold centrifuge for 10 min at 8,500 rpm. The cells were collected in 250 mL flask and resuspended in MSM (mineral salt medium) with 2 % L-Phenylalanine to an initial OD of 0.8 (1.2×10^{7} CFU•ml^{-1}). The MSM medium comprised of 0.015 g $CaCl_2$•$12H_2O$, 0.422 g KH_2PO_4, 0.244 g $(NH_4)_2SO_4$, 0.05 g $MgSO_4$•$7H_2O$, 0.375 g K_2HPO_4, 0.054 g NH_3•Fe•Citrate, 0.015 g NaCl and 0.05 g tryptone in 1 L of distilled water of pH 7.0. The cinnamic acid content was determined 7 d after incubation. The maximum cinnamic acid-producing bacteria was picked up for identification.

Morphological Identification of isolates

Bergery's Manual was used as a standard to identify the cinnamic acid producing bacterial isolates (4). Gram reaction was performed by Gram staining. Different biochemical tests- oxidase, fermentation of glucose, fructose, and sucrose and was Vogues Proskauer test, Indole production, Arginine dihydrolase, Gelatin liquefaction, Catalase, Amylase and Nitrate reduction tests were performed (4).

Molecular identification by 16S rDNA gene sequencing

The genome DNA was extracted following the instructions of the manufacturer by using the Gel Extraction Kit D2500 (Omega, China). The 16S rRNA gene was amplified by polymerase chain reaction (PCR) from the genomic DNA of the strain using universal primers, 7F (5′-CAGAGTTTGATCCTGGCT-3′) and 1540R (5′-AGGAGGTGTCCAGCC GCA-3′). The PCR conditions were as under: Initial denaturation at 94 °C for 3 min, followed by 30 cycles with denaturation at 94 °C for 1 min each, annealing at 55 °C for 1 min, extension at 72 °C for 2 min, and final elongation at 72 °C for 10 min. The amplified product was checked for size and purity on 1 % (w/v) agarose gel. PCR products were sequenced through commercial services provided by Sangon Biotech Co., Ltd. (Shanghai, China). The sequence homology analysis and the homologous sequence obtained was used for Online BLAST of NCBI (24). The MEGA10.0 was used to construct its phylogenetic tree using the Maximum-likelihood model (11).

Cinnamic acid Production

Cinnamic acid formation by strain BW16 was determined by growing cells in MSM liquid medium with the 0.2 %, 0.5 % and 1 % L-Phenylalanine at 150 *ca* for 37 ºC for 72 h. The content of cinnamic acid, L-phenylalanine and biomass was determined every 24 h.

Enzyme Assay

P-hydroxybenzoate hydroxylase is an intracellular enzyme, to obtain crude enzymes extract, cells were cultured aerobically in the LB medium at 37 °C. After the OD_{600} reached at 0.8, the cells were harvested and resuspensions in buffer were sonicated on the ice-water bath and centrifuged at 23,000×g for 10 min. The supernatant (cell crude extracts) was filtered through 0.22 µm microporous filter. The crude enzyme extract was collected and stored at 4 ºC till determination of enzyme activity.

The temperature, pH, and time affected on the reaction was measured in 2.5 mL reaction system (RS) containing 1 mL crude enzyme solution and 1.5 mL of 1.0 % substrate solution. The effects of temperature and pH on the p-hydroxybenzoate hydroxylase was studied by incubating the reaction mixture for 30 min in water bath maintained at 25,30,35,40,45 and 50 °C. For pH effect the reaction mixture contained buffer having pH of 3.0,4.0,5.0,6.0,7.0,8.0,9.0 and 10.0 at 35 ℃ for 30 min. The stability test for the reaction was estimated by allowing the enzyme reaction for 5,10,15,20,25,30, 35 or 40 min at 35 ℃.

Chemical and Statistical analysis

The cell density was monitored at a wavelength of 600 nm by using spectrophotometry. Phenol was determined by standard colorimetric assays at a wavelength of 510 nm, Ultraviolet spectrophotometry was used to determine the Cinnamic acid and its derivatives at a wavelength of 268 nm (12).

Statistical analysis

Statistical significance was determined by one-way analysis of variance (ANOVA) followed by LSD's multiple comparison test and Pearson correlation test, and significance was ascribed at $p < 0.05$. All tests were repeated at least three times and with three parallel treatments.

RESULTS AND DISCUSSION

Soil Physico-Chemical Properties

The soil physico-chemical properties did not much differ after 8- and 14-years' tea plantation ($p > 0.05$, Table 1). After 20 years planting, the pH was significantly decreased, while the available N and organic matter increased ($p < 0.05$) i.e. favourable, growth and development of tea plants (Table 1). However, our previous study showed that the growth of tea plants and tea quality did not improve under this condition, due to negative effects of cinnamic acid concentrations (9). It indicated that cinnamic acid might a limit factor for tea tree growth.

Cinnamic acid contents in rhizosphere soils

The cinnamic acid content in rhizosphere soil samples of tea plantations of 8, 14, 20, 26 and 32 years were determined, and the results are presented in Table 1. The concentration of cinnamic acid increased with the increase of cultural years, the maximum cinnamic acid concentration was 24.55±0.74 mg per kg soil in 32-year soil sample which was significantly higher than other samples ($p < 0.05$). The least abundant was the soils of 8 years (9.74±0.22 mg per kg soil). The concentration of cinnamic acid in 32-year soil was significantly differed from 8,14, 20, 26-year soil ($p < 0.05$), which was 1.3-2.2 times that of 14, 20, 26-year soil and 2.5 times that of 8-year soil (Table 1).

Table 1. Physico-chemical properties of soils with different tea planting years

Soil Chemical properties	Soil planted years				
	8	14	20	26	32
pH	4.78±0.13a	4.56±0.13a	4.31±0.02b	4.18±0.03b	3.91±0.42b
Organic matter ($g \cdot kg^{-1}$)	11.87±0.34a	12.25±0.38a	14.22±0.23b	15.52±0.42b	16.01±0.37b
Available N ($mg \cdot kg^{-1}$)	124.11±2.18a	125.37±3.11a	127.38±3.86b	131.75±3.22b	132.44±6.38b
Available P ($mg \cdot kg^{-1}$)	12.22±0.31a	12.54±0.26a	12.88±0.19a	12.78±0.28a	12.91±0.44a
Cinnamic acid ($mg \cdot kg^{-1}$)	9.74±0.22a	12.24±0.45b	14.32±1.13b	18.88±0.44c	24.55±0.74d

Means standard error (SE) from three replications for each determination.

Screening the harmful bacteria from Rougui tea soil

Sixteen bacterial colonies showing differences in morphological and colony characteristics were selected and picked up from plates inoculated with rhizosphere soil samples of different periods of tea cultivations. Sixteen selected bacterial colonies were transferred individually into broth medium for extending culture and preservation. Then, these cultures were inoculated in MSM medium containing 2 % L-phenylalanine for detecting the cinnamic acid production, respectively. Results presented in Fig. 1 show that the three bacterial isolates namely, BW02, BW07, and BW16 produced cinnamic acid. However, the cinnamic acid accumulation by BW16 was significantly higher than other strains (p <0.05) which ranged between 3.0 and 4.3 times than BW02 and BW07, respectively (Fig. 1).

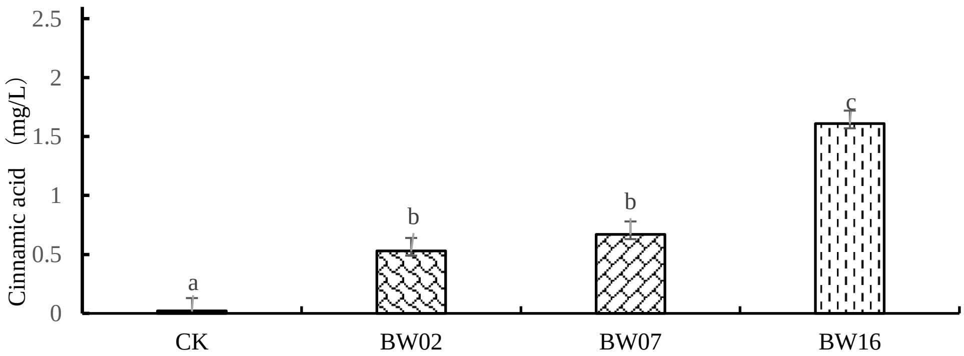

Figure 1. Cinnamic acid accumulation by three strains in MSM medium containing 2 % L-phenylalanine.

Morphological and molecular identification of harmful bacteria

The morphology and biochemical characteristics of strain BW16 are shown in Table 2. The colony morphology of the isolate was round with white color, and most of its cells were arranged in pairs under light microscope (Fig. 2a, b). Biochemical characteristics showed it was Gram-positive bacilli in singles, oxidase-positive, glucose fermenting, fructose fermenting, sucrose fermenting, and was Vogues Proskauer negative, Indole, Arginine dihydrolase, Gelatin, Catalase, Amylase and Nitrate reduction test was positive. The PCR production of 16S rDNA fragment was 1621 bp from Strain BW16 (Fig. 3a). The 16S rDNA ML-tree was divided into three main groups and each group is divided into subgroups (Fig. 3b). ML-tree analysis demonstrated that Strain BW16 belonged to *P. megaterium* (3) and had the highest indent with *P. megaterium* (99 %) which revealed similarity with *P. megaterium* (3).

The rhizosphere soil is the vital environment, where the plant-microbe interactions play major role in plant growth and health (2,6). It is noteworthy that as planting years increased, the soil quality, plant nutrients status, plant resistance, and the benefit of microorganism decreased significantly in the rhizosphere soil environment (30). Then, the population of harmful microbes and their interactions with plants further declines the tea yield and quality. The soil bacteria such as *Burkholderia cepacia* (22), *Xanthomonas axonopodis* (23), *Streptococcus faecalis* (8) and *Pectobacterium athrosepticum*was (28) are important plant pathogens. However, the abundance of these disease-causing microbes in tea soil was less (9). Besides, the studies on screening and identifying the main harmful bacteria in tea soil were rare, and most these studies focused on the benefit bacteria (18,33). Thus, the mechanism of harmful bacteria effects on tea plants remains to be further studied.

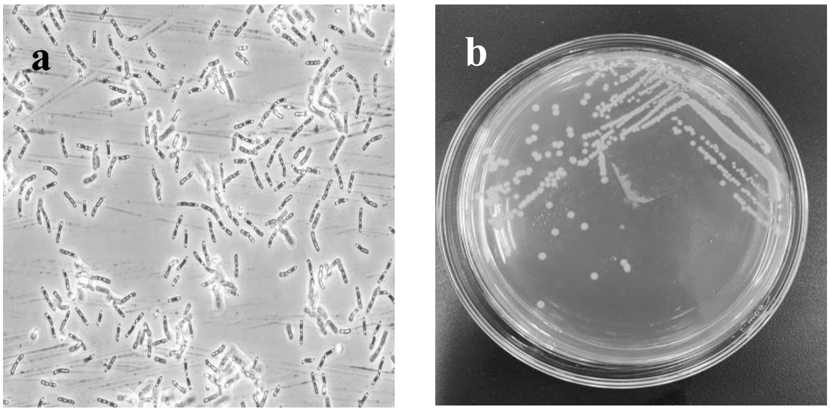

Figure 2. Morphological features (a) and round with white color of strain BW16 on broth medium (b).

Table 2. Physiological and biochemical characteristics of strain BW16.

Characteristic features	Results	Characteristic features	Results
Gram's staining	+	Voges-Proskauer	-
Yellowish pigment	-	Indole	+
Glucose utilization	+	Arginine dihydrolase	+
Fructose utilization	+	Gelatin	+
Sucrose utilization	+	Nitrate reduction	+
Oxidase	+	Penicillin	-
Catalase	+	Carotenoids	-
Amylase	+	Lipoidase	-
Growth in 4°C	-	Growth in 41°C	-

Note: “+” stands for positive and “-” for negative.

Production of cinnamic acid by BW16

Use of phenylalanine in cinnamic acid production by strain BW16 was shown in Fig 4. Fig. 4a showed that the biomass did not increase in each group when phenylalanine was added as sole carbon source. It indicated that BW16 strain could not use phenylalanine for its growth. The phenylalanine decreases rapidly in 7 d with the production of cinnamic acid in each group. The cinnamic acid increased, when phenylalanine was added to the medium, the highest cinnamic acid concentration was 4 g/L which was observed at 1 % phenylalanine supplement ($p < 0.05$). The decrease in biomass and disappearance of phenylalanine in the medium was accompanied by the accumulation of cinnamic acid in BL1, BL2, BL3 group during 7 d cultivation (Fig. 4a, 4c). The result indicated that BW16 did not use cinnamic acid or phenylalanine as sole carbon source (Fig. 4a), and strain BW16 accumulated and excreted significant amounts of cinnamic acid into the medium.

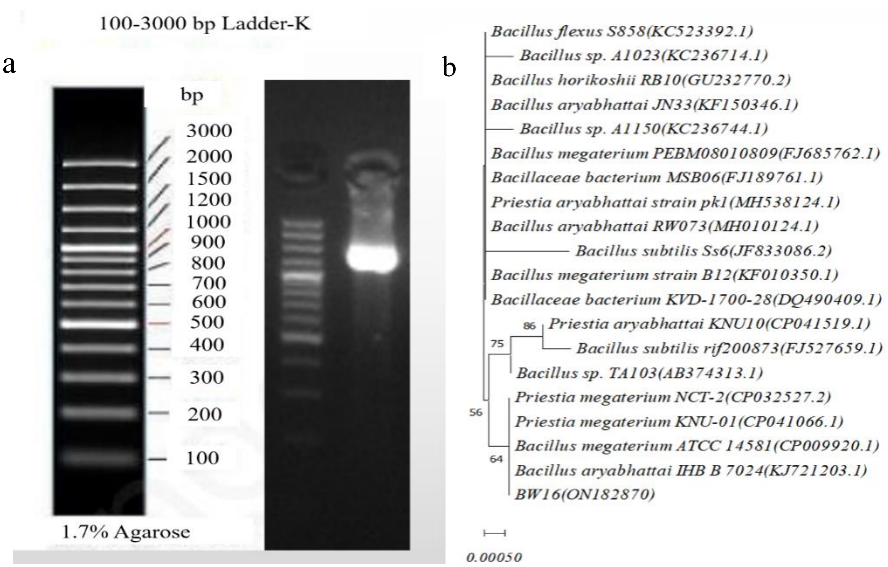

Figure 3. Electrophoresis of PCR products of strain BW16 (a) and phylogenetic tree for the strain BW16 and related bacterial strains (b).

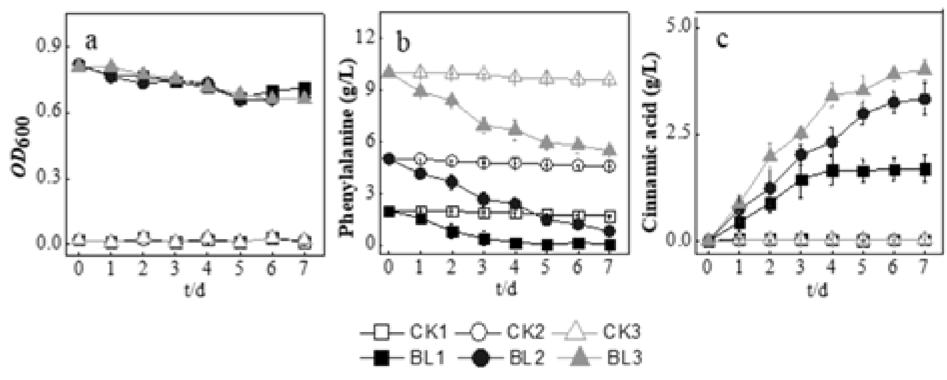

Figure 4. Growth in MSM with L-phenylalanine as sole carbon source (a); the L-phenylalanine utilization (b) and cinnamic acid product (c) of BW16. BL1, BL2 and BL3 represent the MSM containing bacterial suspension was supplemented with 0.2 %, 0.5 % and 1 % L-Phenylalanine, respectively. CK1, CK2 and CK3 represent the MSM without bacteria was supplemented with 0.2 %, 0.5 % and 1 % L-Phenylalanine, respectively.

Conversion of L-phenylalanine to cinnamic acid by BW16

BW16 plays an important role in converting L-phenylalanine to cinnamic acid, resulting in its accumulation at higher doses in the environment for a long incubation time of 7 days. Cinnamic acid has been widely used in health care treatment and biology and has relaxing, anti-bacterial and anti-inflammatory functions (6). Although low levels of cinnamic acid secreted and accumulated by plant roots promotes the plant growth and inhibits the growth of harmful bacteria, concentrations > 0.1 mmol L^{-1} limits plant growth and developmental (5). The concentration of cinnamic acid in sagebrush succession soils is usually higher than 0.1 mmol/L^{-1} (9). The cinnamic acid not only affects the diversity and structure of microbial communities in soil, but also promotes the growth of harmful bacteria, such as *Fusarium oxysporum* and *Phytophthora* (9). The cinnamic acid has the potential to selectively enhance specific microbial communities (especially soil-borne pathogens) and lead to the development of communities with different qualitative and quantitative composition (27). This study showed that the abundance of BW16 increased with the year of planting, which may have led to the accumulation of high concentrations of cinnamic acid.

Properties of phenylalanine ammonia lyase (PAL)

Results presented in Fig. 5a showed the optimum temperature for PAL test, where the relative enzyme activity was highest at 35 ℃, reaching 100 %. The effects of pH on enzyme production at 35 ℃ (Fig. 5b) showed that the enzyme had the highest activity at pH 7, and its enzymatic activity gradually decreased as the pH increased to 8. At pH 7 and 35 ℃, the enzyme activity was high from 0 to 20 min, after which it decreased rapidly, reaching a peak of 91 % at 20 min (Fig. 5c).

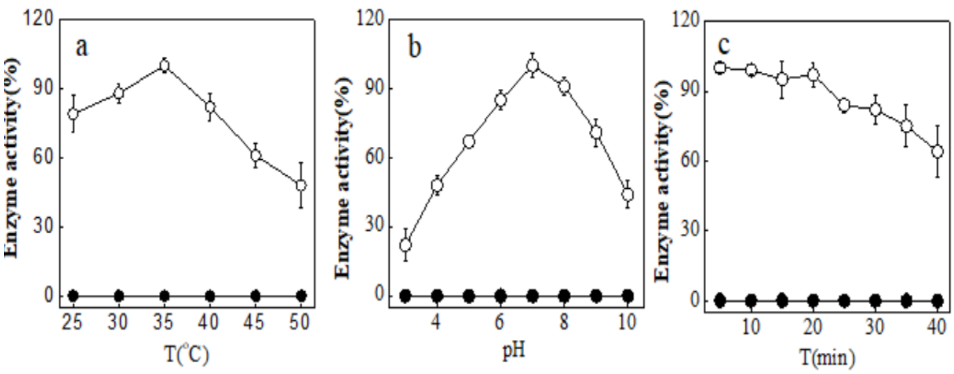

Figure 5. Effects of Temperature (a), pH (b) and incubation time (C) on enzyme production by BW16 in enzyme buffer containing L-phenylalanine.

The accumulation of cinnamic acid in soil is mainly derived from plant PAL, an enzyme widely present in many higher plants with the ability to catalyse the non-oxidative deamination of L-phenylalanine to cinnamic acid and to produce phenylpropanoid metabolites through committed steps (20). However, while enzymes from plants usually only convert L-phenylalanine to cinnamic acid, microbial PALs differ in that they can also produce p-hydroxycinnamic acid by catalysing tyrosine, as well as convert L-phenylalanine to cinnamic acid (10). The crude enzyme assay of BW16 showed that it had the activity to convert L-phenylalanine to cinnamic acid. The highest enzyme activity was found at 35 ºC and pH 7, which was close to the actual tea rhizosphere soil environment and conditions might benefit the strain BW16 to grow and accumulate cinnamic acid. These results were consistent with the study that the growth of *Burkholderia* sp1 was stimulated by addition of a certain concentration of phenolic acids, which was similar to the result that *P. heterophylla* (18) rhizosphere abundance was increased with the phenolic acids accumulation in soil. However, it implied that its biocontrol mechanism may be partly due to its ability to accumulate and tolerate high concentrations of cinnamic acid. Although many studies have shown that *Bacillus.* sp. has a great potential application in plant biocontrol, growth promotion and resistance (18,28), which showed it was beneficial microorganism for most soils. On the contrary, *P. megaterium* might be a harmful microorganism in tea soil, which lead to a high cinnamic acid accumulation.

CONCLUSIONS

We isolated strain BW16, with high cinnamic acid production from the rhizosphere soil of Rougui tea tree and was morphologically and molecularly identified as belonging to the genus *Bacillus*. The abundance of BW16 increased with the year of cultivation, which leads to the accumulation of cinnamic acid in the soil. The crude enzyme of BW16 had the ability to convert L-phenylalanine to cinnamic acid and was most active at 35 ^{o}C and pH 7, which is close to the environmental conditions of tea tree rhizosphere soil. The results provided a basis for further studies on the mechanism of action of pathogenic bacteria, and subsequent to select the antagonists to inhibit the propagation of pathogens, thus effectively reducing the accumulation of cinnamic acid in soil.

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DECLARATION

We declare that all authors of this Ms. have made substantial contributions. We have not excluded any author that substantially contributed to this Ms. We have followed our ethical norms established by our respective institutions.

CONFLICT OF INTEREST

The authors announce that they have no conflict of interest.

ETHICAL APPROVAL

The authors declare that the study was carried out following scientific ethics and conduct. However, this study did not involve any use of animals, hence no ethical approval has been obtained from the concerned committee.

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