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Bioassay of Endophytic Bacteria from Tea (*Camelia sinensis*) against Foodborne Disease Bacteria

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Abstract : Tea (*Camellia sinensis*) is the second largest type of beverage in the world that contain lots of antioxidants and antibacterial. Tea has endophytic bacteria which has ability to inhibit growth of pathogenic bacteria, like Foodborne Disease (FD) Bacteria. The objective of the research was studied the potency of endophytic bacteria as antibacterial against Foodborne Disease bacteria. The step of this research includes collecting sample and isolation, antibacterial assay of endophytic bacteria and identification based on 16S rDNA sequence. A3, A4, A6 and A7 isolates showed the inhibition activity against *Bacillus cereus* (ATCC 11778), *Staphylococcus aureus* (ATCC 25923), *Salmonella thypimurium* and *Eschericia coli* (ATCC 25922). The highest inhibition zone were A6 and A7 isolates against *Salmonella thypimurium* sequentially are 14,6 $\pm$ 3,7 mm and 12,7$\pm$ 5 mm. A6, A3, and A4 isolates were identified as *Alcaligenes faecalis* B IV 2L44 (98.95 %), *Bacillus cereus* GTC 02826^T (99.97 %), and *Bacillus stratosphericus* 41KF2a^T (99.33 %) respectively, but A7 isolate was unidentified bacteria.
Keywords : Antibacterial, *Camellia sinensis*, endophytic bacteria, Foodborne disease.

Introduction

The emergence of antibiotic resistance in pathogenic microorganisms and prevalence of potent mutated strains, have created an alarming situation for both humans and crop plants. Since, natural bioactive compounds are known to control pathogens, there is an urgent need for continuous and rigorous search for novel natural products from different sources including plants, microorganisms and organisms inhabiting to unique niches[1]. The current research focuses on the bioactive compounds obtained from endophytes. Endophytes are microorganisms that live in plant tissues without causing any harm to their hosts. During endophytes colonization the microorganisms resides in almost every internal part of plant ranging from tissues of the underground roots to stem, leaf, flower, fruit and seed[2]. Endophytic bacteria promote host plant growth through direct mechanisms by producing phyotohormones IAA, gibberellins or indirectly by production of antibiotics[3]. Natural products from endophytic bacteria have been observed to inhibit or kill a wide variety of harmful disease-causing agents including as well as bacteria, fungi, viruses, and protozoans that affect humans and animals[4].

Camellia sinensis commonly known as tea is a herb plant of Family *Theaceae* cultivated in South Asia. Tea is the most widely consumed beverage in the world, and its polyphenolic compounds have been found for medinical properties[5]. Tea has endophytic bacteria which has ability to inhibit growth of pathogenic bacteria. Foodborne Disease (FD) is pervasive problem caused by consuming contaminated food or drink. An estimated 13.8 million cases of Foodborne Disease due to known agents, roughly 30 % are due to bacteria[6]. Pathogen

bacteria are the causative agents of foodborne illness in 60 % of cases requiring hospitalization[7]. The two most common types of foodborne disease are intoxication and infection. Foodborne bacterial intoxication is caused by the ingestion of food containing preformed bacterial toxin, such as the toxins produced by *Staphylococcus aureus* and *Clostridium botulinum*, resulting from bacterial growth in the food. Foodborne infection is caused by ingestion of food containing viable bacteria such as *Salmonella* then grow and establish themselves in the host, resulting in illness[8].

The presence of endophytic microorganisms in plants has been demonstrated. Although several fungi and *Actinomycetes* species were reported, a few of bacteria were isolated. Therefore, the present research aimed to study endophytes bacteria of tea plant (*Camelia sinensis*) used as antibacterial against Foodborne Disease bacteria.

Material and Methods

Sampling, Isolation and Characterization of Endophytic Bacteria

Leaves and roots tea (*Camelia sinensis)* were collected from Wonosari Tea Garden, Malang Indonesia. Samples were collected during plant's flowering and placed in sterile bags to transport to Microbiology Laboratory, Mathematics and Natural Sciences Faculty, Biology Department, Brawijaya University. Briefly, 25 g each of root and leaf were carefully weighed from *C. sinensis* plants, washed under running tap water to remove soil particles and epiphytic bacteria. Each root and each leaf was divided into fragments of about 1 cm^2. These portions were further surface sterilized (70 % C_2H_5OH, 1 min, 5 % NaOCl, 3 min and 70 % C_2H_5OH, 30 s) before the samples were rinsed thrice with sterile distilled water. Root and leaf fragments were assepticaly transferred to Erlenmeyer flask containing 0.85 % Sodium chloride. Endophytic bacteria were isolated using a serial dilution of 10^{-1} - 10^{-6} in Nutrient Agar (NA) medium and its incubated at 28 ^oC for 24-48 hours. The bacterial colonies were purified by spread plate method[9]. Gram reaction and cell shape were examined using microscope (Olympus CX 21).

Test Microorganisms

Test miroorganisms were collected from Laboratory Health Center Yogyakarta. The microorganisms used in this research were *Bacillus cereus* (ATCC 11778), *Staphylococcus aureus* (ATCC 25923), *Salmonella thypimurium* and *Eschericia coli* (ATCC 25922). The bacterial cultures were incubated at 37 °C for 24 hours in Nutrient Agar medium (Oxoid, Denmark). Stock cultures were maintained on NA slants at 4 °C and subcultured in Nutrient Broth (NB) at 37 °C prior to each antimicrobial test.

Bioassay of Antibacterial Activity of Bacterial Crude Extracts

All endophytes strain were screened for antibacterial properties against Foodborne Disease bacteria using agar well diffusion method. All endophytic isolates were grown separately in Nutrient Broth (NB) at 30 °C for 48 h. Each bacterial culture (5 µL) with similar optical density added in sterile paper disc for 15 min. Each Foodborne Disease bacterial (10^7 cell/mL; 100 µL) were spread on Nutrient Agar (NA) plates. Endophytic isolates in paper disc were placed on NA plate's surface. Antibacterial activities were detected after an incubation at 37 °C for 48 h. The presence of zone of clearance on plates was used as an indicator of bioactive nature of the strain. Three replicates were carried out for each antibacterial activity test. The diameter of inhibition zone was measured[10]. Data were analyzed using Analysis of Variance (ANOVA). All test were subjected to a 95% confidence limit ($P < 0.05$). Statistical analysis was carried out with Genstat.

Identification of Endophytic Bacteria Based on 16S rDNA Sequence

Endophytic isolates A3, A4, A6 and A7 were grown in Nutrient Broth (NB) for 24 h. Bacteria DNA was extraced using iGenomic soil DNA Extraction Kit (iNtRON Biotechnology Inc) according to their respective manufacturer's instructions. Amplification of 16S rDNA was done using Polymerase Chain Reaction (PCR) thermocycle Amplitron-1 by using primers 27F (5 '-GAG AGT TTG CTG GCT ATC CAG- 3') as a forward primer and 1492R (5 '-CTA CGG CTA TAC TGT CCT GA- 3 ') as a reverse primer[11]. 50 µL reaction mixture consisted of 25 µL (2x GoTaq Green Master Mix), 2 µL of 10 pmol each primer (27F and 1492R), 2 µL of 450 ng DNA template and 19 µL ddH_2O. The thermocycling profile was carried out with 35 cycles,

denaturation at 94 °C for 20 s, annealing at 52 °C for 30 s, extension at 72 °C for 5 min and final extension at 72 °C for 5 min[12]. Amplification products were resolved by agarose-gel electrophoresis (1.5 %), and visualized using a gel documentation system. Sequence of 16S rDNA were purified and sequenced in 1st BASE, Malaysia. Partial sequences was matched with nucleotide database in GeneBank (http://www.ncbi.nlm.nih.gov) using BLAST. Phylogeny tree was constructed by the Neighboor Joining method using program MEGA version 7.0 based on 1000 bootstraps[13].

Results and Discussion

Number of Endophyte Bacteria and Antibacterial Activity Assay

The endophytic bacteria of roots and leaves of tea plants were assessed in surface disterilization protocols. The number of colony-forming units (CFU) per gram fresh weight of endophytic bacteria from tea plants was determined. The resulst showed that number of endophyte bacteria in roots had higher (16,7 x 10^5 CFU/g) than in leaves (3,97 x 10^5 CFU/g). The species specificity of endophytes, the difference in endophytic assemblages in different tissue types might be a capacity for utilizing the substrate along with factors like tissue physiology and chemistry[14].

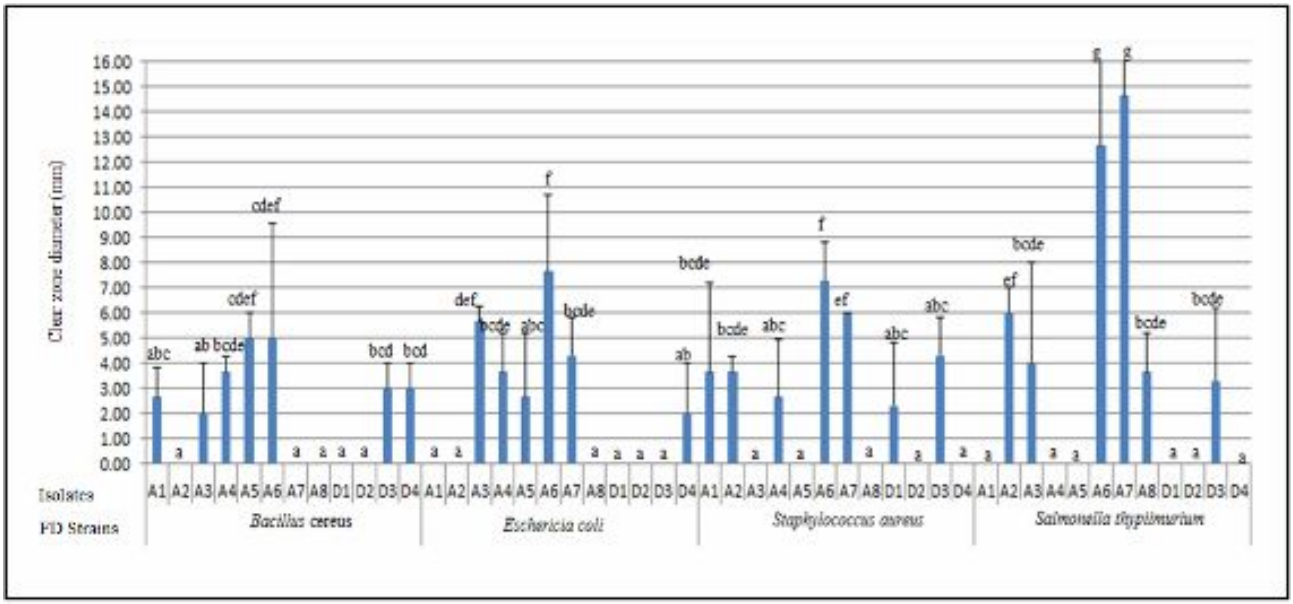

Fig. 1 Antibacterial activity of endophytes bacteria towards Foodborne Disease bacteria

Twelve isolates of endophyte bacteria were found and antibacterial assay was determined based on diameter of clear zone. Antibacterial properties of all bacterial endophytes were assessed against four bacterial test were *Bacillus cereus* ATCC 11778, *Staphylococcus aureus* ATCC 25923, *Salmonella thypimurium*, and *Eschericia coli* ATCC 25922. The isolate which inhibited growth of any of the test bacteria was considered having antibacterial activity and the diameter of inhibition zone was measured (Fig 1). Among 12 isolates, 4 isolates were have highest clear zone diameter ($p < 0.05$). Isolates with the highest diameter were A6, A7, A3 and A4 (Fig 2). Isolates with highest diameter zone of all treatments were A7 and A6 where the clear zone diameter was 14,6 mm $\pm$ 3,7 and 12,7 mm $\pm$ 5 mm, respectively. The diameter of clear zone each endophytic isolates indicated the different capability from each metabolites producing bacteria.

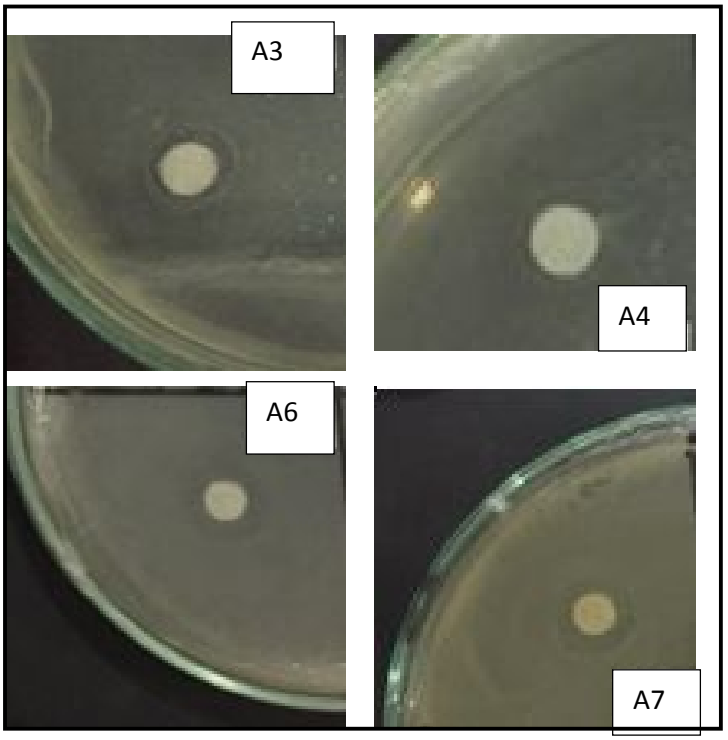

Fig 2. Antibacterial activity clear zone (a) A3, (b) A4, (c) A6, (d) A7 isolates

This study was a part of an endeavour to isolate bioactive bacteria that have antimicrobial properties. Since 96 % of total host bioactive compounds have been isolated from leaf, flower, seed and stem compared to 4 % shares of root tissues therefore, root may harbor more microbial species than the tissues produce higher numbers of bioactive compounds[15]. The bacterial strains secrete different types of natural products to inhibit or kill a wide variety of harmful disease-causing agents including, bacteria, fungi, viruses and protozoans that affect humans and animals[16]. The bacterial secretes 2,4-diacetylphloroglucinol (DAPG), phycocyanin, siderophores, lytic enzymes, chitenase which degrade the cell wall of pathogens and act as natural biological control. In present investigation the strain of A3, A4, A6 and A7 have shown the inhibition zones and indicated a strong antibacterial property among the all isolates.

The Species Potential of Tea Plant Endophyte Bacteria

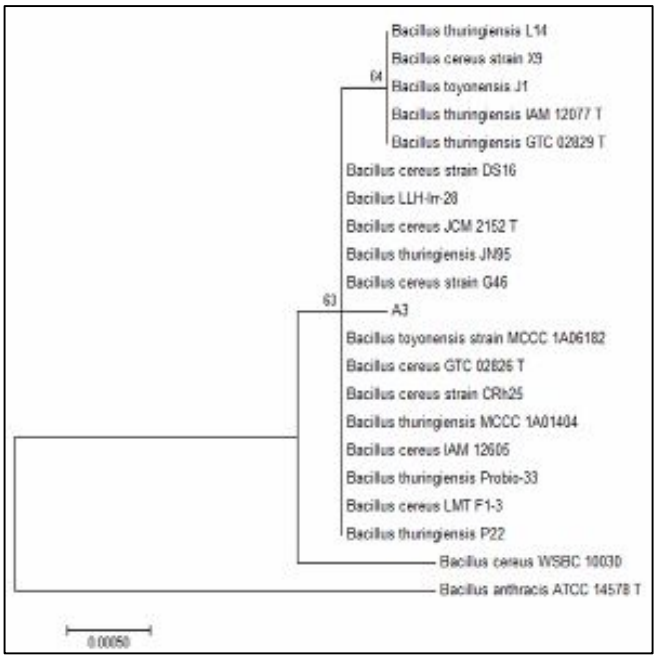

Fig 3. Phylogenic tree of A3 and reference isolates based on 16S rDNA sequence

Based on 16S rDNA sequence of endophytic bacteria in BLAST showed that Isolate A6 has highest potency as anti FB bacteria was identified as *Alcaligenes faecalis* B IV 2L44 has similarity 98.95 %. Isolate A3 was identified as *Bacillus cereus* GTC 02826^{T} has similarity 99.97 %, Isolate A4 was identified as *Bacillus stratosphericus* 41KF2a^{T} has similarity 99.33 %, and isolate A7 was unidentified bacteria. The phylogenetic tree was generated from the distance data using the neighbor-joining algorithm with the Tamura-Nei model in a MEGA 7 program (Fig. 3, 4, 5).

Bacteria belonging to *Bacillus* sp. have been identified as plant-growth-promoting rhizobacteria, and *nifH* genes have been detected in some strain of *Bacillus cereus*[17]. *Bacillus* species are often isolated as endophytes, and since they produce very resistant dormant endospore. Fungicidal and bactericidal compounds have been isolated from most endophytic spesies *like B. subtilis, B. cereus, B. pumilus, B. mycoides and B. sphaericus*[18].

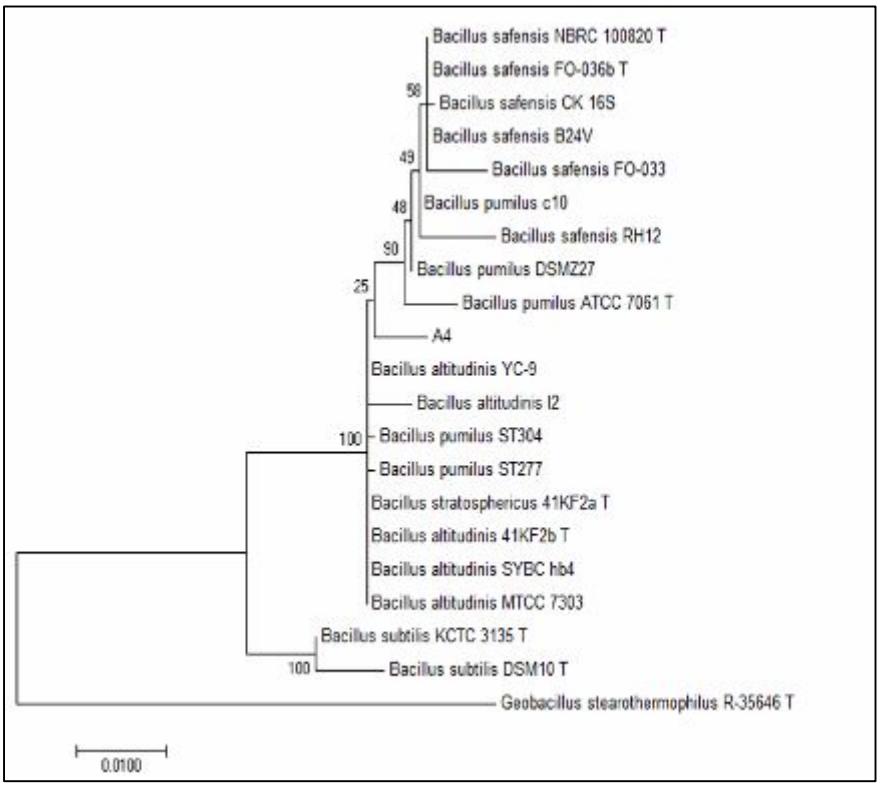

Fig 4. Phylogenic tree of A4 and reference isolates based on 16S rDNA sequence

A6 isolate (*Alcaligenes faecalis* B IV 2L44) and A7 isolate (unidentified bacteria) has a higher potential as an antibacterial. On other hand, study *A. faecalis* A72 showed antimicrobia activity against members of *E. coli* and *S. aureus* which is in agreement with our results[19]. More riable evidence of the endophytic bacteria of *A. faecalis* A15 shown that this strain will colonize the root surface, particularly at the root tips and lateral root junctions and will also enter the epidermal cells[20].

Most of the bacteria in this research had antibacterial activity. These results showed the potential use of endophytic bacteria for biocontrol to protect plants and human from bacterial pathogen. Further studies are needed to separate and extract the active substances from endophytic bacteria.

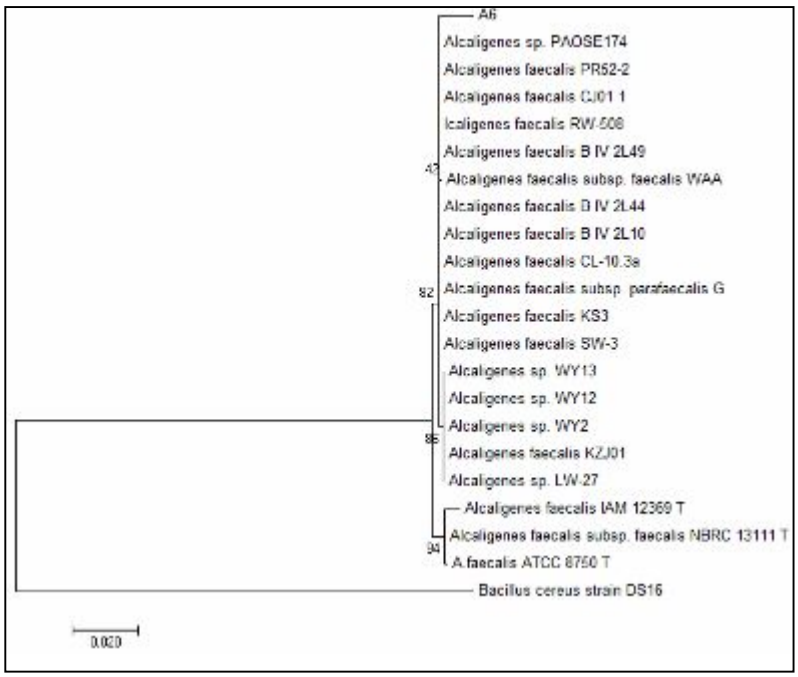

Fig 5. Phylogenic tree of A6 and reference isolates based on 16S rDNA sequence

Conclusion

This study evidenced that from 12 isolates, there were 4 isolates that had the highest antibacterial activity against FD bacteria, A6 (12,7 mm $\pm$ 5 mm), A7 (14,6 mm $\pm$ 3,7), A3 and A4 isolates. Based on 16S DNA sequences, A6 isolate has similarity 98.95 % with *Alcaligenes faecalis* B IV 2L44, A3 isolate has similarity 99.97 % with *Bacillus cereus* GTC 02826^T, A4 isolate has similarity 99.33 % with *Bacillus stratosphericus* 41KF2a^T and A7 isolate was unidentified bacteria.

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