EVALUATION OF THE ANTIMICROBIAL ACTIVITY OF CELLULOLYTIC *BACILLUS* SPP. ISOLATED FROM RICE PADDY FIELDS IN THE PHILIPPINES

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Abstract: Drug-resistant strains are becoming a public health problem as antibiotic-resistant diseases become more prevalent in clinical settings throughout the world. Multidrug-resistant microorganisms are constantly adapting to the effects of currently produced antibiotics by inactivating them via a variety of mechanisms. To address this issue, microbial natural sources isolated from various settings are being used to meet the demand for new antimicrobials. On this study, *Bacillus* species were isolated from rice field soils and their enzyme production and antibacterial activity were determined using plate techniques and agar-well diffusion assays. The results revealed that all isolates synthesized cellulase, with strain BI-AI.1 exhibiting the largest hydrolysis zone of 2.07±1.07 mm. *Bacillus* cell-free supernatant (CFS) from strains NE-AIII.10, BI-BI.7, and BI-CI.2 also demonstrated antimicrobial activity against *Enterococcus faecalis*, *Micrococcus luteus*, and *Candida albicans*. Only NE-AIII.10 and BI-BI.7 inhibited Gram-negative *Klebsiella pneumoniae* (8.25±0.12 mm) and *Aeromonas hydrophila* (2.91±0.72 mm) with *p*-values of 0.00002 and 0.00006, respectively. Interestingly, *Bacillus* BI.CI.2 inhibited methicillin-resistant *Staphylococcus aureus* (MRSA) with a zone of inhibition (ZOI) of 9.83±0.44 mm (p = 0.0005). *Bacillus* CFS Minimum Inhibitory Concentration (MIC) assays revealed the inhibition of test pathogens at varied concentration ratios. Molecular identification of the top strains NE-AIII-10 and BI-BI.7 using 16s rRNA gene sequence analysis showed similarities of the isolates to *Bacillus subtilis*. The findings established the presence of cellulolytic *Bacillus* species in the soils of Philippine rice paddy fields. Additional investigation of the *Bacillus* CFS is required to confirm the presence of the active components responsible for the inhibitory effects against test pathogens.

Keywords: *antibiotic resistance, antimicrobial, cellulolytic Bacillus, cellulase*

1. INTRODUCTION

Antibiotics' benefits are reflected by their extensive use in clinical and agricultural settings, which has resulted in the rise of resistant bacterial species. Antimicrobial resistance develops as a result of microorganisms adapting to currently dispensed antibiotics through a variety of methods, including enzymatic breakdown of antimicrobial agents, genomic sequence modification, and altered membrane permeability to antibiotics (Munitas & Arias, 2016). Due to the increasing usage of antibiotics in the treatment of many communicable diseases and the stagnation of new medication discovery, the quest for novel antimicrobials to combat bacterial infections is considered critical (Chait et al., 2012).

The majority of antibiotics are derived from natural compounds, owing to their capacity to inhibit disease-causing microbes without harming the host. This result to a gain in popularity of these compounds as potential economically viable antimicrobials (Coates et al., 2011). Thus, the search for the most promising molecule derived from a natural product source has gained prominence.

Natural antimicrobials are found in nearly every environment, ranging from phytochemicals in plant extracts and volatile oils (Cowan, 1999; Dorman & Deans, 2000) to extracted compounds from isolated microorganisms in water and soil (Solomon, 2005) and even on resident microbiota (Sun et al., 2015). Natural products, specifically cultivable microorganisms, have been shown to be capable of producing stable enzyme complements (Thakham et al., 2020), indicating their potential as antimicrobials that exhibit antagonistic activity against human pathogens (Mg Mg et al., 2015). *Bacillus*, a genus of endospore-forming rhizobacteria, is regarded as promising candidate for microbial-derived natural products. *Bacillus* species are aerobic and Gram-positive rods that are found throughout nature and are mainly harmless saprophytes (Turnbull, 1996; Ochiai et al., 2001). *Bacillus* is found in a variety of settings, including freshwater, saline water, soil, plants, and animals, as well as the air. They can even live under intense heat and salinity (Chantarasiri, 2015). Studies show that isolated *Bacillus* species from rice field soil may be capable of producing cellulase as a way utilizing of alternative energy sources (Khianggam et al., 2014; Taprig et al., 2015). Notable for their adaptability to virtually any environment, *Bacillus*' capacity to breakdown cellulose can also be applied to other agricultural and industrial processes, including biofuel production, food and feed production, wine and beer production, and even bioremediation (Pokhrel et al., 2014).

With the growing demand for alternative sources of microbial-derived natural products to combat antibiotic resistance, this study, therefore, evaluated the antimicrobial activity of cellulolytic *Bacillus* species isolated from rice paddy field soils in two agricultural provinces in the Philippines against a panel of clinically important human pathogens.

2. METHODOLOGY

2.1 Research design

The antimicrobial potential and enzymatic activity of cellulolytic *Bacillus* (Bergey et al., 1984; Turnbull, 1996) against selected clinical pathogens were evaluated using an experimental research design. The experiment was conducted in four stages: isolation and initial identification of *Bacillus* species to obtain pure cultures; screening of the isolated *Bacillus* species for cellulolytic activity; biochemical and enzymatic analysis of the selected *Bacillus* species; and finally, evaluation of the antimicrobial potential of the top cellulolytic *Bacillus* species' crude enzyme extract against selected test pathogens, comparing their activity with antibiotic control. Each analysis was performed in replicates.

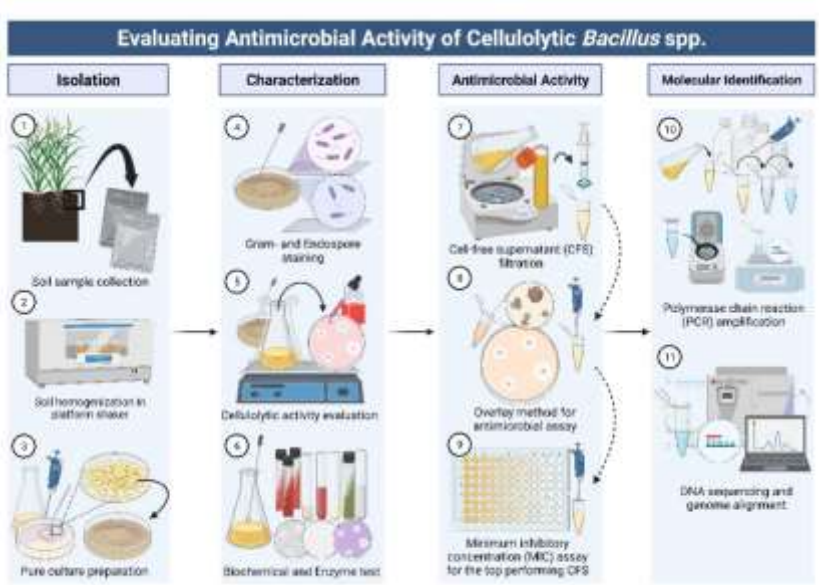

Figure 1. Simplified illustration of the whole methodology process for evaluating the antimicrobial activity of cellulolytic *Bacillus* isolated from rice paddy fields. Figure created using BioRender accessible at BioRender.com

2.2 Soil sampling

A total of approximately ten grams of soil was gathered from the sampling points using a spatula that had been sterilized in 70% ethanol (Ahmed et al., 2013). Random sampling was conducted from three 1-hectare regions in the municipalities of Nabua, Camarines Sur, and Cabiao, Nueva Ecija provinces in the Philippines. The regions were designated as Area A, B, and C, respectively. Separate soil samples were placed in clean polythene bags with appropriate labelling, then placed in an ice cooler maintained at around 10°C and transported chilled to the Research Institute for Science and Technology, Microbiology, and Parasitology Laboratory, Polytechnic University of the Philippines. Samples were stored at 4°C in the refrigerator (Ogunmwonyi et al., 2008; Amin et al., 2015).

2.3 Isolation of Bacillus species

Five grams of each soil samples were placed into a flask containing 100 mL distilled water and homogenized using a platform shaker. Flasks were treated to a 7-minute heat shock using a water bath with a temperature range of 70-80°C (Humam, 2016). Fifty microliters of the soil suspension were pipetted out from the flask and plated onto a freshly prepared tryptic soy agar (TSA) (HiMedia Laboratories, India) plate and then swirled and disseminated in a circular motion on the agar surface using an L-shaped rod. The plates were incubated at 30°C for 24 hours. The morphology of growing colonies was analyzed to determine whether colonies resembled those of *Bacillus* species. White, dry, crusty, large colonies were identified, plated freshly onto TSA plates and incubated at 30°C for 24 hours to obtain pure cultures. All were suitably labeled and subsequently characterized using Gram staining and endospore staining methods.

2.4 Cellulase enzymatic activity assay

Each *Bacillus* isolate was aseptically spot inoculated from the standardized broth cultures onto a carboxymethylcellulose (CMC) agar (containing $NH_4H_2PO_4$ 1 g, KCl 0.2 g, $MgSO_4$ 1 g, TSA 1 g, agar 26 g, CMC 26 g, distilled water 1L) and incubated at 37°C for 48 hours. Following incubation, cultured plates were flooded with 0.1% Congo red solution and shaken for 15 minutes before rinsing with 0.1 M NaCl solution (Kasana et al., 2008; Sriariyanun et al., 2015). The diameter of the hydrolysis zone (HZ) around the colonies cultured on CMC agar was estimated as the end-to-end diameter of the clear zone minus the diameter of the colony. Ten *Bacillus* isolates with the highest overall cellulolytic activity were chosen for further characterization.

2.5 Biochemical tests

For subsequent characterization, selected *Bacillus* species were subjected to biochemical tests, including the Catalase test (Aryal, 2015), the Indole test (MacWilliams, 2009), the Methyl Red test (Giri, 2015), the Voges-Proskauer test (Acharya, 2015), the Citrate test (MacWilliams, 2009), and the Triple Sugar Iron test (Aryal, 2019). The results were classified as positive (+) or negative (-) depending on the presence of the biochemical reaction.

2.6 Hydrolytic enzymatic characterization of bacillus isolates

Along with cellulase activity testing, amylase, chitinase, and protease–production assays were performed. A single loop of *Bacillus* colony from a subculture was inoculated on a starch agar plate (HiMedia Laboratories, India) (HiMedia M107S-500G Starch Agar) for the amylase test, on a colloidal chitin agar plate (containing 0.06 g, K_2HPO_4 0.14 g, KH_2PO_4 0.06 g, $MgSO_4$ x 7 H_2O 0.1 g, agar 6.2 g, NH_2NO_3 0.4 g, NaCl 0.2 g, distilled water 200 mL) for the chitinase test (Murthy & Bleakley, 2012), and on a skimmed milk agar (HiMedia Laboratories, India) (containing skim milk agar 5g, agar 1g, distilled water 100 mL) for the protease test. All agar media were processed in conformity with the manufacturers' preparation formula and existing standards.

After 48 hours of incubation at 37°C, starch agar plates were added with 2-3 drops of iodine solution and placed aside for 15 minutes (Bassiri, 2017). Colloidal chitin agar plates, on the other hand, were incubated for 96 hours (Saima & Roohi, 2013), whereas skimmed milk agar (SMA) plates were incubated for 24-48 hours at 37°C (Panda et al., 2013). A visible halo or hydrolysis zone forming around neighboring colonies indicate bacterial activity and was only reported as positive if present, and as negative if absent.

2.6.1 Cellulolytic bacillus bell-free supernatant (CFS) production and antimicrobial assay

Antimicrobial activity of ten cellulolytic *Bacillus* strains was determined using a bacterial cell-free supernatant (CFS) in agar well diffusion assay. *Bacillus* CFS was obtained by centrifugation (4000 rpm for 30 minutes) of cultured samples from flasks filled with 50 mL antibiotic broth medium (containing 25 g sodium chloride, 30 g tryptic soy broth, 10 g sodium nitrate, and 10 g glucose, and 1L distilled water) (TSB: HiMedia Laboratories, India), incubated for 2-3 days at 35°C in a platform shaker at 150 rpm.

Filtration of the resultant CFSs was performed using a 0.20 µm Minisart ® filter syringe (Salleh et al., 2014).

One hundred microliters (100 µL) of each prepared *Bacillus* CFS were pipetted into wells in Mueller-Hinton agar (MHA) (HiMedia Laboratories, India) plates that had been punched with a 6-mm sterile borer. Each MHA plate was prepared using MHA base agar and 5 mL soft agar, and then inoculated with 50 µL standardized test pathogen using the overlay method (Aween et al., 2012). *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli*, *Vibrio parahaemolyticus*, *Micrococcus luteus*, *Klebsiella pneumoniae*, *Aeromonas hydrophila*, *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Candida tropicalis* were utilized in the agar well diffusion assay. Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli* pathogens with extended spectrum beta-lactamase (ESBL) resistance were included. Plates were incubated for 24 hours at 37°C. Following incubation, zones of inhibition were determined using a Vernier caliper, starting at the borders of the last visible antimicrobial growth and extending up to the diameter of the wells. As antibiotic controls for the susceptibility test, 5g antibiotic discs containing cloxacillin and ofloxacin (HiMedia Laboratories, India) were used.

2.7 Minimum inhibitory concentration (MIC) of bacillis isolates CFS

The Minimum Inhibitory Concentration (MIC) of the top three performing cellulolytic *Bacillus* CFSs was further examined against three pathogens against which they all demonstrated substantial activity in the antimicrobial assay. MIC can evaluate which concentration would the antimicrobial CFSs can inhibit the selected bacterial pathogens. Using a two-fold dilution procedure in a 96-well microtiter plate, sterile Mueller Hinton Broth (MHB) (HiMedia Laboratories, India) was pipetted into each well and 100 µL of supernatant was added. The microtiter plates were then incubated for 24 hours at 37°C (Ramachandran et al., 2014).

2.8 Molecular identification of top performing cellulolytic bacillus species

Bacillus isolates that exhibited inhibitory activities against test strains were cultured in tryptic soy broth (TSB) (HiMedia Laboratories, India) at 37°C for 24 hours. Bacterial cells were harvested, and DNA extracted according to the manufacturer's instructions using the Ultraclean Microbial DNA commercial kit (Mo Bio, Carlsbad, CA). Extracted genomic DNA was employed as a template for 16S rDNA PCR amplification. The universal primers 27 F (5' AGAGTTTGATCMTGGCTCAG 3' and 1429 R (5' GGTTACCTTGTTACGACTT 3') were used. PCR products were sent to Macrogen Korea for DNA sequencing, and the partial sequences were aligned using an open-source bioinformatics software Seaview version 3.2 and MEGA6 software for the likelihood tree. Consensus sequences were run using the National Center for Biotechnology Information's (NCBI) nucleotide BLAST program to explicitly match the partial sequences to *Bacillus* species sequences in the database. The corresponding FASTA data were retrieved, the *Bacillus* isolates partial sequences were aligned, and a tree was created using the neighbor-joining method.

2.9 Statistical analyses

All data were recorded, edited and entered using IBM SPSS Statistics version 23.0 software. The paired samples *t*-test, one-way ANOVA, and Tukey's Honest Significant

Difference post hoc test were used to analyze the data from the cellulase enzymatic activity test. To compare the zones of inhibition of all *Bacillus* cell-free supernatant and antibiotic control, the antimicrobial assay was statistically treated using the *t*-test paired sample for means. The alpha level for all analyses was set to 0.05, with a *p*-value of 0.05 considered significant.

3. RESULTS AND DISCUSSIONS

Bacillus species were presumptively identified based on the colony morphology of growing cultures on a plate. On pure cultivated plates, isolated colonies exhibited an elevated, round shape, off-white, frosty color, with some mucoidal in consistency (Figure 2a). Among the 58 presumptive *Bacillus* isolates from the two sampling sites, 40 isolates were found to have Gram-positive rod-shaped cells and the presence of endospores (Figure 2b) (Figure 2c). The defining characteristics of *Bacillus* spp., as listed by Logan (2011) and as observed in the study, are their Gram-positive cell wall, their capacity to grow aerobically, and their ability to develop resistant endospores when stressed. The total *Bacillus* isolates recovered from the rice paddy soils using the heat shock approach were comparable to those obtained by Smily et al. (2012) who, on the other hand, used MacConkey and blood agar in their study.

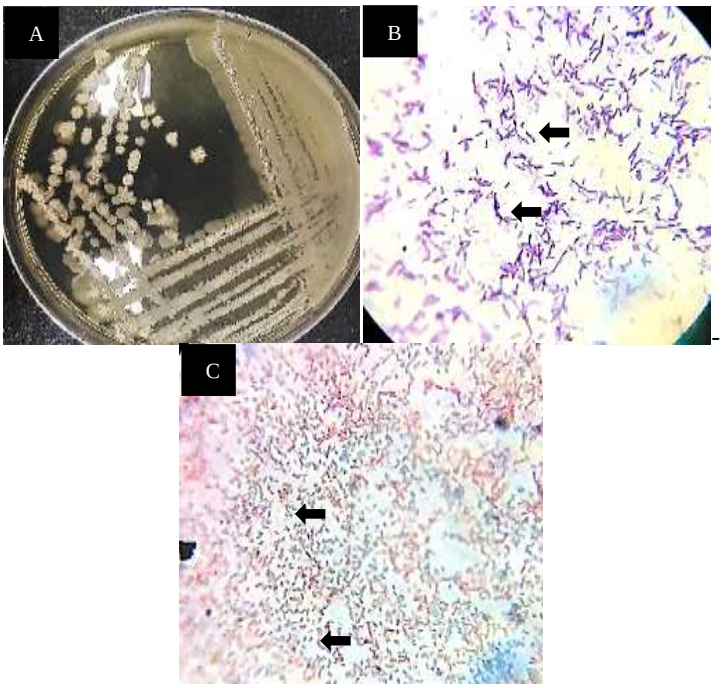

Figure 2. Sample NE.AIII.10 (a) Colony morphology showing a white, raised, and frosty colony (b) Gram-staining showing purple and rod-shaped bacteria and (c) Endospore staining positive result.

Twenty *Bacillus* isolates were selected from each municipality sampling site and subjected to the Carboxymethylcellulose (CMC) assay; all demonstrated cellulolytic activity with varied hydrolytic activities (HC) (Figure 3). Specified unique codes were designated in this study for the sole purpose of systematic identification of the *Bacillus* samples from the sampling points up to the pure cultures. Ten were selected as top performing cellulolytic *Bacillus* species coded as NE.AIII.1 (1.88±1.06 mm), NE.AIII.6 (1.71±0.65 mm), NE.AIII.8 (1.59±0.25 mm), NE.AIII.10 (1.73±0.47 mm), NE.CII.5 (1.94±1.03 mm), BI.AI.1 (2.07±1.07 mm), BI.AI.5 (1.57±0.60 mm), BI.AII.3 (1.85±0.53 mm), BI.BI.7 (1.75±0.77 mm) and BI.CI.2 (1.57±0.52 mm). This finding indicated that *Bacillus* spp. present in rice paddy soil environment is capable of using sugars as an alternative carbon source. It has been known that spore-forming bacteria possess genes for the catabolism of a variety of carbon sources and synthesis of antibiotics (Prescott et al., 2008). This unique characteristic exhibited by spore-forming *Bacillus* was demonstrated earlier in the study of Veith et al. (2004), who discovered the genes encoding the cellulase enzyme of a certain strain of *Bacillus licheniformis*, a finding that has not been previously described in pathogenic species.

The rice field soils could provide a suitable condition for the growth of *Bacillus* spp. This is undoubtedly possible because their physiological abilities can allow the bacteria to thrive in any natural environment (Nicholson, 2002). Moreover, it was found out that most of the rice-associated bacteria (RABs) belonged to the genus of *Bacillus* (Bishnu et al., 2016). This would stretch more opportunities for the *Bacillus* strains obtained from Philippine rice paddy fields to be studied for purposes other than cellulase production, such as amylase extraction, enzyme optimization, and further soil microflora explorations.

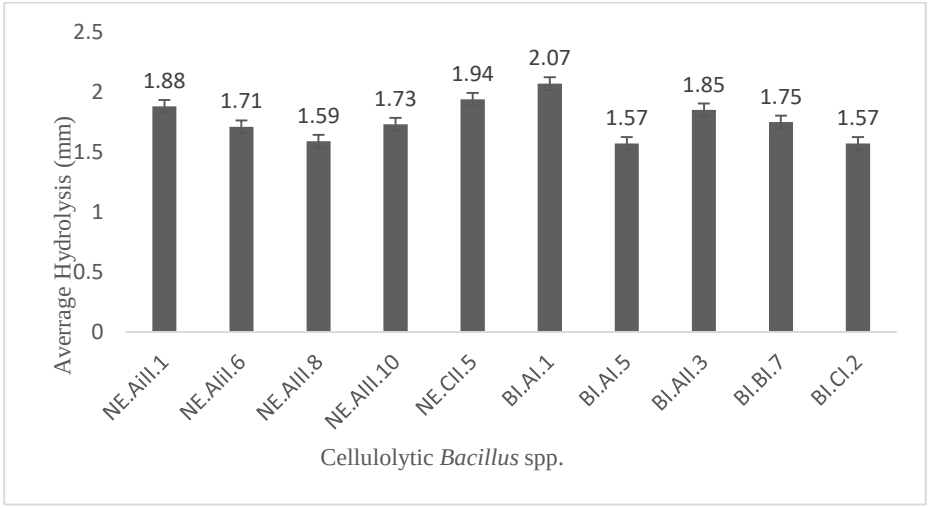

Figure 3. Cellulolytic activity of selected top ten *Bacillus* isolates based on their average hydrolysis zone (HZ).

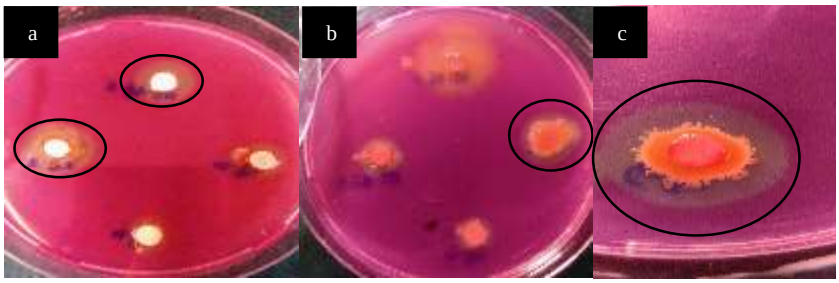

Figure 4. Hydrolysis zone of top three cellulolytic *Bacillus* (a) BI.AI.1 with mean ± SE 2.07±1.07 mm (b) NE. CII.5 with 1.94±1.03 mm (c) NE.AIII.1 with 1.88±1.06 mm.

The quantitative assessment of the cellulolytic activity of *Bacillus* cultures showed that only one bacterial strain from the ten selected isolates displayed remarkable cellulolytic activity in the range of 2.00-2.10 colony ratio (Figure 4a), whereas, the remaining strains exhibited cellulolytic activity in the range of 1.50–1.99 colony ratio.

The hydrolytic activity of the ten *Bacillus* isolates was observed to be comparable to the results generated by Hatami et al. (2008) for cellulolytic bacterial isolates from rice field soils which ranged from 1.38 to 2.33 mm. This reflects the varying capacity of *Bacillus* strains in generating cellulase, an enzyme which hydrolyzes the β 1-4 glycosidic linkages in a cellulose polymer to provide glucose and cellobiose (Pokhrel et al., 2014). Extracellular cellulase complexes on cell surfaces can be used by aerobic bacteria and are recoverable from culture supernatants, hence, they are obtainable for future studies (Lynd et al., 2002). Cellulase complexes, through the interaction of endoglucanase, exoglucanase and α-glucosidase in cellulose hydrolysis, catalyze the majority of reactions that fall under the broad acid-base catalysis category (Behera et al., 2016). The assessment of each individual enzyme activity is beyond the scope of this study and would require a separate in-depth methodology. Likewise, the detection of cellulase from the *Bacillus* isolates obtained from Philippine rice paddy soils suggests a potential source of the enzyme, a positive result which can be employed in various medical, agricultural, and industrial applications, including biofuel production, food and feed manufacturing, wine and brewing, pulp and paper manufacturing, and textile manufacturing (Pokhrel et al., 2014).

Table 1. Results of the biochemical tests.

Sample code	Catalase Test	Indole Test	Methyl Red	Vogues-Proskauer	Citrate Test	Triple sugar iron (TSI) test			
						slant	*butt*	H_2S	CO_2
NE.AIII.1	+	-	-	+	-	-	-	-	-
NE.AIII.6	+	-	-	+	-	-	-	-	-
NE.AIII.8	+	-	-	+	-	-	-	-	-
NE.AIII.10	+	-	-	+	-	-	+	-	-
NE.CII.5	+	-	-	+	-	-	+	-	-
BI.AI.1	+	-	-	+	-	-	-	-	-
BI.AI.5	+	-	-	+	-	-	-	-	-
BI.AII.3	+	-	-	+	-	-	-	-	-
BI.BI.7	+	-	-	+	-	-	-	-	-
BI.CI.2	+	-	-	+	-	-	-	-	-

Note: H_2S refers to Hydrogen sulfide formation, while CO_2 refers to carbon dioxide formation.

Table 2. Results of the enzyme production tests.

Sample code	Amylase Test	Chitinase Test	Protease Test
NE.AIII.1	+	-	+
NE.AIII.6	+	-	+
NE.AIII.8	+	-	+
NE.AIII.10	+	-	+
NE.CII.5	+	-	+
BI.AI.1	+	-	+
BI.AI.5	+	-	+
BI.AII.3	+	-	+
BI.BI.7	+	-	+
BI.CI.2	+	-	+

Table 1 summarizes additional analysis on the biochemical properties of the selected *Bacillus* isolates which can be useful as a starting point for further identification. All strains are positive for the catalase and Vogues-Proskauer (VP) test, but negative for indole, methyl red, and citrate tests. Catalase-positive *Bacillus* taxonomically separates them from their counterpart, *Clostridium*, which are known to be Gram-positive, catalase-negative endospore-forming bacilli (Reiner, 2010; Turnbull, 1996). Indole test is generally negative for *Bacillus* spp., although with some exceptions such as indole-positive *B. alvei* and *B. thiaminolyticus* species. Likewise, Vogues-Proskauer (VP) test is generally positive for *Bacillus* spp., including *B. cereus, B. mycoides, B. thuringiensis, B. firmus* and *B. lentus* among others. Methyl red test were able to show the inability of the strains to metabolize glucose through mixed acid pathway and, similarly, their inability to utilize citrate as observed in citrate test. There were, however, vast account of citrate-positive *Bacillus* in literatures, some of which are well-known, such as *B. cereus* and *B. thuringiensis*. Notably, all isolates aerobically metabolized peptone in the triple sugar iron (TSI) test, except for NE-AIII.10 and NE-CII.5, which were observed to be glucose fermenters. This result is nothing of surprise since *Bacillus* can utilize wide array of carbon sources and the majority are known for their ability to digest glucose (Logan & Berkeley, 1984). These observations on the biochemical tests, although confirmatory, were inconclusive and not species-specific. Species under the genus *Bacillus* are frequently difficult to identify from one another through conventional methods since they share a common pattern of morphological, biochemical and even genetic features unique to their taxa (Celandroni et al., 2016).

Along with the screening of isolated *Bacillus* spp. for cellulase production, other enzymes were studied on the ten selected strains, as detailed in Table 2. The enzymatic profile of the bacteria would allow room for examination of the strains' ability to carry out other metabolic processes that may possibly be unique to an isolate. This is an important analysis for a potential novel strain, although pure enzyme preparation and quantification is beyond the scope of this paper.

The enzyme production assays on chosen *Bacillus* spp. revealed that the isolates were capable of producing amylase (Figure 5a) and protease (Figure 5b), but not chitinase. These findings corroborate those of Powthong and Suntornthiticharoen (2017), who examined the biological enzyme production of different *Bacillus* strains isolated from agricultural soils in Thailand, identifying sample 209 as highly proteolytic (262.24 mm) and sample 290 as amilolytic (682.24 mm) using spot inoculation method. Additionally, all *Bacillus* strains tested negative for the chitinase enzyme. Only *Bacillus circulans* has been formally documented to be chitinolytic, with growth promoted by crab-shell chitin (Thiamthiankul

et al., 2001). It has been hypothesized that the loss of the chiA coding area contributes to *Bacillus* spp. lacking chitinase activity (Waldeck et al., 2006). On the other hand, the generation of alpha amylase by several *Bacillus* species, including *B. subtilis*, *B. licheniformis*, *B. caldolyticus*, and *B. amyloliquefaciens*, has been extensively documented in the literatures (Dibyangana et al., 2014). *Bacillus* spp. protease synthesis has been considerably studied in recent years due to its biotechnological significance in the creation of thermostable extracellular proteases that are easier to extract and purify (Bhich Thuy & Bose, 2011).

The results of agar well diffusion assay using *Bacillus* cell-free supernatant (CFS) showed that there is no statistically significant difference in the efficacy of CFS acquired on the second and third days of culture against the selected pathogens (*p*-value, 0.42). The computed mean of the second- and third-day cultures has a correlation of 92.61 %, a standard deviation of 2.68, and a large statistical variance of 7.21, which is thought to be attributable to the differential in activity of one *Bacillus* sample against distinct target pathogens. Because the *p*-value is > 0.05, the following tables hereby summarize the results of Day 2 and Day 3 CFS cultures against the pathogens. The tables are organized according to the cell wall type of the pathogen being tested as determined by Gram staining.

The mean values for the zone of inhibition of pathogens in millimeters (mean ± SD) following incubation with *Bacillus* CFS are shown in Tables 3 and 4. The majority of samples tested against Gram positive pathogens exhibited a positive response, with BI.AI.5 showing the highest hydrolysis zone measurement (10.80±1.10 mm) against *E. faecalis* and NE-AIII.10 exhibiting a positive response against both *M. luteus* (39.50±1.27 mm) and *C. tropicalis* (11.62±1.49 mm). Only NE-AIII.10 and BI.CI.2 inhibited *S. aureus* growth, with a hydrolysis zone (HZ) of 13.42±1.49 and 12.91±2.10 mm, respectively. On the other hand, all *Bacillus* supernatants were found to be ineffective against gram-negative bacteria such as *E. coli*, *V. parahaemolyticus*, *S. marcescens*, and *P. aeruginosa*. Surprisingly, NE-AIII.10 inhibited *A. hydrophila* (8.25±0.12, *p*-value 0.00002) and *K. pneumoniae* (2.91±0.72, *p*-value 0.00006) significantly more than other samples.

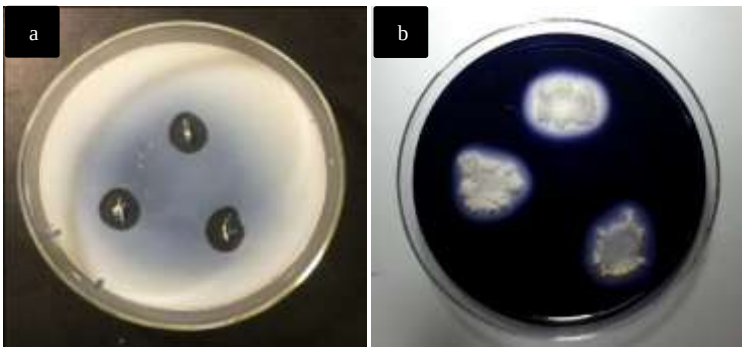

Figure 5. Observed hydrolysis zone (HZ) or halo formation around the *Bacillus* NE.AIII.6 colonies in a skimmed milk agar (a) and BI.CI.2 on starch agar plate (b).

Table 3. Average measurements of gram-positive pathogens' zone of inhibition in millimeters (mean ± SD) against *Bacillus* cell-free supernatant (CFS) cultures.

	S. aureus	**p-value**	***E. faecalis***	**p-value**	***M. luteus***	**p-value**	***C. tropicalis***	**p-value**
NE.AIII.1	-	-	-	-	17.47±1.05	0.4883	10.72±1.12	0.2173
NE.AIII.6	-	-	9.92±0.66	0.0170*	32.33±2.67	0.2498	9.96±1.48	0.1720
NE.AIII.8	-	-	7.91±1.17	0.0061*	32.73±4.67	0.1949	11.10±1.78	0.2991
NE.AIII.10	13.42±1.49	0.2818	9.41±0.63	0.0137*	39.50±1.27	0.2095	11.62±1.49	0.2555
NE.CII.5	-	-	10.40±1.02	0.0142*	23.11±1.26	0.1561	8.85±0.87	0.1398
BI.AI.1	-	-	9.22±2.83	0.0144*	21.91±0.44	0.1474	10.50±0.32	0.4233
BI.AI.5	-	-	10.80±1.10	0.0172*	34.28±2.43	0.2688	-	-
BI.AII.3	-	-	7.65±0.62	0.0092*	18.62±0.02	0.4296	10.10±0.40	0.0812
BI.BI.7	-	-	8.03±0.30	0.0111*	18.47±3.63	0.0565	8.83±0.42	0.2551
BI.CI.2	12.91±2.10	0.2553	9.54±0.43	0.0145*	37.02±04.05	0.2107	9.95±1.66	0.2396
control	16.24±6.96		21.08±3.45		17.24±6.90		15.14±4.00	

Note: Statistically significant values $p \leq 0.05$ are marked with asterisk (*) with H_o = mean difference is zero. Positive control used is a 5µg cloxacillin disk.

Table 4. Average measurements of gram-negative pathogens' zone of inhibition in millimeters (mean ± SD) against *Bacillus* cell-free supernatant (CFS) cultures.

	E.coli	***Vibrio parahaemolyticus***	***Serratia marcescens***	***P. aeruginosa***	***A. hydrophila***	***K. pneumoniae***
NE.AIII.1	-	-	-	-	-	-
NE.AIII.6	-	-	-	-	-	-
NE.AIII.8	-	-	-	-	-	-
NE.AIII.10	-	-	-	-	8.25±0.12	2.91±0.72
NE.CII.5	-	-	-	-	-	-
BI.AI.1	-	-	-	-	-	-
BI.AI.5	-	-	-	-	-	-
BI.AII.3	-	-	-	-	-	-
BI.BI.7	-	-	-	-	-	-
BI.CI.2	-	-	-	-	-	-
control					9.92±2.31	10.35±3.02

Note: Significant *p*-values are 0. 00002 and 0.00006 for NE-AIII.10 against *A. hydrophila* and *K. pneumoniae* pathogens, respectively. Positive control used is a 5µg ofloxacin disk.

It was observed in this study that the *Bacillus* CFSs were effective against *M. luteus* and some strains of *S. aureus*, however, were all negative against *E. coli* and *P. aeruginosa*. The obtained results have a slight difference with Ahmed et al. (2013)'s work on *Bacillus* spp. isolated from rhizospheric soil, by which when tested with live *Bacillus* spp., a clear zone appeared around *E. coli* and *S. aureus* colonies but not against *M. luteus* and *P. aeruginosa*. Earlier studies discovered that *Bacillus* isolates were actually more effective against *S. aureus* than *E. coli* (Tantiado et al., 2016). It was later conjectured that the effectivity of *Bacillus* strains against Gram-positive bacteria more than the Gram-negative bacteria, is most likely attributable to their Bacteriocin Type A, which specifically targets voltage-dependent holes in the cell's cytoplasmic membrane (Humam, 2016). Prescott et al. (2008), however, asserted that the *Bacillus* spp. can also inhibit *E. coli* and *S. aureus* via formation of bacitracin. Moreover, because certain *Bacillus* species are bacteriocins, their cationic peptides may have hydrophobic or amphiphilic characteristics, which are frequently used to target the bacterial cell membrane. Cationic peptides create channels

for ions to travel through and/or damage cytoplasmic membranes. Antimicrobial peptides (AMPs) produced by *Bacillus* should be able to cross the Gram-negative bacteria' negatively charged outer wall and can be inactivated in several ways, including proteolytic degradation via polysaccharide binding, modification of the bacterial outer membrane, or pumping of AMPs out of the cell via ABC transporters and/or resistance to efflux pumps. On the other hand, Gram-positive bacteria are easily inhibited by bacteriocin-like compounds, which are metabolites with antagonistic properties against a variety of diseases. Furthermore, the use of *Bacillus* antimicrobial peptides does not result in the development of cross resistance among the pathogens involved. This qualifies the *Bacillus* AMPs for the drug discovery challenge (Priest, 1977).

The results of the minimum inhibitory concentration (MIC) assay on three *Bacillus* species, NE-AIII.10, BI-BI.7, and BI-CI.2, indicate that chosen isolates require specific dilutions to inhibit the growth of pathogenic strains. At a minimum dilution of $1x10^{-1}$ mg mL^{-1} or a 1:1 ratio, all samples inhibited *E. faecalis*. On the other hand, MIC analysis of the three *Bacillus* CFS strains against *M. luteus* reveals that they were able to totally inhibit pathogens at a 1:32 ratio even at $1x10^{-5}$ dilution. When tested against *C. tropicalis*, samples BI-BI.7 and NE-AIII.10 inhibited pathogen growth at $1x10^{-5}$ dilution, whereas BI-CI.2 inhibited pathogen growth only at $1x10^{-2}$ dilution. Using taxonomic identification of their phylogenetic relationship, selected top performing strains NE-AIII.10 and BI-BI.7 demonstrated that both isolates are close relatives of *Bacillus subtilis* (Figure 6).

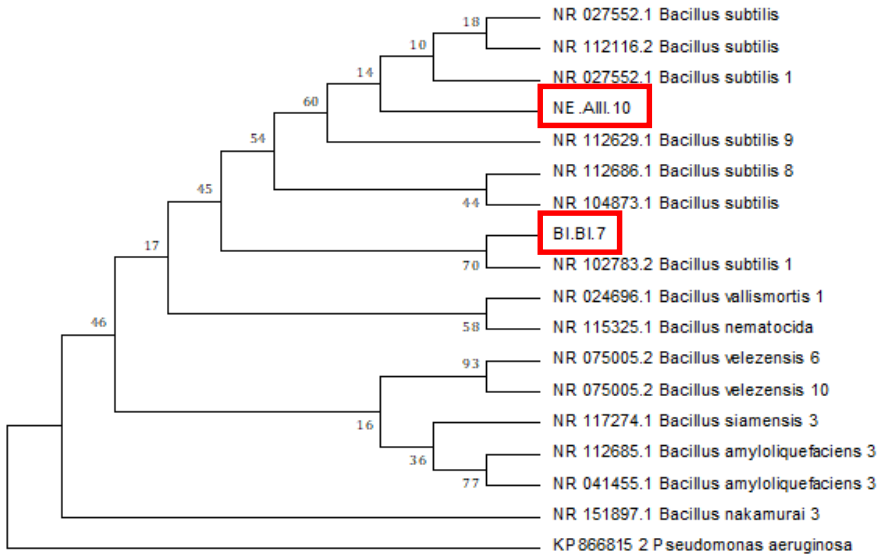

Figure 6. Phylogenetic tree of the top performing *Bacillus spp.*, NE-AIII.10 and BI-BI.7 strains, based on antimicrobial assay. Their phylogenetic relation to close neighbours resembles that of *Bacillus subtilis* strain.

Further investigation of the top five *Bacillus* CFS antimicrobial activity against Methicillin-resistant *Staphylococcus aureus* (MRSA) and Extended spectrum beta-lactamases (ESBL) *Escherichia coli*, using the agar well diffusion assay, revealed no inhibition against Gram-negative bacteria, consistent with the initial results. Similarly, they were unable to inhibit the MRSA bacterium, with the exception of sample BI.CI.2, which was compared to a positive control of Cloxacillin (p = 0.0005 at the 0.05 alpha level) and Ofloxacin (p = 0.0003). On initial investigation, the BI.CI.2 sample shown substantial inhibitory effect against *S. aureus*. Thus, among the five *Bacillus* isolates tested in this investigation, sample BI.CI.2 is regarded a unique *Bacillus* isolate capable of producing antimicrobial chemicals that inhibit the MRSA strain. Although a filtered *Bacillus* supernatant appears to have a narrow spectrum of activity against various diseases, it may yet be capable of inhibiting multidrug resistant bacteria. As a result, data demonstrates that rice paddy fields may be a source of novel antimicrobials required to combat the growth of antibiotic-resistant strains.

4. CONCLUSIONS

Bacillus strains isolated from the soils of Philippine rice paddy fields were confirmed to generate enzymes necessary to utilize cellulose as a source of alternative energy. This can be used to further the biotechnological application of cellulase enzyme in a variety of industrial contexts. Due to their ability to synthesize novel peptide compounds, cultivable *Bacillus* spp. may also serve as a new source of natural antimicrobials capable of inhibiting multidrug resistant bacteria. The majority of CFSs inhibited Gram positive pathogen development, but only one selected strain inhibited methicillin resistant *S. aureus* (MRSA), both of which are remarkable feats when employing crude enzyme extract. With a single strain inhibiting both Gram-positive and Gram-negative bacteria successfully, future investigations may examine a broader range of environmental source and/or target diseases.

5. RECOMMENDATIONS

Optimization of *Bacillus'* crude enzyme extracts and antibacterial properties may be a worthwhile endeavor in the future, and it is proposed that *Bacillus* culturing for the formation of cell-free supernatant (CFS) be thoroughly investigated. Because the results of Day 2 and Day 3 culture observations were not statistically significant, this study merged them; subsequent investigations would require a longer or shorter time. Other methods for determining *Bacillus* antibacterial activity exist in the literature, and the use of supernatant is just one of them. External parameters such as the incubation period, the amount of *Bacillus* cell biomass, the temperature, and the amount of microbial pathogen biomass are believed to contribute to the efficacy of filtered *Bacillus* supernatant and should thus be always considered. Further identification of *Bacillus* isolates to the species level is still advised using molecular analysis.

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