

Characterization of bacterial endophytes from Myanmar medicinal plants for antimicrobial activity against human and plant pathogens

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This research aimed to investigate the antagonistic activity of the bacterial endophytes from Myanmar medicinal plants. Thirty-one bacterial isolates were isolated from Myanmar medicinal plants: *Tinospora cordifolia* (Wild.) Miers., *Catharanthus roseus* G. Don., *Tectona hamiltoniana* Wall. and *Boscia variabilis* Collett & Hemsl. (Capparaceae). Dual culture and agar well diffusion methods were used for antimicrobial assay. One endophyte from *Catharanthus roseus* and two bacterial isolates from *Boscia variabilis* Collett & Hemsl. (Capparaceae) had not only the antibacterial activity towards the human pathogenic bacteria but also the antifungal activity against the plant pathogenic fungi. From 16S rRNA sequencing, one strain from *Catharanthus roseus* G. Don. was *Bacillus amyloliquefaciens* DSM7 and two antagonistic strains from *Boscia variabilis* Collett & Hemsl. (Capparaceae) were *Bacillus subtilis* subsp. *subtilis* str. 168 and *Bacillus amyloliquefaciens* DSM7, respectively. The best medium for the maximum production of the bioactive compounds was *Bacillus* medium supplemented with the 4% of starch and 0.3% of peptone for *B. amyloliquefaciens* DSM7 and *B. subtilis* subsp. *subtilis* str. 168 showed the maximum antimicrobial compounds production when it was incubated in the medium amended with 3% of starch and 2% of peptone. The optimum conditions for the the maximum production of the antimicrobial compound were the medium pH of 6 at 35 °C after two days of incubation for *B. amyloliquefaciens* DSM 7 and *B. subtilis* subsp. *subtilis* str. 168 secreted the maximum concentration of the bioactive compounds at pH 7.5 and 35°C on second day incubation period. In conclusion, the isolated endophytic bacteria showed the strong antimicrobial activity towards the pathogenic microbes and they could be used in medicine and agriculture as well.

Keywords: Antibacterial. Antifungal. Culture conditions. Endophytes. Medicinal plants.

INTRODUCTION

Myanmar belongs to with enormous biodiversity of medicinal plants. Among them, *Tinospora cordifolia* (Wild.) Miers., *Catharanthus roseus* G. Don., *Tectona hamiltoniana* Wall. and *Boscia variabilis* Collett & Hemsl. (Capparaceae) have a wide array of bioactive principles and they have been proven medicinally important plants.

Endophytes are the microorganisms that live inside the plant tissues and they have the activity which can suppress the growth of human pathogenic bacteria and plant pathogens. They are symbiotic microbial organisms

that inhabit the interior of plants without causing an apparent harm to the host (Bandara, Seneviratne, Kulasoorya, 2006). Endophytes belong to diverse groups of bacteria and fungi (Hirsch, Braun, 1992). Because natural selection favors the evolution of beneficial endophytic strains, several endophytes were found to secret secondary metabolites that protect plants against insect pests, pathogenic organisms as well as herbivores (Saikkonen *et al.*, 2004), thus, endophytes represent a promising source of novel, biologically active metabolites for pharmacological and agricultural applications (Dreyfuss, Chapela, 1994; Schulz *et al.*, 2002). Biochemical research revealed that a wide variety of natural products can be obtained from endophytic microbes (Ahmed *et al.*, 2012). Natural products from endophytic fungi were observed to inhibit many

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pathogenic organisms including bacteria, fungi, viruses and protozoans.

Medicinal plants are gaining global attention because the herbal drugs are most effective, easily available and with negligible side effects. The world population uses the medicinal plants in health care with homeopathy. It is important to note that some of the endophytic microorganisms can produce the same secondary metabolites as that of the plant thus making them a promising source of novel compounds (Ahmed *et al.*, 2012). Although it has been known for a long time, their significance becomes evident only more recently when it was shown that they play specific roles as for instance, protecting the plant hosts against pathogens. Many endophytes have antimicrobial activity. (Seo *et al.*, 2010) found that some endophytic bacteria isolated from young radishes can be used as biocontrol agents against human and plant pathogens.

Low yield, fungal attack, pathogenic microbial attack and biotic or abiotic stress are often the problems faced in agriculture sector both in developing and developed countries. The beneficial properties of endophytes can be exploited to tackle the issues related to cultivated crop plants and to provide further benefit in agricultural sector (Rosenblueth, Martínez-Romero, 2006).

Researchers have been studying the endophytic bacteria and fungi from the medicinal plants for the last many years. However, the antimicrobial activity of the endophytic bacteria from Myanmar medicinal plants used in this experiment has not yet been sufficiently researched. To our knowledge, this is the first study to use Myanmar medicinal plants mentioned here for evaluating the antimicrobial activity of the endophytic bacteria against the plant pathogens.

MATERIAL AND METHODS

Sample collection from some medicinal plants

Firstly, the leaf, bark and the stems of *Tinospora cordifolia* (Wild.) Miers. (Family: Menispermaceae, Genus: *Tinospora*), *Catharanthus roseus* G. Don. (Family: Apocynaceae, Genus: *Catharanthus*), *Tectona hamiltoniana* Wall. (Family: Lamiaceae, Genus: *Tectona*) and *Boscia variabilis* Collett & Hemsl. (Capparaceae) (Family: Closteroviridae, Genus: *Boscia*) were collected from Kankaw, Kyaukse, Myanmar. Healthy plant leaf samples were selected and taken in order to avoid contamination by plant pathogens. Collected plant leaf samples were brought to the laboratory within 24 hours and processed for microbial isolation following guidelines

of (Nalini *et al.*, 2005). The surface decontamination method described by (Fisher, Petrini, Scott, 1992) was used with some minor changes. Firstly, they were washed with tap water and were immersed in 70% ethanol for 1-3 minutes, following soaking in aqueous 70% sodium hypochlorite for 3-5 minutes. And then, the parts of the plants were sterilized in 70% ethanol for 2-5 minutes and finally rinsed with sterilized distilled water for 5 times. After all these steps, the plant samples were dried under aseptic condition (40 °C in hot air oven) and kept for the continuous use.

Isolation of the endophytes from the selected medicinal plants

For isolation of the endophytic bacteria, the sterilized plant parts were rolled many times on one part of the culture plate and it was kept as the positive control and negative control was also kept without any swab to know whether the surface sterilization was complete or not. They were incubated on the various media such as nutrient medium, *Bacillus* medium, starch casein agar (SCA), water agar and Tap Water Yeast Extract medium (TWYE) at 35 °C for two weeks. The colonies that appeared around the plant samples were picked up with a sterile needle. And then, the collected bacterial isolates were sub-cultured on the medium to get the pure colonies. The resulting pure isolates were kept in a sterile 20% glycerol solution at -80 °C for further study.

Production of the hydrolytic enzymes of the bacterial endophytes

Amylase activity was determined in well-established plate assay based on the appearance of clear zones around the bacterial isolates which were incubated on nutrient medium containing 1% starch. After 24 h of incubation at 35 °C, the plate was flooded with iodine solution which allowed the visualization of clear halos around the colonies (Kim, Yang, Kim, 2003)

The production of cellulase enzymes was assayed on indicator plates containing 0.5% carboxymethylcellulose (CMC). Plates were incubated at 35 °C for 72 h and then they were stained with 1 g/L Congo red solution for 30 min and were extensively washed with 0.9% NaCl. The formation of clear zones around the colonies indicated CMC degradation (Palumbo, Kobayashi, 2000).

The protease activity was studied by the nutrient medium containing 1% gelatin, following the spraying of the plate with 1% iodine after overnight incubation to see the clear zone around the colony. And, esterase activity was investigated on nutrient medium supplemented with

tween 80 and the positive result was regarded as the appearance of a halo zone around the colony after 3 days of incubation (Mehmood *et al.*, 1999).

Study on antibacterial activity of the isolated bacterial endophytes

The bacterial isolates were screened for the antimicrobial activity against the human pathogenic bacteria such as *Salmonella typhi*, *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis*, *Candida albican*, *Bacillus cereus* and *Pseudomonas aeruginosa* by dual culture and agar well diffusion methods. Firstly, each endophytic bacterium isolate was cultured in SCA broth for two days and the pathogenic bacteria were inoculated in Muller Hinton Broth for six hours respectively. Then, each endophytic bacterial broth culture was centrifuged at 12,000 rpm for 30 minutes to separate the pellet and supernatant. At that time, the pathogenic bacterial broth was swabbed on each Muller Hinton Agar plate and 100 µL of the supernatant was added into each well of the plates which were punched. In this antibacterial assay, tetracycline was used as a positive control and distilled water was used as a negative control. After 24 h incubation period, the inhibition zone was measured and recorded the antimicrobial activity as zone diameter in milliliter. All the experiments were carried out in triplicate and repeated for three times to get the accurate data.

Determination of the antifungal activity of the isolated bacterial endophytes

The endophytic bacterial isolates were investigated for the antifungal activity against the plant pathogenic fungi by dual culture and agar well diffusion methods. Firstly, each endophytic bacterium isolate was cultured in SCA broth for three days and *Rhizotonia solani*, *Fusarium oxysporum* and *Pythium* sp., *Metarhizium* and other isolated plant pathogenic fungi were also inoculated in Potatoo Dextrose Broth for three days respectively. Then, each bacterial broth culture was centrifuged at 12,000 rpm for 30 minutes and the supernatant was collected. The PDA plates were swabbed with the fungal broth and kept to dry for a few minutes. Then, the plates were punched and 100 µL of the supernatant was added into each well of the plates which were swabbed with each pathogenic fungal broth. Nystatin dissolved in distilled water was used as a positive control. After 3 days of incubation at 25 °C, the inhibition zones were measured and recorded. The effective strains which show the antimicrobial activity were selected and continued to study.

Characterization of the potent endophytic bacteria

The three potent bacterial strains were characterized by morphological and biochemical tests according to Bergey's Manual of Systematic Bacteriology (Holt *et al.*, 1986). The genomic DNA was extracted from each of potent bacterial strain and it was amplified by using polymerase chain reaction (PCR). The PCR cocktail consisting of distilled water 4.9 µL, Taq polymerase 0.3 µL, Taq buffer 1 µL, dNTPs 0.8 µL, genomic DNA 1 µL, 10F primer (5' CTACCAGGGTATCTAAT 3') 1 µL and 800R primer (3' AGTTTGATCCTGGCTC 5') was used for amplification of 16S rRNA gene fragment, and the size of the amplified DNA fragment was checked by 1% agarose gel electrophoresis by using 100 bp DNA ladder. Then, the purified PCR products were sent to the company (Macrogen, South Korea) for 16S rRNA sequencing. The alignment and assessment of the gene sequences data were performed by comparing with the sequences available in Gene Bank database of NCBI, using the algorithm BLAST program. Those sequences with highest identity were selected and aligned in the multiple-sequence alignment program, Clustal X (Thompson *et al.*, 1997), and the phylogenetic tree was constructed according to Neighbour-joining method by MEGA 6.0 (Tamura *et al.*, 2013).

Optimum medium for the maximum production of bioactive compounds

Three potent bacterial strains were inoculated into the different media such as Starch Casein Agar, *Bacillus* medium and nutrient agar at 35 °C for two days. Then, they were centrifuged at 12000 rpm for 30 min and 100 µL of each representative cell free supernatant was added into each well of the Muller Hinton agar and PDA plates which were swabbed with the human pathogenic bacteria and plant pathogenic fungi respectively. The antibacterial and antifungal activities were noted as inhibition zone in millimeter (Sierra, 1957).

Incubation time for the optimum antimicrobial compounds production

Each selected potent endophytic bacterium was inoculated in *Bacillus* medium broth at 35 °C for two weeks. At every 24 hr interval, the flasks were harvested daily and they were centrifuged at 12000 rpm for 30 minutes. And then, 100 µL of the supernatant was taken and examined the antibacterial and antifungal activity by agar well diffusion method previously mentioned.

Carbon and nitrogen sources

The isolated strains were incubated into the basal medium (*Bacillus* medium) supplemented with different carbon sources named sucrose, starch, dextrose, glucose and mannitol and various nitrogen sources such as peptone, KNO₃, Casein, yeast extract and (NH₄)₂SO₄. For the optimum concentration of the carbon and nitrogen nutrients, different amounts of 0.15, 0.3, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5 % of each carbon and nitrogen sources were added into the basal medium (Haggag, El Soud, 2012) and they were incubated at 35 °C for three days. And then, the antibacterial and antifungal activities of each collected supernatant were examined by agar well diffusion method.

pH and temperature

For the optimum pH examination, *Bacillus* medium was used with different pH of 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 and the optimum temperature was also studied from the range of 15 to 50 °C at the difference of 5 °C for three endophytic bacteria (Merlin *et al.*, 2013). The antimicrobial and antifungal activities were also studied against human pathogenic bacteria and plant pathogenic fungi by agar well diffusion method. All the experiments were done in three replicates for three times to obtain the accurate and specific data.

RESULTS AND DISCUSSION

Isolation of endophytes from the selected medicinal plants

Total thirty one bacterial strains was isolated from the four medicinal plants. Six bacterial strains were obtained from *Tinospora cordifolia* (Wild.) Miers., another six different strains were discovered from *Catharanthus roseus* G. Don., five potent bacterial endophytes were isolated from *Tectona hamiltoniana* Wall. and fourteen bacterial strains were obtained from *Boscia variabilis*

Collett & Hemsl. (Capparaceae). Although other fungal strains also appeared on the SCA and TWYE media, they were not collected for this experiment because this research was aimed to investigate the antimicrobial activity of the endophytic bacteria.

The hydrolytic enzyme producing activity of microbes

From this experiment, it was found that almost all of the endophytes had the protease and amylase producing activities. But, these isolated bacteria did not show the potent activity for degradation of cellulose and they did not have esterase activity as well. So, it can be assumed that these endophytic bacteria can also be applied in food, detergent, and cosmetic industries that use the protease and amylase enzymes.

Antibacterial activity of the endophytes against the human pathogenic bacteria

After studying the antimicrobial activity of the isolated strains against the human pathogenic bacteria by dual culture method and agar well diffusion method, it was found that all the isolated endophytes did not have the antagonistic activity. From the total thirty one isolated bacteria, one microorganism isolated from *Catharanthus roseus* G. Don. and four bacterial strains from *Boscia variabilis* Collett & Hemsl. (Capparaceae) had the activity which can inhibit the growth of the human pathogenic bacteria and plant pathogenic fungi. As illustrated in Table I, TB3, TM12 and TM13 had the best antibacterial activity among the isolated bacterial endophytes, nearly 20 mm in zone diameter on the plate.

Antifungal Activity of the endophytic bacteria isolated from the medicinal plants

Antifungal activity of the isolated bacteria was also investigated daily by dual culture and agar well diffusion

TABLE I - Antibacterial activity of the isolated bacterial strains against the human pathogenic bacteria (Zone diameter in mm)

Endophytes	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>S. typhi</i>	<i>B. cereus</i>
TB3	17	18	20	20
TM9	10	13	10	15
TM12	18	20	20	20
TM13	18	20	18	20
TM14	12	20	15	20

TABLE II - Antifungal activity of the isolated bacterial endophytes against the plant pathogenic fungi (Zone diameter in mm)

Endophytes	<i>Metarhizium</i>	<i>Pythium</i> sp.	Or 3.2	PF1	PF4
TB3	25	23	25	35	30
TM9	14	15	16	32	15
TM12	26	23	28	32	35
TM13	24	23	20	35	35
TM14	24	20	25	32	35

methods. Among the five antagonistic strains, TB3, TM12, and TM13 showed the greatest antifungal activity against the four target plant pathogenic fungi and the data were illustrated in Table II. These strains had better antifungal activity against the pathogenic fungi isolated from the infected potato (about 35 mm in diameter).

Identification of the endophytic bacteria

Total base nucleotide of 16S rRNA gene sequences was analyzed by multiple alignments using Clustal X of MEGA 6 program. Based on the phylogenetic tree and 16S rRNA sequencing, the nearest homolog genus-species with the beneficial strain isolated from *Catharanthus roseus* G. Don. was found to be 97% similarity to *Bacillus amyloliquefaciens* DSM7 and two bacterial isolates from *Boscia variabilis* Collett & Hemsl. (Capparaceae) had 99% identity with *Bacillus amyloliquefaciens* DSM7 and *Bacillus subtilis* subsp. *subtilis* str. 168 respectively. Their evolutionary relationship with other *Bacillus* species was illustrated in Figure 1.

Cultural optimization of endophytic bacteria for fermentation processes

From this study, it was clearly seen that *Bacillus* medium was the best medium for optimum antimicrobial compound production.

The optimum secondary metabolite production of each selected bacterium was investigated daily for two weeks. All of three strains isolated from Myanmar medicinal plants showed the most efficient antimicrobial and antifungal activities with their growth in *Bacillus* medium and it was seen that the optimum incubation time for the production of the bioactive compounds was two days of incubation for antimicrobial activity and antifungal activity was the optimum after three days of fermentation (Figure 2).

The best carbon and nitrogen sources for the maximum production of the antimicrobial compound were also studied. All the endophytic strains gave the

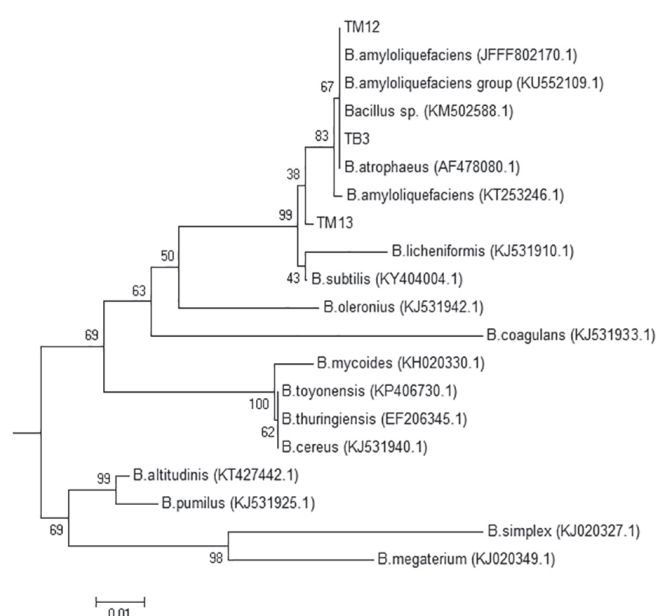


FIGURE 1 - Evolutionary relationships of taxa. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.36747719 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 20 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 363 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

optimum antimicrobial activity against plant and human pathogens when they were inoculated in the basal medium with starch and peptone (Figure 3). From the maximization of the carbon and nitrogen concentration, it was found that 3.5-4 % of starch and 0.3% of peptone for *B. amyloliquefaciens* DSM7 from *Boscia variabilis* was the optimum composition for the production of the antimicrobial compounds and it was shown in Figure 4.

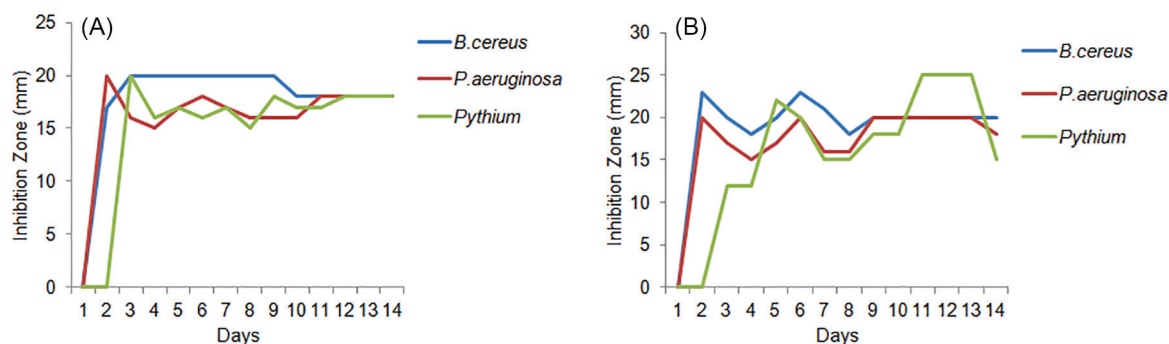


FIGURE 2 - Antimicrobial activity of the bacterial endophytes against *B. cereus*, *P. aeruginosa*, and *Pythium* at different incubation periods (A) *B. amyloliquefaciens* DSM7 (B) *B. subtilis* subsp. *subtilis* str. 168

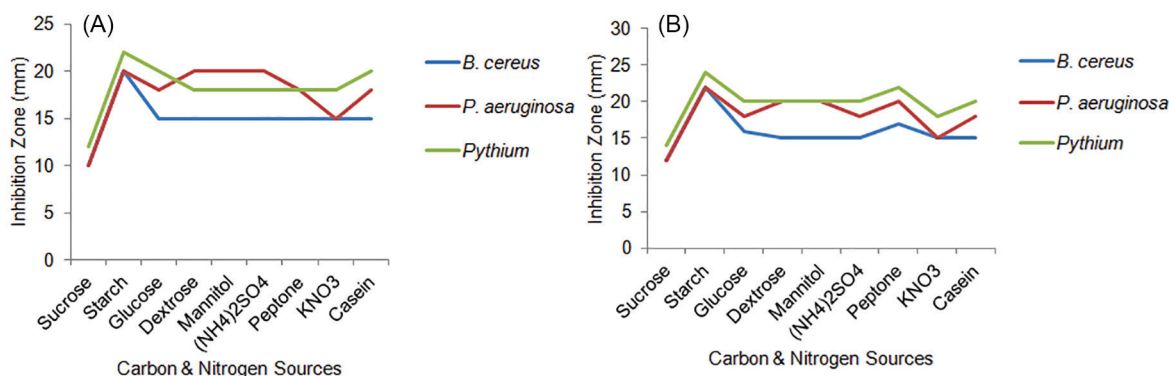


FIGURE 3 - The effect of carbon and nitrogen sources on the antimicrobial activity of the endophytes against *B. cereus*, *P. aeruginosa*, and *Pythium* (A) *B. amyloliquefaciens* DSM7 (B) *B. subtilis* subsp. *subtilis* str. 168

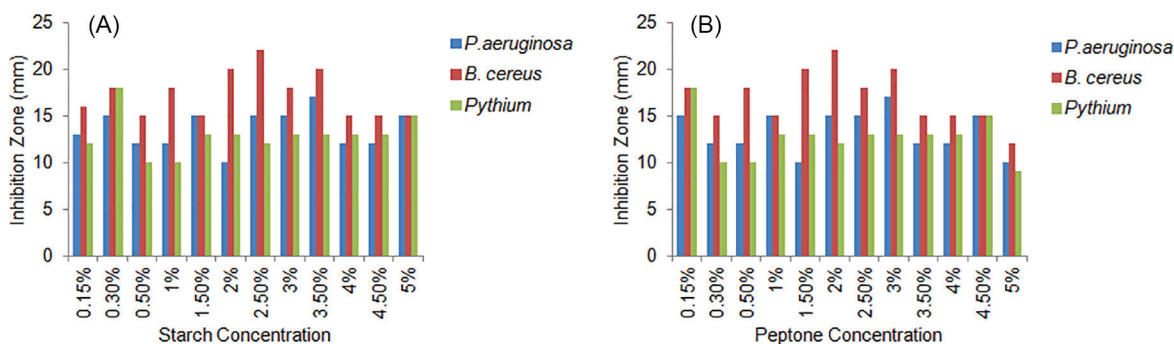


FIGURE 4 - The effect of starch and peptone compositions on the antimicrobial activity of *B. amyloliquefaciens* DSM7 against different pathogens.

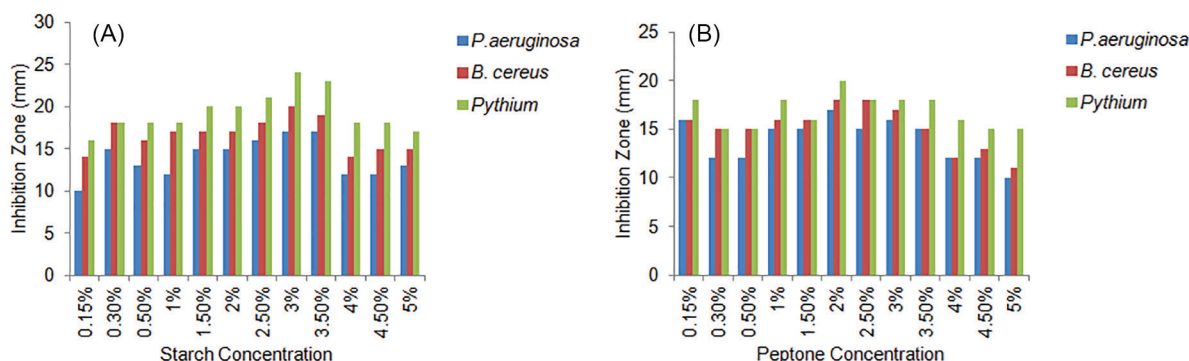


FIGURE 5 - The effect of starch and peptone compositions on the antimicrobial activity of *B. subtilis* subsp. *subtilis* str. 168 against different pathogens.

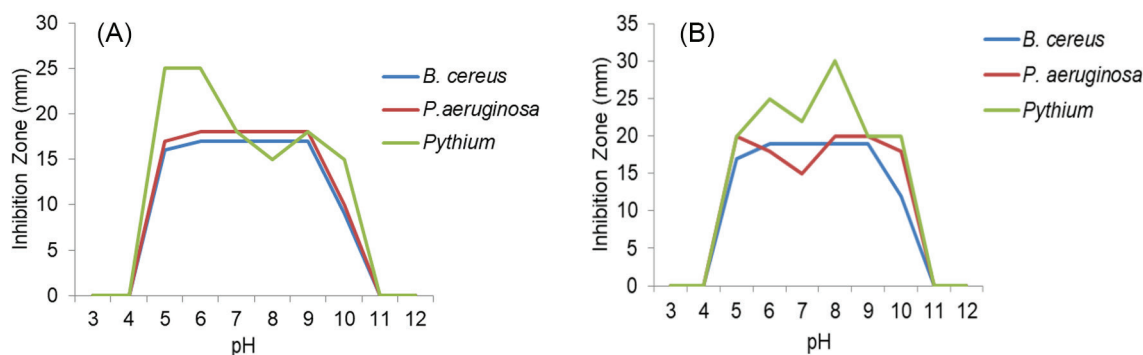


FIGURE 6 - The effect of pH on the antimicrobial activity of *B. amyloliquefaciens* DSM7 and *B. subtilis* subsp. *subtilis* str. 168 against *B. cereus*, *P. aeruginosa*, and *Pythium* (A) *B. amyloliquefaciens* DSM7 (B) *B. subtilis* subsp. *subtilis* str. 168.

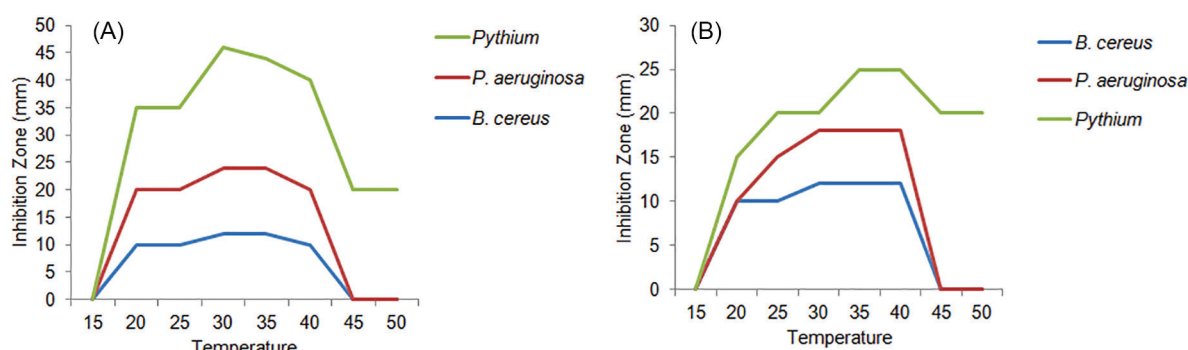


FIGURE 7 - The effect of temperature on the antimicrobial activity of *B. amyloliquefaciens* DSM7 and *B. subtilis* subsp. *subtilis* str. 168 against *B. cereus*, *P. aeruginosa*, and *Pythium* (A) *B. amyloliquefaciens* DSM7 (B) *B. subtilis* subsp. *subtilis* str. 168

And then, *B. subtilis* subsp. *subtilis* str. 168 secreted the maximum amount of secondary metabolites into the medium supplemented with 3% of starch and 2% of peptone respectively (Figure 5).

The effect of pH and temperature on the production of antimicrobial compounds

The optimum pH for maximum production of the antimicrobial compounds by *B. amyloliquefaciens* DSM7 was 7.5 and *B. subtilis* subsp. *subtilis* str. 168 had the strongest antimicrobial activity at pH 6 (Figure 6). Both of these two strains did not show the antifungal activity at pH 3 and 4 and so it could be discussed that the beneficial endophytic strains could not produce the secondary metabolites for antimicrobial and antifungal activities in acidic condition. In concern with the temperature, *B. amyloliquefaciens* DSM7 showed the optimum temperature at 30-35 °C and *B. subtilis* subsp. *subtilis* str. 168 had the optimum antimicrobial compounds producing activity if it was inoculated at 35 °C (Figure 7). Both of these two strains did not show the antimicrobial activity against the human pathogens and plant pathogens at 15 °C incubation because they could not completely grow at this temperature. And,

although both strains did not have the antibacterial activity against the human pathogenic bacteria if they were incubated at 45 °C and 50 °C, they still produced the antifungal compounds at these temperatures.

CONCLUSION

The main conclusion that can be drawn was that two Myanmar medicinal plants named *Catharanthus roseus* G. Don. and *Boscia variabilis* Collett & Hemsl. (Capparaceae) consisted of the effective endophytic bacteria which could secrete the bioactive compounds (secondary metabolites), which can suppress both the human pathogenic bacteria and the plant pathogenic fungi. And, these beneficial endophytic strains could be used not only in medical field but also in agriculture as biofungicide. Since they were isolated from the medicinal plants, it was clearly concluded that they will not give the side effects to humans by using them.

THE AUTHORS' CONFLICT

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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