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Characterization of Endophytic Bacteria, *Bacillus Amyloliquefaciens* for Antimicrobial Agents Production

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Abstract. Endophytic bacteria are able to produce bioactive compounds for various biotechnological applications. The antimicrobial activity of endophytic bacteria, which were isolated from the medical plant *Memecylon edule* Roxb, *Tinospora cordifolia* Miers, *Phyllodium pulchellum* (Benth) Desv and *Dipterocarpus tuberculatus* Roxb in Thailand, were determined using agar well methods against four bacteria, *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and fungal pathogen, *Candida albicans*. These bacterial strains were investigated the efficiency of solvents for extraction of antimicrobial agents. The extract of UD25 isolate obtained from diethyl ether and chloroform were effective in the inhibition of *S. aureus*; it had the bacteristatic activity. The crude extract of diethyl ether and ethyl acetate could inhibit the growth of *E. coli* and *B. cereus* giving the bactericidal activity. The suitable solvent of other four isolates was ethyl acetate. The minimum inhibitory concentration, MIC, by microdilution broth test showed the lowest concentration of crude extract that inhibited the growth of pathogenic microorganisms in the range of 0.156 - 2.5 mg/ml. The minimum bactericidal concentration, MBC, was in the range of 0.312-5 mg/ml. The most effective strain, UD25, was examined morphological and biochemical properties; the 16S rDNA gene analysis was also performed. It was identified as *Bacillus amyloliquefaciens*.

Keywords: Endophytic bacteria, Antimicrobial activity, Bactericide, Bacteristatic

1. Introduction

Endophytes are microorganisms that reside in the tissues of living plants. They are potential sources of novel nature product for exploitation in medicine, agriculture and industry [1]. Endophytes provide a broad variety of bioactive secondary metabolite were applied in a wide range of areas as agrochemicals, antibiotics, immunosuppressants, antiparasitics, antioxidants and anticancer agents [2]. There are many kinds of endophytes such as fungi, bacteria and actinomycetes. To date, many strains of endophytic bacteria have been reported such as *Azorhizobium, Bacillus, Bradyrhizobium, Gluconacetobacter, Klebsiella, Burkholderia, Enterobacter, Pseudomonas, and Streptomyces* [3]. *Bacillus amyloliquefaciens* is a Gram-positive, spore forming bacteria, and is closely related to *Bacillus subtilis* and other members of the *B. subtilis* group [4]. The genome of the plant-associated *B. amyloliquefaciens* GA1 contained three gene clusters directing the synthesis of the antibacterial polyketides macrolactin, bacillaene and difficidin [5]. Endophytic bacteria from the medical plant of *Andrographis paniculata* showed activity against both Gram-positive and Gram-negative bacteria pathogens [6]. Secondary metabolites produced by endophytic bacteria *Bacillus pumilus* MAIIIM4A showed a strong inhibitory activity against the fungi *Rhizoctonia solani*, *Pythium aphanidermatum* and *Sclerotium rolfsii* and LC-MS/MS was used to identify the active fraction assigned as punilacidin [7]. The aim of this study was determination organic solvents that gave high potential extracts,

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which inhibited the growth of indicator microorganisms. The bacterial strain was identified by 16S rDNA gene sequence compared with morphological and biochemical properties.

2. Materials and Methods

2.1. Pathogen Microorganisms and Culture Condition

Indicator bacteria (2 Gram-positive; *Staphylococcus aureus* and *Bacillus cereus*, and 2 Gram- negative; *Escherichia coli* and *Pseudomonas aeruginosa*) and a fungal specie (one yeast; *Candida albicans*) were from the department of Microbiology in faculty of Science and the department of Medical technology in faculty of Associated Medical Science, Khon Kaen University. The stock culture at -20°C of 5 indicator microorganisms were streak on nutrient agar plate; NA (BioMARK Laboratories, India). After 18-24 h incubation at 37°C (for bacterial strains) and 30°C (for yeast strain), a loopful of each indicator strains was added into nutrient broth (NB) (for bacterial strains) and Yeast Extract Malt Extract broth (YMB). The culture broth was incubated by shaking in suitable temperatures.

2.2. Isolation of Active Compounds

Inoculation of each endophytic bacterial 5 isolates (UD25, UD41, UD136, UD205 and UD306) were in 125 ml Erlenmeyer flasks containing 25 ml nutrient broth with a shaking (150 rpm) at 30°C up to 24 h. These seed cultures were then transferred to 1L Erlenmeyer flask containing 475 ml nutrient broth with a shaking (150 rpm) at 30°C up to 48 h. The culture (cell and supernatant) were extracted with organic solvent (1:1v/v) from non-polar to polar (diethyl ether; D, chloroform; C, and ethyl acetate; E, respectively). The crude extract was recovered and then organic solvent was evaporated. The dry weigh of crude extract was performed and dissolved in dimethyl sulfoxide (DMSO) for antimicrobial activity.

2.3. Antimicrobial Activity

Antimicrobial activity was assay by Agar well diffusion test with pathogen microorganisms as mentioned above. The culture of indicator strains was diluted in sterile distilled water until turbidity equal to 0.5 McFarland ($1x10^8$ CFU/ml). The final inoculum was spread with sterile cotton swab on Muller Hilton Agar; MHA (BioMARK Laboratories, India). Crude extract concentration was 20 mg/ml was added (50µl) into wells (6 mm) formed by cork borer on the MHA layer. The plates were incubated in suitable temperatures for 24-48 h; the zone of inhibition was measured and recorded [8].

2.4. Minimum INHIBITORY Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Indicator microorganisms were suspended in sterile water obtained a turbidity of 0.5 McFarland, and then was diluted 200 fold ($1x10^6$ CFU/ml) in NB medium (for bacteria) and YM medium (for yeast). The 50 µl of indicator strains were added in 96-well microtitre plates (Zellkutur Testplatte, Switzerland). The NB medium was used to dilute two-fold of crude extract giving final concentration from 20-0.156 mg/ml, then each concentration of 50 µl was added into indicator strains suspension in 96-well microtitre plates and incubated at 35°C for 18 h. After that, the 10 µl of 0.18% resazurin were added and incubated for 2 h in order to determine viable cells of indicator microorganisms. Crude extract has the ability of pathogens inhibition; the color of resazurin does not change (blue or purple). If it cannot inhibit pathogens, the pathogens will oxidize the resazurin (pink). The lowest concentration of crude extract showed inhibition of pathogens growth defined as MIC value. MBC was determined by subculturing from the negative results (blue or purple resazurin) on NA agar and incubated at 35°C for 24 h; no growth of pathogens showing 99% is definition of MBC value.

2.5. Identification of Endophytic Bacteria

The bacterial strain UD 25 was characterized by morphological and biochemical tests according to Bergey's Manual of Systematic Bacteriology [9]. The DNA of UD25 was extracted from cell suspension in 1 ml TE buffer (pH 8.0). The cell suspension was boiled for 10 min to release the DNA [10], chilled on ice for 10 min. After that, the suspended was centrifuged at 10,000 rpm for 5 min and kept for supernatant. The DNA template was added to 45 µl of DreamTaq PCR Master Mixture (Fermentas) using the primer pair of

27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3'). The PCR reaction was performed by PTC-200 Peltier Thermal cycler (DNA Engina). The amplification condition were as follows 95°C for 3 min (denaturation); 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 2 min; and final extension at 72°C for 10 min. PCR product of around 1.5 kb was checked by agarose gel electrophorysis. The PCR product was purified by Gel Extraction Kit (Thermo scientific) and analyzed DNA sequencing (1st BASE sequencing Int). The sequence was compared to the NCBI nucleotide database (www.ncbi.nih.gov/blst) and multiple sequence alignment was done using the CLUSTALW (http://www.ebi.ac.uk/Tools/msa/clustalw2).

3. Results and Discussions

3.1. Determination of Antimicrobial Activity in Various Organic Solvents

Agar well diffusion assay, 5 bacterial isolates producing antimicrobial agents were investigated a suitable organic solvent extraction, diethyl ether, chloroform and ethyl acetate. Four of 5 antimicrobial isolates, except UD25, displayed inhibitory activity against microorganisms only from crude extracts of ethyl acetate. Crude extract of UD25 from diethyl ether had efficiency against *B. cereus* and *E. coli* to 26 mm and 16 mm of inhibition zone, respectively. For *S. aureus*, no clearance of inhibition zone (30 mm) was observed in all crude extracts (Fig 1), indicating that the concentration of crude extract was too low. The inhibition of *P. aeruginosa* and *C. albican*s growth were found in the ethyl acetate extract obtained from the UD136 and UD205 isolates, respectively; whereas the crude extract of UD25 from all organic solvents could not inhibit the growth of both pathogens. As previous report heterotropic bacteria, *B. pumilis* (S6-05) had the broadest range of inhibition in Gram positive and Gram negative pathogens [11]. The results implied that solvent extraction had different roles to recover different compounds, which had effective inhibition in various pathogenic microorganisms. In addition, the amount of crude extracts from each solvent is one of the factors used in determining the choice of solvent for extraction. Diethyl ether gave yield of the crude extract about 25 mg per 500 ml culture while chloroform and ethyl acetate gave more than 2-fold (approximately 45-50 mg) of the extracts. The MIC and MBC values were determined to compare the effectiveness of the extracts.

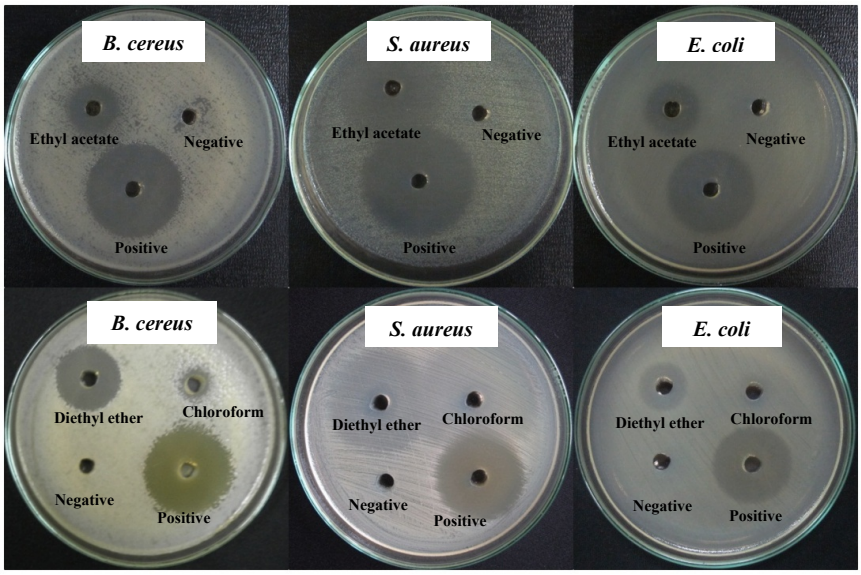

Fig. 1: Antimicrobial activity of UD25 strain against *B. cereus*, *S. aureus* and *E. coli*; DMSO was represented as negative; positive was tetracycline 20 mg/ml.

3.2. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Five endophytic bacterial cultures extracted with tree types of organic solvents (diethyl ether, chloroform and ethyl acetate) were tested with the pathogenic microorganisms that showed inhibition from previous results. The result showed that *E. coli* was inhibited by crude extracts of UD25 from diethyl ether and ethyl acetate, which was effective in killing bacteria (bactericidal) and in inhibition effect (bacteristatic),

respectively (Table 1). The *S. aureus* was compared the effective of the inhibition between crude diethyl ether and chloroform of UD25. The crude chloroform was more efficiency. The *B. cereus* was killed (bactericidal) by crude diethyl ether and crude ethyl acetate of UD25 as the MBC values were shown in 2 fold of the MIC values. Crude ethyl acetate of UD136 has efficiency against *P. aeruginosa*. For the *C. albicans* inhibition, the effective extracts were crude ethyl acetate of UD136 and UD205. These results were related to the inhibition from agar well diffusion. The inhibitory effects on gram-positive and gram-negative bacterial growth were relatively low concentration of MIC values ranging in 0.156-0.625 mg/ml. This showed high effective extract with low concentration compared to the previous reports. For instance, the minimum inhibitory concentration of *M. jodocodo* against *E.coli* was 2.75 mg /ml while that of *T. robustus* against *M. bourtardi* was 15.75 mg/ ml [12]. The values of MBC in the range of 0.312-5 mg/ml were effective in killing some pathogen strains.

Table1: Antimicrobial activity of endophytic bacteria by microdilution broth test (minimum inhibitory concentration, MIC and minimum bactericidal concentration, MBC).

Crude extract	*E. coli*		*S. aureus*		*P. aeruginosa*		*B. cereus*		*C. albicans*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
UD 25 - C	-	-	0.156	5	-	-	-	-	-	-
UD 25 - D	0.312	0.625	0.625	5	-	-	0.156	0.312	-	-
UD 25 - E	0.156	2.5	-	-	-	-	0.312	0.625	-	-
UD 41-E	0.156	5	-	-	-	-	0.312	1.25	-	-
UD 136- E	-	-	-	-	0.625	2.5	-	-	2.5	2.5
UD 205- E	-	-	-	-	-	-	0.156	2.5	2.5	2.5
UD 306- E	-	-	-	-	0.625	5	-	-	2.5	5

The values shown are mg/ml concentration of extracts against 5 indicator strains, (-) no tested inhibition, C is chloroform, D is diethyl ether, E is ethyl acetate.

3.3. Identification of Endophytic Bacterial Strain UD25

Morphological characterization of the strain UD25 was gram positive bacteria, rod-shape, and endospore forming. This strain produced catalase, amylase, acetoin and acid from glucose, and manitol, growth in 6.5% NaCl. The biochemical properties categorized to strain *Bacillus* in Bergey's Manual of Systematic Bacteriology [9]. Comparison of 16S rDNA amplified gene to sequence of GenBank was shown 97% identify to the *Bacillus amyloliquefaciens* and the phylogenetic tree of the strain UD25 was closely related group as *Bacillus amyloliquefaciens* (Fig 2). A *Bacillus* strain producing a bacteriocin-like substance was characterized and showed high similarity sequence with *Bacillus amyloliquefaciens* [13].

4. Conclusion

Endophytic bacteria can produce active compounds with various activities. The present study was found that the crude extracts of UD25 from diethyl ether and chloroform were effective in the growth inhibition of Gram-negative and Gram-positive bacteria. However, the crude ethyl acetate of UD25 had high potential activity, as well as the other four isolates which used ethyl acetate for suitable extraction of active compound. This implied that the common organic solvent of extraction was ethyl acetate giving antimicrobial agents. The effective strain UD25 was indicated 97% identity to the *Bacillus amyloliquefaciens*. It has been reported that a *Bacillus amyloliquefaciens*.

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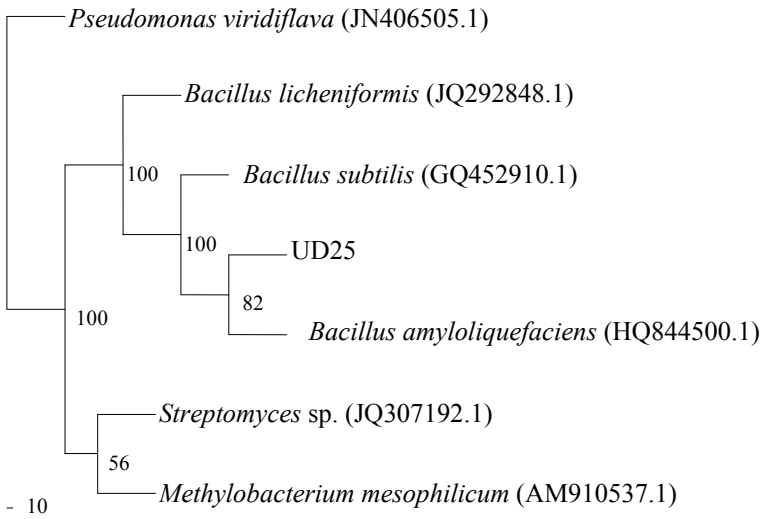

Fig. 2: Phylogenetic tree from analysis of 16S rRNA gene sequence of the strain UD25 using neighbor joining approach. Each number on a branch indicates the bootstrap confidence values correspond to the scale bar of branch lengths. GenBank accession numbers of nucleotide sequences are shown along with the name of bacterial species.

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