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Endophytic *Bacillus* species isolated from mangrove plants, and their antagonestic effects against some pathogenic bacterial strains

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Abstract

This study aimed to isolate bacterial endophytes with potential antimicrobial effects from five mangrove plants and to investigate the stability of the most active metabolites under different PH and temperature.

Bacterial colonies were isolated from the collected plant parts. Bacterial strains with potential antagonistic interactions were subjected to idnetification by VITEK 2 system or sequencing of the 16S rRNA.MIC values of the crude substances were determined against *Bacillus cereus, Streptococcus uberis, Escherichia coli, Klebsiella pneumonia* and *Salmonella typhiumurium* using the the serial dilution technique.The stability of the most active crude extracts was examined under different, PH (2-7) and temperature(60 -90 C).

Twenty one of the isolated strains showed potential antagonistic effects. Of these, 16 identified as *Bacillus* species. Best antimicrobial effects were recorded for *Bacillus tequilensis* against, *B. cereus* and *S. typhimurium* (MIC value=65µl/m1), *S.uberis* (MIC value 78µl/ml), *E.coli* and *K.pneumonia* (MIC value=125µl/m1).Both *Bacillus subtilis* and *Brevibacillusbrevis* inhibited growth of *S. typhimurium* with MIC value of 78 and 60 µl/m1 respectively. The metabolites of *B.subtilis* and *B.tequilensis* appeared to be stable under PH2-9. Product of *B.subtilis* showed stability under high temperature (90 C) against *S. typhimurium*.

The isolated endophytes possessed wide range of antimicrobial activities against the selected pathogens. *Bacillus tequilensis* and *B.subtilis* produced the most active metabolites. This is the first report on isolation of endophytic strains of *B.tequilensis*. The crude extracts obtained from *B.tequilensis* and *B.subtilis* in this study could be further developed as food preservatives.

Keywords: Antimicrobials,mangroves,Bacillus,Biotechnology

Introduction

Endophytes are microorganisms including bacteria that live in the intercellular spaces of plant tissues causing no clear damage to their host. Bacterial endophytes provide a broad variety of bioactive metabolites with unique structure that could be developed and utilized for various applications [1].The mangrove habit has proved to be a rich source of endophytes. They occupy different parts of the trees through development of various physiological modifications to meet with the requirements of their challenging environment. Bacterial endophytes colonize an ecological niche similar to that of phytopathogens would serve as biocontrol agents [2].Thus, interactions of endophytes under these conditions may lead to the production of wide group of agents with interesting pharmaceutical applications [3]

Bacillus species are Gram-positive bacteria known to colonise a wide variety of environments due to their ability to form highly resistant endospores with huge genetic and metabolic diversity. They become an interesting targets for different biotechnological applications [4].A diverse array of antimicrobial peptides with several different basic chemical structures, have been produced by different strains of the genus *Bacillus*. Some studies have revealed that *Bacillus subtilis* can suppress infection against *Escherichia coli*, *Salmonella enterica*, *Chlostrodiumperfringens* and *Citrobacterrodentium* [4,5]. Antimicrobial substances produced by *Bacillus mycoides* and *Paenibacillus* sp were found to be effective against wide range of pathogenic bacteria such as *S. aureus, Clostridium sporogenes, Lactobacillus* sp., *and Listeria* sp.[4, 6].

Based on the unique characterisitc of the *Bacillus* sp.as a ubiquitous, commensal and transient organisms, some researchers stated that *Bacillus* specieshave functioned as probiotics by producing substances that play a key role in mitigating a systemic pro-inflammatory and autoimmune state, detoxification of the gastrointestinal system [7].Several reports confirmed the isolation of a bioactive bacteial metabolic substances from *Enterococcus faecium Enterococcus faecium SH 528,Pediococcus pentosaceus SH 740, Bacillus licheniformis* and *Bacillus thuringiensis*. Of these isolates,some showed antimicrobial effects on *Lactobacillus plantarum, Enterococcus facealis, Listeria monocytogens,Salmonella paratyphi* and *Clostridium perfringensBacillus* sp, *Staphylococcus* sp, *Streptococcus* sp and *Listeria* sp and *E.coli* [8, 9, 10, 11,12].

Mangrove environment and their endophytes are a rich source with diverse inhabitant of microorganism that need be discovered and heavily investigated as an endless source of novel constituents with diverse applications including of course antimicrobial agents.
In my previous report [1], isolation and identification of some bacterial endophytes with antimicrobial propoerties were highlighted using the fully automated VITEK 2 system. This report highlights isolation of endophytic *Bacillus* starins from diffrent mangrove species using both VITEK and 16S rRNA. The report also describes antimicrobial properties of the isolates against some pathogenic bacterial strains and initial characterization of their harvested crude bioactive proteins-like substances.

Materials and methods

Collection of plant material

Plant parts including leaves and roots from five mangrove plants were collected from a mangrove habitat in Setiu wet land mangrove plantation in Terengganu, Malaysia. The collected mangroves were *Acanthus ilicifolius, Bruguiera cylindrica, Bruguiera gymnorrhiza, Ceriops decandra* and *Lumnitzera racemosa.* All samples were transfered immediately to our laboratory and stored at 4 C for further process.
Culuring, isolation and identification of the endophytic bacteria:
Healthy and mature plant parts were carefully chosen. Leaves, stems, roots and in some cases flowers and fruits from different sites of each plant were randomly collected for the study. The materials were rinsed gently in running water. All the process was performed in sterilized condition under laminar air hood. Preparation, culturing and isolation of endophytic bacterial strains was done as described before [1]. In brief: all the materials were treated with 75 % ethanol for 1 minute followed by immersion in sodium hypochlorite for 10 minutes and again with 75 % ethanol for 30 second. Later they were rinsed three times with sterile water, dried and sliced into small segments (3-5 mm). Five fragments of each samples were plated on nutrient agar (Difco agar) and incubated at 37 C for 48 h. The mixtures were examined for any antagonistic effects. The inhibitor strains were then subjected to a serial purification process to obtain pure culture using the dilution-streaking technique on the same media. Bacterial strains with potential antagonistic interactions were selected for further test.

Determination of potential antagonistic interactions

Potential antagonistic interactions of the isolated endophytic bacterial strains were obtained using the deferred-antagonism plate assay. Bacterial strains appeared to be producing inhibitory substances were inoculated along a striaght line through the centre of Difco agar plates by using sterile cotton-tipped swabs dipped in overnight broth cultures. The plate was incubated for 24-h at 37 C to allow bacterial growth and production of inhibitory substances. Then the indicator strains were inoculated perpendicular to the producing bacteria with sterile cotton-tipped swabs dipped in overnight broth cultures. The plate contents were incubated for a further 24 h at 37 C before they were examined for the presence of inhibition zones at the intersections of the streak.

Identification of the bioactive producer strains

Only bacterial strains with clear antagonistic interactions were stained, morphologically characterized and further identified using the fully automated VITEK 2 system. Producer strains that could not be idenfied by VITEK 2 were subjected to sequencing of the 16S rRNA for identification. In breif: DNA template was prepared using 10µl of pure culture from the liquid media transferred into a sterile microcentrifuge tube and centrifuged at 12000rpm for 5 minutes. The cell pellets produced was then heated in microwave for about 10 seconds for six times followed by the addition of PCR grade water [13]. The prepared DNA was kept in -20 C prior further PCR analysis.

Polymerase Chain Reaction (PCR)

The gene fragment was amplified using forwards and reversal primer of 8F (5'-AGAGTTTGATCCTGGCTCAG-3')and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') amplified by Standard My Taq TM Mix, a ready-to-use setting up for PCR reaction that allows a fast cycling with greater efficiency and reproducibility. The amplification was carrried out using the mixure of, template (1ul), primer (1µl), MyTaq Mix (2X) (25µ), distiled water (50µl). The mixure was cycled in MJ Mini Personal Therma Cycler (Bio-RAD) under the following conditions: Initiation at 94 C for 3 minutes, 45 cycles of denaturation at 94 Cfor 1 min, annealing at 55 Cfor 1 min and extension at 72 Cfor 1 min. The final extension was set at 75 Cfor 5 min followed by 4 Cfor indefinitely. The quality of the PCR product was evaluated via 1% agarose gel electrophoresis. The PCR products were send to firstbase for sequencing.

Sequences Analysis

ThePCR products were sent for sequencing to the 1st Base Company. The sequencing data were then edited using MEGA ver 6.0 Software and compared to NCBI database using NCBI BLAST programhttp://www.ncbi.nlm.nih.gov/guide/sequence-analysis, [14].

Production and precipitation of Antimicrobial substances by the isolated producers

Bacterial endophytes were primarily screened for production of antimicrobial substances following the deferred-antagonism plate assay method using five test organisms: *Bacillus cereus, Streptococcus uberis, Escherichia coli,Klebsiella pneumonia* and *Salmonella typhiumurium.*
To produce and extract the antimicrobial substances of the isolated endophytes, each strain was cultivated separately in tryptic soya broth (TSB) for 24h at 30 C, centrifuged at 16 000 x g for 15 min. The supernatant was then concentrated with ammonium sulfate to 80% saturation and precipitated at 4 C. The precipitated crude proteins-like substances were pelleted by centrifugation at 16000 x

g for 20 min at 4 C and sucked up from the bottom of the centrifuge tube (4 ml) and re centrifuged again for 10 min. The upper layer was carefully sucked up.The remaining crude protein extract (2 ml) was re-suspended in 2 ml of 50 mM phosphate buffer (pH 7.0). The stock solution (I ml of crude extract dissolved in I ml of 50 mM phosphate buffer) was used for the subsequent bioassay tests.

Determination of antimicrobial activity of the crude bacterial proteins

The antibacterial effects of the crude proteins-like extracts were determined using the serial dilution technique described by Eloff [15], using 96-well micro-plates. Two ml cultures of four bacterial strains: Gram-positive *Bacillus cereus* and*Streptococcus uberis* and three Gram-negative: *Escherichia coli*, *Klebsiella pneumonia*and *Salmonella typhimurium* were prepared and placed in a water bath overnight at 37 C. The overnight-cultures were diluted with sterile MH broth (1 ml bacteria/ 50 ml MH broth) and adjusted to an optical density of 0.4.

100 µl of the stock solution (initial concentration of 1 ml/m1) were two-fold serially diluted with 100 µl sterile distilled water in a sterile 96-well micro-plate from well A to H before adding 100µl of each of the test bacterial strains .The plates were covered and incubated overnight at 37 C. To indicate bacterial growth, 50 µl of 0.2 mg/ml of Resazurin blue was added to each well and the plates incubated at 37 C for 30 min. Bacterial growth in the wells was indicated by a pink colour, whereas blue wells indicated inhibition by the tested substances.

Effects of, PH, tepmrature, types of salt precipitation and saturation percentage on the activity of the harvested crude proteins-like substances

The susceptibility of the crude protein extracts obtained from the producer strains to pH and temperature was determined by adjustment of three samples of the stock solution (I ml of crude extract dissolved in I ml of 50 mM phosphate buffer) to PH: 2, 7 and 9 individualy using 1N NaOH. To evaluate temperature stability, another samples were incubated at 60 &90 separately in water bath for 15 min and rapidly chilled on ice.

For efects of salts and saturation percentages, each of the producer starins was cultivated as mentioed above. The crude proteins-like extracts was obtained by the same way using amonium sulfate (30%, 50% and 100% saturation) and sodium sulfate 90% saturation.All the newely harvested and treated samples were subjected to the Micro-dilution assay as described earlier for the determination of their antimicrobial effects against the indicator strains used.

Results

Isolation and identification of bacterial endophytes

A total of 30 bacterial endophytes were isolated and purified from the leaves of the five Malaysian mangrove plants.The removal of surface microbial flora was achieved by the surface sterilization process including sequential immersion in 70% ethanol,10 % sodium hypochlorite and sodium bicarbonate. The colonies were purified and subjected to morphological characterization prior to further identification process.Sixteen of the isolated strains showed positive antagonistic effects against the indicator strains when evaluated using the deferred-antagonism plate assay (Figure 1).

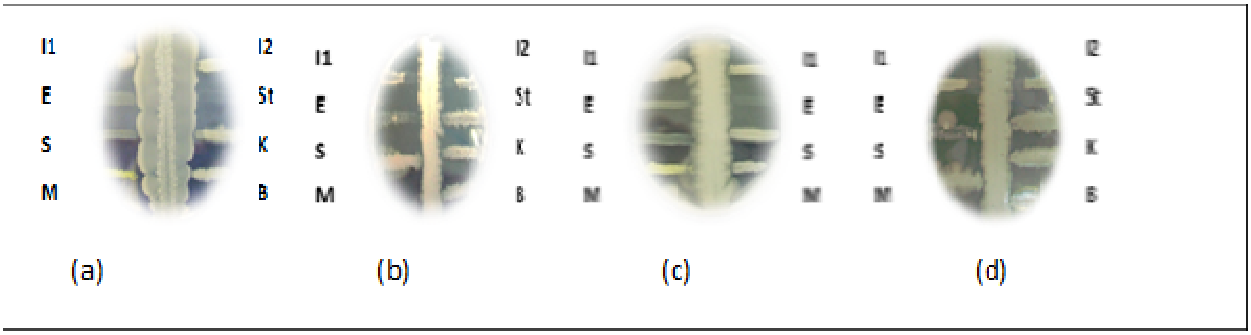

Figure 1. Best antagonitic effects of four of the isolated strains against indicator strains as determined using the deferred-antagonism plate assay. Producer starins: (a) Pa, (b)Pb, (c) Pe, (d) Pf. Indicator strains: (E)*E. coli*, (S)*Streptococcus uberis*, (M) *Micrococcus* sp., (11) *Brevibacillus* sp., (I2) *Bacillus* sp. (K) *Klebsiellapneumoniae*, (St) *Salmonellatyphiumurium,* (B) *Bacillus cereus* .

The isolated strains with potential antagonistic effects were subjected to VITEK 2 fully automated system machine for identification. The results with some of the relevant biochemical reactions are given in Table (1). Two of the potential producer strains were unidentified by VITEK2. These two strains (Pa, Pb) were subjected to 16S rRNAsequence determination for identification. The 16S rRNA sequence of each strain (retrieve from FIRSTBASE) was used to identify the bacteria species from DNA data base in NCBI. Accordngly, the two bacteria were identified to be: Pa=*Bacillus subtilis* subsp. *natto* BEST195 DNA, and Pb= *Bacillus tequilensis* strain Y11 16S ribosomal RNA gene, partial sequence (Table 1).

Table 1 Identification of the isolated endophytic bacteria using the VITEK 2 compact and sequencing of the 16Sr RNA genes used for the identification of the two bacteria Pa and Pbas they were unidentified by the VITEK system..

Isolate	Source	Morphological characterisitc		VITEK 2 identification	Confidence	Probability%	Analysis time/h	Sequencing of the 16Sr RNA genes of Pa and Pb		
		Gram staining	Shape					Accession number	Best match in blast analysis	Identity Score (%)
Pg	*Acanthus ilicifolius*	+	bacilli	*Brevibacillusbrevis*	Excellent	96	6	-	-	-
Pa	*Bruguierac ylindrica*	+	bacilli	Unidentified	Unknown	-	14	AP011541.2	*Bacillus subtilis* subsp. *natto* BEST195 DNA, complete genome	100
C6	*B.cylindrica*	-	bacilli	*Rhizobium radiobacter*	Excellent	98	14	-	-	-
C10	*B.cylindrica*	-	bacilli	*Pantoea*sp	Excellent	97	5	-	-	-
11	*B.cylindrica*	+	bacilli	*Brevibacillusbrevis*	Excellent	96	5	-	-	-
Pb	*Ceriopsdecandra*	+	bacilli	Unidentified	unknown	-	5	JX077105.1	*Bacillus tequilensis* strain Y11 16S ribosomal RNA gene, partial sequence	99
Pc	*C.decandra*	+	bacilli	*Bacillus cereus*	Excellent	99	14	-	-	-
Pd	*C.decandra*	+	bacilli	*Brevibacillusbrevis*	Good	92	5	-	-	-
C7	*C.decandra*	+	bacilli	*Bacillus cereus*	Excellent	95	6	-	-	-
C10	*C.decandra*	-	bacilli	*Pantoea*sp	Good	91	5	-	-	-
14	*C.decandra*	+	bacilli	*Bacillus cereus*	Excellent	96	5	-	-	-
112	*C.decandra*	+	bacilli	*Bacillus cereus*	Good	91	5	-	-	-
113	*C.decandra*	+	bacilli	*Brevibacillusbrevis*	Good	92	6	-	-	-

Table 1 continued

114	*C.decandra*	+	bacilli	*Bacillus cereus*	Good	94	14	-	-	-
Pe	*C.decandra*	+	bacilli	*Bacillus cereus*	Good	90	10	-	-	-
16	*C.decandra*	+	bacilli	*Bacillus cereus*	Good	91	6	-	-	-
13	*Bruguieragymnorrhiza*	+	bacilli	*Bacillus cereus*	Excellent	96	5	-	-	-
C3	*B.gymnorrhiza*	+	bacilli	*Bacillus cereus*	Excellent	97	14	-	-	-
C8	*B.gymnorrhiza*	-	bacilli	*Sphingomonasp aucimobilis*	Good	91	14	-	-	-
Pf	*Lumnitzerarace mosa*	+	bacilli	*Bacillus cereus*	Good	90	6	-	-	-
19	*L.racemosa*	-	bacilli	*Aeromonassobria*	Acceptable	87	6			

Production and precipitation of antimicrobial substances by the isolated producers

Crude proteins from the identified isolates were harvested and tested for minimum inhibitory concentration against the indicator strains. The tested substances possessed different antibacterial activities with MIC values ranging from 60 to 500 µl/ml(Table 2).
Based on the MIC values obtained, crude proteins-like substances produced by the bacteria Pb (identified as *Bacillustequilensis*isolated from *C.decandra*) showed the best performance against all the tested pathogenic bacterial strains with low MIC value against *B. cereus* and *S. typhimurium* (MIC value=65µl/m1), *S.uberis* (MIC value 78µl/ml), *E.coli* and *K.pneumonia*(MIC value=125µl/m1). Bacterial Pa (identified as *Bacillus subtilis* isolated from *B.cylindrica*) and Pg (identified as B*revibacillusbrevis* isolated from *A.ilicifolius*) also inhibited *S. typhimurium* with MIC value of 78 and 60 µl/m1respectively. Similar activities also recorded for C3 and 14 (*Bacillus cereus* isolated from *B.gymnorrihizaC.decandra* respectively) with MIC values of 65 and 78 µl/m1 respectively against both*B. cereus* and *S. typhimurium* .

Table 2-Antimicrobial properties (expressed as MIC values (µl/m1) of the crude bioactive extracts obtained from the potential producers against five pathogenic bacteria. This was detected using the Microdilution assay. Values are presented as means±SD.

Producers	MIC values µl/ml				
	Bacteria tested				
	Bc	Ec	Kp	Su	St
Pg	250±0.2	250±0.1	na*	na	60±1.8
Pa	125±1.0	250±2.6	250±1.2	125±0.2	78±2.4
C6	250±1.4	350±2.1	350±1.4	250±0.3	250±0.4
C10	250±0.9	na	na	250±1.3	250±1.0
11	250±0.8	250±1.1	250±1.2	250±0.2	125±0.6
Pb	65±4.1	125±5.0	125±3.1	78±0.4	65±1.7
Pc	125±1.8	250±2.4	250±3.1	250±0.5	78±2.1
Pd	250±0.2	450±3.1	350± 2.8	250±1.1	60±1.8
C7	125±0.7	na	na	na	250±0.5
C10	125±0.6	125±0.3	na±0.8	250±0.4	250±0.6
14	78±1.8	250±2.4	250±3.1	420±1.3	78±2.1
112	125±1.8	300±2.4	250±3.1	150±1.2	78±2.1
113	250±0.2	250±0.5	250±0.1	125±0.8	60±1.8
114	125±1.8	250±2.4	250±3.1	250±0.7	78±2.1
Pe	125±0.8	125±0.6	250±0.9	125±0.5	65±1.8
16	125±0.3	250±0.4	250±0.6	150±4.1	125±2.1
13	78±0.1	125±0.7	350±0.3	125±0.3	65±0.2
C3	65±0.4	125±0.2	250±0.9	125±0.6	65±3.0
C8	na	125±4.1	500±0.9	na	na
Pf	125±0.2	125±1.2	250±1.9	125±0.4	125±0.8
19	78±1.7	250±0.2	350±0.3	78±0.2	78±0.5
Gentamicin sulfate (positive control)	2.1.±1.3	8.3±1	1.9±2.4	2.3± 0.7	1±0.4

Bc=Bacillus cereus, Ec= Escherichia coli, Kp=Klebsiella pneumonia Su= Streptococcus uberis, St= Salmonella typhiumurium. *na =not activeat the highest concentration tested (500 µl/ml).

Effects of, PH, temperature, precipitating salts used, and saturation percentage on the activity of the harvested crude proteins-like substances

Antimicrobial properties of the most active harvested crude proteins-like substances obtained from the three producers: Pa (*B.subtilis*), Pb (*B.tequilensis*) and Pg (*Brevibacllusbrevis*) were determined to evaluate their stability against different temperature and PH level using the micro-dilution assay. Crude proteins-like substances obtained from the three strains using sodium sulfate and three different saturation of ammonium sulfate were also tested (Table 3). The bioactive substances produced by Pa and Pb appeared to be more stable under the three PH level used (PH2,7 and 9) with the best activity around PH7. Product of Pa also showed more resistant to temperature especially against *B.cereus* and*S. typhimurium*. It was interesting to observed that its activity against *S. typhimurium*at a temperature of 90 C was still maintained with MIC value of 63µlm1^{-1}. For salt precipitation, ammonium sulfate appeared to be the best withsaturation of 50

and 100%. However, the crude substances produced by Pb could still be precipitated with 90% saturation of sodium sulfate but their activities remained lower compare to the crude substances precipitated by using ammonium sulfate.

Table 3-Effects of PH, Temperature and salt precipitation on the antimicrobial properties (expressed as MIC values (µl/ m1) of the crude bioactive extracts obtained from the most active producer: Pa,,Pb and Pg against five pathogenic bacteria. This was detected using the Microdilution assay.

Test condition		Mic valuesµl/ m1 obtained by crude proteins from the producers strains														
		Pa					Pb					Pg				
		indicator strains used					indicator strains used					indicator strains used				
		Bc	*Ec*	*kp*	*Su*	*St*	*Bc*	*Ec*	*kp*	*Su*	*St*	*Bc*	*Ec*	*Kp*	*Su*	*St*
PH	PH2	500	na*	500	63	125	125	500	na	500	na	na	250	na	na	150
	PH7	125	250	250	125	63	65	125	125	65	65	250	250	na	na	65
	PH9	60	na	250	125	63	65	250	na	125	65	250	na	na	na	65
Temperature	60 C	250	-	-	-	63	-	500	-	-	-	-	-	-	-	-
	90 C	-	-	-	-	63	-	-	-	-	-	-	-	-	-	-
Precipitation with Ammonium sulfate	30%	78	350	195	125	234	65	250	na	na	313	na	na	na	na	125
	50%	125	250	234	125	117	65	125	125	65	78	250	250	na	na	65
	100 %	125	250	250	125	80	65	125	125	65	332	250	125	na	na	65
precipitation with Sodium sulfate	90%	-	-	-	-	-	391	490	391	350	490	-	-	-	-	

Bc=Bacillus cereus, Ec=Escherichia, Kp=Klebsiella pneumonia Su= Streptococcus uberis, St= Salmonella typhiumurium. *na =not activeat the highest concentration tested (500 µl/ml).

Discussion

The VITEK 2 is an automated microbial identification system that provides accurate and reproducible results as shown in multiple independent studies. With its colorimetric reagent cards, and associated hardware and software advances, the VITEK 2 offers a state-of-the-art technology plat form for phenotypic identification methods. The system was confirmed to provide accurate identification reports without the need for further assessment [16, 17]. However, lack of information in the database in some cases and /or low reactivity of the strains within the incubation time may lead to failure of identification[18]. This may justify that two of our isolated strains were unidentified.

Sequence analysis of the 16S r RNA was proven to be an effective method which has been widely used to identify bacterial species and perform taxonomic studies [19]. In this study, results of the sequencing of thee 16Sr RNA led to the identification of the two analyzed bacteria strains to be, *Bacillus subtilis* and *Bacillus tequilensis*. Based on the identification systems used in this study, results showed that most of the isolated endophytic bacteria (with few exceptions) are belong to the genus *Bacillus*.

Bacillus species are widely distributed in the environment including growth in terrestrial and mangrove plants as endophytic colonies [1]. *Bacillus* species in this study showed broad spectrum of activities against the indicator strains. Crude bioactive substances from *B.subtilis* and *B.tequilensis* showed the best activities and stability over different PH and temperatures.

B.tequilensis was first isolated as Gram-positive, spore-forming *Bacillus* and identified as new species named *B.tequilenesis*[20]. However, isolation of endophytic*B.tequilensis* from the mangrove plant *Ceriopsdecandra* in this study was unprecedented and therefore, could be recorded as a novel findings as no report to our knowledge indicated isolation of this species as endophytic bacterium.

Bacillus species are an important source of fine biochemicals with diverse array of antimicrobial properties[21]. During recent investigation into biological activities of *B.subtitlis*, Permpoonpattana[22] stated that *Bacillus subtilis* can suppress infection against *Escherichia coli* 070:K80, *Salmonellaenterica*, *Chlostrodiumperfringens* and *Citrobacterrodentium*.Both Grams negative and Gram-positive bacteria produce proteinaceous antibacterial compounds, which constitute a heterologous subgroup of ribosomally synthesized antimicrobial peptides such as bacteriocins. These products can be exploited by food processors to provide an additional barrier to undesirable bacterial growth in foods [23]. This is in agreement with the observed activities by the metabolites of the isolated *Bacillus* species in this study. Our results mayopen a window for further utilization of these productsas a good candidate forpossible exploitation as food preservative agents.

B. subtilis species are confirmed to be in most cases unharmed to mammals, including humans, and is commercially important as producer of a high and diverse metabolites used for different applications. They were reported used as antibiotics, enzymes, antigens and vaccines [24].This author also concluded that

B.subtilis could be utilized as potential probiotic candidate due to their beneficial properties. Antimicrobial properties observed by the endophytic*B.subtilis* in this study are in agreements with this and confirming the beneficial properties of this species as potential food preservatives producers.Alteration of pH is known to cause significant differences on properties of antimicrobial agents. In this study, the most active bacterial strains showed better performance in the PH around 7. However, antimicrobial agents produced by the *B.subtilis* against *B.cereus* and *S .typhimurium*showed more stability with PH ranging between 7-9. This is in line with previous report on the effects of PH on antagonistic activity of *Bacillus* species [6]. These authors stated that, antagonistic activity of *B. subtilis* strain L10 was higher when tested under PH condition of, 7.3 and 8.

For the temperature, most of theobsereved activities were lost or decreased when subjected to a temperature above 60 C . However, the product of *B.subtilis* against *S.typhimurium* maintained its activities even at higher temperature. Shin [9], reported characterization of proteins-like products isolated from some bacterial strains. They stated that some of the isolated proteins were highly thermostable, maintaining antibacterial activities even after incubation at above 95 C. It is very interesting that in this study, crude proteins-like substances harvested from *B.subtilis* maintained activity against *S.typhimurium* with a rising temperature up to 90 C. This is a promising characteristic for the harvested substances to be utilized for various relevant applications.

Conclusion

In this study, a total of 30 bacterial endophytes were isolated and purified from five mangrove plants. Most of the isolates were identified to be *Bacillus* species. The most active strains were *Bacillus subtilis,B.tequilensis* and *Brevibacillusbrevis*. This is the first report on isolation of an endophytic*B. tequilensis*from a mangrove plant. The three strains showed activities against the different indicator strains used with the best activities recorded for *B.tequilensis* and *Bacillus subtilis*. The products of these two strains will be further investigated for possible exploitation as food preservatives.

Conflict of Interest

The authors have no conflict of interest.

Author's contributions

IMS: Conceived of the study, lead the project, combined the results and have the manuscript prepared and written. LS: Isolation of bacterial endophytes and determination of the antagonistic effects. J: 16S RNA analysis. NA: Identification using VITEK 2 system.

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