

Antibacterial Activity and Tannin Tolerance of *Bacillus* spp. Isolated from Leaves of Miang (*Camellia sinensis* (L.) Kuntze var. *assamica* (J.W. Mast.) Kitam.)

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Abstract: Sixteen *Bacillus* isolates were obtained from leaves of Miang plant (*Camellia sinensis* (L.) Kuntze var. *assamica* (J.W. Mast.) Kitam.) collected from Miang gardens in Chiang Mai and Phrae provinces of Thailand. All bacterial isolates were identified within 3 species including *B. licheniformis*, *B. siamensis* and *B. tequilensis* based on 16S rRNA gene sequence. The culture broth of *B. siamensis* ML122-2, ML123-1 and ML124-1 could inhibit growth of *Staphylococcus aureus* ATCC 25923 and methicillin resistant *S. aureus* DMST 20625 while *B. licheniformis* ML071-1, ML073-1, ML075-1 and ML076-2 could inhibit growth of *B. cereus* TISTR 687 and *S. aureus* ATCC 25923 with the inhibitory value ranging between 242.4 - 363.6 and 265.2 - 340.9 AU/ml, respectively. Moreover, *B. siamensis* ML122-2 could tolerate tannin, 1% (w/v). Accordingly, *B. siamensis* ML122-2, ML123-1 and ML124-1 and *B. licheniformis* ML071-1, ML073-1, ML075-1 and ML076-2 may involve in biological control of Miang fermentation process.

Key words: Biocontrol, fermentation, Miang, peptide, tea.

1. Introduction

Bacillus species is a bacterium in the family Bacillaceae that is defined as a Gram-positive, rod shape and endospore forming bacteria that can be obligate aerobe or facultative anaerobe. Endospore formation makes them resistant to nutritional and environmental stresses [1]. *Bacillus* plays a vital role in many aspects including decomposition, pathogenicity and industries [2]. It can be found in environment such as soil, dust, aquatic ecosystem, vegetation, foods as well as gastrointestinal tracts of animals [3]. Some species are harmful such as *B. cereus* and *B. anthracis*, which have been reported as the causative agents of food poisoning and anthrax disease, respectively [4]. In contrast, some species of *Bacillus* are widely used in food industry such as *B. clausii*, *B. coagulans*, *B. licheniformis* and *B. subtilis*, which have been previously reported as probiotic bacteria [5], [6].

“Miang” or Assam tea (*Camellia sinensis* (L.) Kuntze var. *assamica* (J.W. Mast.) Kitam.) is a local plant in northern Thailand. For centuries, local people have collected Miang leaves to make “Fermented Miang”, a kind of chewing refreshment product. Previous studies found that lactic acid bacteria (LAB) such as *Lactobacillus plantarum*, *L. casei*, *L. pentosus*, *L. panthesis* and *Leuconostoc mesenteroides* play an important role in Miang fermentation [7], [8].

LAB and *Bacillus* spp. have been reported to possess antibacterial activity against closely related strains as well as some pathogenic bacteria involving *Bacillus* spp., *Staphylococcus* spp. and *Listeria* spp. [3], [9]. Some reports demonstrated that *Bacillus* spp. can produce antimicrobial peptide, a kind of antimicrobial substance generally produced by ribosome synthesis, which usually refers to bacteriocins or bacteriocin-like inhibitory substances (BLIS).

Therefore, *Bacillus* spp. isolated from Miang leaves that exhibited some bioactive properties may demonstrate the relationship between microorganisms found in fermented Miang and on Miang leaves during Miang fermentation. This study aims to evaluate some biological properties of *Bacillus* spp. isolated from Miang leaves in order to rule out the role of the microbes on Miang fermentation process.

2. Methodology

2.1. Collection of Miang Leaves

Miang leaves were harvested from the area of northern Thailand including Chiang Mai (18°47'N 98°59'E) and Phrae (18°8'N 100°8'E) provinces. Each Miang leaf was soaked in sterile NaCl, 0.85% (w/v), prior to isolation of bacteria within 24 hours.

2.2. Isolation of *Bacillus* spp.

Each sample of Miang leaf was spread on tryptic soy agar (TSA) (Merck, Germany) prior to incubation at 37°C for 24-48 hours. The representative colonies with characteristic of *Bacillus* species such as Gram-positive, rod shaped and endospore forming were selected and re-streaked on TSA before storage at -20°C in tryptic soy broth (TSB) (Merck, Germany) containing 20% (v/v) of glycerol.

2.3. Identification of *Bacillus* spp.

All bacterial isolates were identified using morphology and biochemistry determination including Gram stain, endospore formation, catalase and oxidase production. The molecular identification was accomplished by 16S rRNA gene sequencing. Briefly, the chromosomal DNA of each isolate was extracted according to the method of Pitcher *et al.* [10] with some modifications. The 16S rRNA gene was amplified by polymerase chain reaction (PCR) in a thermal cycler (Labcycler, SensoQuest, Germany) using universal bacterial primers, 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3') [11]. The amplification reactions were performed following the method of Schulze-Schweifing *et al.* [12]. The PCR products were purified and sequenced by a sequencing service (First BASE Laboratories Sdn Bhd., Malaysia). The 16S rRNA gene sequences were investigated by comparing with GenBank and EzBioCloud databases, and phylogenetic tree was analyzed using neighbor-joining method [13] with a MEGA 7 software [14].

2.4. Production of Antimicrobial Substances

Bacteriocins or BLIS production was performed according to modified method of Zendo *et al.* [15]. Each *Bacillus* strain was cultivated in TSB and TSB supplemented with 1% (w/v) of CaCO₃ in order to neutralize acidity arising from organic acid produced. The initial pH of both media was adjusted to 7. The cultures were subsequently incubated at 37°C for 48-72 hours. Each bacterial culture was centrifuged at 4°C 6,000 rpm for 5 mins prior to filtration using nylon syringe filter, pore size 0.45 µm. The filtrates were kept at -20°C.

2.5. Determination of Antibacterial Activity by an Agar Well Diffusion Method

The test pathogenic bacteria including *B. cereus* TISTR 687, *Escherichia coli* ATCC 25922, *E. coli* O157:H7 DMST 12743, methicillin resistant *S. aureus* DMST 20625, *Salmonella* Typhi DMST 22842, *Shigella*

dysenteriae DMST 1511, *S. aureus* ATCC 25923 and *Vibrio cholerae* DMST 2873 were cultivated in Mueller Hinton Broth (MHB) (Merck, Germany) at 37°C for 24 hours. Each culture broth was adjusted its turbidity equivalent to a No. 0.5 McFarland standard prior to swab onto Mueller Hinton Agar (MHA) (Merck, Germany).

Each filtrate prepared in 2.4 was tested against pathogenic bacteria mentioned above by an agar well diffusion method according to the method of Gómez-Sala *et al.* [16]. The swabbed agars were punctured by Pasteur pipettes to obtain small wells with diameter of 6 mm each. Afterwards, the *Bacillus* filtrate, 33 µl, was added into each well. Gentamicin (Sigma-Aldrich, USA), 0.1 and 50 mg/ml (MRSA), were used as positive control. All test plates were incubated at 37°C for 24 hours. The inhibitory clear zone was observed and measured using a Vernier caliper. The antagonistic activity in arbitrary unit/ml (AU/ml) was calculated according to the equation below [17]:

$$\text{Arbitrary activity units (AU/ml)} = \frac{\text{Diameter of the zone of inhibition (mm)}}{\text{Volume taken in the well (}\mu\text{l)}} \times 1000$$

2.6. Tannin Tolerant Determination

Each bacterial isolate was cultured on TSA supplemented with tannic acid, 1-3% (w/v). The tannic acid solution was prepared by adjusting the pH to 7.0, filtering through 0.45 µm filter and sterilization prior to addition into the melted TSA (1% final concentration). Growth of each isolate was observed subsequent incubation at 37°C for 48 hours [18].

3. Results

3.1. Isolation and Identification of Bacteria from Miang Leaves

Ten Miang leaves were collected from two sampling sites including Chiang Mai (Code ML07) and Phrae (Code ML12) provinces between March 2016 and March 2018. Miang leaves were harvested at an altitude of more than 500 m above sea level.

Sixteen bacterial isolates showed different colony morphologies of *Bacillus*. All isolates presented Gram-positive, rod shape, endospore forming, catalase and oxidase positive. Miang leaves harvested from Chiang Mai demonstrated a maximum of two different morphological *Bacillus* colonies. Afterwards, all isolates were identified using 16S rRNA gene analysis. The isolated bacteria were identified as *B. tequilensis*, *B. siamensis* and *B. licheniformis*, which had the similarities of 16S rRNA gene related to their type strain between 99.2 and 99.9%. The phylogenetic tree comparing the 16S rRNA gene sequence between each *Bacillus* strain and their closed species is shown in Fig. 1.

3.2. Determination of Antibacterial Activity

The culture filtrate of *B. siamensis* ML122-2, ML123-1 and ML124-1 could inhibit growth of *S. aureus* ATCC 25923 and MRSA DMST 20625 with the inhibitory clear zones ranging between 8.0 and 12.0 mm (242.4-363.6 AU/ml) while all *B. licheniformis* isolated could prohibit growth of *B. cereus* TISTR 687 and *S. aureus* ATCC 25923 with clear zone of inhibition from 9.0 to 11.3 mm (265.2-340.9 AU/ml). In contrast, the culture filtrates of all *B. tequilensis* could not inhibit test pathogenic bacteria. The pH of culture broths were ranging between 6.86 and 7.42. However, antibacterial activity of each *Bacillus* isolate cultured in TSB supplement with 0.1% (w/v) of CaCO₃ was decreased. *B. licheniformis* strains could only inhibit growth of *B. cereus* TISTR 687 and *S. aureus* ATCC 25923 with the inhibitory clear zones from 8.8 to 9.8 mm (265.2-272.7 AU/ml). The pH of culture broths were ranging between 7.16 and 7.71 (Table 1 and Fig. 2). For

positive control, gentamicin demonstrated zone of inhibition against test pathogenic bacteria between 9.4 and 16.0 mm (284.8-484.8 AU/ml).

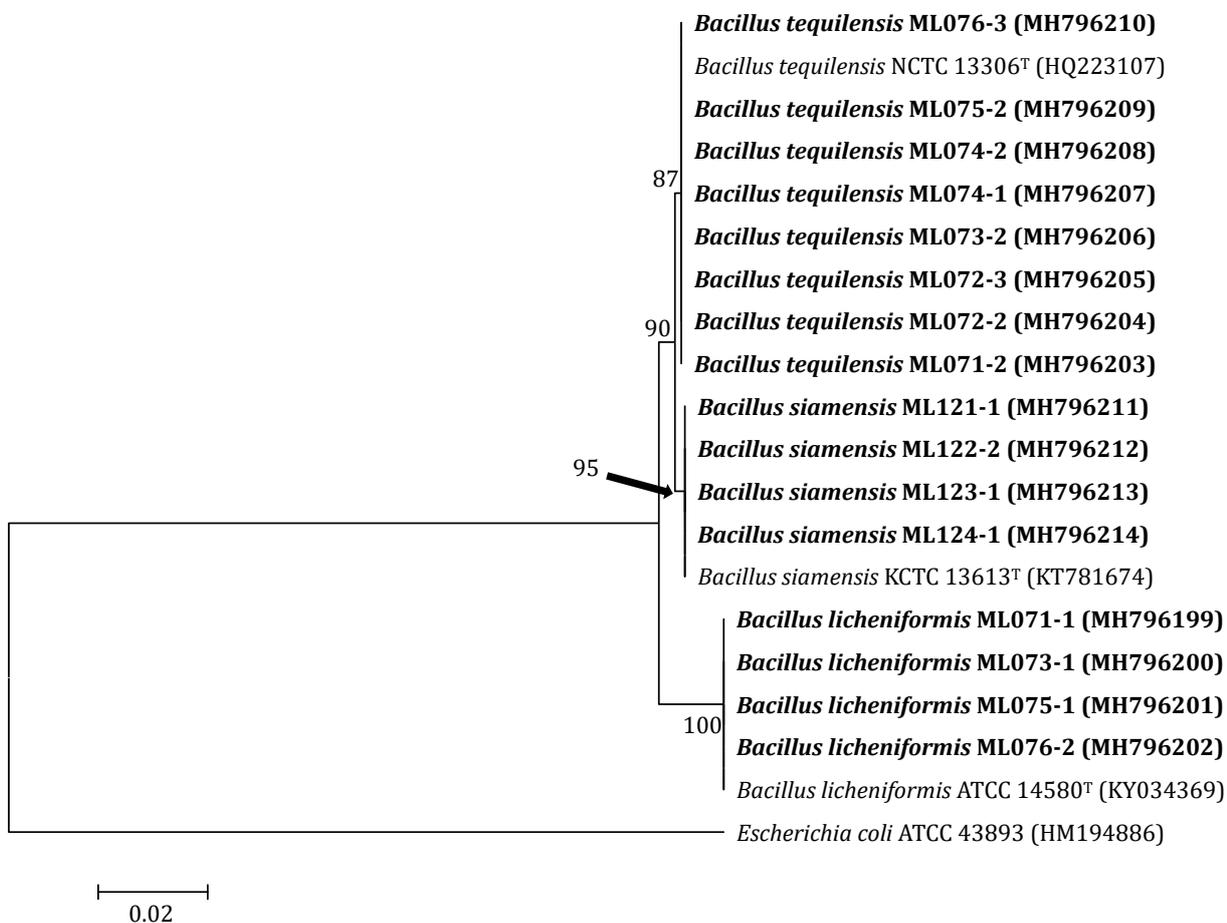


Fig. 1. Phylogenetic tree represented the relationship of each isolate with their type strain. The branching pattern was established using the neighbour-joining tree based on the 16S rRNA gene. Bootstrap values above 50%, based on 1,000 replications, are shown at the nodes. Bar, 0.02 substitutions per nucleotide position. *Escherichia coli* ATCC 43893 (HM194886) is presented as outgroup sequence.

Table 1. Effect of *Bacillus* Culture Filtrates on Test Pathogenic Bacteria

Culture medium	<i>Bacillus</i> strain	Culture pH	Arbitrary activity units (AU/ml)		
			<i>B. cereus</i> TISTR 687	<i>S. aureus</i> ATCC 25923	MRSA DMST 20625
TSB	ML071-1	7.03±0.02	280.3±10.7	0	0
	ML073-1	7.02±0.00	295.5±10.7	0	0
	ML075-1	7.12±0.01	340.9±10.7	272.7±0.0	0
	ML076-2	6.86±0.01	333.3±0.0	0	0
	ML122-2	7.42±0.00	0	242.4±0.0	363.6±0.0
	ML123-1	7.36±0.00	0	0	280.3±10.7
	ML124-1	7.36±0.00	0	0	340.9±10.7
TSB + 0.1% (w/v) CaCO ₃	ML071-1	7.64±0.00	272.7±27.7	0	0
	ML073-1	7.71±0.00	295.5±26.2	0	0
	ML075-1	7.69±0.01	268.9±7.6	265.2±10.7	0
	ML076-2	7.58±0.00	265.2±8.7	0	0

Data were expressed as mean \pm standard deviation (n=3), the initial pH value of TSB and TSB containing 0.1% (w/v) CaCO₃ media were 7.03 \pm 0.01 and 7.16 \pm 0.00, respectively.

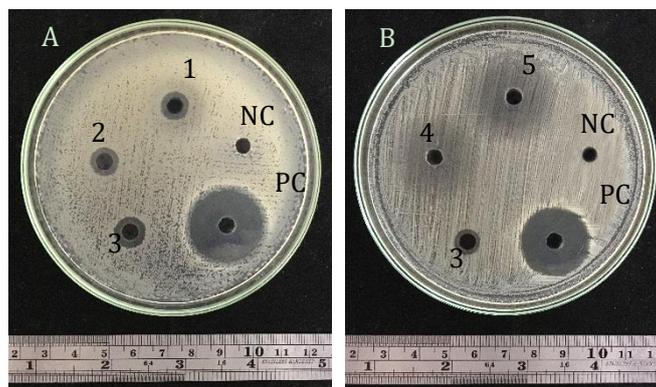


Fig. 2. Zone of inhibition of test *Bacillus* strain cultivated in TSB supplemented with 0.1% (w/v) CaCO₃ against *B. cereus* TISTR 687 (A) and *S. aureus* ATCC 25923 (B); ML071-1 (1), ML073-1 (2), ML075-1 (3), ML122-2 (4), ML121-2 (5), TSB + 0.1% (w/v) CaCO₃ (NC) and 0.1 mg/ml gentamicin (PC).

3.3. Evaluation of Tannin Tolerance

Only *Bacillus siamensis* ML122-2 could grow on the medium containing 1% (w/v) of tannic acid. The bacterium was growth inhibited when concentration of tannic acid was higher. Growth of *B. siamensis* strains on TSA containing tannic acid are presented in Fig. 3.

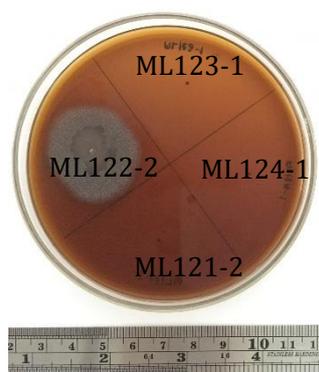


Fig. 3. Growth of *Bacillus* strains on TSA supplemented with 1% (w/v) of tannic acid.

4. Discussion

The genus *Bacillus* is widely distributed in the environment [19] including Miang plant. *Bacillus* spp. have been found to play a vital role in health and economy such as *B. cereus* is the cause of food poisoning while *B. subtilis* has been reported as probiotic bacteria [5]. Besides, *Bacillus* spp. also have the potentiality to produce plant growth promoter and biological substances to inhibit harmful insects and germs [20]. In terms of antimicrobe, *Bacillus* spp. could produce antimicrobial substances including peptide and lipopeptide antibiotics, and bacteriocins [3]. Smitha and Bhat [21] found that *B. licheniformis* BTHT8 produced bacteriocin BL8 that could inhibit growth of closely related species such as *B. cereus* and *Clostridium perfringens*. Similarly, *B. subtilis* SN7 was reported to produce bacteriocin, a broad-spectrum activity against pathogenic bacteria [22]. Recently, *B. siamensis* was reported to produce BLIS, a

broad-spectrum antibacterial activity and it was considered as a probiotic bacterium to control bacterial infection in fish farming [23]. However, the antibacterial activity of *Bacillus* isolates was decreased after growing in the medium supplemented with CaCO₃. Zendo *et al.* [15] suggested that Ca²⁺ suppress bacteriocin production. Besides, Ca²⁺ can react with cell membrane that leads to loss of bacteriocin secretion ability.

Accordingly, *B. siamensis* and *B. licheniformis* isolated from Miang leaves may involve in Miang fermentation by inhibiting the growth of food spoilage microbes. Moreover, *Bacillus* spp. has also been reported to enhance growth of probiotics such as *Lactobacillus* [24]. *Bacillus* spp. can produce endospore, which helps them to resist to heat and acid. Additionally, the tannin tolerance also enhances their ability to withstand tannin-rich substrate especially in Miang fermentation process.

5. Conclusions

Bacillus spp. found on Miang leaves can inhibit growth of some pathogenic bacteria, narrow or broad-spectrum properties depend on species of *Bacillus*, by the release of the antimicrobial substances. They have ability to tolerate tannic acid which is abundant in Miang leaves. Generally, fermented Miang contains mixed microbial cultures mainly lactobacilli which are probiotics. This report reveals that *Bacillus* spp. found on Miang leaves may not only function as biological control during Miang fermentation but also act as probiotics which will be further elucidated.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

PR and NT designed the experiments and analyzed the data. PR isolated and identified microorganisms from Miang leaves. PR and NT prepared and edited manuscript.

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