ISSN: 2279 - 0594

Journal of Biomedical and Pharmaceutical Research

Available Online at www.jbpr.in

CODEN: - JBPRAU (Source: - American Chemical Society)

Volume 5, Issue 1: January-February: 2016, 76-88

Research Article

Biodiversity, enzymatic and antimicrobial activities of bacterial endophytes in selected local medicinal plants

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Received 28February 2016; Accepted 14March 2016

ABSTRACT

Endophytic bacteria existing in healthy medicinal plants of *Ocimumbasilicum, Cymbopogoncitratus*, *Morindacitrifolia*and*Triticumaestivum*were isolated to evaluate their diversity, enzyme production and antimicrobial potentials of their bioactive compounds. The molecular characterization of their 16S rRNA gene indicated 41 isolates to have 98-100% similarity with the respective organisms of 14 genera and 32 species. The dominant genera are *Pseudomonas* and *Bacillus*. Antimicrobial activities of the ethylacetateextracts of their metabolites revealed 20 isolates (48%) that inhibit at least two of the nine pathogens tested with inhibition zone from 7.3 to 15.0 ±0.5 mm. Isolates-*Bacillus mycoides* OBR-1, *Acinetobacterbaumannii* LGL-1, *Sphingomonasyabuuchiae*TAR-1 inhibited at least five pathogens and they are considered to be the most active with zone of inhibition from 7.7 to 15.0 ±0.5 mm. In addition, eight isolates were found to produce all the four enzymes-cellulase, xylanase, amylase and pectinase with *B. cereus* and *B.weihenstenphenensis* to be the most active producers of the enzymes. Also, *C. citratus*was found to have been mostly colonized by *Bacilli* species with high potentials to produce cellulase, amylase, pectinase and xylanase enzymes. Thus, this study revealed bacterial endophytes with ability of producing antimicrobial substances of pharmacological importance.

Key words: Bioactive compounds; 16S rRNA gene; endophytes

INTRODUCTION

Medicinal plants are natural sources for valuable novel bioactive compounds,including their derivatives. In recent years, researchers have explored not only the plants but, the microorganisms harboured by these plants called endophytes. Endophytes are found in the interstitial space of plants without causing any symptoms or apparent harm to the host. Recent studies have shown endophytic bacteria to play an important role in resistance to diseases, promote growth and mediate some beneficial role between the endophyte and its host (1, 2). Endophytic bacteria produce novel metabolites exhibiting a variety of biological activities against different pathogens (3). As a result, endophytes have become increasingly popular for isolation and characterization of several bioactive compounds (1, 4). Recently, anticancer enzyme, L-asparaginase and taxol-anticancer drug were isolated from endophytes(5-7). Preveena and Bhore (8) identified several bacterial endophytes with antibacterial activities from the medicinal plant *Tridaxprocumbens* Linn., which has wound healing properties. Hence, a number of researchers have suggested the hypothesis that endophyte-host plant association, particularly with medicinal plants, may have influenced the ability of endophytes to produce similar beneficial compounds as the host plant. Also, questions such as “What are the possible associations between microbiota and plants?” and “What are they doing there and how do they respond to environmental changes and interact with each other?” have often been proposed and attempts to provide answers to these questions are on-going(9).

Consequently, many plants have been explored for their different endophyte communities. It has been estimated that there is approximately 300,000 plant species on earth, and each

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individual plant could potentially host a large number of endophytes(9). The diversity of endophytes is evident as proposed by Loh, Tan (10)in which 71 different endophytic bacteria were isolated from 1055 plants species. Lilley, Fry (11) further isolated 23 different genera of endophytic isolates from sugar beet plant. In our separate study, we discovered twenty-nine culturableendophytic bacteria species from *Aloe vera* plant (12). To further validate the hypothesis, four local medicinal plants (*Ocimumbasilicum, Cymbopogoncitratus*,*Morindacitrifolia* L. and *Triticumaestivum*) were selected for endophyte profiling and to establish their valuable properties.

Ocimumbasilicum, commonly known as basil, originates from India is used widely in Indonesian cuisines as a flavouring. *In vitro* studies have established that lipid compounds in basil have potent antioxidant, antiviral, as well as antimicrobial properties.In India, it is traditionally used for supplementary treatment of stress, asthma and diabeteswith recent studies revealing its essential oil showing antifungal and insect-repelling properties (13). *Cymbopogoncitratus* (Lemon grass), is another medicinal plant with known medicinal properties. *In vitro* studies using leaf extracts showed cytoprotective, antioxidant, anti-inflammatory as well as antifungal properties (14). The essential oil from this plant, citronellol, has antihypertensive properties and is known to be active against dermatophytes such as *Trichophyton-mentagrophytes*, *T. rubrum*, *Epidermophyton-floccosum*and *Microsporumgypseum*(15). The third medicinal plant selected for this study, *Morindacitrifolia* L., belonged to the family *Rubiaceaea*, and is known locally as 'mengkudu' or 'Noni'. *M. citrifolia*has wide range of uses in traditional medicine, having been used as a treatment for dysentery, heartburn, high blood pressure, cancer, as well as for respiratory infections and tuberculosis (16). Also, *T. aestivum* (wheatgrass) extracthas been found to possess superoxide scavenging and ferric reducing power(17) and ability to inhibit oxidative DNA damage has also been demonstrated by Falcioni (18).This study reports our findings of the population structure of endophytic bacteria in *O. basilicum*, *C. citratus*, *M. citrifolia* and *T. aestivum*, and the evaluation of their enzymatic and antimicrobial potential of pharmacological importance.

MATERIALS AND METHODS

Isolation of endophytic bacteria:

The medicinal plants (asymptomatic plants) were collected from Sungai Buloh horticulture field area (3.235283 N, 101.568342 E), Selangor, Malaysia. Five plants each were dug neatly from different points and transferred into sterile biosafety bags and brought to the Microbiology Laboratory for immediate analysis. The surface tissue sterilization was performed as stated earlier in our previous studies (12). The disinfected leaves, stems and roots were rinsed three times in sterile distilled water and drained. The tissues were cut longitudinally with a sterile scalpel and laid, with the exposed inner surface facing downwards, on Nutrient agar. The inoculated plates were incubated for 36-48 h at 30 ± 2 ºC and pure cultures were established. In addition, the uncut surface-disinfected tissues, and the last rinsing water were also inoculated onto separate Nutrient agar plates. This was to validate the effectiveness of the surface sterilization procedure (control) as bacteria growth in the control agar plates within 24 h of incubation (30± 2 ºC) indicated ineffective surface-sterilization.

Molecular characterization and biodiversity:

The genomic DNA of the endophytic bacteria was extracted using methods described by (19). This was extracted using the QIAamp DNA Mini Kit by QIAGEN. The 16S rRNA gene was amplified using the forward primer [8F: 5'-AGAGTTTGATCCTGGCTCAG-3'] and reverse primer [1492R: 5'-GGTTACCTTGTTACGACTT-3'] (20). The PCR conditions were performed as stated in previous study (12). The PCR products were purified using NucleoSpin Gel and PCR Clean-up kit by Macherey-Nagel. The 16S rRNA gene sequencing was performed by First-BASE Laboratories Pte. Ltd. The isolates were identified based on hits analysis from mega blast (highly similar sequences) output of the BLASTN program at (http://ncbi.nlm. nih.gov). The DNA sequences were deposited at NCBI and accession numbers obtained as shown inTable 1. To build the phylogenetic tree, sequences of the 16S rRNA gene were aligned using the multiple sequence alignment program (MUSCLE) (21) and the

phylogenetic analysis performed using Maximum Likelihood methods (MEGA6) (22)with Bootstrap analysis performed using data resampled 1,000 times.

Determination of enzymatic production:

The agar diffusion assay was performed to detect extracellular cellulase, xylanase, amylase and pectinase production of the endophytic bacteria (23). To assay for cellulase production, the isolates were grown on cellulase activity indicator medium [Nutrient Agar medium containing, 0.5 % (w/v) carboxyl methylcellulose, and 1.5 % agar (w/v)]. Spot-dot inoculation was performed for the detection of activity. Plates were incubation at 30 ± 2 ºC for 18-24 h. To visualize the halos formed due to cellulase, the plates were flooded with 0.5 % Congo red solution for 30 min, rinsed with water and then rinsed twice with 1M NaCl. Colonies positive for extracellular cellulase activity were surrounded by a yellow halo against a red background. To detect xylanase production, the isolates were grown on xylanase activity indicator medium [Nutrient Agar medium containing, 0.5 % (w/v) oat spelt xylan and 1.5 % agar (w/v)]. Plates were incubated at 30 ± 2 ºC for 18-24 h.

To visualize the halos formed due to xylanase activity, the plates were flooded with 0.5 % Congo red solution for 30 min, rinsed with water and then rinsed twice with 1M NaCl. Colonies positive for extracellular xylanase activity were surrounded by a yellow halo against a red background. To detect amylase production, the isolates were grown on amylase activity indicator medium [Nutrient Agar medium containing, 0.5 % (w/v) starch powder and 1.5 % agar (w/v)]. Plates were incubated at 30 ± 2 ºC for 18-24 h, a clear zone surrounded by black coloration indicated the activity of amylase when rinsed with 0.1M Iodine solution. To detect pectinase production, cultures were grown on pectinase indicator medium [Nutrient Agar medium containing 0.7 % (w/v) sodium polypectate and 1.5 % agar (w/v)]. Plates were incubated at 30 ± 2 ºC for 18-24 h. To visualize the halos formed due to pectinase activity, the plates were flooded with 10 % of a saturated solution of copper acetate (Cu ($C_2H_5O_2$) for 30 min. After excess stain was washed off, a halo against a blue background became visible.

Determination of antimicrobial activity:

The metabolites of the bacterial endophytes were extracted with ethyl acetate solvent as described earlier (12), and the antimicrobial activities of the extracts were tested against bacteria and yeast pathogenic strains, which include-*Pseudomonas aeruginosa* ATCC 10145*, Staphylococcus aureus* ATCC 33591*, Bacillus cereus* ATCC 14579*, Salmonella* TyphimuriumATCC 14028*, Proteus vulgaris*-ATCC 8427*, Klebsiellapneumoniae* ATCC 10031, *Escherichia coli* ATCC 25922*, Streptococcus pyogenes* ATCC 12384 and *Candida albicans* ATCC 90028 using agar disc diffusion method (12, 24). All pathogens were obtained from the Microbiology Laboratory of Monash University Malaysia. The bacteria pathogens were pre-cultured overnight in Mueller Hinton broth at 35 ± 2 ºC, and 5 ml of the culture were centrifuged at 6,000 × *g* for 5 min. The pellets were re-suspended in sterile distilled water and the cell density was subsequently adjusted to 0.5 McFarland standard. The inoculum suspension was then seeded on the plate of Mueller Hinton agar for antimicrobial assay.

The ethyl acetate crude extracts (20 mg mL^{-1}) of the endophytic bacteria were impregnated onto sterile discs and placed on seeded agar plates. The plates were incubated for 48 h at 35 ± 2 ºC and the annular zone of inhibition was measured. The experiment was performed in triplicates. Chloramphenicol (30µg) and Gentamicin (10µg)standard antimicrobial discs were used as positive control.

Statistical analysis:

One-way ANOVA was used to analyse all data obtained. The analysis was carried out using the Statistical Package for Social Science (SPSS) version 20 and means were compared using Tukey'sStudentized Range Test ($HSD_{(0.05)}$). Differences were considered statistically significant at $p < 0.05$.

RESULTS

Diversity of endophytic bacteria:

The results of microbial diversity analysis revealed mixed composition of the endophytic communities from the roots, stems and leaves of *O. basilicum, C. citratus*, *M. citrifolia* and *T. aestivum*. Eight endophytic bacteria were isolated from *O.*

basilicum belonging to four genera and eight species; 16 isolates from *C. citratus* (five genera of 14 species); nine isolates from *M. citrifolia* (six genera of eight species) and eight isolates from *T. aestivum* (seven genera of eight species) (Table 1). The relative abundance of species isolated from each tissue can be depicted as seen in Figure 1, with *C. citratus* having the highest *Firmicutes* (*Bacilli* species) followed by *Proteobacteria* from both the stem and leaf of *M. citrifolia* and *C. citratus* respectively. The phylogenetic tree revealed the relatedness of the species from each tissue of the respective plants (Figure 2-5).

Antimicrobial activity:

The antimicrobial assay revealed that of the 41 isolates tested, 20 were positive for antimicrobial activity against at least two of the pathogens tested (Table 2). Isolates with strong antimicrobial activities were mostly isolated from *C. citratus,* with six of the 16 isolates indicating antimicrobial activities towards two to four pathogens tested, and inhibition zones from 7.3 to 15.0 ± 0.5 mm. Three of the isolates- *Bacillus mycoides* OBR-1, *Acinetobacterbaumannii* LGL-1 and *Shingomonasyabuuchiae* TAR-1 inhibited at least five pathogens and were considered as the most active with zones of inhibition from 7.7 to 15.0 ± 0.5 mm (Table 2).

Production of valuable enzymes:

The production of the enzymes (cellulase, xylanase, amylase and pectinase) by endophytic bacteria were investigated. Of the 41 endophytic bacteria isolates screened, eight isolates produce all the four enzymes assayed (Table 3). Three of these isolates each, were from both *C. citratus* (LGR-10, LGR-3 and LGR-1) and *T. aestivum* (TAR-5, TAL-2 and TAL-3), and one isolate each from *M. citrifolia*-(MCS-1) and *O. basilicum*-(OBS-1). Six of these isolates belonging to the genus, *Bacillus*. However, *B. weihenstenphenensis* TAR-5 and*B.cereus*LGR-3 produce the most active enzymes for all the tested enzyme with annular halo zone from 4 to 6 mm (Table 3). More also, 27 isolates showed activities for either cellulase, xylanase, amylase or pectinase, while four isolates do not produce any of the enzyme tested.

Table 1: Similarity values (identity %) based on 16S rDNA sequences obtained to identify endophytic bacteria from *Ocimumbasilicum*, *Cymbopogoncitratus*, *Morindacitrifolia* and *Triticumaestivum* plants. The accession numbers for the isolates were assigned by NCBI.

Isolates	Nearest relatives[a]	Accession no	Query cover %	Identity %
OBS-1	*Bacillus thuringiensis*	KT253971	99	99
OBL-1	*Pseudomonas parafulva*	KT253972	99	100
OBR-1	*Bacillus mycoides*	KT253973	99	100
OBR-2	*Hydrogenophagadefluvii*	KT253974	98	99
OBR-3	*Pseudomonas monteilii*	KT253975	99	100
OBS-2	*Pseudomonas putida*	KT253976	99	99
OBS-3	*Pseudomonas fulva*	KT253977	99	99
OBS-4	*Pantoeavagans*	KT253978	99	100
LGL-1	*Acinetobacterbaumannii*	KM401858	100	100
LGL-2	*Citrobacterfarmeri*	KM401859	100	98
LGL-3	*Pseudomonas plecoglossicida*	KM401860	100	99
LGL-4	*Enterobactercancerogenus*	KM401861	100	99
LGL-5	*Pseudomonas monteilii*	KM401862	100	99
LGS-1	*Bacillus aerophilus*	KM401863	99	99
LGR-1	*Bacillus thuringiensis*	KM401864	99	98
LGR-2	*Bacillus anthracis*	KM401865	99	99
LGR-3	*Bacillus cereus*	KM401866	100	99
LGR-4	*Bacillus bataviensis*	KM401867	100	99
LGR-5	*Bacillus niacini*	KM401868	100	99
LGR-6	*Bacillus stratosphenicus*	KM401869	99	99
LGR-7	*Enterobacter cloacae*	KM401870	99	98
LGR-8	*Bacillus oleronius*	KM401871	99	98
LGR-9	*Enterobacter cloacae*	KM401872	99	98
LGR-10	*Bacillus stratosphenicus*	KM401873	98	99
MCR-4	*Pseudomonas knackmussii*	KT253979	99	99
MCS-1	*Enterobacterasburiae*	KT253980	100	99
MCS-2	*Pectobacteriumcypripedii*	KT253981	100	99
MCS-3	*Stenotrophomonasmaltophilia*	KT253982	100	99
MCS-4	*Pseudomonas denitrificans*	KT253983	99	99
MCS-6	*Bacillus anthracis*	KT253985	98	99
MCS-7	*Erwiniabillingiae*	KT253986	100	98
MCL-1	*Enterobacterasburiae*	KT253987	100	99
MCL-3	*Pantoeavagan*	KT253988	100	99
TAL-1	*Delftiatsuruhatensis*	KJ780828	99	99
TAL-2	*sphingobacteriumcladoniae*	KJ780824	100	99
TAL-3	*Klebsiellavariicola*	KJ780825	99	99
TAL-5	*Pseudomonas putida*	KJ780826	99	99
TAL-6	*Pseudomonas entomophila*	KJ780827	100	99
TAR-1	*Sphingomonasyabuuchiae*	KJ780829	99	99
TAR-4	*Enterobacterasburiae*	KJ780823	99	99
TAR-5	*Bacillus weihenstenphenensis*	KJ780830	100	99

[a]Closest relative species in the 16S rRNA gene sequences database. OBR, OBS and OBL; LGR, LGS and LGL; MCR, MCS and MCL; TAR and TAL represent isolates from the root, stem and leaf of *O. basilicum*, *C. citratus*, *M. citrifolia* and *T. aestivum*respectively.

Table 2: Antimicrobial activities of endophytic bacteria from *O. basilicum, C. citratus*, *M. citrifolia* and *T. aestivum* plants.

Isolates	*P. aeruginosa*	*S.* Typhimurium	*P. vulgaris*	*K. pneumoniae*	*E. coli*	*S. aureus*	*S. pyogenes*	*B. cereus*	*C. albicans*
	Annular zone of Inhibition (mm)								
OBR-1	9.7±0.5^{a}	-	12.0±0.5^{c}	12.3±0.5^{c}	11.0±0.5^{b}	-	-	8.5±0.5^{a}	9.7±0.5^{bc}
OBR-3	-	-	-	-	8.0±0.5^{a}	-	-	-	9.0±0.5^{b}
OBS-1	-	-	-	-	15.0±0.5^{d}	-	-	11.0±0.5^{c}	12.0±0.9^{d}
OBS-3	-	-	-	-	-	-	-	11.0±0.5^{c}	8.7±0.5^{ab}
LGL-1	-	-	-	7.7±0.5^{a}	10±0.5^{ab}	8.0±0.5^{a}	-	12.0±0.5^{d}	7.7±0.5^{a}
LGL-2	-	9.3±0.5^{a}	-	-	-	12.0±0.5^{b}	-	12.5±0.8^{d}	11.0±0.5^{c}
LGL-3	-	-	-	-	-	-	11.0±0.5^{c}	12.0±0.5^{d}	9.0±0.5^{b}
LGL-4	-	-	-	13.0±0.5^{c}	-	-	8.7±0.5^{b}	12.3±0.5^{d}	13.7±0.5^{e}
LGS-1	-	-	-	-	14.0±0.5^{c}	-	-	11.0±0.5^{c}	-
LGR-5	-	-	7.3±0.5^{a}	-	-	8.3±0.5^{a}	7.3±0.5^{a}	-	11± 0.5^{c}
MCS-1	-	9.7 ±0.5^{a}	-	10.7 ±0.5^{b}	-	-	-	12.0±0.5^{d}	-
MCS-2	-	-	11±0.5^{b}	12.0±0.5^{c}	-	-	11.0±0.5^{c}	11.3±0.5^{c}	-
MCS-4	-	-	12.0±0.5^{c}	9.7±0.5^{b}	-	-	-	11.0±0.5^{c}	-
MCL-1	9.0 ±0.5^{a}	-	-	-	-	-	-	9.7±0.5^{b}	-
MCR-4	-	-	-	-	10±0.5^{ab}	-	7.7±0.5^{a}	8.0±0.5^{a}	-
TAR-1	-	12 ±0.5^{b}	7.0 ±0.5^{a}	8.7 ±0.6^{ab}	12 ±0.5^{b}	15 ±0.5d	10±0.5^{bc}	10±0.8^{bc}	10±0.5^{bc}
TAR-4	-	-	-	11 ±0.5^{bc}	7.3±0.6^{a}	8.0 ±0.5^{a}	-	9.0 ±0.5^{b}	-
TAL-1	-	-	-	9.7 ±0.57^{b}	11 ±0.5^{b}	11 ±0.5^{b}	-	10±0.5^{bc}	-
TAL-2	-	-	7.7 ±0.6^{a}	-	-	7.7 ±0.6^{a}	-	-	-
TAL-5	-	-	7.0 ±0.5^{a}	-	-	-	9.0 ±0.5^{b}	9.0 ±0.5^{b}	9.0 ±0.5^{b}
Chloramphenicol (30µg)	-	28.0±0.5^{d}	24±0.5^{d}	30.0±0.5^{e}	26.0±0.5^{f}	26.0±0.5^{d}	26.0±0.5^{d}	26.0±0.5^{f}	16.0±0.5^{f}
Gentamicin (10µg)	18.0±0.5^{b}	20.0±0.5^{c}	26.0±0.5^{e}	22.0±0.5^{d}	20.0±0.5^{e}	20.0±0.5^{c}	30.0±0.5^{e}	23.0±0.5^{e}	28.0±0.5^{g}

Data are mean ±SD values. One-way ANOVA was used to analyse data using Tukey'sStudentized range test. Values are statistically significant at $p<0.05$. OBR, LGR, LGS, LGL, MCR, MCS, MCL, TAR and TAL represent isolates from the root, stem and leaf of *O. basilicum*, *C. citratus*,*M. citrifolia*and *T. aestivum*, respectively. (-) represent no inhibition. (a-g) represent statistical significant (Tukey's key). However, the metabolites of 21 isolates; OBR-2, OBS-2, OBS-4, OBL-1, LGL-5, LGR-1, LGR-2, LGR-3, LGR-4, LGR-6, LGR-7, LGR-8, LGR-9, LGR-10, MCS-3, MCS-6, MCS-7, MCL-3, TAR-5, TAL-3 and TAL-6 do not indicate any inhibition.

Table 3: Detection of enzymatic production of endophytic bacteria isolates from *O. basilicum, C. citratus*, *M. citrifolia* and *T. aestivum* plants.

ISOLATES	CELLULASE	XYLANASE	AMYLASE	PECTINASE
C. citratus				
LGL-1	+	-	+	+++
LGL-2	+	+	-	+
LGL-3	-	-	-	+
LGL-4	+	-	-	-
LGL-5	+	-	-	+
LGR-1	+	+	+	+
LGR-2	++	+	+	-
LGR-3	++	++	+++	+++
LGR-4	+	++	+	-
LGR-5	-	++	-	-
LGR-7	+	+	+	-
LGR-8	+	+	-	-
LGR-10	+	+++	+	+
LGS-1	-	+	+	-
LGS-3	-	-	-	-
M. citrifolia				
MCS-1	+	+	+	+
MCS-2	+	-	-	+
MCS-3	-	-	-	-
MCS-4	+	+	-	-
MCS-6	+	-	+	-
MCS-7	+	-	+	-
MCL-1	+	+	+	-
MCL-3	+	-	+	-
MCR-4	+	+	+	-
O. basilicum				
OBS-1	+	++	++	+
OBS-2	-	-	-	+
OBS-3	-	-	-	+
OBS-4	-	-	-	-
OBR-1	+	+	-	+++
OBR-2	-	-	-	-
OBR-3	+	-	-	+
OBL-1	+	-	-	+
T. aestivum				
TAR-3	+	+	-	+
TAR-4	+	+	+	-
TAR-5	++	+++	+++	+++
TAL-1	-	-	-	+
TAL-2	+	++	+	+
TAL-3	++	++	++	++
TAL-5	+	+	-	+
TAL-6	+	+	-	-

Size of halos formed around bacterial colonies on agar media and symbols; -, +, ++, and +++ indicate no, low (2 mm annular halo zone), weak (4 mm annular halo zone) and strong (6 mm annular halo zone) enzymes activities, respectively. OBR, OBS and OBL; LGR, LGS and LGL; MCR, MCS and MCL; TAR and TAL represent isolates from the root, stem and leaf of *O. basilicum*, *C. citratus*, *M. citrifolia* and *T. aestivum*respectively.

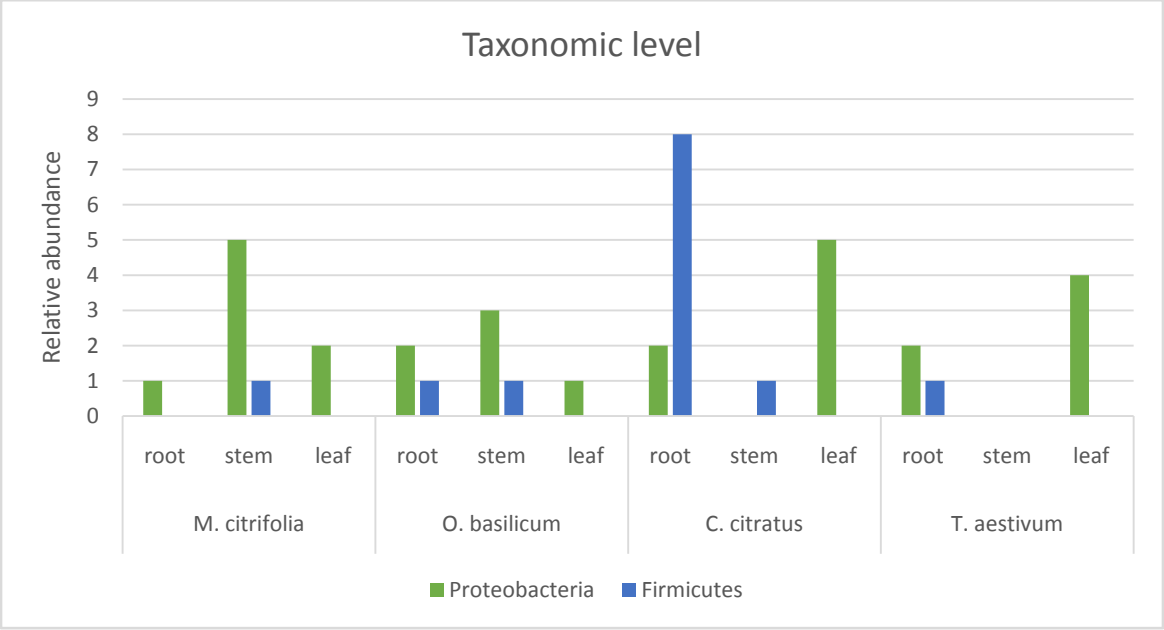

Figure 1: Taxonomic level of isolates from *M. citrifolia*, *O. basilicum*,*C. citratus*and *T. aestivum*plants.

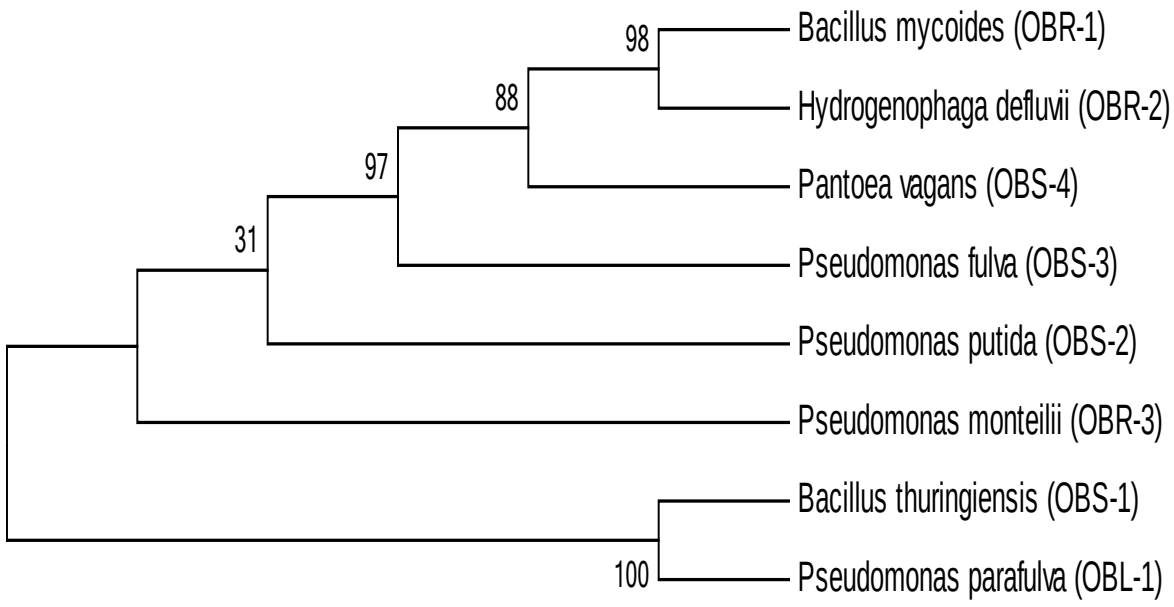

Figure 2: Molecular Phylogenetic analysis of endophytic bacteria from *O. basilicum*. Numbers above each node are confidence levels (%) generated from 1000 bootstrap trees. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model (21). The bootstrap consensus tree inferred from 1000 replicates (36) is taken to represent the evolutionary history of the taxa analyzed (22).

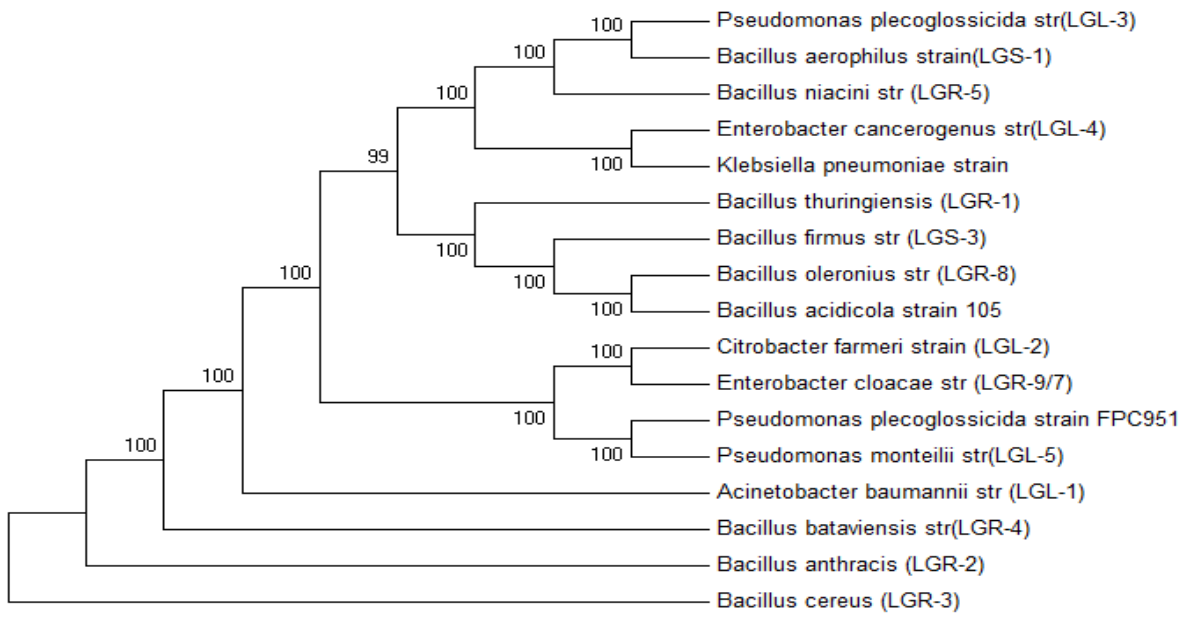

Figure 3: Molecular Phylogenetic analysis of endophytic bacteria from *C. citratus*. Numbers above each node are confidence levels (%) generated from 1000 bootstrap trees. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model (21). The bootstrap consensus tree inferred from 1000 replicates (36) is taken to represent the evolutionary history of the taxa analyzed (22).

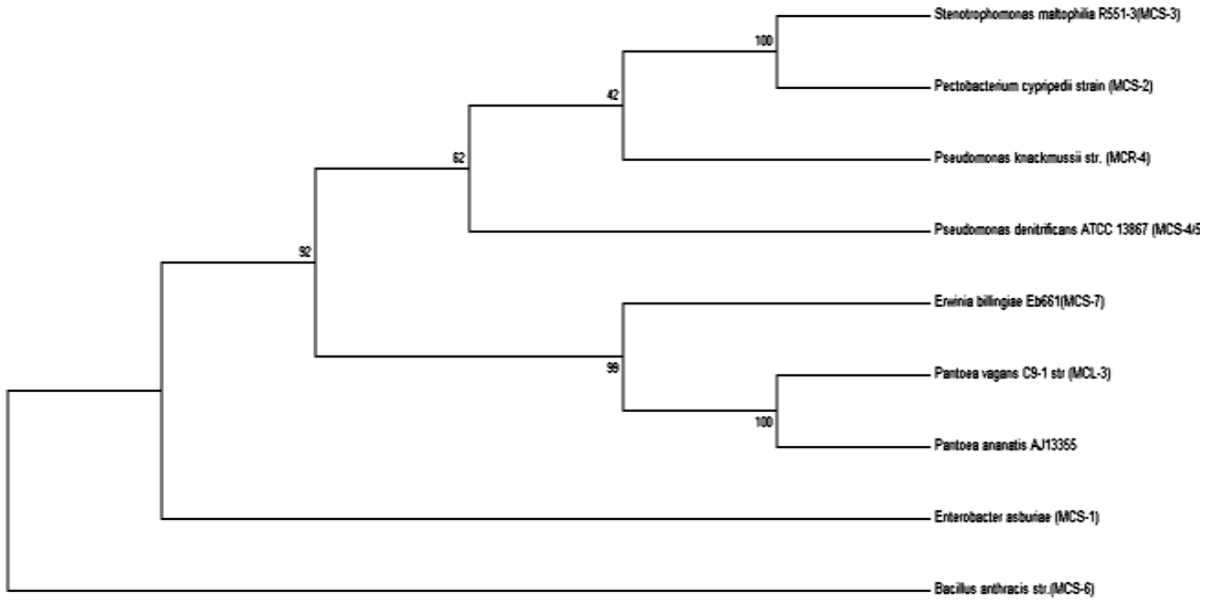

Figure 4: Molecular Phylogenetic analysis of endophytic bacteria from *M. citrifolia*. Numbers above each node are confidence levels (%) generated from 1000 bootstrap trees. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model (21). The bootstrap consensus tree inferred from 1000 replicates (36) is taken to represent the evolutionary history of the taxa analyzed (22).

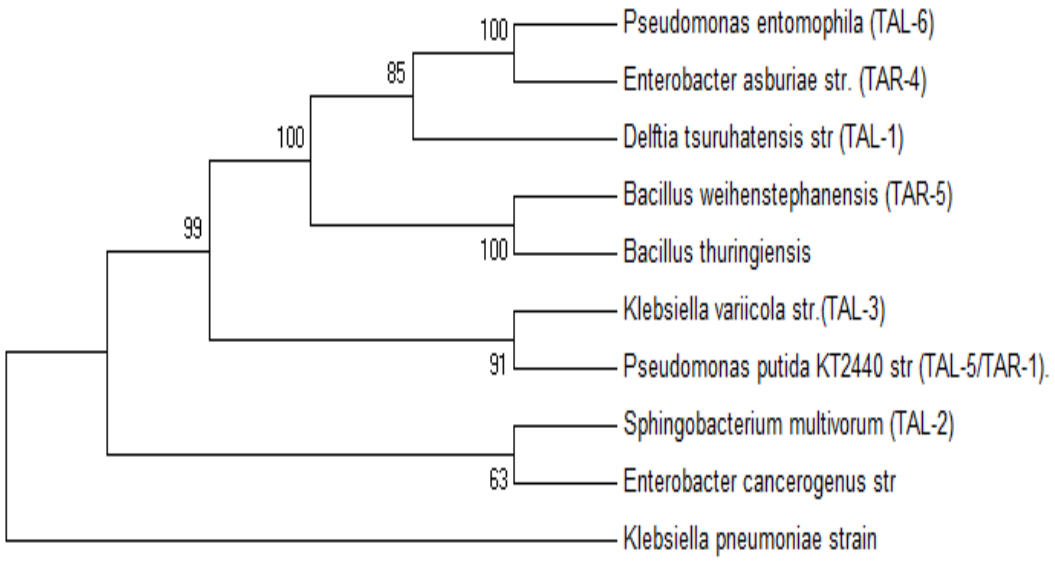

Figure 5: Molecular Phylogenetic analysis of endophytic bacteria from *T. aestivum*. Numbers above each node are confidence levels (%) generated from 1000 bootstrap trees. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model (21). The bootstrap consensus tree inferred from 1000 replicates (36) is taken to represent the evolutionary history of the taxa analyzed (22).

DISCUSSION

Diversity of endophytic bacteria:

The diversity analyses suggested that all four medicinal plants were colonized predominantly by *Pseudomonas* sp. and *Bacillus* sp.as the two genera were present in all plants tissues (Table 1). In our previous studies of some medicinal plants in the same location, similar results were obtained, where we found genus-*Pseudomonas*, *Bacillus* and *Enterobacter* predominating the tissues of the plants (12). The phylogenetic tree equally revealed the evolutionary history of the isolates in each plant and clusters can be seen indicating interactions within them and their hosts (Fig. 2-5). In studies conducted by other researchers, endophytes that were isolated in different plants from different locations are in different numbers. Sturz, Christie (25)noted that the endophyticpopulation of bacteria were different in different host plants, they identified 31 bacteria species from 14 different genera which were recovered from within the foliage, roots and nodules of red clover plants. Jalgaonwala, Mohite (26) isolated 78 bacterial endophytes and 140 fungal endophytes from the root and stem tissues of medicinal plants-*Pinusglabra* (Spruce Pine), *Eucalyptus globulus* (Blue Gum) and *Curcurma longa* (Ginger).

However, recent studies have indicated *Pseudomonas*, *Bacilli*, *Pantoea* and *Enterobacter* as the most common bacterial endophytes in plants (27) and our study have equally confirmed this. In addition, we isolated and identified *Hydrogenophagadefluvii*, *Erwiniabillingiae*, *Acinetobacterbaumannii* and *Citrobacterfarmeri* as endophytic bacteria and obtained more diverse bacterial endophytes in *M. citrifolia* stem and high diversity of *Bacilli* species in *C. citratus* root.

Antimicrobial activity:

The Gram-positive pathogen (*B. cereus*) and yeast pathogen (*C. albicans*) were more susceptible to the antimicrobial metabolites of the endophytic bacteria than the Gram-negative pathogens. *B. cereus* was inhibited by 17 isolates and *C. albicans* was inhibited by 11 isolates, with zones of inhibition from 8.0 to 12.0 ± 0.5 mm and 7.7 to 13.7 ± 0.5 mm respectively (Table 2). On the

contrary, *P. aeruginosa* and *S.* Typhimurium seemed to be more resistant to the metabolites produced by the endophytic bacteria as they were inhibited only by two and three isolates, respectively (Table 2). This agreed with previous studies that showed antibiotics produced by *Bacillus* spp. to be more effective on Gram-positive bacteria(25, 26). However, we found some isolates active against some Gram-negative bacteria such as *E. coli*, *K. pneumoniae*, *P. vulgaris*, *P. aeruginosa* and *S.* Typhimurium. Hence, we suggest that isolates-*B. mycoides* OBR-1, *A. baumannii* LGL-1 and*S. Yabuuchiae*TAR-1 have potential for production of antimicrobial substances that can be of use in the agro-allied and pharmacological industries. Secondly, it can help in the fight against phytopathogens, thus improve crop yield. The bioactive substance produced by these microbes can also be of benefitin fighting fungal disease caused by *C. albicans*. Therefore, we suggest for further work on isolation and characterization of these bioactive compounds.

Production of valuable enzymes:

Our results demonstrated that some endophytic bacteria from *C. citratus*and*T. aestivum*have the ability to degrade plant polymers especially endophytic bacteria *B. stratosphenicus*LGR-10, *B. cereus* LGR-3,*A. baumannii*LGL-1, *B. weihenstenphenensis*, and *Klebsiellavariicola*as they have strong indication for production of cellulase, xylanase and pectinase (Table 3).These enzymes have potential applications in various industries including pulp and paper, biofuel production, textile, food and agricultural industries. For instance, cellulase combined with xylanase, can be used for deinking of different types of paper wastes(28-30). Also, cellulase, xylanase, and pectinase as an enzyme complex, are used as macerating enzymes for extraction and clarification of fruit and vegetable juices to increase the yield of juices in the food industry(31). Bioconversion of lignocellulosic materials into useful and renewable biofuel can be achieve by the use of cellulase.

Previous researchers, have also demonstrated bacterial endophytes producing cell wall degrading enzymes (pectinase, xylanase, cellulase) at apices of root hairs as reported byAl-Mallah, Davey (32), they suggested that the enzymes degrading the cell wall at the apices of root hairs in *Trifoliumrepens* L. with cellulase-pectinase mixture enabled nitrogen-fixing nodule formation with *Lotus*- specific *R. loti*. In a related work ofVerma, Kumar (33)showed the presence of different levels of cellulase and pectinase activities in different isolates suggesting their potential for inter and intracellular colonization. Our work also revealed the enzyme production ability of *K. variicola* which may require further investigation. Though, *K. pneumoniae* and *K. oxytoca* have been found in a variety of plant hosts and are able to fix atmospheric nitrogen into a form that can be used by plants (34, 35).

Conclusively, our findings revealed that the four medicinal plants; *C. citratus*, *M. citrifolia*, *O. basilicum*and*T. aestivum*were predominantly populated by *Proteobacteria* and *Firmicutes* such as *Pseudomonas*, *Bacilli*, *Pantoea*and *Enterobacter* as summarized in Fig. 1. *M. citrifolia*and *T. aestivum*showed most diverse bacteria endophytes with seven genera and eight species. However, *C. citratus*was mostly colonized by *Bacilli* sp., rich in cellulase, amylase, pectinase and xylanase enzymes production. Even though that the plant was obtained in the same locality with *M. citrifolia*,*O. basilicum* and *T. aestivum*. This outstanding colonization by *Bacilli* sp. require further investigations. Similarly, the endophytic bacteria isolateswere found to produce metabolites with broad spectral antimicrobial activities, and Gram-positive pathogens were more susceptible than the Gram-negative pathogens. In our study, *B. cereus* and *C. albicans* were found to be more susceptible to the metabolites extracted. Hence, isolates such as; *B. mycoides* OBR-1, *A. baumannii* LGL-1 and*S.Yabuuchiae*maybe said have potentials for production of antimicrobial substances for both pharmacological and agricultural applications. The characterization of their bioactive compounds may be a lead to novel bioactive compounds. We therefore suggest the characterization of their bioactive compounds for further investigations.

ACKNOWLEDGEMENTS

We wish to acknowledge the assistants provided by MsAmreetaSarjit of Microbiology Teaching Laboratory of Monash University Malaysia for the provision of bacteria pathogens used for this project. This work was supported by scholarship of Higher Degree for Research (HDR) School of

Science, Monash University Malaysia and scholarship of Tertiary Education Trust Fund of Federal Republic of Nigeria.

Conflict of interest

The authors report no conflict of interest and are responsible for the content and writing of the manuscript.

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