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Antimicrobial activity of the Rhizospheric *Bacillus* species isolated from Potato (*Solanum tuberosum)* Organic Farm Soils in the Philippines

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Abstract

The purpose of this study is to determine the potential of rhizospheric bacteria belonging to the genus *Bacillus* isolated from the organic soil of *Solanum tuberosum* (potato) as an untapped and promising source of novel antimicrobials to combat infections, particularly multidrug-resistant strains. The rhizospheric *Bacillus* species were isolated using serial dilution and aerobic cultivation. Hydrolytic exoenzyme production was determined using plate techniques, whereas antimicrobial activity was determined using the cross-streak method and agar-disc diffusion assay. The data indicate that the *Bacillus* isolates possess antimicrobial property against gram-positive bacterial pathogens. The activities were compared to those of the antibiotic Rifampicin as a control. Notably, several *Bacillus* isolates inhibited the growth of methicillin-resistant Staphylococcus aureus (MRSA). The top performing *Bacillus* isolates were identified by 16s rRNA gene sequence analysis, which showed the similarities of the isolates to known soil-associated and plant-growth-promoting species; *B. velezensis, B. mojavensis, B. subtilis, B. sonorensis, B. tequilensis, B. clausii, B. amyloliquefaciens, B. altitudinis,* and *B. siamensis* from those sequences available in GENBANK.

The present investigation establishes the presence of antagonistic *Bacillus* species in *S. tuberosum's* rhizosphere. The findings may form the basis for further investigation of the active compounds produced by the isolates and the mechanisms underlying their antimicrobial activity, while optimizing the culture medium for efficient production of potent antimicrobial compounds to combat infectious agents may further be investigated.

Keywords: Natural product discovery, multi-drug resistant pathogens, *Bacillus* species, 16s rNA gene sequence analysis, agar-disc diffusion, hydrolytic exoenzymes

Introduction

Multi-drug resistant (MDR) pathogens are the leading causes of infections worldwide (Valle et al., 2016). The majority of the hospital-acquired related infections is caused by the bacterial pathogens; *Enterococcus* spp., *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa,* and *Enterobacter species,* collectively called as ESKAPE (Fair and Nitzhak, 2014; Pendleton et al., 2013; Boucher et al., 2009; Karlowsky et al., 2017). Bacterial infections caused by these MDR strains can result in a variety of diseases, including pneumonia, bronchitis, acute water diarrhea, influenza, and tuberculosis, all of which are major causes of morbidity and

mortality globally (World Health Organization, 2014.). MDR infections develop resistance to first-line antimicrobials as a result of their increased virulence; adhesion and invasion factors, endotoxins, exotoxins, capsule formation, to name a few (Peterson and Barron, 1996). Additionally, the treatment of MDR infections resulted in extended hospital stays for patients, increasing their medical expenses and risk of nosocomial infection exposure (Tanwar et al., 2014). This problem was exacerbated by improper and abusive antimicrobial usage in agriculture (Wall et al., 2016; Economou and Gousia, 2015), industry, and medical settings (Fair and Tor, 2014), which resulted in the development of drug resistance. Consequently, treatment delays, inadequate sanitation, and suboptimal control techniques have facilitated the establishment and spread of MDR microorganisms in the community and health care settings (Fair and Tor, 2014; Giske et al., 2008).

Despite the threat posed by multidrug-resistant organisms, treatment options remain available. Antibiotics of last resort, such as polymyxins, tigecycline, and later generation carbapenem (Huttner et al., 2012; Giamarellou, 2006; Meletis, 2016; Boucher et al., 2009; Grill and Maganti, 2011), as well as combination drug therapies (Worthington and Melander, 2013), are still effective in the treatment of MDR-associated infections. Increased doses of broad-spectrum antimicrobials continue to be considered (Huttner et al., 2012; Giamarellou, 2006; Meletis, 2016; Boucher et al., 2009; Worthington and Melander, 2013). However, prolonged therapy with high dosages of antibiotics and other methods of dealing with MDRs has been shown to have harmful effects, including liver, renal, and nervous system failure (Falagas and Kashiakou, 2006). While present levels of antimicrobial production are insufficient to meet rising demand, it is critical to develop new antimicrobials that are effective, less toxic, and cost-effective.

Plants (Valle et al., 2016; Ginovyan et al., 2017; Swany et al., 2017), fungi (Pereira et al., 2013), and animal tissues and secretions (Periyasamy et al., 2012; Kumari et al., 2019) are all potential sources of antimicrobials. However, emphasis is placed on microbial-derived natural compounds, notably those generated from bacteria, due to their inherent capacity for rapid population growth and powerful antimicrobial synthesis in vitro (Sekurova et al.,2019). Among microbe species, *Streptomyces*, *Pseudomonas*, and *Bacillus* are the most well-known producers of antimicrobials (Falkinhami III et al., 2009). There are numerous types of bacteria capable of producing antimicrobial chemicals, but growing research has been directed toward *Bacillus* species (Shahcheraghi et al., 2015).

Bacillus species are Gram-positive, rod-shaped, aerobic or facultatively anaerobic endospore-forming bacteria that are capable of surviving in a broad variety of conditions (Slepecky and Hemphill, 2006). Their widespread distribution is a result of their generation of endospores, which are the bacteria's dormant form and are resistant to a wide variety of conditions, including desiccation, high acid levels, and fluctuating pH and temperature. *Bacillus* members spread by endospores in a variety of settings, including water (Sarkar et al., 2019), air (Pangallo et al., 2009), animal gut (Tam et al., 2006), and plant soil rhizospheres (Tam et al., 2006). (Podile and Kishore, 2006). *Bacillus* species can also exist as saprophytes, or organisms that survive in decomposing organic matter (Jensen et al., 2003; Bottone, 2010). *Bacillus* species predominate in the rhizospheres of numerous plants (the region of soil around the roots of the plants), where they are extremely well suited to survive under severe environmental conditions due to their spore-forming ability (Pandey and Palni, 1997; Amin et al., 2015; Vasudevan et al., 2015). Numerous investigations on the genus's ability to synthesize antimicrobials demonstrate that *Bacillus* spp. can be isolated from the rhizospheres of a variety of plants (Qin et al., 2017; Montealegro et al., 2003). While *Bacillus* spp. grows and behaves as a facultative anaerobe in aerobic settings, they can also thrive and function as a facultative anaerobe in habitats with changing oxygen levels (Cawoy et al., 2011; Clements et al., 2002).

Numerous studies have been conducted on the diverse biological features of *Bacillus* spp. (Yilmaz et al., 2006), including antibacterial (Mondol et al., 2013), antifungal (Oyedele and Ogunbanwo, 2014), antiviral (Steller et al., 1999), and anti-amoebic (Lebbadi et al., 1994) activities. Clearly, members of the genus *Bacillus* may be considered interesting candidates for natural product development for disease control. However, only a few research have attempted to isolate antibiotic-producing *Bacillus* species from organic farms, specifically from the roots of potato plants grown in the Philippines. Thus, this work investigated the antimicrobial activity of *Bacillus* species isolated from the roots of the potato, *Solanum tuberosum*, against pathogenic microorganisms of medical importance. This work has the potential to unlock the potential of rhizospheric bacteria in terms of the finding of novel sources of antimicrobials to battle multidrug-resistant strains.

Materials and Methods

Soil Sampling Site and Collection

The soils were collected from the rhizosphere of *S. tuberosum* at an organic potato farm (Figure 1) in Barangay Paoay, Atok, Benguet, the Philippines (16°37′39″N 120°46′03″E), also regarded as the vegetable capital of the Philippines (Lu et al., 2010). The soil samples were collected in the manner described by Rahman et al., (2012). The *S. tuberosum* plant and soil surrounding the roots were unearthed and placed in a sterile polyethylene zip-lock bag, chilled, and sent to the Research Institute for Science and Technology, Microbiology and Parasitology Laboratory for identification and further processing.

Isolation and Microscopic Characterization of Rhizospheric Bacillus species

The soil suspension was prepared by dissolving ten (10) grams of soil in ninety (90) mL sterile distilled water in a 250 mL Erlenmeyer flask and shaken for thirty (30) minutes under the platform shaker. After adequately diluting and mixing the soil, ten (10) mL of the suspension was added to another 250 mL Erlenmeyer flask containing ninety (90) mL of sterile distilled water and shaken for another thirty (30) minutes on a platform shaker. The flask containing the soil solution was then placed on a water bath for nine (9) minutes to eliminate non-spore producing vegetative bacteria and to ensure *Bacillus* spp. isolation (Ramhan et al., 2012; Panda et al., 2013).

The soil suspension from the flasks was serially diluted (10^{-1} to 10^{-6}), then 100 L of the soil suspension at dilutions ranging from 10^{-4} to 10^{-6} was plated onto a fresh Tryptic Soy agar plate and incubated for 24 hours at 30°C. Fifty (50) mixed culture plates were prepared and placed in the incubator for 24-48 hours at 37°C (Lechiga et al., 2015). *Bacillus* colonies were observed on mixed culture plates using the bacterial morphology characterization method outlined in Bergey's Manual of Determinative Bacteriology, Vol. III, The Firmicutes (Whitman 2009). *Bacillus* colonies are described as flat and uneven in shape, with a mucoid or smooth consistency and a greyish white cream tint with a glass appearance. Presumptive *Bacillus* colonies were subcultured onto a fresh sterile TSA plate using the quadrant-streak method and incubated at 37°C for 24 hours. Slant inoculation was used to make stock cultures of *Bacillus*-suspected colonies, which were then preserved in mineral oil (Sandle, 2014).

Gram staining and endospore staining were used to characterize each of the presumptive *Bacillus* isolates (Sutton, 2010; Acharya, 2015). Gram staining distinguishes gram-positive and gram-negative bacteria, whereas endospore staining differentiates bacteria capable of producing spores from bacteria that do not create spores (Acharya, 2015). Bacterial isolates with rod form, gram positive, and endospore positive phenotypic traits were chosen and retained for further examination, with a focus on their ability to produce hydrolytic exoenzymes and antimicrobial properties.

Figure 1. Potato organic farm in Baranagy Paoay, Atok, Benguet, the Philippines (16°37′39″N 120°46′03″E).

Table 1. Summary of the phenotypic characteristics for the isolation of Bergey's Manual of Determinative Bacteriology, Vol. III, The Firmicutes (Whitman 2009)

Phenotypic Characteristics	Expected Observation
Gram stain	Gram-positive, large rod-shaped cells
Endospore	Positive, either central, terminal, subterminal
Catalase	Positive
Motility	Positive
Colony	Large colonies; confluent growth, dry or moist, undulate, crusty colonies.

Evaluation of Hydrolytic Enzymes produced by Rhizospheric Bacillus species

In vitro production of hydrolytic exoenzymes by presumptive *Bacillus* species was examined. The set of tests was conducted on the assumption that bacteria with a high level of enzyme synthesis and hydrolysis could produce antibiotic compounds (Shivaramaiah et al., 2011). All experiments were conducted using newly grown *Bacillus* cultures that had been diluted in fresh Tryptic Soy broth to match the 0.5 McFarland standard (TSB). To evaluate enzyme production, each isolate was stabbed onto the appropriate medium containing the substrates. All experiments were performed in triplicate, and the clearance zone around the colony was measured and recorded using a digital Vernier caliper.

Detection of Proteolytic Activity

The protease enzyme activity was determined using a modified skim milk agar consisted of (2.8g) skim milk powder, (0.5g) casein enzyme hydrolysate, (0.25g) yeast extract, (0.1g) dextrose, (1.5g) agar, and 100ml distilled water with a final pH of 7. The standardized *Bacillus* spp. was stabbed aseptically into skim-milk agar using an inoculating needle onto the plates. The cultures were incubated at a temperature of 28–35°C for 24 hours. A clean zone surrounding the bacterial colony suggests a favorable enzyme activity (Montealegro et.al., 2003).

Detection of Cellulolytic Activity

Cellulase activity was observed in *Bacillus* spp. isolates using Sakamoto and Toyohara's (2009) methods. Ten *Bacillus* spp. strains were cultivated on a modified Carboxymethyl Cellulose (CMC) agar medium containing 0.2g NH4H2PO4, 0.04g KCl, 0.2g MgSO4* 7H20, 0.6g TSA, 3g technical grade Carboxymethyl cellulose, and 3g agar diluted in 200ml distilled water (Samira et al., 2011). Standardized *Bacillus* species was inoculated into the CMC plate by stabbing the plate with sterile inoculating needle and then incubated at 25°C -30°C for about two (2) days. Following incubation, the medium was filled with 0.1% (w/v) Congo red, a staining reagent, and let to stand for twenty (20) minutes with intermittent shaking. To enhance visibility of the inhibitory zone, 1% sodium chloride was added onto the surface of the plates. The presence of zones of clearance around the colony suggests that bacteria are capable of degrading cellulosic matter.

Detection of Lipolytic Activity

The production of lipase was determined using the Tween 20 agar medium described by Gopinath et al (2005). Peptone, NaCl CaCl2H2O agar, and Tween-20 were used to make the culture medium. Standardized bacterial cultures were stabbed onto the plate and incubated for 72-hours at 37 ˚C. The presence of a crystallized precipitate surrounding the colony shows indicates positive lipolytic activity.

Detection of Amylolytic Activity

The *Bacillus* cultures were screened for amylolytic activity by starch hydrolysis test on starch agar plate containing 0.6 g beef extract, 2 g soluble starch, 2.4 g agar in 200 ml of distilled water (Abd-Elhalem, et al., 2015). The pure isolated colonies were stabbed on starch agar plates with starch as the only carbon source. After incubation at 37ºC for 48 hrs, the individual plates were flooded with Gram's iodine solution,to produce a deep blue colored starch-iodine complex. A zone of clearance encircling the bacterial colony indicates a positive reaction.

Molecular Identification of Rhizospheric Bacillus species by 16s rRNA gene sequence Analysis

The 16s rRNA gene sequence analysis was used to identify the bacterial isolates molecularly (Miranda, Martins, & Clementino, 2007). The agar blocks containing the *Bacillus* isolates' pure cultures were shipped to MACROGEN, South Korea for sequencing. The partial sequences of *Bacillus* spp. were assembled and modified using bioinformatics software Seaview version 3.2 and MEGA 6.

Consensus sequences were submitted to BLAST (Basic Local Alignment Search Tool) in order to identify closely related type strain sequences stored in the database. Sequences having a query coverage of at least 95% were obtained from GenBank (Altschul et al., 1997). The results were downloaded as FASTA files and aligned using Seaview version 3.2 against the *Bacillus* spp. 16s rRNA gene sequences. The maximum-likelihood tree was generated using the MEGA 6 program (Tamura et al., 2013), and the phylogenetic tree of the samples' closely related species was constructed using the neighbor-joining method (Saitou and Nei, 1987). For the neighbor-joining tree construction, the bootstrap value was set at 20,000.

Preliminary Assay for Determining Antimicrobial Activity of Rhizospheric Bacillus species by Cross-streak method

A preliminary assay using the cross-streak method was used to determine whether *Bacillus* isolates exhibit an antagonistic effect on the indicator pathogens: *Staphylococcus aureus* and *Escherichia coli* (Lertcanawanichakul et al., 2015). Standardized *Bacillus* species cultures were smeared on both sides of fresh Mueller Hinton agar (MHA) plates using a sterile swab and incubated for 72 hours. This was done to enable the bacteria to thrive and create diffusible antibiotic substances on the agar medium. Following incubation, indicator strains were swabbed individually in a straight line perpendicular to the *Bacillus* species growth lines. MHA plates were incubated at 37°C for 24 hours. The zones of inhibition from the streaking line of indicator pathogens were observed on the plates and were measured and recorded in millimeters using a Vernier caliper (mm). The top ten *Bacillus* species were identified based on their average zone of inhibition and were chosen as candidates for the production of crude extracts for antimicrobial assays

Production of Bacillus Extracts and Evaluation of the Antimicrobial Activity of Rhizospheric Bacillus spp. by Agar-Disc Diffusion method

Bacillus species extracts were prepared by culturing each standardized *Bacillus* species individually for five days in a 250 mL Erlenmeyer flask containing TSB. The turbidity of the culture media was observed to be suggestive of bacterial isolates growing and multiplying. The liquid cultures were then frozen for 24 hours in a freezer and thawed using ultrasonication at 40Khz for 10 minutes (Garcia-Vaquero et al., 2018). The thawed liquid cultures were centrifuged and the supernatant filtered using a 0.2 um syringe filter following sonication. Each of the *Bacillus* cell-free supernatants was combined with an equal volume of ethyl acetate in a filter flask and shaken for one hour on an orbital shaker. A separatory funnel was used to filter and separate the solution. The ethyl acetate layer was recovered and dried using a simple distillation procedure (Lv et al., 2017). The crude extract was prepared by resuspending the extracted material in the same solvent and placing it in sterile glass tubes. The crude extracts were utilized in an agar disc diffusion assay to determine antibacterial activity (CLSI, 2016; Ogunbanwo et al., 2003). One-hundred microliters (100 uL) of the crude extract was poured over sterile 6mm filter paper and left to dry. Freshly prepared MHA plates were swabbed with previously specified indicator microorganisms and dried disc containing the extract was impregnated onto the plates. The test strains were collected from the Philippine Network for Microbial Culture Collections (PNMCC) and the DLSU Microbial BioBanks (Table 2). Each test was repeated three times, and the zone of inhibition surrounding the disc was quantified and compared to the zones exhibited by the antibiotic control.

Biochemical Characterization of Top-performing Rhizospheric Bacillus species

To further characterize the *Bacillus* isolates demonstrating antimicrobial activity, the top-performing isolates were subjected to a series of biochemical tests, including Catalase, Indole, Methyl Red, Voges Proskauer, Citrate utilization, Urease, SIM, and Triple Sugar Iron (Garcia, 2010). Each test was performed in triplicate.

Table 2. Test strains used for the antimicrobial testing of Rhizospheric *Bacillus* species extract.

Indicator Strains	Accession Number/ Antibiotic Susceptibility Profile
Aeromonas hydrophila	BIOTECH 10089
Pseudomonas aeruginosa	BIOTECH 1335
Serratia marcescens	BIOTECH 1748
Escherichia coli	BIOTECH1634
Salmonella typhi	BIOTECH 1756
Streptococcus mutans	BIOTECH 10231
Staphylococcus aureus	BIOTECH 1582
Enterococcus faecalis	BIOTECH 10348
Micrococcus luteus	BIOTECH 1753
Candida tropicalis	BIOTECH 2085
Methicillin-resistant *Staphylococcus aureus* A1 (MRSA)	Trimethoprim-sulfamethoxazole, Cefoxitin, Oxacillin, Penicillin (Valle et al., 2016).
Escherichia coli (ESβL +)	Ampicillin, Cefepime, Cefotaxime, Ceftadizime, Ceftriaxone (Valle et al., 2016).

Results and Discussion

Isolation and Microscopic Characterization of of Rhizospheric *Bacillus* species

Soil samples collected from an organic potato farm in Atok, Benguet, Philippines yielded a total of 632 isolates, of which 54 (8.45%) were identified presumptively as *Bacillus* species based on their colonial, morphological characteristics. AThe isolation of *Bacillus* species from soil samples is summarized in Table 3.

Table 3. Summary of the Isolation of Rhizospheric *Bacillus* species from *S. tuberosum*

Microscopic characterization	Results
Total number of isolates	632
Total number of presumptive *Bacillus* species	54
Shape	
Large rods	51
Rods appearing in chains	2
Long rods appearing in chains	1

For the observation and isolation of *Bacillus* species, a total of 632 bacteria were cultivated in a suitable culture medium. From the 632, 54 were identified as presumptive *Bacillus* species. Fifty-one (51) of the 54 presumptive *Bacillus* species were found to be large rods, while two exhibited rod shapes in chains, and one exhibited a long rod form in chains. The gram-stain and endospore stain were both positive on all 54 isolates (Figure 2). Based on the overall number of bacterial isolates in this investigation, it is evident that the rhizosphere of *S. tuberosum* has an abundance of aerobic microbial communities. Similar investigations have discovered a significant number of isolates from soil samples, indicating that the majority of bacteria are adaptable to soil settings (Li et al., 2018; Lou et al., 2018), including endospore-forming *Bacillus* species. Numerous other factors contributed to the richness of microorganisms in organic farm soils, including the use of naturally rich in microbes livestock manure and plant residues as fertilizers in organic farms (Alef and Nannipieri, 1972; Rillig and Mummey, 2006).

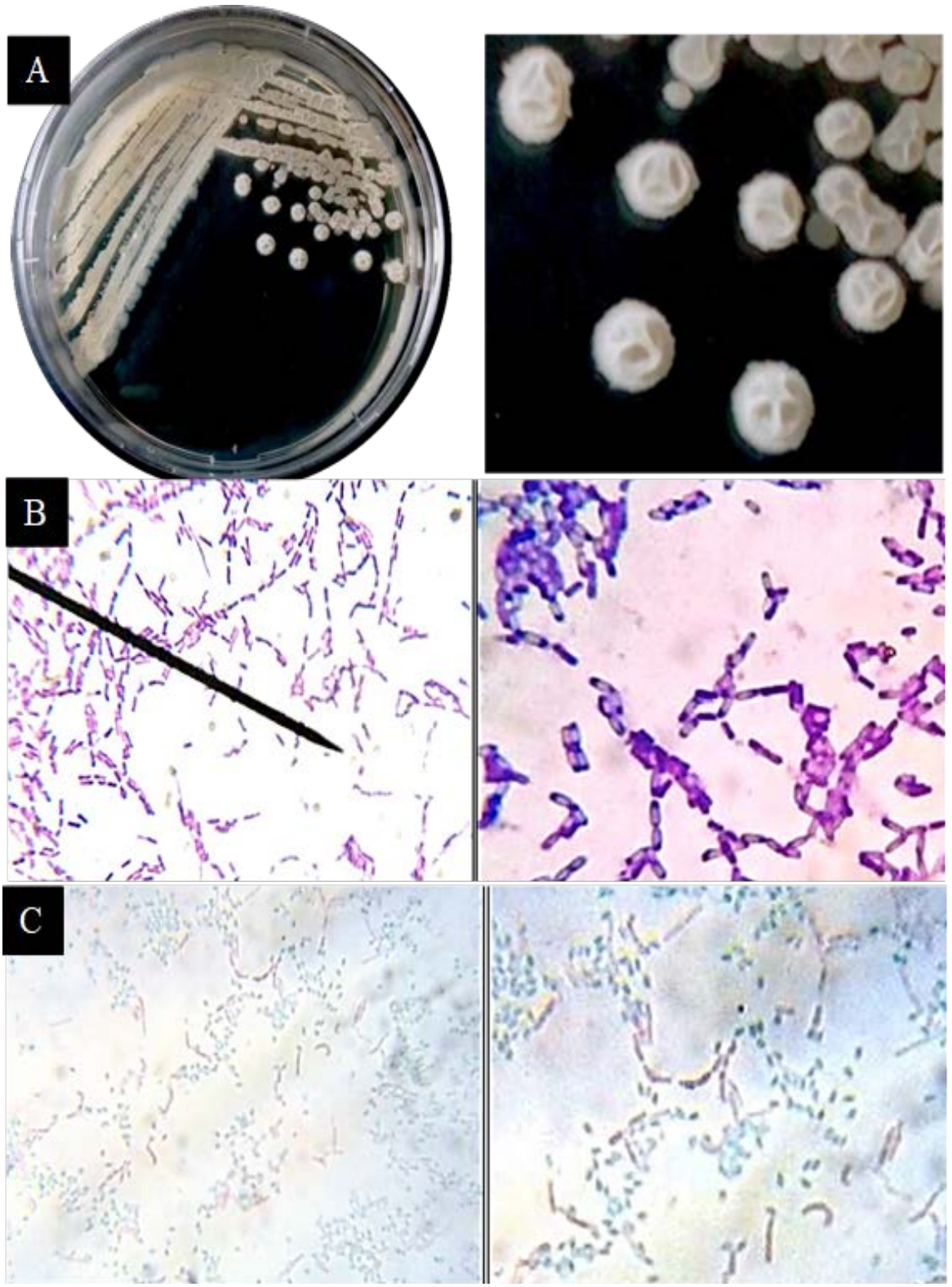

Figure 2. *Bacillus* species colony and microscopic examination; A. *Bacillus* species colony observed on TSA plate with a magnified photo (right), B. Gram-staining results of isolated *Bacillus* species, and C. *Bacillus* species endospore staining photographs.

The current investigation isolated 54 presumptive *Bacillus* species, 51 of which had broad rod morphologies. This finding corroborates previous research on the isolation of soil-associated *Bacillus* species with enormous rod shapes, such as *B. megaterium* (Dobrzanski et al., 2018), *B. subtilis* (van Dijl and Hecker, 2013), and *B. cereus* (Vilain et al., 2006). Numerous *Bacillus* species have been isolated from the rhizosphere due to their ability to produce endospores, which are a dormant, resistant form of spore-forming bacterium (Knaysi, 1948). These bacteria have been revealed to have biotechnological qualities that promote plant growth and protect plants from pathogenic microbes and pests (Beayregard et al., 2013). (Garcia-Faile et al., 2015; Kang et al., 2015). Various studies have isolated *Bacillus* species from organic soils and investigated their ability to promote plant growth (Panneerselvam et al., 2019; Garcia-Faile et al., 2015; Kang et al., 2015). Interestingly, many of the *Bacillus* species described have been commercially used as biofertilizers, where they have been shown to promote plant growth and crop yields. Their impacts on plant development have also been reported, even in extreme environmental situations such as droughts (Radhakrishnan et al., 2017).

Evaluation of Hydrolytic Enzymes produced by Rhizospheric Bacillus species

The ability of 54 presumptive *Bacillus* species to produce hydrolytic enzymes in vitro was assessed. The hydrolytic enzyme evaluation on appropriate media is summarized in Figure 3. All 54 *Bacillus* isolates demonstrated positive hydrolytic enzyme activity, varied in intensity according to the observed zone of clearance on the plates (Figure 4). The isolate 2POTS14 had the highest total hydrolytic enzyme activity among the 54 samples (51.2975 mm). Isolates 2POTS33 (79 mm), 2POTS14 (67.71 mm), 2POTS10 (38.21 mm), and 2POTS32 (48.23 mm) displayed the highest activity for protease, amylase, lipase, and cellulase, respectively. The twenty *Bacillus* isolates with the highest mean value for all hydrolytic enzyme activity were chosen for molecular identification and subsequent evaluation for antimicrobial extract production.

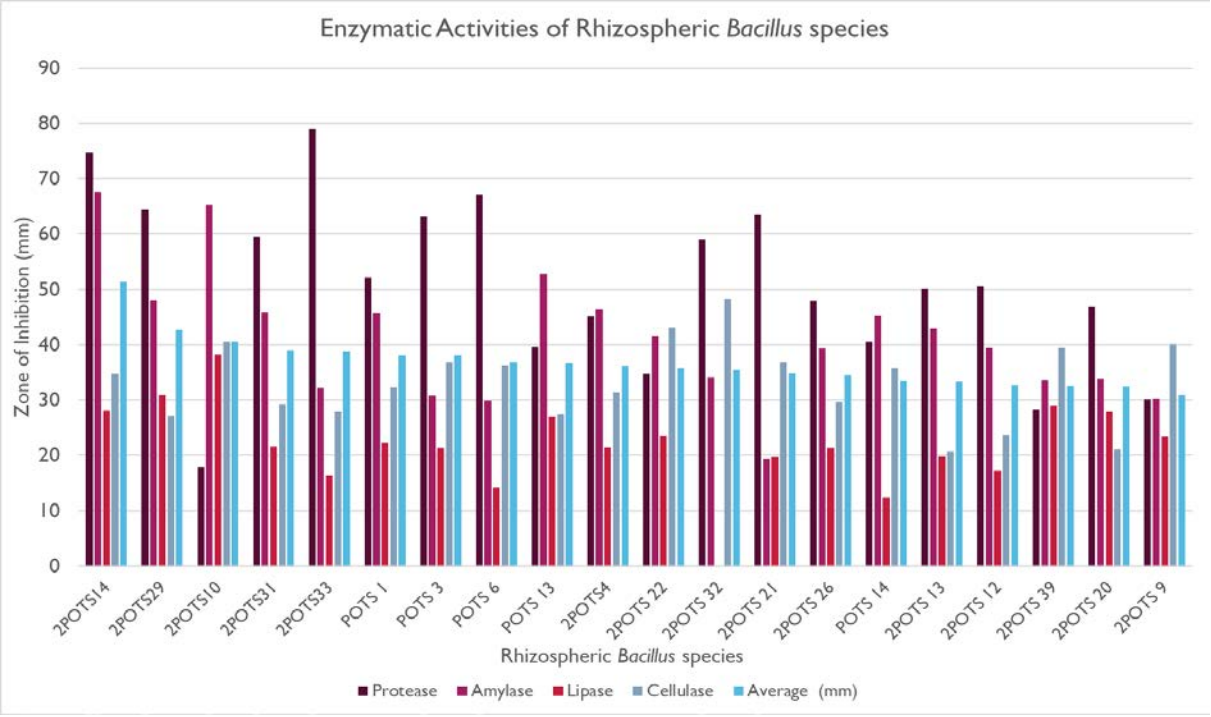

Figure 3. Bar graph of the overall hydrolytic exoenzyme production produced by *Bacillus* species.

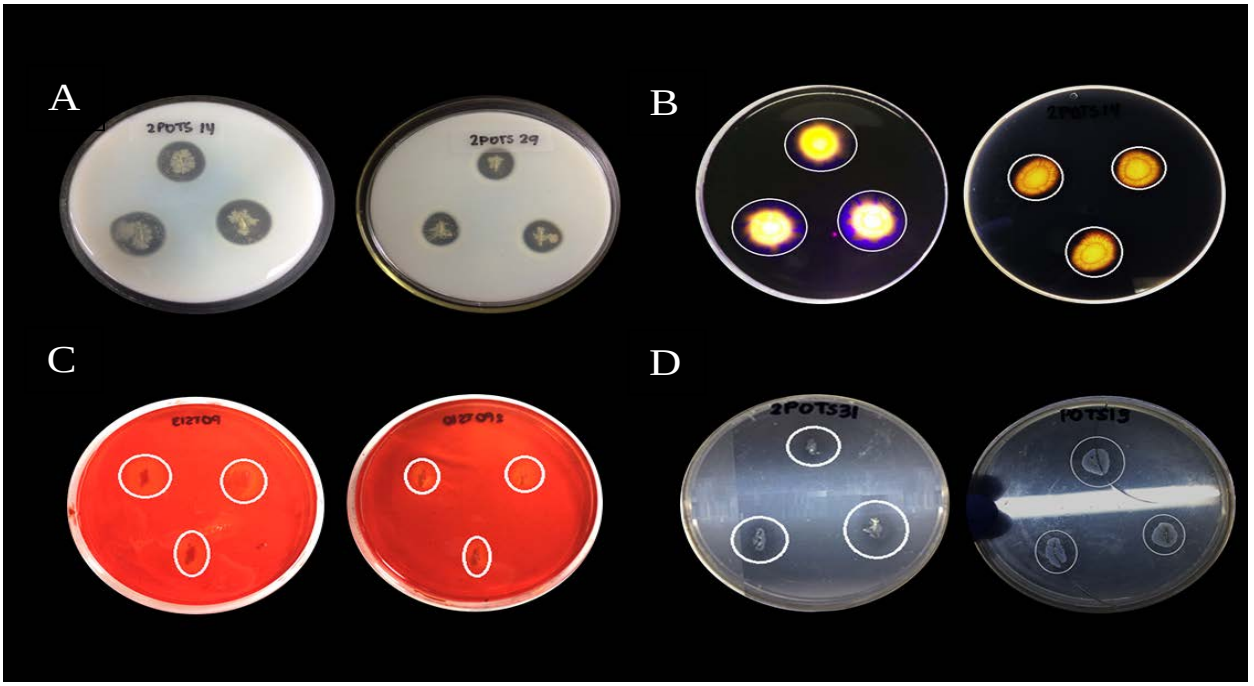

Figure 4. Hydrolytic exoenzyme produced by the rhizospheric *Bacillus* species; A. protease activity on skim-milk agar plate, B. amylase activity on starch agar plate, C. cellulase on CMC agar plate, and D. lipase activity on Tween-20 agar plate. The zone of clearance around the colony represents positive enzyme production.

The ability of isolated *Bacillus* species to produce hydrolytic exoenzymes was examined in different culture conditions on plates. The findings indicate that the isolates are capable of producing enzymes such as protease, lipase, cellulase, and amylase, all of which have significant industrial and biotechnological applications. Numerous studies have examined *Bacillus* species enzyme production in a variety of industries, including textiles (Araujo et al., 2008), food (Raceendran et al., 2018), pharmaceuticals (Mane and Tale, 2015), cosmetics (Babizhayev, 2006), and biopolymers (Raceendran et al., 2018). (Hiraishi and Taguchi, 2009). Significant attention is paid to *Bacillus* species' enzyme synthesis, as these lytic exoenzymes have enormous promise for disease control in agricultural and clinical contexts (Kishore and Pande, 2007; Bal et al., 2009). Similar studies have been conducted on isolated *Bacillus* species to assess both exoenzyme production and antimicrobial activity (Begley et al., 2009; Podile and Prakash 1996; Arguelles et al., 2013; Powethong and Suntornthiticharoen, 2017), providing strong support for the notion that enzyme-producing bacteria can also produce antimicrobial substances.

Bacillus species secrete exoenzymes such as lipase, protease, amylase, and cellulase, which aid the bacteria in degrading and breaking down large substances in their environment and converting them to smaller units (Powethong and Suntornthiticharoen, 2017). This ability enables soil-associated *Bacillus* species to consume organic compounds available in the soil and incorporate them into their biochemical activities (Eckert et al., 2013). As a result, it is discovered that the chemicals created by bacteria degrading organic matter via the production of lytic exoenzymes assist plants specifically in terms of nutrient intake and assimilation (Richardson et al., 2009).

Molecular Identification of Rhizospheric Bacillus species by 16s rRNA gene sequence Analysis

Bacillus species from the rhizosphere were identified molecularly using 16s rRNA gene sequence analysis. Table 4 summarizes the examination of the isolates' sequences and their associated identity as compared to sequences available in NCBI GenBan. A neighbor-joining tree was created using the *Bacillus* isolates sequences the sequences available in the database (Figure 5).

Table 4. Identities of rhizospheric *Bacillus* species based on the similarities of the isolates' 16s rRNA partial gene sequences from NCBI GENBANK

	NCBI BLAST HITS				
***Bacillus* strain Code**	**Organism**	**Query Coverage**	**E-value**	**Identity**	**NCBI Accession (Gene ID)**
POTS1	*Bacillus velezensis*	99%	0	99.86%	KY694464.1
POTS3	*Bacillus velezensis*	99%	0	99.86%	KY694464.1
POTS6	*Bacillus mojavensis*	99%	0	99.67%	MH211387.1
POTS13	*Bacillus velezensis*	99%	0	100.00%	MT114570.1
2POTS4	*Bacillus velezensis*	99%	0	100.00%	MT114570.1
2POTS21	*Bacillus mojavensis*	99%	0	99.93%	MH211387.1
2POTS32	*Bacillus subtilis*	98%	0	99.93%	KJ721209.1
2POTS12	*Bacillus sonorensis*	99%	0	99.80%	MH371778.1
2POTS13	*Bacillus tequilensis*	99%	0	99.93%	MK785130.1
2POTS26	*Bacillus mojavensis*	95%	0	99.80%	MH211387.1
2POTS14	*Bacillus clausii*	99%	0	98.71%	MH114929.1
2POTS29	*Bacillus amyloliquefaciens*	99%	0	99.80%	MH144237.1
2POTS10	*Bacillus amyloliquefaciens*	99%	0	99.80%	KC250199.1
2POTS31	*Bacillus altitudinis*	98%	0	99.80%	MF425586.1
2POTS33	*Bacillus altitudinis*	99%	0	99.67%	MF425586.1
2POTS20	*Bacillus siamensis*	95%	0	99.60%	MN240927.1
POTS14	*Bacillus velenzensis*	99%	0	99.80%	MT114571.1
2POTS9	*Bacillus velenzensis*	100%	0	99.87%	MH718826.1
2POTS39	*Bacillus subtilis*	100%	0	99.93%	MH371779.1
2POTS22	*Bacillus subtilis*	99%	0	99.80%	KF601955.1

The 16s rRNA gene sequencing analysis has been the most common tool used for the identification of clinical and environmental isolates of the members of the genus *Bacillus* to the species level (Sacchi et al., 2002; Miranda et al., 2007; Janda and Abbott, 2007). The advantages of the use of this gene for identification over the other markers and genes have been documented (Patel, 2001). The twenty (20) *Bacillus* isolates exhibited the highest enzyme activities were identified as *Bacillus velezensis* (30%), *Bacillus subtilis* (15%), *Bacillus mojavensis* (15%), *Bacillus altitudinis* (10%), *Bacillus amyloliquefaciens* (10%), *Bacillus clausii* (5%), *Bacillus siamensis* (5%), *Bacillus sonorensis* (5%), and *Bacillus tequilensis* (5%) based on their partial 16s rRNA gene sequence similarities with those *Bacillus* type strains found in GENBANK. The high identity score and E-value of 0 imply that the sequences in the database are highly similar. The present findings regarding the identification of *Bacillus* species and their isolation from soil environments are consistent with previous

research, demonstrating the rhizospheric *Bacillus* species' ability to promote plant growth and their biocontrol potential against pathogens (Lu et al., 2017; Qin et al., 2017; Sung-Hun and Doo-Hyun, 2008; Kim et al., 2015; Islam et al., 2019; Palmisano et al., 2001; van Dijl and Hecker, 2013; Martins et al., 2013).

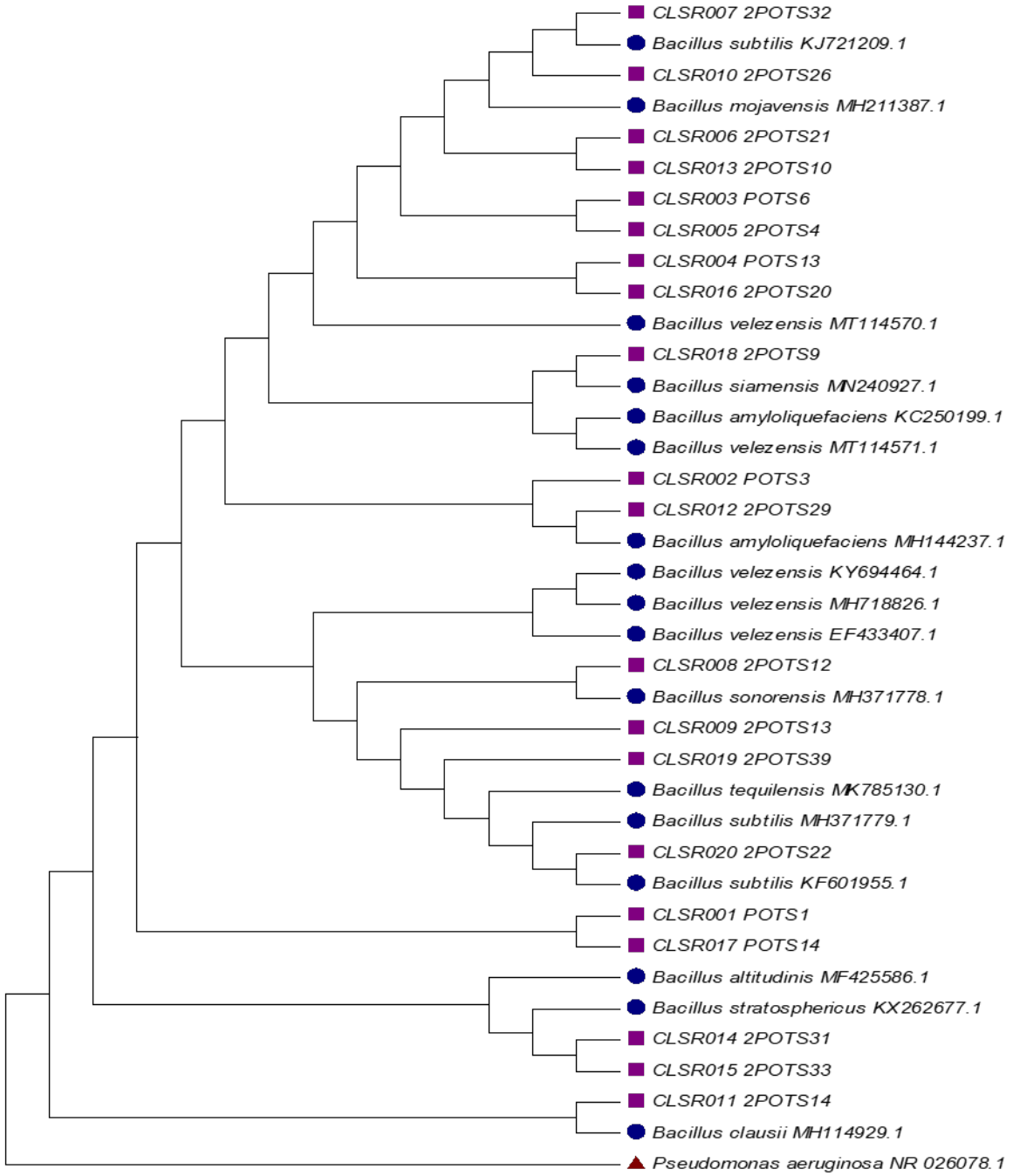

Figure 5. Neighbor-joining tree constructed using the Rhizospheric *Bacillus* species contigs with the reference sequences available at NCBI GENBANK. *Pseudomonas aeruginosa* NR026078.1, a gram-negative bacterium, was used as an outgroup.

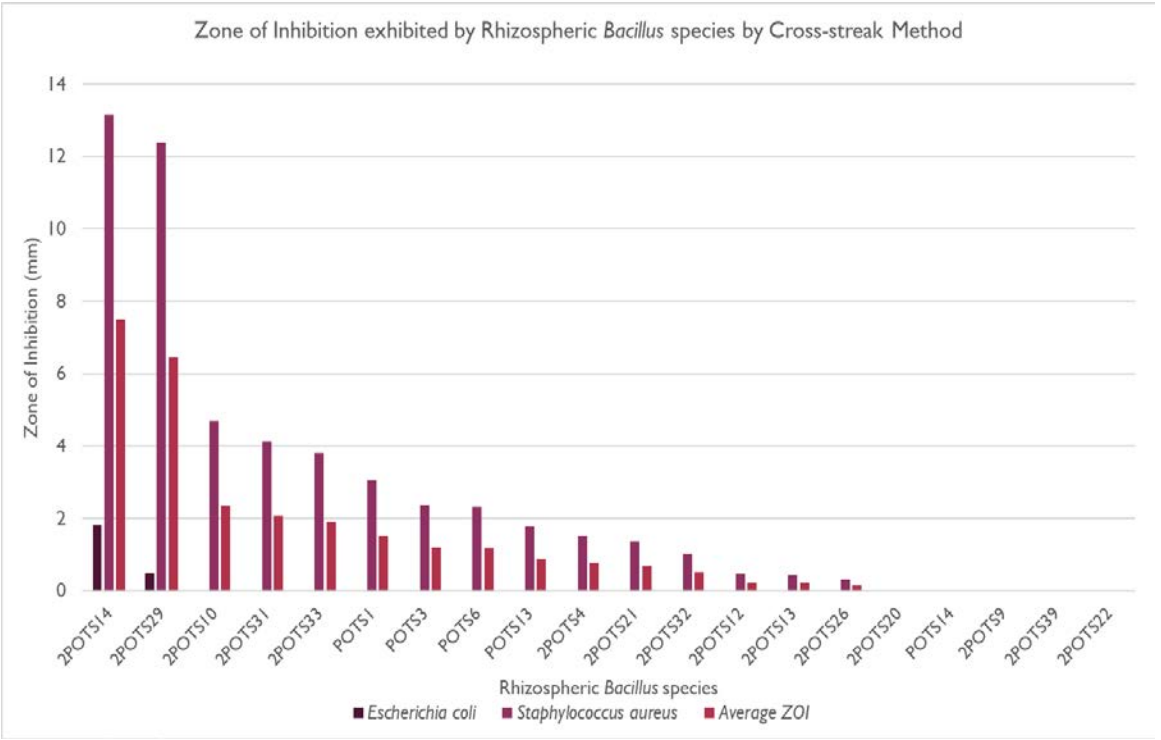

Figure 6. Bar graph showing the overall activities exhibited by the *Bacillus* isolates by the cross-streak method.

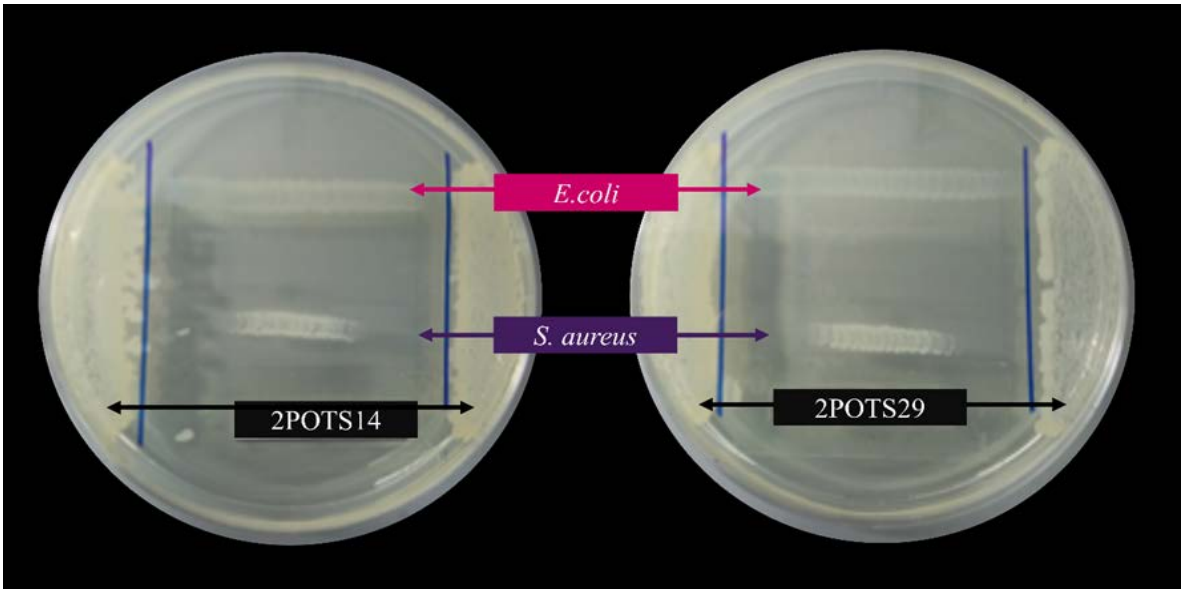

Figure 7. Preliminary evaluation of antimicrobial production of rhizospheric *Bacillus* species on agar plate by cross-streak method.

Preliminary Assay for Determining Antimicrobial Activity of Rhizospheric Bacillus species by Cross-streak Method

The isolated *Bacillus* species were evaluated for their potential antimicrobial production. Out of 20 isolates of *Bacillus*, 15 (75%) exhibited observable antagonistic effects against the indicator

strains. *Bacillus* isolates exhibited inhibitory activity mostly against gram-positive bacterium *S.aureus* (Figure 6) . Only 2POTS14 *B. clausii* and 2POTS29 *B. amyloliquefaciens* showed an observable inhibitory effect against *E. coli* (Figure 7). Based on the average inhibitory activities against indicator strains, the top 10 *Bacillus* species were selected for the production of *Bacillus* antimicrobial extracts and evaluated for their antimicrobial activities.

The cross-streak method was initially used to assess the *Bacillus* isolates' antimicrobial potential against indicator pathogens. This approach enables isolates to create antimicrobial substances that are diffusible in agar medium (Arasu et al., 2008). *Bacillus* isolates exhibited inhibitory activity against gram-positive *S. aureus* in this study. This finding is consistent with a previous study examining the anti-gram-positive activity of *Bacillus* isolates using the cross-streak method (Kvanç et al., 2014). The top ten *Bacillus* isolates were chosen based on the cross-streak method's average zone of inhibition.

Evaluation of Antimicrobial Activity of Rhizospheric Bacillus spp. by Agar-Well Diffusion method

The agar-disc diffusion assay was used to determine the antimicrobial activity of ten *Bacillus* species found in the rhizosphere against indicator pathogens (Figure 8). Inhibitory activities against gram-positive indicator pathogens were consistently detected, however none of the *Bacillus* isolates inhibited P. aeruginosa. It is worth noting that six of the isolates inhibited the MRSA strain, which is resistant to antibiotics. Only 2POTS14 was capable in inhibiting the drug-resistant *E.coli* EBSL (+). The isolates' activity were compared to the antibiotic Rifampicin (Figure 9).

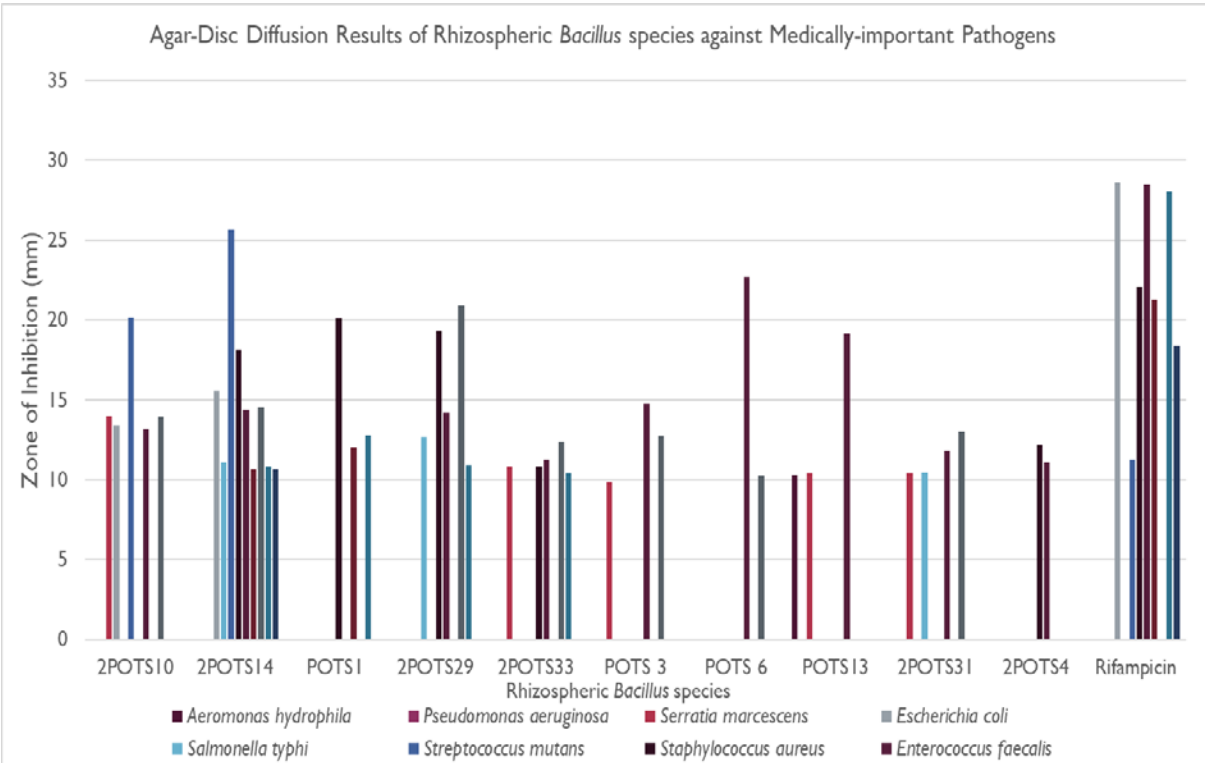

Figure 8. Bar graph of the overall antimicrobial activities of the rhizospheric *Bacillus* species against test pathogens. Rifampicin was used as an antibiotic control.

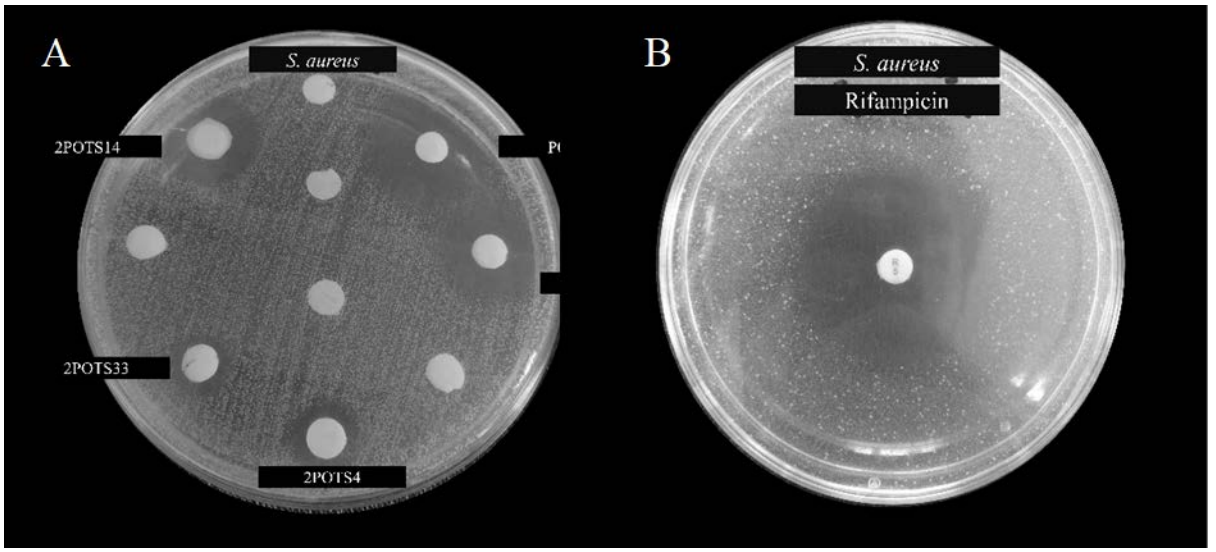

Figure 9. Zone of inhibition observed using *Bacillus* isolates' crude-extract against test strain; A. *S. aureus* and B. Rifampicin.

Solvent extraction and concentration were used to culture the isolates for the purpose of producing antimicrobial extracts. The disc diffusion method was used to determine the dried extracts' inhibitory activity on indicator pathogens. *Bacillus* isolates displayed varying degrees of inhibitory activity depending on the target pathogens, with the majority of activities shown against gram-positive bacteria in this study. It is noteworthy that several isolates inhibited drug-resistant MRSA, whereas isolate 2POTS14 *B. clausii* was shown to be antagonistic against both drug-resistant MRSA and ESBL+ *E.coli* . Similar studies have established *Bacillus* species' anti-MRSA activity (Chalasani et al., 2015; Nasfi et al., 2018). *Bacillus* isolates' anti-gram-positive activity is ascribed to their ability to produce peptides termed bacteriocins via ribosome synthesis which are secreted in the culture medium where the *Bacillus* species are cultivated. It is well established that bacteriocins inhibit closely related species (Jack et al., 1995). Several investigations, however, have established that bacteriocins have anti-gram-negative action in vitro (Ghodhbane et al., 2015; Field et al., 2012). These polypeptides have bactericidal, bacteriostatic, or a combination of antimicrobial activity against target microorganisms (da Silva et al., 2014). Bacteriocins have a variety of mechanisms of action, including actions on the cell envelope (Cotter et al., 2013), inhibitory effects on peptidoglycan production (Breukink and de Kruijff, 2006), and pore-forming properties that result in cell lysis and death (Breukink and de Kruijff, 2006). (Machaidze and Seelig, 2003). The manufacture of these strong antimicrobial polypeptides has been carried out utilizing strains of soil-associated microbes, but most frequently spore-forming *Bacillus* species due to their aerobic nature, non-fastidious behavior, and ease of laboratory cultivation (Ansari et al., 2012; Moshafi et al., 2011). For these reasons, *Bacillus* members are a prospective choice for the development of novel antimicrobials against drug-resistant diseases.

Biochemical Characterization of Rhizospheric Bacillus species

The top ten rhizospheric *Bacillus* species producing antimicrobial properties were further characterized biochemically (Table 5). The Indole and Voges Proskauer assays were negative for all ten isolates. Seven isolates reacted positively to methyl red, whereas five reacted positively to citrate utilization. Only two strains were capable of producing urease. In the SIM media, all isolates were motile, with the exception of one (POTS 6), which generated hydrogen sulfide. The ten showed con-

siderable variation in their ability to ferment carbohydrates with 2POTS10 *B. amyloliquefaciens*, resulting in an alkaline slant, an acid butt, and gas production.

Table 5. **Summary of the biochemical characterization of the top 10 *Bacillus* isolates.**

***Bacillus* isolates**	**Cata-lase**	**Indole**	**Methyl Red**	**Voges Proskauer**	**Citrate**	**Urease**	**SIM**	**Triple Sug-ar Iron**
2POTS14	+	-	+	-	+	-	M	K/A
2POTS29	+	-	+	-	+	+	M	A/A
2POTS10	+	-	+	-	+	+	M	K/A, G
2POTS31	+	-	+	-	-	-	M	K/A
2POTS33	+	-	+	-	+	-	M	K/A
POTS 1	+	-	-	-	-	-	M	K/K
POTS 3	+	-	-	-	-	-	M	K/K
POTS 6	+	-	-	-	-	-	H2S , M	K/A
POTS 13	+	-	+	-	+	-	M	K/K
2POTS4	+	-	+	-	-	-	M	K/K

(+) positive reaction, **(-)** negative reaction, **M**= motile, **H2S**= hydrogen sulphide production, **K**= alkaline, **A**= acid, **G**= gas formation.

Conclusions

MDR infections are becoming more prevalent globally, and their reported resistance to several antimicrobials has resulted in increased morbidity and mortality. Thus, the scientific community must continue its search for safe, environmentally friendly, and cost-effective antimicrobial sources. More importantly, these substances' sources must be readily available, viable, and simple to create, all of which are features of the majority of microbial sources of natural products. The purpose of this study was to determine the antimicrobial activity of rhizospheric *Bacillus* species isolated from *Solanum tuberosum* organic agricultural soils containing.

Bacillus species isolated from organic potato farm soils produced enzymes of biotechnological and industrial significance. Antimicrobial activity was seen against indicator pathogens of clinical significance, including drug-resistant bacteria. It is evident that the rhizosphere region of plants, more precisely the rhizosphere of *S. tuberosum*, might be regarded as a promising environment for the discovery of microbial natural products. The 16s rRNA gene sequence analysis of the isolates showed their identities as members of the genus *Bacillus*, which are all soil-associated species. It is critical to identify the chemical components that operated as an inhibitory agent against the pathogens examined and be tested against various drug-resistant bacteria. Additionally, optimization of the culture medium used to produce antimicrobial extracts may be explored in order to ensure efficient antimicrobial production.

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