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Isolation and Screening of *Bacillus* sp. to Act as Plant Growth Promoting Bacteria

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Abstract: *Bacillus* sp. has been widely known to produce various bioactive metabolites related to plant growth. *Bacillus* sp. has also been known as plant growth promoting bacteria (PGPB). However, the ability of *Bacillus* sp. isolated from banana rhizosphere that produce a wide range of enzymatic reactions was not known. The aim of this study, is to isolate *Bacillus* sp. from banana rhizosphere with potential to secrete cellulase, mannanase, xylanase, indole-3-acetic-acid, and gibberellic acid. In this study, 68 isolates of *Bacillus* sp. were isolated from the rhizosphere of banana plantation using soil suspension technique. Isolated *Bacillus* sp. was then subjected to screening of their secondary metabolites such as cellulase, mannanase, xylanase, indole-3-acetic-acid, and gibberellic acid production before potential isolates were chosen and identified using their 16S rRNA and later subjected to seed germination testing. It was observed that *Bacillus* sp. isolated produces most of cellulase but did not have much affinity to produce gibberellic acid as only 5 *Bacillus* sp. were observed producing gibberellic acid. Three of the potential *Bacillus* sp. were selected and identified as *Bacillus pumilus* strain PNO21, *Bacillus amyloliquefaciens* strain PNO7 and *Bacillus wiedmannii* strain PNO28. Seed germination test also revealed that these 3 *Bacillus* sp. did not inhibit the seeds germination.

Keywords: *Bacillus* sp., secondary metabolites, Malaysia soil, seed germination test, plant growth promoter

1.0 Introduction

Rhizosphere is well known for being a place where abundance of microbial activities took place due to its nutrient rich ecosystem. This ecosystem is made up of interactions between microorganisms, plants, and other higher organisms in the soil. Soil bacteria are known to be one of the most capable microorganisms in producing various secondary metabolites, which are important for their growth.

One of the most commonly studied species of soil bacteria is *Bacillus subtilis*, which is used as a model organism for sporulation and formation of biofilm (Kovács, 2019). *Bacillus subtilis* is made up of four original phylogenetically and phonetically homogeneous species of *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, and *B. pumilus* (Caulier et al., 2019). Studies shown that most of the members from the genus Bacillus produce various kinds of secondary metabolites such as antifungal, antibacterial, and lots more (Stein, 2005; Kaspar et al., 2019). These secondary metabolites are the polyketides, terpenes, siderophores, and ribosomally and nonribosomally synthesized peptides (Harwood et al., 2018). While numerous natural products have been identified from *B. subtilis* species complex, the diversity of secondary metabolite production from various isolates of *Bacillus* sp. has not been properly studied to understand their functions.

Nowadays, agricultural researches are more targeted towards environmentally friendly alternatives for controlling plant pathogens and also to improve crop productions (Miljakovi´c et al., 2020). *Bacillus* spp. have been well studies to be an excellent producer of biocontrol properties either by promoting plant growth or by reducing plant diseases caused by both plant pathogenic microorganisms (Fan et al., 2017). This has made Bacillus as an alternative for natural plant growth enhancement agrochemical. According to Ongena and Jacques (2008), bioactive compounds from the families of surfactins, fengycins, and iturins are produced by *Bacillus* sp. The aim of this study was to bioprospect for *Bacillus* sp. isolated from locally soil for their bioactivity.

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2.0 Materials and methods

2.1 Isolation of *Bacillus* sp. from soil sample

From the soil samples collected MARDI Organic Plot (coordinate 2.992973, 101.698674), 10 g of the soil sample was diluted in 100 ml of sterile distilled water and put on a heating block at 80^{o}C for 10 mins to eliminate the vegetative cells that present in the soil samples (Manzum and Al Mamum, 2018). One milliliter of the suspension was then pipetted into a conical flask containing 99 ml of sH2O and agitated for 1 min before 150µl was pipetted onto Luria Bertani Agar (LBA) plate and incubated at 28±2^{o}C for 24-48 hrs. Colonies of bacteria emerged were selected and grew onto a new LBA plate.

2.2 Screening of *Bacillus* sp. for extracellular hydrolysis enzymes activities

Bacillus sp. obtained were screened for extracellular hydrolysis enzyme activities (manannase, xylanase and cellulase) using minimal medium agar containing yeast extract, 1.0 g; bacteriological peptone, 1.0 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $(NH_4)_2HPO_4$, 1.0 g; agar, 15.0 g; and substrate, 1.0 g which contained Megazyme: AZO-Carob-Galactomannan, AZO-CM-Cellulose and AZO-Xylan (oat) in 1000 ml of distilled water with pH adjusted to 7 (Jeffrey, 2011).

2.3 Screening for indole-3-acetic acid (IAA) activities

Method used by Iqbal and Hasnain (2013), was the followed with modification. Bacterial cell suspension was incubated at 30 ± 2^{o}C for 48 hrs and density of the culture broth was adjusted to 10^6 cfu/ml. Two milliliters of culture broth was pipetted into a new microcentrifuge tube and centrifuged at 10,000 rpm for 30 mins. Salkowski's reagent was mixed at the ratio of 2:1 with the supernatant and allowed to stand in dark for 30 mins after the centrifugation. Colour changes to pink indicate the presence of IAA. Amount of IAA presence was measured by Nanodrop spectrophotometer at wavelength of 530 nm. Standard curve was constructed using synthetic IAA (Sigma) to determine the amount of IAA secreted by the *Bacillus* sp.

2.4 Screening for gibberellic acid (GA_3) activities

Method proposed by Pandya and Desai (2014) was followed without any modification. Standard curve was constructed using synthetic GA_3 (Sigma) and was measured using Nanodrop spectrophotometer at the wavelength of 254 nm.

2.5 Identification of *Bacillus* sp. using 16S rRNA region

DNA isolation was conducted using Qiagen DNeasy DNA extraction kit protocol as suggested by the manufacturer. Polymerase Chain Reaction parameter and conditions used was as described by Jeffrey (2011), with only modification on the primers used. Primers used in this study was, universal primers F8 (AGA GTT TGA TCM TGG CTC) and rP2 (ACG GCT ACC TTG TTA CGA CTT). Polymerase Chain Reaction products obtained were later subjected to purification using Vivantis GF-1 Gel DNA recovery kit according to the protocol stated by the manufacturer. Purified PCR products were later sent for sequencing at Apical Sdn. Bhd., Selangor. Sequencing results obtained were then blast with the databases from National Center for Biotechnology Information (NCBI).

2.6 Seed germination test

Brassica sp. seed were used for the germination test. Twenty seed were used for each treatment. The seeds were pre-soaked with *Bacillus* sp. culture broth for 30 min before being transferred to filter paper in petri dishes. Five milliliters of Bacillus sp. broth were used to wet the filter papers for constitutive of 3 days. Percentage of germination was calculated based on the percentage of seed germinated after 3 days. Sterile distilled water used acted as control and all tests were conducted in triplicates.

3. Results and Discussions

3.1 Isolation and screening of *Bacillus* sp. for selected enzymes and phytohormones

It has been well known that *Bacillus* sp. can be found abundant in soil. Sixty-eight isolates of *Bacillus* sp. were isolated from soil samples collected with the average cfu/g of soil of 3.5 x10^7. Screening for cellulase, xylanase and mannanase activity showed that 63, 40 and 46 isolates of Bacillus sp produces these enzymes respectively. From the test it is also noted that only 5 and 10 isolates from the total 68 isolates produces gibberellic acid (GA_3) and indole-3-acetic acid (IAA) respectively. This showed that not all *Bacillus* sp. have the potential of secreting these valuable phytohormones.

Table 1: Enzymatic activity produces by *Bacillus* sp.

Enzymatic activity (number of isolates)				
Cellulase	**Mannanase**	**Xyalanase**	**indole-3-acetic acid**	**Gibberellic acid**
64	46	40	10	5

The ability of *Bacillus* sp. to produce cellulase was well documented (Balla et al., 2022; Dobrzyński et al., 2022). Dobrzyński et al. (2022) isolated *Bacillus* sp.8E1A with a good cellulolytic property. Balla et al. (2022) also noted that *Bacillus* sp. does have potential in producing cellulases.

Table 2: Enzymatic activities of 3 selected potential *Bacillus* sp.

Isolate	Enzymatic activity (Mean ± standard deviation cm)			Enzymatic activity (Mean ± standard deviation µg/ml)	
	Cellulase	**Mannanse**	**Xyalanase**	**Indole-3-acetic acid**	**Gibberellic acic**
PNO21	2.50 ± 0.08	1.63 ± 0.21	2.17 ± 0.12	31.93 ± 1.44	26.27 ± 0.34
PNO7	2.08 ± 0.15	1.27 ± 0.05	1.57 ± 0.09	28.77 ± 0.84	25.00 ± 0.53
PNO28	2.37 ± 0.09	1.17 ± 0.12	2.23 ± 0.17	29.63 ± 0.34	23.30 ± 0.43

It was observed that isolate PNO21 was the best potential *Bacillus* sp. among the three selected *Bacillus* sp. Suliasih and Widawati (2020) stated that *Bacillus siamensis* isolated from peat soil produce 13.29 µg/ml of Indole-3-acetic acid after optimization done. However, this is very much less than the amount of Indole-3-acetic acid produce by three of the potential *Bacillus* spp. in this study.

3.2 Identification of Bacillus sp.

Three of the *Bacillus* sp isolates were identified as *Bacillus pumilus* (PNO21), *Bacillus amyloliquefaciens* (PNO7) and *Bacillus wiedmannii* (PNO28) Table 3.

Table 3: Identification of three potential *Bacillus* sp.

Isolate	ID
PNO21	*Bacillus pumilus*
PNO7	*Bacillus amyloliquefaciens*
PNO28	*Bacillus wiedmannii*

3.3 Seed germination test

Seed germination rate of Brassica seeds was not retarded by the used of *Bacillus* sp. suspension. All seeds showed 100% germination at the end of the test except for *Bacillus wiedmannii* strain PNO28. Table 4 showed the germination rate of the three *Bacillus* spp. against control. Hsiao et al. (2023), showed that cabbage seeds inoculated with *B. amyloliquefaciens* PMB05 was able to inhibit the growth of black rot disease on cabbage. While another researcher showed that tomato seed inoculated with *Bacillus pumilus* gave a 86.7% of seed germination (Cabra Cendales et al., 2017. This is however is way lower than what was observed in this study where 100% of seed inoculated with *Bacillus pumilus* strain PNO21 germinated.

Table 4: Germination rate of selected *Bacillus* sp. against control

Isolate	Germination rate (mean ± SE %)
Bacillus pumilus strain PNO21	100.00 ± 0.00
Bacillus amyloliquefaciens strain PNO7	100.00 ± 0.00
Bacillus wiedmannii strain PNO28	95.00 ± 4.08
Control	100.00 ± 0.00

4.0 Conclusions

Preliminary screening of utilization of *Bacillus* sp. as a plant growth promoting bacteria showed that three of the selected Bacillus sp. known as *Bacillus pumilus* strain PNO21, *Bacillus amyloliquefaciens* strain PNO7 and *Bacillus wiedmannii* strain PNO28 display the ability to secrete cellulase, mannanase, xylanase, Indole-3-acetic acid (IAA) and Gibberellic acic (GA_3). Further seed germination test also suggested that these bacteria did not inhibit the growth of the seeds and suggested the potential of utilizing these *Bacillus* sp. for further study in enhancing growth and yield of the plant.

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