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Enzymatic Activity of Rhizobacillus Isolated from Tomato Rhizosphere

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Authors' contributions

This work was carried out in collaboration between both authors. Author KJA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors EDF and KJA managed the analyses of the study. Author KJA managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Aim: The aim of this study was to isolate *Rhizobacillus* from tomato rhizosphere and its screening for the production of hydrolytic enzymes to be used as a biocontrol agent.
Place of Study: The study was carried out in the Department of Microbiology, Faculty of Science, Ekiti State University, Ado Ekiti, Nigeria in August 2018.
Methodology: Isolation was of *Rhizobacillus* carried out from rhizospheric soil of tomato plant at agro-zone along Afao road Ado Ekiti using pour plate method. Enzyme assay was carried out on the bacterial isolates, to examine their ability to produce hydrolytic enzyme required for biocontrol of phytopathogenic fungi. Isolates were tentatively identified with the help of Bergy's Manual of Systemic Bacteriology.
Results: Ten strains were tentatively identified as *B. brevis, B. circulans* BC1, *B. macquariensis* BM1, *B. macquariensis* BM2, *B. macerans, B. macquarensis* BM3, *B. alcalophilus B. macerans* BC11, *B. circulans* BC3 and *B. macerans* BC9. All the Isolates demonstrated the ability to produce hydrolytic enzymes with the highest activity recorded in *Bacillus macquariensis* BM2 (60.28 µmol)

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for chitinase, *Bacillus macerans* BC9 (11.14 µmol) for Protease, *Bacillus macquariensis* BM2 (150.00 µmol) for Glucanase, and *Bacillus circulans* BC1 (46.45 µmol) for cellulase respectively. In conclusion the *Bacillus* strains isolated from rhizosphere are promising and could be used in bioprocessing technology to produce hydrolytic enzymes for the purpose of biocontrol in management of phytopathogenic fungi.

Keywords: Hydrolytic enzymes; rhizosphere; bacillus strains; biocontrol; rhizobacteria; rhizobacillus.

1. INTRODUCTION

Rhizosphere is best described as the soil region subject to the influence of plant roots exudates and their associated microorganisms. The constant presence of root exudates in rhizosphere allow activity of different microorganisms. The microorganisms present in this region produce various secondary metabolites that influences plant protection through direct interaction with the roots. [1]. Exudates, from the plant are released into the soil via the root which serves as nutrient to different microorganisms thereby controlling the populations, types and association of microorganisms found in the vicinity of roots of different plants. [2] The chemical compounds in exudates include the secreted ions, free oxygen and water, enzymes, mucilage, carbon containing primary and secondary metabolites [3]. The functions of most root exudates have not been established; however there is evidence that certain compounds are involved in lubrication of media and in positive and negative interactions (via attractant and repellent compounds) with indigenous microbes and other plant species. The production and release of root exudates to the root environment and the levels of excreted exudates vary with the physiological state of the plant and nutrient availability [4]. This zone encompasses several component regions of various layers of the root cells that can be colonized by microorganisms (endorhizosphere), the root surface (rhizoplane) and the media directly surrounding the root containing root-associated microorganisms (ectorhizosphere). It is important to note that the boundaries of these component regions within the rhizosphere are not always distinct [5]. Rhizosphere was described as the sole region subjected to influence of plant-exudate that permits the numerous activities of plant growth promoting bacteria such as *Bacillus*. Rhizobacteria that wield advantageous effect on plant growth and development and colonize the roots of the plants or rhizosphere are termed plant growth promoting rhizobacteria (PGPR) [6]. PGPR play significant role in inhibiting fungal pathogen and also considerable enhancement of plant growth [7]. Interestingly the mechanism responsible for imparting plant growth promoting properties to the plant growth promoting rhizobacteria is not fully comprehended [8]. It may be attributed to the ability to produce plant hormones indoleacetic acid (IAA), gibberellic acid, cytokinins, and ethylene; fix nitrogen; suppress the growth of phytopathogenic microorganisms by production of siderophore, -1,3-glucanse, chitinases, antibiotics, and cyanide; and solubilise phosphate and other nutrients. PGPRs effectively establish themselves in soil ecosystem with their high compliance during a wide variety of environments, faster rate of growth and biochemical adaptability to metabolize different natural and xenobiotic compounds [9].

Members of the genus *Bacillus* are generally found in soil and most of these bacteria have the power to disintegrate proteins, namely proteolytic activity. Several *Bacillus* strains such as *B. subtilis*, *B. cereus* and *B. amyloliquefaciens* and *B. amyloliquefaciens* has the ability to protect plants from deleterious pathogens. They were first isolated in 1943 and named after its ability to produce amylase. It is known to produce several antibiotics and is often found in soil and associated with plants [10].

Thus, the aim of this study was to isolate rhizobacillus from tomato rhizosphere and screen them for the production of hydrolytic enzymes for the purpose of using them as a biocontrol agent.

2. METHODOLOGY

2.1 Collection and Treatment of Sample

The sample was collected at an agric zone (Afao-road) Ado-Ekiti and was transferred in a sterile polythene bag for further analysis in the Laboratory.

2.2 Sample Treatment

Exact 0.1 g of rhizospheriic soil sample was measured into a boiling test tube containing 9ml

of distilled water. The mixture was then boiled in a water bath at 80° C for 20 minutes to kill non spore former that might be present in the sample.

2.3 Isolation of *Bacillus* from Treated Sample

Poured plate method was used to carry out the isolation of *Bacillus* spp present in the sample. About 0.1ml of the treated sample (soil) was dispensed into the sterile 90 mm petri dish after which the already prepared nutrient agar was poured, covered, and gently mixed to allow homogenization. The plate was incubated at 37^0C for 24 hours. To obtain a single pure colony after 24 hours' of incubation, the culture plates were checked for visible growth. The colonies with distinct growth were then sub–cultured into freshly prepared nutrient agar by the streaked method. Agar plates the sub-cultured were further inoculated at 37^0C for 24 hours.

2.4 Enzymatic Analysis

2.4.1 Chitinase assay

Culture filtrate was centrifuged and the supernatant was used immediately for enzyme activity and served as the enzyme solution [11]. About 1ml of chitin solutions was mixed with 1 ml of enzyme solution then incubated for 30mins.The reaction was stopped by boiling for 3 mins in water bath by the addition of 1ml of dinitrosalicylate (DNS) reagent. The absorbance of reaction mixture was measured in a spectrophptometer (6850 UV/vis Jenway) at 520 nm.

2.4.2 Glucanase assay

Glucanase was assayed by incubating 500 uL of 5.0% Laminarin in 50 mM acetate buffer pH 4.8 with 200 uL enzyme solution at 45 °C for 30 min and determination of reducing sugars with DNSA The amount of reducing sugars was calculated as mmol of glucose per min per ml [12].

2.4.3 Protease assay

Protease activity was determined by Anson method [13]. About 1 mL of 1.5% casein solution, pH 7.0 was placed at 37^0C and, then, 1 mL of properly diluted enzyme sample was added. The reaction was incubated for 10 min prior to the addition of 2 mL of 0.4 Mtrichloroacetic acid. The solution with precipitates was altered and to 0.5 mL of the clear filtrate 2.5 mL of 0.4 M Na_2CO_3 and 0.5 mL of Folin reagent were added. After further 10 min of incubation, the color density developed was determined at 660 nm. One unit was defined as 1 µmol of tyrosine released per minute by 1 mL of enzyme.

2.4.4 Cellulase assay

Cellulase was measured according to the method of Ghose [14]. A 900 uL of 1% CMC solution was added with 100 uL enzyme solution in a test tube. About 1.5 mL DNS reagent was added and incubated at 50°C in water bath. The absorbance was measured at 540 nm. One unit of cellulase activity was defined as the amount of enzyme that liberates 1 micromole of reducing sugars equivalent to glucose per min under the assay conditions.

3. RESULTS AND DISCUSSION

3.1 Isolation of Rhizobacillus

Five groups of microorganisms belonging to ten strains of *Bacillus* were isolated and identified using biochemical reactions during this study. They include *B. alcalophilus, B. brevis B. circulans, B. macquariensis* and *B. macerans* (Table 1). Isolates were characterized according to the Bergey's Manual of Determinative Bacteriology [15]. Microscopic studies revealed that the cells were rod-like Gram positive and spore-forming under aerobic conditions. The isolated Baciili were facultative anaerobes. The biochemical studies showed that some organisms were motile, gram-positive, catalase positive, citrate positive, glucose positive, for *B. alcalophilus, B. brevis and B. circulans.* All showed positively to Methyl red except for *B. macquariensis* and Citrate utilization were positive except for *B. alcalophilus.* The biochemical results of this work is similar to that of Tariq *et al.,* [16], who identified various *Bacillus* spp. such as *Bacillus subtilis, Bacillus polymyxa* and *Bacillus megateriu.* and observed them to be gram positive, motile, indole negative, MR positive, VP positive, citrate positive, oxidase negative, glucose positive, lactose positive, sucrose positive and urease positive. Isolates were gram positive rod and this is line with Berkeley and Ali, [17] who reported that *Bacillus* is a genus of rod-shaped gram positive bacteria and are usually either obligate or facultative aerobes under stressful environmental conditions and can produce oval endospore that can stay dominant for extended periods.

Table 1. Results of various biochemical tests

Motility	Citrate	Glucose fermentation	Methyl red	Urease	V. P test	Indole	Gas production	Nitrate reductance	NaCl 6.5%	Gram reaction	Tentative Identification
Motile	+ve	-Ve	+ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve Rod	*B. brevis*
Motile	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve Rod	*B. macerans*
Non Motile	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve Rod	*B. macquariensis* BM1
Motile	+ve	-ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	+ve Rod	*B. circulans*
Motile	+ve	+ve	-ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve Rod	*B. macquariensis* BM2
Non motile	+ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve Rod	*B. macquarensis* BM3
Non motile	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve Rod	*B. alcalophilus*
Motile	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve Rod	*B. macerans* BC9
Motile	+ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve Rod	*B. macerans* BC11
Motile	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve Rod	*B. circulans* BC3

-ve = No reaction, +ve = There is reaction

3.2 Enzymes Activities

3.2.1 Chitinase activities

Fig. 1 showed the result of chitinase activity of Bacilli isolated from rhizosphere of tomato plant. *Bacillus macquariensis* BM2 has the maximum activity (60.28 µmol) followed by *Bacillus circulans* BC3 that has (46.45 µmol) activity and the least activity was recorded in isolate *Bacillus macquarensis* BM3 (3.19 µmol). This report is similar to the work of Kumar *et al.,* [18] who reported that *B. circulans* and *B. licheniformis* produce the enzyme chitinase that degrade chitin. The recent discovery of suitable strategy in disease management is the use of microorganism. The use of biological control agents, such as rhizobacteria belong to plant growth promoters (PGP), has been described as good methods. Plant growth promoting rhizobacteria (PGPR), such as *Bacillus* strains, are part of major root colonizers [19,20] and can elicit plant defenses [21] Cell wall degrading enzymes especially chitinase have been isolated from *Bacillus* species. Many strains of *Bacillus* can produce a high level of chitinolytic enzymes [22]. Moreover, many studies have shown that chitinase is involved in antiphytopathogen activity and can enhance the pesticidal activity of *Bacillus* sp. The characteristics of lytic enzymes is important in disease management, although the certain or specific quantities of enzymes require to enhancing antifungal ability was not known but there are evidences that lytic enzymes in their combination would be effective in disease controlling practices. Chitinases have been described as enzymes that have a broad range of biotechnological applications such as production of fungal protoplasts, crustacean chitin waste management, production of single cell protein and chitooligosaccharides for various applications and biocontrol of fungal plant pathogens [23]. The significant of an organism that can produce chitinase cannot be over emphasized in the disease controlling strategies especially disease cause by phytopathogenic fungi. This is because the cell wall of these fungi contains chitin that helps in their proliferation and disease development within a suitable host.

3.2.2 Glucanase activity

Fig. 2 showed the result on glucanase activity of bacillus isolates. The *B. macquariensis* BM1 and *Bacillus circulans* had the highest activity (150 µmol) followed by *B. macerans* BC11 and *B. circulans* BC1 (26.28 µmol) had the lowest activity. In the Production of glucanase, results show that all the isolated bacillus are good

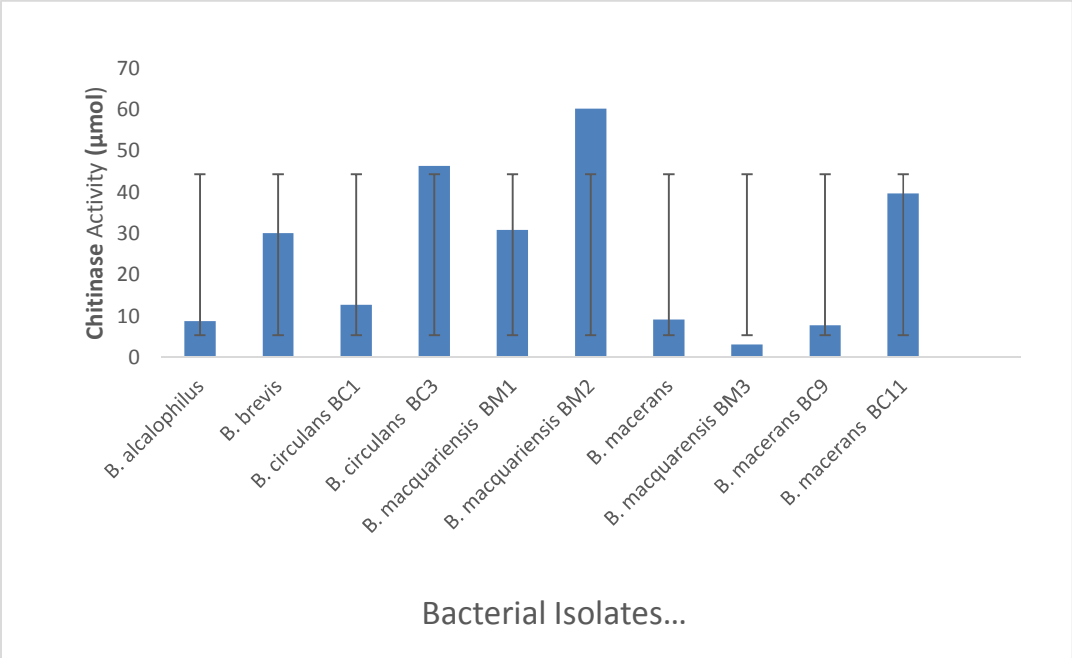

Fig. 1. The Chitinase activity of Bacilli isolated from tomato rhizosphere
Error bar represents standard deviation of enzyme activities

producers; Among the strains, synthesis of glucanase level was quite good as compare to other enzymes. In the previous study, biocontrol agents such as *Serratia marcescens. P. cepacia* [24], secrete β-1,3 glucanase capable of degrading β-1,3-glucan, which is one of the major components of fungal cell walls. As examples, isolates related to *Bacillus* spp. [25] produce chitin-degrading enzymes while *Bacillus subtilis* AF1 displays some fungitoxicity through the secretion of N-acetyl glucosaminidase and glucanase [26]. Enzyme β -1, 3-glucanase has been known to playing significant role in biocontrol of plant pathogenic fungi. Although in some cases both chitinase and β -1, 3-glucanase produced by bacterial strains have biocontrol activity but the production of β -1, 3-glucanase singly by *Bacillus* spp had proved to have killing effect on the fungal cell wall. The production of lytic enzymes during mycoparasitism may play a role in the biocontrol of fungal pathogens [27].

3.2.3 Protease activity

Fig. 3 showed the result protease activity of the isolated bacilli. Isolate *Bacillus macerans* BC11 has the highest activity (11.40 µmol) followed by the isolate *Bacillus alcalophilus* has the lowest protease activity (3.73 µmol). The production of protease by *Bacillus* spp had been reported by Fergus [28] Protease is a part of antimicrobial metabolite secreted by bacteria in fighting against phytopathogenic fungi in disease management. Protease enzyme is very important not only in the industries but in agricultural practice. It contributes to the soil fertility and enhances plant growth. The significant of protease in biocontrol has been proved in the absence of other enzymes such as chitinase and lipase [29]. This implies that when an organism possesses enough proteases without the presence of other enzymes it would be useful in management of disease caused by phytopathogens.

3.2.4 Cellulase activity

Fig. 4 showed the result on cellulase activity of Bacillus isolates. The highest isolates activity is observed in *Bacillus alcalophilus* with the activity of (66.36 µmol) and the least activity was recorded for isolate *Bacillus macquariensis* BM2 (10.30 µmol). The isolation of protease producing

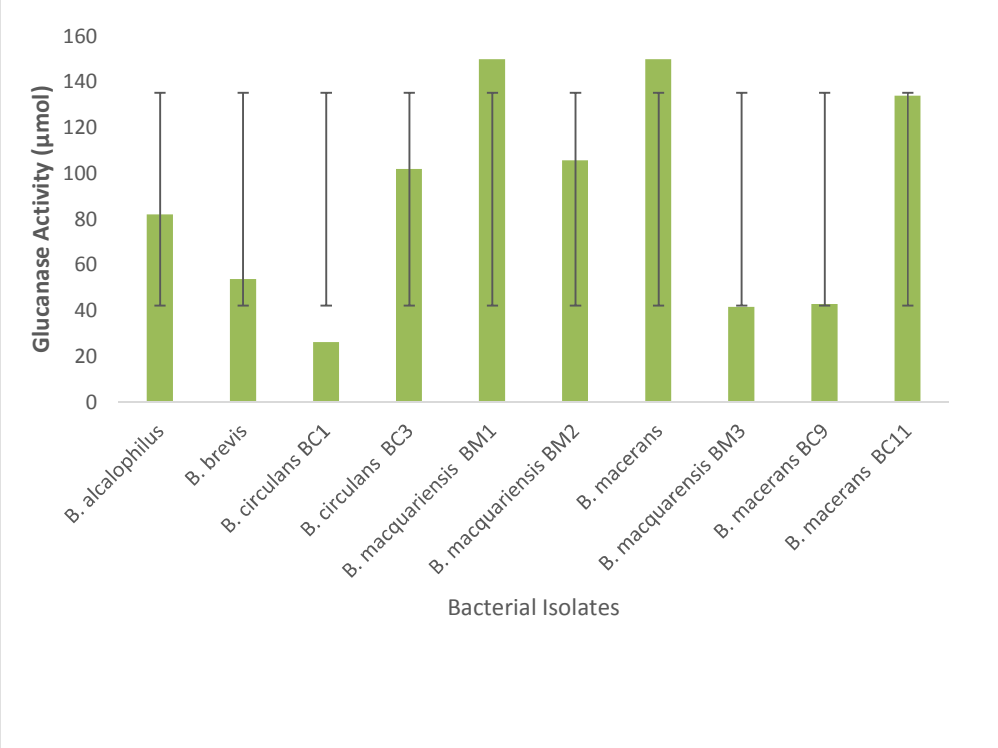

Fig. 2. The Glucanase activity of Bacilli isolated from tomato rhizosphere
Error bar represents standard deviation of enzyme activities

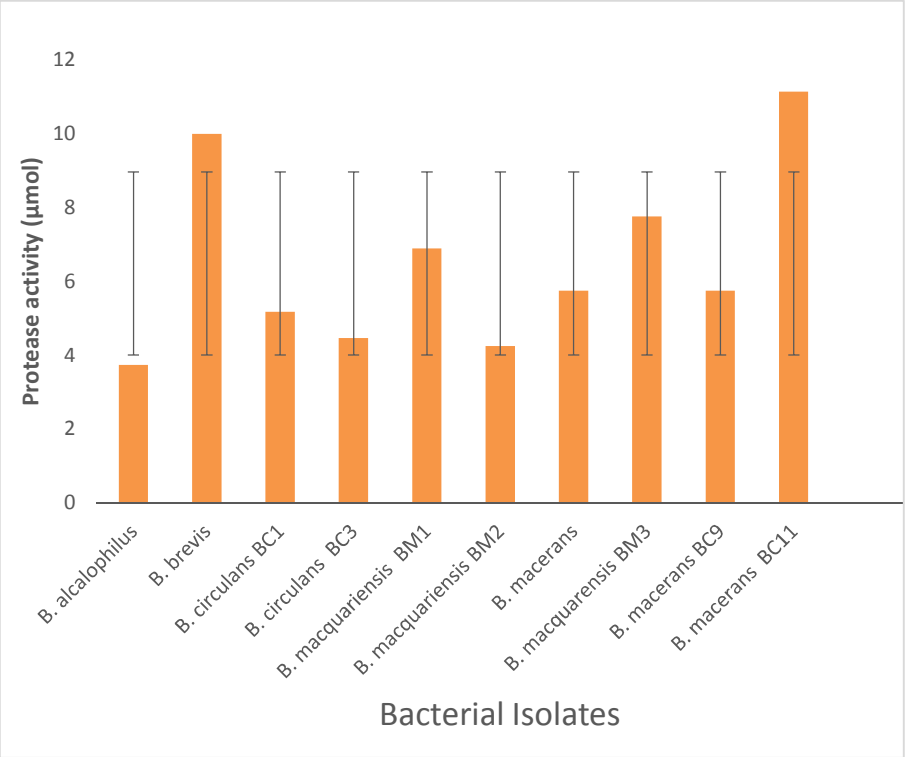

Fig. 3. Protease production by the isolated Bacilli from tomato rhizosphere
Error bar represents standard deviation of enzyme activities

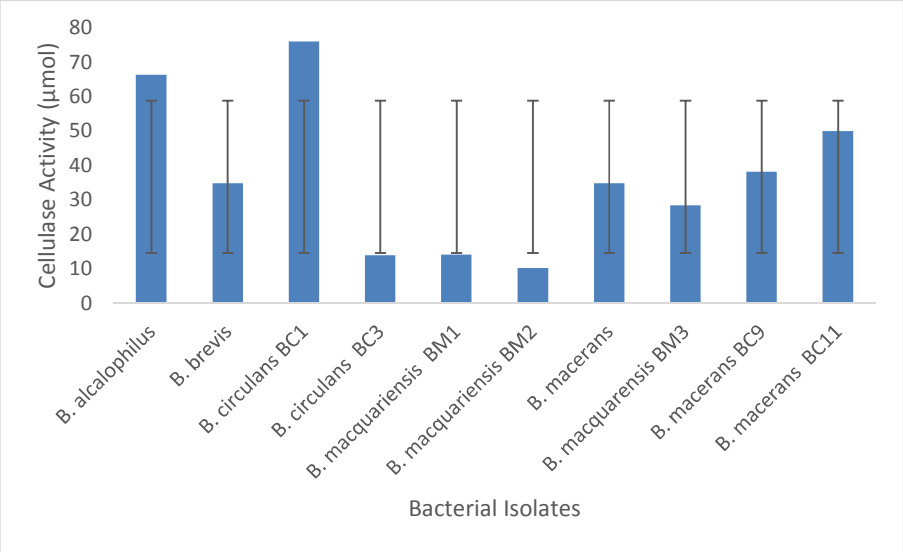

Fig. 4. Cellulase production by the isolated Bacilli from tomato rhizosphere
Error bar represents standard deviation of enzyme activities

Bacillus has been reported by other researchers[28]. Bacteria employed the enzyme cellulase to break down the cellulose in the cell wall of phytopathogenic fungi. In agriculture apart from biocontrol mechanism where cellulase activities seem to be more pronounced, Cellulase has been described as plant growth-promoting traits secreted by certain *Bacillus* spp [30].

4. CONCLUSION

There is need to look for alternative method of controlling fungi disease spreading in agricultural practices that will not have any harmful effect on the farmer and environment. These microorganisms especially *Bacillus* isolated from rhizosphere are promising. They could be used in bioprocessing technology especially when the focus is on the ability of such Bacillus to produce hydrolytic enzymes that has the potential to degrade cell wall of phytopathogenic fungi.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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