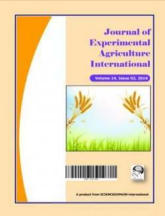

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Screening of Bacteria as Antagonist against *Xanthomonas oryzae pv. oryzae*, the Causal Agent of Bacterial Leaf Blight of Paddy and as Plant Growth Promoter

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Authors' contributions

This work was carried out in collaboration between all authors. Author NAA designed the study, performed the statistical analysis, wrote the protocol, and the first draft of the manuscript. Authors KS and EMH managed the analyses of the study. Authors RO and HMS managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The aim of this study is to screen and characterize bacteria isolated from different sources that has potential as antagonistic bacteria against *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), the causal agent of bacterial leaf blight of paddy and as a plant growth promoter. Ninety three bacteria were successfully isolated from different sources including compost, rhizosphere, water, roots and leaves from paddy field. By using chloroform vapours method, only 16 bacterial isolates showed positive antagonistic activity indicated by inhibition zone around bacterial colony against *Xoo* on nutrient agar plate. Twelve antagonistic bacteria were able to produce protease indicated by clear halo zone around bacterial colony on skim milk agar. Fourteen antagonistic bacteria were able to produce cellulase indicated by clear zone against red colour of congo red. Fifthteen antagonistic bacteria

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were able to produce lipase indicated by crystallize zone around bacterial colony. All antagonistic bacteria were able to produce siderephore which exhibited by orange halo zone around bacteria colony on CAS agar. All 16 antagonistic bacteria were able to produce IAA indicated by red colour development of antagonistic bacterial culture supernatant mixed with Salkowski reagent. Only 13 antagonistic bacteria were able to solubilize phosphate shown by clear halo zone around the bacteria growth on NBRIP agar. All antagonistic bacteria were able to fix nitrogen indicated by colour change of Nitrogen free agar from green to blue. Molecular identification by 16s rRNA amplification successfully identified the antagonistic bacteria as *Bacillus* sp. (5)*, Acinetobacter* sp. (2)*, Bacillus licheniformis* (1)*, Pseudomonas putida* (1)*, Burkholderia cepacia* (1)*, Bacillus amyloliquefaciens* (1)*, Staphylococcus warneri* (1)*, Pantoea vagans* (1)*, Pantoea* sp. (1)*, Oceanobacillus oncorhynchi* (1) *and Paenibacillus cineris* (1)*.*

Keywords: Bacterial leaf blight; Xanthomonas oryzae pv. *oryzae*; *antagonistic activity; hydrolytic enzyme; plant growth promoter.*

1. INTRODUCTION

Rice is the staple food for more than half of the world population [1]. One of the major problems in paddy field is diseases caused by fungi, bacteria and viruses. These paddy diseases may cause considerable crop losses [2]. The most important bacterial paddy diseases is the Bacterial Leaf Blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae (Xoo)* bacteria [3]. BLB incidence was reported from most rice growing areas in Malaysia [4]. According to investigations carried out in recent years, the disease has become economic importance in many other countries of East, South and Southeast Asia [5]. The disease reduces grain yield to varying levels depending on the stage of the crop, degree of cultivar susceptibility and a great extent to the conduciveness of the environment in which it occurs [6]. BLB is a serious disease of the crop which ranged of yield loss estimated from 50 to 100% [7,8,9].

At present, planting resistant varieties has been proven to be the most efficient, most reliable, and cheapest way to control *Xoo*. Other disease control options include use balanced amounts of plant nutrients, especially nitrogen; ensure good drainage of fields and nurseries, keep fields clean, remove weed hosts and plow under rice stubble, straw, rice ratoons and volunteer seedlings, which can serve as hosts of bacteria, allow fallow fields to dry to suppress disease agents in the soil and plant residues. BLB management tactics, such as resistant cultivars, are the most economical strategy for disease management, although they have only been partially successful because of an enormous diversity in the pathogens, while others, such as agro-chemicals, are harmful to the environment. Therefore, biological control assumes a special significance in being an ecologically conscious, cost-effective alternative strategy for BLB management without the negative effect of synthetic chemicals that can cause environmental pollution and may induce pathogen resistance [10]. This can also be used in integration with other strategies to afford greater levels of protection and sustain crops yield. Antagonistic bacteria are considered ideal biological control agents for obvious reasons like rapid growth, easy handling and aggressive colonization of the rhizosphere. Bacterial antagonistic have been evaluated with various degree of success for the suppression of rice disease of fungal origin [11].

Rice production has become important in order to fulfill the rice demand for the growing population. However, BLB disease which caused by *Xoo* leads to yield losses. Therefore, an effective control agent is required to control BLB. Compost, rhizosphere, water, roots and leaves comprise a large population of beneficial microorganism with huge potential to be explored not only as antagonist bacteria against *Xoo* but also as plant growth promoter. Therefore, the aim of this study is to screen and characterize bacteria isolated from different sources which has potential as antagonistic bacteria against *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), the causal agents of bacterial leaf blight of paddy and as a plant growth promoter.

2. MATERIALS AND METHODS

2.1 Pathogen Inoculum Preparation

The *Xanthomonas oryzae* pv. *oryzae* TK040 (KY848237) was obtained from Bacteriology Laboratory, Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia.

The strain was sub-cultured in Potato Sucrose Agar (PSA) plate, PSA slant and stored in 20% glycerol at -80℃ for long term preservation. Preparation of PSA media: Peptone (10 g/L), Sucrose (10 g/L), Bacteriological Agar (16 g/L), Cyclohexamide (0.05 g/L), Cephalexin (0.04 g/L) and Kasogamycin (0.02 g/L). For inoculums preparation, the purified *Xoo* culture was inoculated on PSA plate and incubated at 28℃ for 48 hours. Then, *Xoo* was diluted with sterile distilled water. Cell suspensions were adjusted to 10^8 colony forming unit (CFU/mL) using a spectrophotometer at 600 nm wavelength with optical density volume of 0.6.

2.2 Isolation of Bacteria from Compost, Water, Rhizosphere, Roots and Leaves

Bacteria were isolated from multiple sources including compost, rhizosphere, water and rice plant components. Bacterial isolates from compost were obtained from UPM compost (UPMC), JITU compost (JITUC), chicken dung compost (TAC), green field compost (GFC), SRI1 compost, SRI2 compost, SRI3 compost and SRI4 compost. One sample of rice plant, rhizosphere and water (in the rice plot) was collected from three different locations (Block B, Block C and Blok K) of rice field in Tali Air 2 Sungai Burung, Kampung Sungai Burung, Tanjung Karang, Selangor, Malaysia. Isolation was done using dilution plate technique: (i) 5 g of compost was added to 95 mL sterile distilled water in a sterile flask, (ii) 10 mL water (in the rice plot) was added to 90 mL sterile distilled water in a sterile flask, (iii) 10 g rhizosphere was added to 90 mL sterile distilled water in a sterile flask, (iv) 1 g of surface sterile roots and leaves (healthy young leaves) mashed with mortar was added to 99 mL sterile distilled water in a sterile flask. All suspensions were shaken on incubator-shaker at 150 rpm for 30 minutes. After that, sample suspensions was withdrawn (1 mL) and added to sterile distilled water (9 ml) and shaken for 1 minute. Each sample suspension was subjected to serial dilution up to 10^{-8}. Serial dilution (0. 1 mL) was dispensed using pipette on nutrient agar (NA), then the suspension was spread using glass rod: (i) 10^{-5} to 10^{-7} dilution for compost and rhizosphere and (ii) 10^{-3} to 10^{-5} dilution for water, roots and leaves and incubated at 28℃ for 2 days. The experiment was performed with a completely randomized design with 3 replications. Individual bacterial colonies with different morphological characteristics were obtained by sub-culturing and were stored in 20% glycerol at -80℃ for long term preservation.

2.3 Screening for Antagonistic Activity

Antagonistic activity screening was performed based on chloroform vapour method [12]. Antagonistic bacteria candidate were inoculated vertically at centre of NA and incubated for 5 days at 28℃. Five days grown bacteria were killed by inverting petri dishes over chloroform for 3 minutes. The plates in which bacteria were grown were flooded with 2 mL of pathogen cell suspension (10^8 CFU/mL), air dried and incubated for 1-2 more days. Effectiveness of strains as antagonist was evaluated by measuring the inhibition zones around antagonistic bacteria by subtracting bacteria colony growth length at the centre plate from the combined inhibition zone length and bacteria colony growth length. The experiment was performed with a completely randomized design with 3 replications.

2.4 Antagonistic Bacteria Inoculum Preparation

Antagonistic bacteria were sub-cultured in NA plate, NA slant and stored in 20% glycerol at -80℃ for long term preservation. For inoculum preparation, the purified antagonistic bacterial culture were inoculated on NA plate and incubated at 28℃ for 24 hours. Then, antagonistic bacteria were diluted with sterile distilled water. Cell suspensions were adjusted to 10^8 colony forming unit (CFU)/mL) using a spectrophotometer at 600 nm wavelength with optical density volume of 0.6

2.5 Production of Various Hydrolytic Enzymes

Positive antagonistic bacteria from the previous experiment were evaluated for hydrolytic enzyme activity such as protease, cellulase and lipase. All diameters that indicated positive results were measured by subtracting the bacterial colony diameter from the combined positive zone diameter and the bacterial colony diameter.

2.5.1 Screening for protease activity

Antagonistic bacterial suspension (10 µl) was dispensed using pipette onto sterile filter paper (6.00 mm) that was placed on skim milk agar plate [13]. The plates were then incubated at

30℃ for 24 hours. After that, clear halo zone (mm) around the colony was measured for positive result. The experiment was performed with a completely randomized design with 3 replications. Skim milk agar preparation: Skim milk powder (28 g/L), Casein enzymic hydrolysate (5 g/L), Yeast extract (25 g/L), Dextrose (1 g/L) and Bacteriological Agar (15 g/L).

2.5.2 Screening for cellulase activity

Antagonistic bacterial suspension (10 µl) was dispensed using pipette onto sterile filter paper (6.00 mm) that was placed on NA media with 1% carboxymethylcellulose. The plates were then incubated at 37℃ for 48 hours. After that, plate flooded with 0.1% congo red reagent and left for 20 minutes. The plate was washed with 1 M NaCl. Clear zone against red colour of congo red indicated positive test. The experiment was performed with a completely randomized design with 3 replications.

2.5.3 Screening for lipase activity

Antagonistic bacterial suspension (10 µl) was dispensed using pipette onto sterile filter paper (6.00 mm) that was placed on agar medium for lipase enzyme activity that was prepared with slight modifications [14]. The plates were then incubated at 30℃ for 48 hours. Positive test were indicated by the crystallized zone around bacterial colony. The experiment was performed with a completely randomized design with 3 replications. The agar medium was prepared using Peptone (10.0 g/L), NaCl (5.0 g/L), $CaCl_2$ (0.1 g/L) and Bacteriological agar (15.0 g/L). After the medium was autoclaved, it was cooled to about 50℃ and 10 mL of autoclaved Tween 20 was added. The final pH of this medium was 7.4.

2.6 Plant Growth Promoting Activities

Active isolates with antagonistic potential against *Xoo* were further evaluated for their plant growth promoting activities such as siderophore production, IAA production, phosphate solubilizing and nitrogen fixation. All diameters that indicated positive results were measured by subtracting the bacterial colony diameter from the combined positive zone diameter and the bacterial colony diameter. Nitrogen fixation ability were observed based on qualitative scale.

2.6.1 Screening for siderophore production activity

Antagonistic bacterial suspension (10 µL) was dispensed using pipette onto sterile filter paper (6.00 mm) that was placed on Chrome azurol S (CAS) agar plate [15]. The plates were then incubated at 30℃ for 5 days. Development of yellow-orange halo zone around the bacterial growth was considered as positive for siderophore production and the diameter were measured. Experiment was performed with a completely randomized design with 3 replications. CAS agar was prepared from 4 solutions. Solution 1 (Fe-CAS indicator solution) was prepared by mixing 10 ml of 1 mmol/L $FeCl_3.6H_2O$ (in 10 mmol/L HCl) with 50 mL of an aqueous solution of CAS (1.21 g/L). The resulting dark purple mixture was added slowly with constant stirring to 40 mL of aqueous solution of hexadecyltrimethylammonium bromide (1.821 g/L). The yielded of dark blue solution which was autoclaved, then cooled to 50℃. The entire reagent was freshly prepared for each batch CAS agar. Solution 2 (buffer solution) was prepared by dissolving 30.24 g of piperazine-N, N-bis (2-ethane sufonic acid) (PIPES) in 750 mL of salt solution containing 0.3 g K_2HPO_4, 0.5 g NaCl and 1.0 g NH_4Cl. The pH was adjusted to 6.8 with 50% (w/v) KOH, and water was added to bring the volume 800 mL. The solution was autoclaved after adding 15 g of agar then cooled to 50℃. Solution 3 contained 2 g glucose, 2 g mannitol, 493 mg $MgSO_4.7H_2O$, 11 mg $CaCl_2$, 1.17 mg $MnSO_4.2H_2O$, 1.4 mg H_3BO_3, 0.04 mg $CuSO_4$. $5H_2O$, 1.2 mg $ZnSO.7H_2O$, 1.0 mg $NaMoO_4$. $2H_2O$ in 70 mL water, autoclaved, cooled to 50℃. Solution 4 was 30 mL filter sterilized 10% (w/v) casamino acid. Finally, solution 3 added to solution 2 along with solution 4, solution 1 was added last, with sufficient stirring.

2.6.2 Screening for IAA production activity

Bacterial isolates were grown in NB media and incubated in incubator shaker with 150 rpm agitation at room temperature (28 ± 2℃) for 24 hours. Bacterial culture (1 mL) was inoculated into 100 mL of sterile NB amended with 5 mL L-TRP solution and grown for 48 hours. The broth was centrifuged at 12000 rpm for 5 minutes and 1 mL of supernatant was added to 2 mL Salkowski reagent. The colour density of the mixture (red colour development which indicated IAA production) was measured using UV spectrophotometer at 530 nm absorbance [16]. The amount of IAA production was determined

based on standard curve. Salkowski reagent preparation was prepared by 2% of 0.5M $FeCl_3$ in 35% perchloric acid. Standard curve preparation by 100 mg/L of an IAA solution was prepared by dissolving 0.005 g of IAA in 50 mL of ethyl acetate solution. From the prepared solution, 0.25 to 3.75 mL of aliquots was mixed with 25 mL of ethyl acetate to make 1 to 15 mg concentration of IAA/mL, respectively. The experiment was performed with a completely randomized design with 3 replications.

2.6.3 Screening for phosphate solubilisation activity

Antagonistic bacterial suspension (10 mL) was dispensed using pipette onto sterile filter paper (6.00 mm) that was placed on National Botanical Research Institute's phosphate (NBRIP) agar plate [17]. The plates were then incubated at 28℃ for 7 days. Phosphate solubilization was assessed by measuring the clear halo zone. The halo zone was calculated by subtracting bacterial colony diameter from the combine halo zone and bacterial colony diameter. The experiment was performed with a completely randomized design with 3 replications. NBRIP preparation: Glucose (10 g/L), $Ca_3(PO4)_2$ (5 g/L), $MgCl_2.6H_2O$ (5 g/L), $MgSO_4.7H_2O$ (0.25 g/L), KCl (0.2 g/L), $(NH_4)_2SO_4$ (0.1 g/L), Bacteriological Agar (15/L g).

2.6.4 Screening for nitrogen fixation activity

Antagonistic bacterial suspension (10 µL) was dispensed using pipette onto sterile filter paper (6.00 mm) that was placed on Nitrogen free media [18]. After 7 days of incubation period at 28±2℃, the isolates ability to fix nitrogen was observed by green to blue coloration of the nitrogen free medium. The experiment was performed with a completely randomized design with 3 replications. Nitrogen free media preparation: DL-malic acid (5 g/L), K_2HPO_4 (0.5g/L), $MgSO_4.7H_2O$ (0.2 g/L), KOH (4 g/L), NaCl (0.1 g/L), $CaCl_2$ (0.02 g/L), Bacteriological Agar (16 g/L), Bromothymol blue (0.5% solution in 0.2 M KOH) (2 ml/L), FeEDTA (1.64% solution) (4 ml/L), Trace element solution (2 ml/L) and Vitamin solution (1 ml/L). Trace element solution preparation: Na_2MoO_4 (1 g/L), $MnSO_4.H_2O$ (1.175 g/L), H_2BO_3 (1.4 g/L), $CuSO_4.5H_2O$ (40 mg/L) and $ZnSO_4.7H_2O$ (120 mg/L). Vitamin solution preparation: Biotin (100 mg/L) and Pyridoxine (200 mg/L).

2.7 Identification of Antagonistic Bacteria Using Molecular Method

Antagonistic bacterial isolates were grown in Nutrient Broth at room temperature with 180 rpm agitator for 2 days. Fresh bacteria pellet were collected by centrifugation. Genomic DNA extraction was performed using genomic DNA Mini Kit (GeneAid). Amplification of the 16s rRNA was performed by Polymerase Chain Reaction (PCR) using primer 8F (5'AGAGTTTGATCCTGGCTCAG3') and 1492R (5'GGTTACCTTGTTACGACTT3') primer pair synthesised by First Base Labroratories Sdn Bhd, Selangor, Malaysia. The PCR reaction mixture (25 µL) consisted of genomic DNA (3 µL), Dream Taq Green PCR Master Mix (Thermo Scientific) (12.5 µL), nucleus free water (8.5 µL) and 10 µm of each primer (0.5 µL). The thermal cycler (MJ Mini Personal Thermal Cycler, Bio-Rad) was set with the following temperature profile: an initial denaturation at 95℃ for 3 minutes followed by 34 cycles of denaturation at 95℃ for 1 minute, annealing at 57℃ for 1 minute, and extension at 72℃ for 1 minute, and final extension at 72℃ for 10 minutes [19]. PCR products were analyzed by agarose gel electrophoresis, 2% agarose gel were prepared in 1XTAE Buffer which were stained with Fluoro Safe DNA Stain (1st base). PCR products (5 µL) were separated by electrophoresis on 2% agarose gels in 1XTAE buffer at 100 V and monitored the progress of the gel. A 100 bp and 1 kb ladder were used as a marker. Gel current was stopped after the gel was run ¾ lengths. Gel was visualized under UV light and photographed by using Bio Rad™ gel documentation system. The PCR product was purified using the Qiaquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced by First Base Laboratories Sdn Bhd, Selangor, Malaysia. The multiple sequence and pair wise alignments were constructed by CLUSTAL W Bioedit software. The nucleotides sequences were subjected to Basic Local Alignment Search Tool (BLAST) analysis (http://www.ncbi.nih.gov/index/html.) for identification and all sequences were submitted to NCBI GenBank database.

2.8 Statistical Analysis

All data were analyzed by Statistical Analysis System (SAS, version 9.3, 2014). Data were subjected to the analysis of variance procedure (ANOVA), differences among treatment means

were determined using Tukey's Test comparison method at probability level of 0.05.

3. RESULTS AND DISCUSSION

3.1 Isolation of Bacteria from Compost, Water, Rhizosphere, Roots and Leaves

There was significant difference in the population of bacteria from different compost (Table 1). SRI2 compost recorded highest population of bacteria which is 7.0 x 10^8 CFU/g and chicken dung compost stated lowest population of bacteria which is 1.4 x 10^8 CFU/g. Bacterial diversity during composting and by-product compost was well established [20], therefore, abundant of bacteria were able to be isolated from compost. Bacterial populations for samples collected from paddy field were shown in Table 2. No significant difference evaluated from the same sample from different block. However, type of samples (water, rhizosphere, roots and leaves) in each block recorded means of bacterial population with a significant different. The highest bacterial population was recorded from the rhizosphere compared with other samples and endophytic bacteria from leaves sample recorded the lowest population. Rhizosphere supports large and active microbial population capable of exerting beneficial, neutral and detrimental effects on the plants [21]. Separation of bacterial isolates was performed based on morphological characteristics such as colour, configuration, margin and elevation. A total of 93 isolates were isolated from different sources obtained from this study. Highest isolates (46) were isolated from compost samples followed by other sample; leaves (13), soil (12), water (12) and roots (10).

3.2 Screening for Antagonistic Activity

A total of 93 strains isolated from this study were isolated from different sources. However, only 16 isolates demonstrated positive result as antagonist against *Xoo* (Table 3). Positive antagonistic activity indicated by inhibition zone production on agar plate (Fig. 1). Antagonistic activity from isolates indicated by inihibition zone production was ranged from 3.33 to 15.00 mm. There was significant difference on antagonistic activity against *Xoo* between each of different isolates. UPMC10 strain showed the highest potential as antagonist compared to other isolates which demonstrated 15.00 mm inhibition zone against *Xoo*. Lowest potential as antagonist was recorded by SRI2-1 strain, which demonstrated only 3.33 mm inhibition zone. Antagonistic activity screening is an essential step for a preliminary assessment to identify potential antagonistic isolates that produce antibacterial effect towards the target pathogen. In this study, chloroform vapour method proved that this method was able and successfully ascertained the potential candidates of antagonistic bacteria. In previous study, the same method was used to assess the antagonistic activity of *B. subtilis* B2G, *Streptomyces setoni* RP87 and fluorescent Pseudomonads against *Ralstonia solanacearum*, plant pathogen of bacterial wilt disease of potato, tomato and eggplant [22]. In sustainable agriculture, certain plant pathogens can be controlled by biological agents like plant growth promoting bacteria (PGPB) and at the same time, PGPB was used as bio-fertilizer [23]. There are a lot of PGPB strains that reported to suppress numerous of plant pathogen, reduce the disease incidence, stimulate the plant growth factor and supplies the nutrition for the growth of plant [23-26]. Therefore, it has been considerable research interest in the potential use of antagonistic bacteria as PGPB [27,28]. It has been reported that, *P. fluorescens* PDY7 able to control BLB and promoting the growth of rice variety IR24 [29].

Table 1. Population of bacteria from different compost

Source	Bacterial population (CFU/g) x 10^8
UPM	2.10 ^{c}
JITU	2.20 ^{c}
Green Field	2.30 ^{c}
Chicken Dung	1.40 ^{d}
SRI1	4.00 ^{b}
SRI2	7.00 ^{a}
SRI3	1.70 ^{c,d}
SRI4	2.30 ^{c}

** Means in column followed with different letter (s) are significantly different (Tukey's Test P= .05)*

3.3 Hydrolytic Enzyme Production

Isolates tested positive in antagonistic activity screening (16 strains) against *Xoo* were demonstrated positive reactions in different hydrolytic enzyme production test (protease, cellulase and lipase). All 16 strains produced at least one hydrolytic enzyme. Indications for positive results off all enzyme production tests

Table 2. Population of bacteria in water, rhizosphere, roots (endophytic) and leaves (endophytic) from different block on paddy field

Location/ Source	Water (CFU/ml) x 10^6	Rhizosphere (CFU/g) X 10^6	Roots (endophytic) (CFU/g) X 10^6	Leaves (endophytic) (CFU/g) X 10^6
BLOCK B	17 ^{aB}	440 ^{aA}	21 ^{aB}	1.70 ^{aC}
BLOCK C	18 ^{aB}	420 ^{aA}	23 ^{aB}	1.80 ^{aC}
BLOCK K	15 ^{aC}	400 ^{aA}	27 ^{aB}	1.80 ^{aD}

** Means in column followed with different lower case letter (s) are significantly different (Tukey's Test P= .05)*
** Means in row followed with same different upper case letter (s) are significantly different (Tukey's Test P= .05)*

are shown in Table 4. Combination of hydrolytic enzymes or other antagonistic mechanism result in higher level of antagonism than that obtained from a single mechanism alone [30]. Studies have shown that the antibacterial effect of bacteria is generally due to either individual or joint production of antibiotics, bacteriocins, siderophores [31], lysozymes and proteases and alteration of pH by organic acid production [32]. Previous study reported that PGPB isolates that suppressed plant pathogen produced various hydrolytic enzymes [33]. There were a lot of reports on PGPB as a growth promoter and biocontrol agents against plant pathogen that were able to produce hydrolytic enzymes [34]. It have been reported that esterase, lipase and protease are hydrolytic enzymes which are involved in the suppression of pathogenic growth and subsequent reduction in damage to plants [35]. However, inhibition due to such compounds was highly dependent on the experimental conditions, which were different *in vitro* and *in vivo* [36].

Fig. 1. Bacterial isolate showed antagonistic activity against *Xoo* on nutrient agar

3.3.1 Screening for protease activity

Only 12 antagonistic bacteria out of 16 isolates were able to produce protease indicated by clear halo zone around bacteria colony on skim milk agar (Fig. 2). There was significant difference on protease activity among antagonistic bacteria which ranged from 2.00 to 16.33 mm. Bacterial isolate SRI2-1 showed highest protease activity (16.33 mm) and lowest shown by bacterial isolate UPMC10 (2.00 mm).

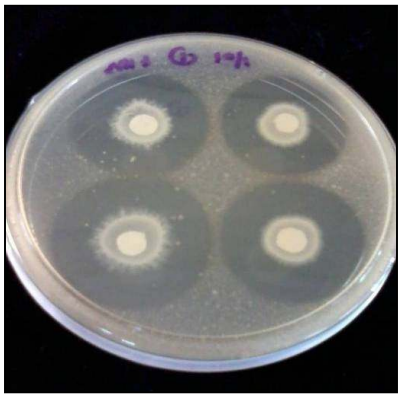

Fig. 2. Antagonistic bacterial isolate showed positive protease activity by producing clear halo zone around bacterial colony on skim milk agar

Table 3. Antagonistic Activity of Antagonistic Bacteria against *Xoo* (positive reaction zone, mm)

Isolate	Antagonistic Activity
UPMC10	15.00 ± 0.58 ^{a}
LBC2	10.33 ± 1.76 ^{b}
JITUC7	10.00 ± 0.58 ^{b}
TAC2	10.00 ± 0.58 ^{b}
LBB3	9.67 ± 0.33 ^{b}
SBC1	9.33 ± 0.33 ^{b}
SRI3-2	9.00 ± 1.16 ^{bc}
SRI4-3	7.67 ± 0.88 ^{bcd}
SRI2-2	7.00 ± 0.58 ^{bcde}
LBB2	5.33 ± 0.33 ^{cde}
SRI4-1	4.67 ± 0.33 ^{de}
HBC2	4.33 ± 0.33 ^{de}
LBC1	4.33 ± 0.67 ^{de}
SRI1-2	3.67 ± 0.33 ^{e}
SRI1-3	3.33 ± 0.33 ^{e}
SRI2-1	3.33 ± 0.33 ^{e}

** Means in column followed with different letter (s) are significantly different (Tukey's Test P= .05)*

Table 4. Protease, cellulase and lipase activity of antagonistic bacteria (positive reaction zone, mm)

Isolate	Protease	Cellulase	Lipase
UPMC10	2.00 ± 0.00 ^{d}	-	16.50 ± 1.5 ^{a}
LBC2	-	3.00 ± 0.58 ^{bc}	8.00 ± 0.41 ^{cdef}
JITUC7	14.67 ± 1.20 ^{ab}	3.25 ± 0.25 ^{bc}	12.75 ± 1.11 ^{ab}
TAC2	11.33 ± 0.33 ^{abc}	4.25 ± 0.63 ^{bc}	3.75 ± 0.25 ^{fg}
LBB3	10.00 ± 2.08 ^{bc}	5.25 ± 0.25 ^{bc}	7.25 ± 1.32 ^{defg}
SBC1	12.67 ± 1.33 ^{abc}	5.00 ± 0.71 ^{bc}	11.50 ± 0.87 ^{bcd}
SRI3-2	8.67 ± 1.67 ^{c}	9.00 ± 0.71 ^{a}	12.00 ± 0.91 ^{bc}
SRI4-3	15.33 ± 0.33 ^{a}	3.75 ± 0.25 ^{bc}	11.00 ± 0.71 ^{bcd}
SRI2-2	12.00 ± 0.00 ^{abc}	3.50 ± 0.29 ^{bc}	11.00 ± 1.08 ^{bcd}
LBB2	-	4.50 ± 0.87 ^{bc}	-
SRI4-1	14.67 ± 0.88 ^{ab}	4.00 ± 0.58 ^{bc}	8.75 ± 1.11 ^{bcde}
HBC2	11.67 ± 0.33 ^{abc}	2.50 ± 0.29 ^{c}	5.75 ± 0.48 ^{efg}
LBC1	-	3.50 ± 0.50 ^{bc}	3.00 ± 0.00 ^{g}
SRI1-2	-	5.50 ± 0.50 ^{b}	6.50 ± 0.65 ^{efg}
SRI1-3	12.00 ± 1.00 ^{abc}	-	8.50 ± 0.87 ^{bcde}
SRI2-1	16.33 ± 0.33 ^{a}	2.50 ± 0.29 ^{c}	4.00 ± 0.00 ^{fg}

***** *Means in column followed with different letter (s) are significantly different (Tukey's Test P= .05)*
** - : negative result for the respective test*

3.3.2 Screening for cellulase activity

Only 14 antagonistic bacteria were able to produce cellulase. Cellulase activity among antagonistic bacteria varies which evidence by clear zone against red color of congo red range from 2.50 to 9.00 mm (Fig. 3). There was significant difference on cellulase activity among antagonistic bacteria. Lowest cellulase activity was shown by bacterial isolate HBC2 (2.50 mm) and highest was by bacterial isolate SRI3-2 (9.00 mm).

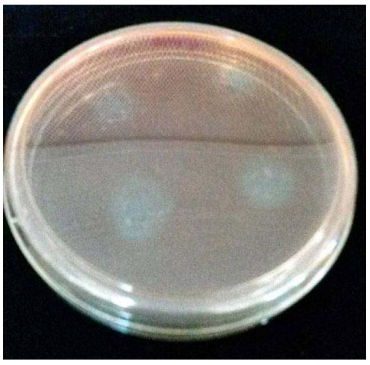

Fig. 3. Antagonistic bacterial isolate showed positive cellulase activity showed by clear zone against red colour of congo red

3.3.3 Screening for lipase activity

From 16 antagonistic bacteria, 15 were able to produce lipase. Lipase activity among antagonistic bacterial ranged from 3.00 to 16.50 mm indicated by crystallize zone around bacterial colony (Fig. 4). There was significant difference on lipase activity among antagonistic bacteria. Bacterial strain UPMC10 recorded highest lipase activity (16.50 mm) and lowest by LBC1 strain (3.00 mm).

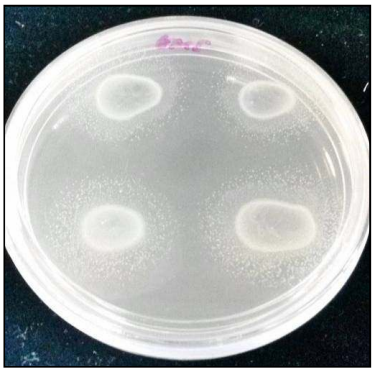

Fig. 4. Antagonistic bacterial isolate showed positive lipase activity by producing crystallize zone around bacterial colony

3.4 Plant Growth Promoting Potential

The screening results for plant growth promoting traits are depicted in Table 5. All 16 antagonistic bacteria were positive in the siderophore production, IAA production and nitrogen fixation activity. Only 13 of 16 antagonistic bacteria were positive in the phosphate solubilizing activity.

Table 5. Siderophore and IAA production; Phosphate solubilizing and nitrogen fixation activity of antagonistic bacteria

Isolate	Siderophore production (mm)	IAA production (mg/L)	Phosphate solubilisation (mm)	Nitrogen fixation
UPMC10	6.00 ± 0.57 ^{cd}	4.42 ± 0.26 ^{c}	11.00 ± 0.00 ^{ab}	Positive
LBC2	6.67 ± 0.33 ^{bc}	4.57 ± 0.68 ^{c}	11.33 ± 0.33 ^{a}	Positive
JITUC7	10.00 ± 0.57 ^{ab}	4.16 ± 0.05 ^{c}	12.33 ± 0.88 ^{a}	Positive
TAC2	10.00 ± 0.00 ^{ab}	1.56 ± 0.36 ^{ef}	-	Positive
LBB3	4.67 ± 1.86 ^{cdef}	3.65 ± 0.12 ^{cd}	5.67 ± 0.33 ^{c}	Positive
SBC1	7.33 ± 0.33 ^{bc}	0.99 ± 0.17 ^{ef}	5.00 ± 1.00 ^{cd}	Positive
SRI3-2	11.00 ± 0.57 ^{a}	1.82 ± 0.23 ^{ef}	-	Positive
SRI4-3	5.00 ± 0.57 ^{cde}	2.33 ± 0.10 ^{cd}	1.00 ± 0.00 ^{f}	Positive
SRI2-2	4.67 ± 0.33 ^{cdef}	0.44 ± 0.04 ^{f}	2.67 ± 0.67 ^{def}	Positive
LBB2	5.00 ± 0.00 ^{cde}	14.37 ± 0.65 ^{a}	8.67 ± 0.67 ^{b}	Positive
SRI4-1	4.00 ± 0.00 ^{cdef}	0.33 ± 0.03 ^{f}	3.33 ± 0.33 ^{cdef}	Positive
HBC2	4.00 ± 0.57 ^{cdef}	0.76 ± 0.04 ^{f}	-	Positive
LBC1	2.67 ± 0.67 ^{def}	11.52 ± 0.36 ^{b}	10.67 ± 0.33 ^{ab}	Positive
SRI1-2	1.67 ± 0.33 ^{ef}	0.71 ± 0.04 ^{f}	5.33 ± 0.33 ^{c}	Positive
SRI1-3	1.33 ± 0.33 ^{f}	3.40 ± 0.06 ^{cd}	2.33 ± 0.33 ^{f}	Positive
SRI2-1	2.00 ± 1.00 ^{ef}	0.81 ± 0.24 ^{ef}	4.00 ± 0.00 ^{cde}	Positive

** Means in column followed with different letter (s) are significantly different (Tukey's Test P= .05)*
** - : Negative result for the respective test*

3.4.1 Screening for siderophore production activity

All 16 antagonistic strains were able to produce siderephore which exhibited by orange halo zone around bacteria colony on CAS agar (Fig. 5). There was significant difference on siderophore production among antagonist bacteria which ranged from 1.33 to 11.00 mm. Lowest siderophore production (1.33 mm) was recorded by strain SRI1-3 and highest by SRI3-2 (11.00 mm). It was known that microorganism that can produce siderophore provided Fe nutrition to enhance plant growth when iron element bioavailability was low [37]. It was also known for more than three decades that different bacterial species were capable to improve plant growth, contributed into plant Fe nutrition and promoted roots and shoots growth by producing siderophores [38]. Siderophore is particularly important when evaluating the potential of a strain for biocontrol [39]. Siderophores have been suggested to be an environmentally friendly alternative to hazardous pesticides [40]. The biological control mechanism depended on the role of siderophore as competitors for Fe in order to reduce Fe availability for the phytopathogen [41]. Siderophores produced by numerous bacteria had a significant role in the biocontrol and negatively affected the growth of several pathogens [41,42]. Siderophores also have role in induced systemic resistance (ISR) in plants [43].

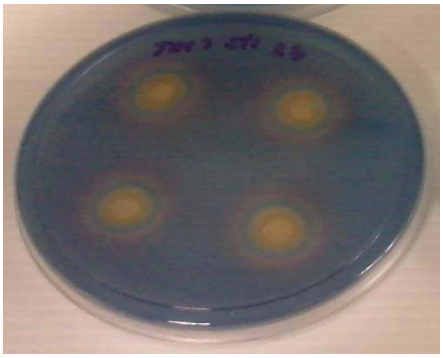

Fig. 5. Antagonistic bacterial isolate showed positive siderophore production activity as indicated by orange halo zone around bacterial colony on CAS agar

3.4.2 Screening for IAA production activity

All 16 antagonistic bacteria were able to produce IAA which ranged from 0.33 to 14.37 mg/L (Fig. 6). There was significant difference on IAA production among antagonistic bacteria. Isolate LBB2 showed the highest value for IAA production at 14.37 mg/L, where isolate SRI4-1 recorded the lowest value at 0.33 mg/L. IAA is one of the most important phytohormone for plant growth and function as important signal molecule in the regulation of plant development. IAA also has been speculated to improve the fitness of plant-microbe interactions [44]. From previous studies, it showed that many

plant-associated bacteria have the ability to produce IAA take part in the most important role in plant growth promotion by stimulating plant roots development and improving absorption of water and nutrients from soil [45,46]. The IAA-producing bacteria encouraged adventitious root formation, produced the greatest roots and shoots weight [47]. Therefore, it was possible that PGPB strains that produced IAA affected plant hormones levels [48]. IAA has been shown to have an important role not only in plant development but also in activation of the plant defense system [49]. Moreover, several recent reports indicate that IAA can also be a signaling molecule in bacterial communication [50]. It has been reported that strains of IAA production had more active metabolism resulting in tolerance to stress environments [51].

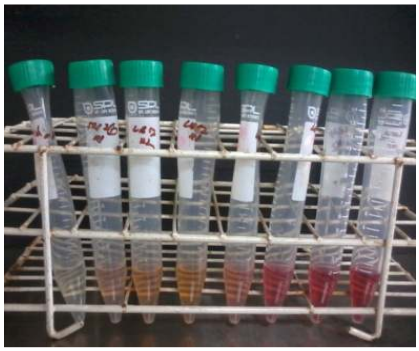

Fig. 6. IAA production activity indicated by red colour development of antagonistic bacterial culture supernatant mixed with Salkowski reagent

3.4.3 Screening for phosphate solubilization activity

Out of 16 isolates, only 13 antagonistic bacteria were able to solubilize phosphate (Fig. 7). There was significant difference on phosphate solubilizing activity among antagonist bacteria. Bacterial isolate JITUC7 recorded the highest phosphate solubilizing activity by 12.33 mm clear halo zone indication around the bacterial growth while the lowest recorded by bacterial isolate SRI4-3 was 1.00 mm. In rhizosphere, bacteria secreted organic acids which results in phosphate solubilization from insoluble complexes, making it available for plant uptake [52]. Therefore, it have been reported that phosphate solubilizing bacteria (PSB) induced plant growth promotion [53]. Plant root-associated PSB have been considered as one of the possible alternatives for inorganic phosphate fertilizers for promoting plant growth and yield [54]. Increased plant growth and phosphate uptake have been reported in many crop species as a results of PSB inoculants [55,56]. Current research suggested that the inoculation of crops with phosphate solubilizing microbes (PSM) had the potential to reduce application rates of phosphate fertilizers by 50% without significantly reducing crop yield [57].

Fig. 7. Antagonistic bacterial isolate showed positive phosphate solubilizing activity by producing clear halo zone around the bacterial colony on NBRIP agar

3.4.4 Screening for nitrogen fixation activity

All 16 antagonistic bacteria were able to grow on nitrogen free agar plate and showed positive results (Fig. 8). Bacteria which were able to fix nitrogen particularly important in organically managed soils, which typically have lower proportion of nitrogen in available forms [58]. Free-living nitrogen fixing bacteria may fix significant amounts of nitrogen (0-60 kg N $ha^{-}1$) [59]. Nitrogen fixing bacterial strains has been reported to influenced root and shoot length, seedling vigor, higher plant height and dry biomass of some crop plants [60]. Inoculation with nitrogen fixing bacteria also significantly increased chlorophyll content, and the uptake of different macro- and micro-nutrient contents [60]. Associative diazotrophic microorganism can contribute at least 20-40% of the plant N requirement of numerous non leguminous crops through N_2 fixation process [61]. *Azospirillum* and rhizobacteria inoculation can supply a large amount of the total plant N requirement through biological N_2 fixation (BNF) process [62]. There are studies showing that N-fixing bacteria, free-living as well as *Rhizobium* strains, can stimulate the growth of non-legumes such as radish and rice [63], in this way contributing to reduced dependence on N based fertilizers [64].

Table 6. Molecular identification of potential bacteria as antagonistic

Isolate	Source	Isolate name	Genbank accession no	% similarity	Genbank reference
UPMC10	Compost	*Bacillus licheniformis*	KT958890.1	99%	KJ190320.1
LBC2	Leaves	*Acinetobacter sp.*	KT958889.1	100%	JQ912623.1
JITUC7	Compost	*Acinetobacter sp.*	KT958891.1	99%	KF771255.1
TAC2	Compost	*Bacillus sp.*	KT958892.1	100%	FM992836.1
LBB3	Leaves	*Pseudomonas putida*	KT958893.1	100%	KC622047.1
SBC1	Rhizosphere	*Burkholderia cepacia*	KT958894.1	100%	AB252073.1
SRI3-2	Compost	*Bacillus amyloliquefaciens*	KT958895.1	100%	KJ123715.1
SRI4-3	Compost	*Staphylococcus warneri*	KT958896.1	100%	LK934697.1
SRI2-2	Compost	*Bacillus sp.*	KU221021.1	100%	KJ563082.1
LBB2	Leaves	*Pantoea vagans*	KT958897.1	100%	HG421010.1
SRI4-1	Compost	*Bacillus sp*	KT958898.1	100%	KR259213.1
HBC2	Water	*Bacillus sp.*	KT958899.1	100%	KJ130061.1
LBC1	Leaves	*Pantoea sp.*	KT958900.1	100%	KF358308.1
SRI1-2	Compost	*Oceanobacillus oncorhynchi*	KT958901.1	100%	KF600569.1
SRI1-3	Compost	*Paenibacillus cineris*	KT958902.1	100%	KF979149.1
SRI2-1	Compost	*Bacillus sp.*	KT958903.1	100%	KF872711.1

3.5 Identification of Antagonistic Bacteria Using Molecular Method

All the antagonistic bacteria candidates were subjected to 16s rRNA amplification for molecular identification. All the isolates were succesfully amplified and produced DNA fragment sizes of approximately 1500 bp. The antagonistic bacteria were succesfully identified by 16s rRNA amplification (Table 6). The antagonistic strains belong to *Bacillus* sp. (5), *Acinetobacter* sp. (2), *Bacillus licheniformis* (1), *Pseudomonas putida* (1), *Burkholderia cepacia* (1), *Bacillus amyloliquefaciens* (1), *Staphylococcus warneri* (1), *Pantoea vagans* (1), *Pantoea* sp. (1), *Oceanobacillus oncorhynchi* (1) and *Paenibacillus cineris* (1). In this study, the potential antagonistic bacterial isolates have been identified from various genera to species. Bacteria genera including *Azotobacter*, fluorescent *Pseudomonas* species, *Rhizobium* and *Bacillus* are widely used as biological control agents [65]. *Pseudomonas* and *Bacillus* was reported to enhanced plant growth in addition to disease control [66]. In this study, most of the antagonistic bacteria against *Xoo* belong to the genus *Bacillus*. Among terrestrial bacterial strains, the genus of *Bacillus* has been studied due to its ability to produce different structure of inhibitory compounds [67]. Most of these inhibitory agents have been categorized in peptide derivative family [67,68]. *Bacillus* spp. and its related genera have been identified as potential biocontrol agent as they produce wide range of cyclic lipopeptides active against various microorganisms [69]. *Bacillus* spp. was also known to produce a wide range of secondary metabolites such as antibiotics, non-volatile and volatile compounds [70] and lytic enzymes [71]. Both *Bacillus* and *Paenibacillus* species were well known for the antagonizing ability against the pathogens under *in vitro* and *in vivo* conditions [72,73].

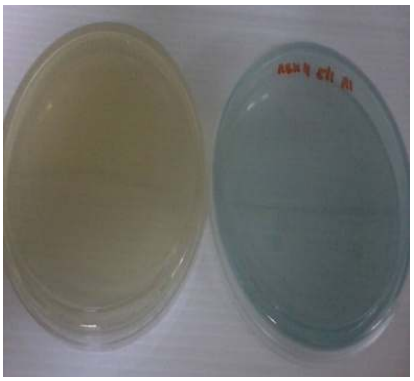

Fig. 8. Nitrogen fixation activity indicated by colour change of agar from green to blue

4. CONCLUSION

This study confirmed that bacteria have potential as antagonistic against *Xoo*. Antagonistic bacterial isolates also posses other characteristics such as hydrolytic enzyme (protease, cellulase, and lipase) production, siderophore production, IAA production, phosphate solubilization and nitrogen fixation.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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