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Selection of Potential Bacteria in Termite Nest and Gut for Sustainable Agriculture

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Abstract

In this study, bacteria with the best abilities in cellulose degradation, siderophore production, phosphate solubility, and *Pythium parasitica* inhibition were selected from termite nests and guts. The isolate BTNASP 5-2, BTPK 5-3 and BTNA 5-1 from termite guts exhibited highest in siderophore production index (SPI) (4.16 ± 0.21), phosphate solubilizing index (PSI) (2.10 ± 0.14) and percentage inhibition of radial growth (PIRG) (67.07 ± 4.02 %), respectively. The BGNACMC 4-3 isolated from termite nest gave the highest cellulolytic index (CI) of 5.17 ± 0.24. Bacterial classification was performed using 16s rRNA gene sequencing. The isolates BTPK 5-3, BGNACMC 4-3 and BTNA 5-1 were found closely related to *Bacillus cereus*, whereas the bacterial isolate BTNASP 5-2 was closely related to *Bacillus subtilis*. It is also suggested that the *Bacillus cereus* exhibited a variety of biological activities, denoting the highest cellulase, phosphate-solubilizing and antifungal activities, while *Bacillus subtilis* produced only a siderophore. The results obtained suggest that the bacteria selected will be used to develop bio-compost to promote plant growth, leading to sustainable farming.

Keywords: *Bacillus*, Termite nest, Termite gut, *Phythium parasitica*

Introduction

According to the report by the Food and Agriculture Organization (FAO), the International Fund for Agricultural Development (IFAD), the United Nations Children's Fund (UNICEF), the World Food Programme (WFP) or World Health Organization (WHO) indicated that an estimated 3.8 million hungry people in 2022 were less than that of 2021. It is estimated that nearly 600 million people will still face hunger in 2030 [1]. Nonetheless, the impacts on food agricultural crop production and food security related to the growing global population, the Covid-19 pandemic, global warming and war in Ukraine, etc., collectively increased demand for agricultural crop production. It is therefore imperative to produce sufficient agricultural crops to meet the needs of the world's population, without compromising the safety of the environment and human health. Serious health and environmental problems are caused by the use of chemical fertilizers and pesticides around the world. This problem therefore requires safe alternative measures towards reaching the Sustainable Development Goal 2 (SDG 2) targets in terms of helping supporting sustainable food production, maintaining ecosystems and continually improving land and soil quality. Therefore, it is necessary to develop alternative fertilizers that are low-cost, efficient and environmentally friendly [2].

Therefore, the use of biofertilizers and biocontrol agents instead of chemical fertilizers in food agricultural crop production is necessary. Biological fertilizers are made from living microorganisms that can produce nutrients or prone to provide beneficial nutrients plant growth and health as they indeed improve the biological and physical state of the soil [3,4]. Microorganisms found in biofertilizers are able to nitrogen, dissolve phosphate, oxidize sulfur, produce plant hormones, decompose organic compounds and inhibit plant pathogens in the soil [5]. The termite comb (fungus comb, termite nest, fungus garden) is

a nest for termites from the sub-family Macrotermitinae (Isoptera: Termitidae), a porous structure resembling a sponge. The wavy shape looks like a brain or coral-like, some resemble a honeycomb. Termite comb is created from termite excrement. In the nest, there is a substrate for the growth of fungi *Termitomyces* spp. [6].

Previous research studies have isolated plant growth-promoting bacteria from termite mound nests: increase fertilizer in the soil and stimulate plant growth by *Pseudomonas monteillii* (*Fluorescent pseudomonads*), *B. endophyticus* TSH42 and *B. cereus* TSH77 [7,8]. *Chloroflexi* [9], *Planctomycetes* [10], *Thiobacillus* and *Rhizobium* [11] can perform nitrogen fixation. *Streptomyces* spp. strain M56 [12], *B. endophyticus* TSH42 [8], *B. cereus* TSH77 [8], *B. methylotrophicus* [13] and *Staphylococcus saprophyticus* [13] can inhibit plant pathogens. Moreover, *Bacillus* and *Alcaligenes* produce indole acetic acid siderophores, ammonia and HCN by Devi and Thakur [14]. In addition, previous studies showed that cellulolytic bacteria from termite guts were the genera *Bacillus*, *Bacteroidetes*, *Bifidobacterium*, *Brevibacterium*, *Cellulomonas*, *Clostridium*, *Enterobacter*, *Eubacterium, Fusobacterium, Lactobacillus*, *Microbacterium*, *Peptococcus*, *Peptostreptococcus*, *Ruminococcus*, *Staphylococcus* and *Termitomyces* [15-18]. In addition to cellulolytic activity, the genus *Bacillus* was capable of degrading lignin. Ali *et al.* [19] reported that the bacteria *Paenibacillus lactis*, *Lysinibacillus macrolides*, *Stenotrophomonas maltophilia*, *L. fusiformis* and *Bacillus cereus* isolated from the gut of *Psammotermes hypostoma* Desneux play vital roles in cellulose decomposition. In Thailand, Sujada *et al.* [20] isolated antimicrobial actinobacteria found the genera *Streptomyces*, *Amycolatopsis*, *Pseudonocardia*, *Micromonospora* and *Nocardia*. Properties of microorganisms, used as biofertilizers, include nitrogen fixation, phosphate solubilization, sulfur oxidization, plant hormones production, plant pathogens inhibition and organic compounds decomposition [3,21]. Furthermore, microorganisms used as biocontrol agents have been involved in the production of antibiotic compounds, siderophores and antifungal enzymes, promoting systemic resistance and plant pathogens suppression [3,21]. Currently, the terms biofertilizers and biological control agents are most commonly used. They are classified in a group called plant-growth-promoting rhizobacteria (PGPR) [3]. In Thailand, a few reports were found to isolate bacteria from termite nests and guts. There will be different types and characteristics of bacteria.

To solve the problem of using pesticides and chemical fertilizers in production of agricultural products. Therefore, we aimed to isolate and select bacteria from termite nests and guts that have the ability as plant growth-promoting bacteria (PGPB). It includes development of inoculum and potential biofertilizer formula by bacteria selected from termite nests and guts to promote plant growth and control rot disease in kale leading to sustainable agriculture.

Materials and methods

Collecting samples from termite nests and guts.

A total of 5 points of termite nest and gut were collected from Don Sai Sub-district, Photharam District, Ratchaburi Province, Thailand as shown in **Table 1** and **Figure 1**.

Table 1 Sampling points for isolation of termite nests and guts.

Points	GPS No	Location
1	592903 / 1505784	Don Sai Subdistrict, Photharam District, Ratchaburi Province, Thailand
2	593060 / 1505671	
3	592907 / 1505796	
4	592908 / 1505820	
5	592912 / 1505815	

Figure 1 Map of 5 points of termite nests and guts.

Preparation of termite mound nests and guts

The bacteria were isolated from termite nests and guts using the modified of [22,23,19].

Termite nests solution

One hundred grams of each termite nests were soaked in 70 % ethanol for 30 s and rinsed thoroughly with sterile distilled water. Termite nests samples were diluted in 900 mL of 0.85 % NaCl and isolated bacteria in Medium-2 medium added Carboxymethylcellulose (CMC), Pikovskaya's Agar medium (PVK), Chrome Zurol S (CAS) (Fluka chemicals) Agar medium and Nutrient Agar (NA) medium.

Termite guts solution

A total of 100 worker termites from each termite nests were soaked in 100 mL of 70 % ethanol for 30 s and washed thoroughly with sterile distilled water. Worker termites had to be cut off the head with a syringe and allowed it to air dry for 1 min. Then the termite guts were pulled out and macerated in 900 mL of 0.85 % NaCl. The bacteria were isolated in Medium-2 medium added CMC, PVK agar medium, CAS agar medium and NA medium.

Isolation of bacteria from termite nests and guts

Sample solutions of termite nests and guts were cultured in a specific medium to select for PGPB as follows:

Cellulose-degrading bacteria

Cellulase-producing bacteria were isolated according to the method of [22,24], the optimal dilution of termite mound and gut samples were inoculated in flask with 50 mL Medium-2 broth (K_2HPO_4 0.2 g/L, KH_2PO_4 0.2 g/L, $MgSO_4$ 0.2 g/L, NaCl 0.2 g/L, $NaNO_3$ 1 g/L, $CaCO_3$ 0.01 g/L, Yeast extract 0.5 g/L containing 0.5 % CMC as carbon source, with adjusted pH of 7.0. The culture flasks were incubated in a shaker at 150 rpm at 37 °C for 14 days. Cellulose-degrading bacteria were selected by taking 1 mL of culture solution and spreading it in CMC agar plates medium containing Medium-2 and 1.5 % agar, medium pH was adjusted to 7.0 and incubated at 37 °C for 4 days. The single colony was isolated by cross streaking on a new freshly prepared CMC agar plates. The isolates were stored at −80 °C in sterile 20 % glycerol before the analyses.

Cellulose degrading bacteria activity (Cellulolytic activity) was tested by modifying the method of [23,25]. The isolated bacteria were cultured in tubes containing 3 mL of Nutrient Broth (NB) medium and incubated at 35 ± 2 °C for 3 days. The culture bacterium was adjusted the turbidity to an OD_{600} nm of 0.1. The 3 µL of optimal culture was dropped onto CMC agar plates medium and incubated at 35 ± 2 °C for 3 days. The observation for clearing of the zone of CMC hydrolysis was carried out by dropping 0.1 % Congo red to flood on the plate for 15 min and washed with 1M NaCl solution for 15 min. The clear zone was measured and was then used to calculate the Cellulolytic Index (CI) value as follows:

$$\text{CI} = \frac{\text{Zone diameter} - \text{Colony diameter}}{\text{Colony diameter}}$$

Phosphate-solubilizing bacteria

The isolates of phospho-solubilizing bacteria [26] were performed on PVK agar medium. Each 0.1 mL of the optimal dilution of termite nest and gut solution was added to the PVK agar medium by spread plate technique and incubated at 35 ± 2 °C for 5 days. The single colony that showed a clear zone around them on PVK agar medium was selected. The selected colony was then kept at −80 °C as stock in sterile 20 % glycerol before the analyses.

Phosphate solubilizing activity was tested by Nacoon *et al.* [27]. The bacteria were cultured in tubes containing 3 mL of NB medium and incubated at 35 ± 2 °C for 3 days. After the OD_{600} of the culture was adjusted to 0.1, the 3 µL of bacterial culture were spotted onto PVK agar medium and performed in triplicate. The plates were incubated at 35 ± 2 °C for 3 days. The clear area around the bacterial culture droplets was measured. Phosphate solubilizing index (PSI) was calculated using equation [28] as follows:

$$\text{PSI} = \frac{\text{Colony diameter} + \text{Halo zone diameter}}{\text{Colony diameter}}$$

Siderophore-producing bacteria

The siderophore production was assayed on CAS agar medium and hexadecyltrimethylammonium bromide (HDTMA) (Fluka chemicals) as indicators. Preparing CAS agar was prepared according to the method of [29-31]. The bacterial isolation for siderophore production involves some steps, the first step, is the CAS assay solution (blue dye) was prepared as follows [29]: Solution 1 contains 50 mL of 0.12 % of CAS, solution 2 contains 10 mL of 0.01M $FeCl_3.6H_2O$ in 10 mL of 10 mM HCl and solution 3 contains 40 mL of 0.18 % HDTMA. The mix solution was prepared by mixing 50 mL of solution 1 with 9 mL of solution 2 and slowly adding 40 mL of solution 3 while stirring. The CAS assay solution was sterilized and cooled to 50 °C. This mix solution was freshly prepared. The final step, the CAS agar plate was prepared by dissolving 28 g of NA medium (Himedia) in 900 mL distilled water, then cooled to 50 °C. One hundred mL of the CAS assay solution was added and poured in already prepared plates. The optimal culture dilution was inoculated on a CAS agar plate by spread plate technique and incubated at 35 ± 2 °C for 3 days. The selected colony showed the color halo around the colony from greenish blue to yellow-orange.

The siderophore production assay was tested by a modified method of [30]. Each of the selected isolate was cultured in NB medium for 3 days at 200 rpm and 35 ± 2 °C. The culture was adjusted to 0.1 at optical density at 600 nm. Three µL of bacterial medium was added to CAS agar plates in 3 replicates per plate and incubated at 35 ± 2 °C for 3 days. The diameter of clear yellow orange around the bacterial colony and bacterial colony was measured. The siderophore production index (SPI) was calculated by equation as follows:

$$\text{SPI} = \frac{\text{Colony diameter} + \text{Halo zone diameter}}{\text{Colony diameter}}$$

Evaluation of the ability of antagonistic bacteria to inhibit the growth of the pathogen P. parasitica

Efficiency test on growth inhibition of *P. parasitica* fungi causing Chinese-kale damping-off from Plant Protection Research and Development Office, Department of Agricultural, Thailand. The dual culture test was conducted according to the method of [32]. *P. parasitica* was cultured on Potato Dextrose Agar (PDA) medium for 3 days and antagonistic bacteria isolated from termite nests and guts were cultured in NA medium for 3 days. The pathogen-test grown assay on a PDA plate was performed using sterilized a cork borer with a diameter of 0.6 cm and placed in the middle of a petri dish containing Plate Count Agar (PCA) medium. One loop of antagonist-isolated bacteria cultured was then spotted on the plates at a distance of 3 cm from *P. parasitica* in 4 spots (**Figure 2(B)**). Non-cultured antagonist bacteria plate was used as a control (**Figure 2(A)**). After 3 days at room temperature, *P. parasitica* fungal radius without any bacteria antagonist was measured in the test and control plates.

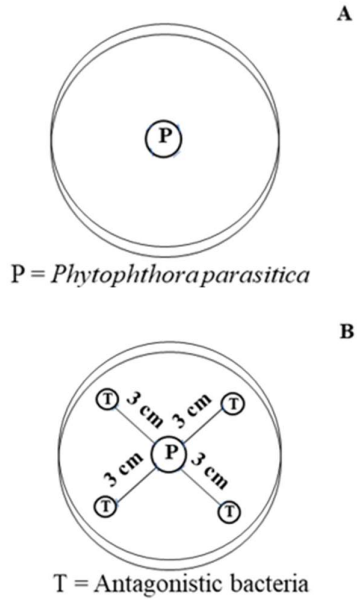

Figure 2 Test of the ability of antagonistic bacteria to inhibit the growth of pathogen *P. parasitica* (A) Control plate (B) Test plate.

The measured values were used to calculate the percentage inhibition of radial growth (PIRG) of bacteria against *P. parasitica* using the following formula [33]:

$$PIRG = ((RC - RT)/RC) \times 100$$

RC was a mean of the mycelial radius of *P. parasitica* mycelium without antagonistic bacteria.
RT was a mean of the mycelial radius of *P. parasitica* mycelium with antagonistic bacteria.

Bacterial identification

Genomic DNA (gDNA) of the selected bacterium was delivered to Humanizing Genomics, Macrogen Inc., Seoul, Republic of Korea, for 16S rRNA gene sequencing. The gDNA was extracted and purified using an A.B.T.^{TM} genomic DNA mini extraction kit, CAT Number I01-01-10 (Applied Biotechnology, Egypt) following the manufacturer's instructions. The presence of the 16S rRNA region in each bacterial isolate was confirmed by enriching PCR products with universal primers: 27F primer (5'-AGAGTTTGATCMTGG-3') and 1492R primer (5'-TACGGYTACCTTGTTACGACTT-3'), covering nearly full-length of 16S rRNA gene (~ 1400 bp). Sequencing primers, 785F primer (5'-GGATTAGATACCCTGGTA- 3') and 907R primer (5'CCGTCAATTCMT TTRAGT TT 3') were used to sequence a DNA fragment and reveal its DNA sequence identity. The sequences of each bacterial isolate were subjected to BLAST (Basic Local Alignment Search Tool) analysis at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and deposited in GenBank (https://submit.ncbi.nlm.nih.gov/subs/ genbank/). A phylogenetic tree depicting interrelated sequences was conducted through MEGA11 [34]. The evolutionary history was inferred using the Neighbor-Joining method [35] and the evolutionary distances were computed using the Kimura 2-parameter method [36].

Statistics analysis

The data were analyzed with one-way analysis of variance (ANOVA) using Statistical Package for Social Sciences (SPSS) Statistics for Windows Version 29.0.2.0 (Armonk, NY: IBM Corp, USA). Duncan's multiple range test method was used to compare of different treatments at a confidence level of 95 %.

Results and discussion

Isolation and characteristics of bacteria from termite nests and guts

Cellulose-degrading bacteria

The bacteria were isolated from termite nests and guts from Don Sai Sub-district, Photharam District, Ratchaburi Province in the amount of 5 points as shown in **Figure 3**. The selection of bacteria from 5 points of termite nests from Don Sai Sub-district, Photharam District, Ratchaburi Province as shown in **Table 1** and **Figure 1**. The characteristics of the termite nests from 5 points showed that the dark color was points 3, 4 and 5. The presence of dark color in termite nest has a large amount of organic substances, which is consistence with previous research in that termite nest is considered as a "gold mine" of bacterial communities due to termite mounds were contained clay particles and organic carbon, which were bound by termite secretions, feces, excretions or saliva [21,20,37]. The organic substances found in termite mound nest were created from biochemical processes in the termites of digestive tracts [38]. Due to the termite nest is rich in organic substances such as primary macronutrients such as carbon (C), phosphorous (P) and potassium (K), including secondary macronutrients such as calcium (Ca), zinc (Zn), iron (Fe), magnesium (Mg) and copper (Cu) [21,39]. It was found that only the fourth sample was darker than the other termite nest samples. The dominant termite species distribution in Kanchanaburi province of Thailand were *Odontotermes feae*, *Globitermes sulphureus* and *O. proformosanus* [40]. Therefore, termite species collected from Ratchaburi province are the same as those found in Kanchanaburi province because of the 2 adjacent provinces. Cellulose-degrading bacteria were isolated on Medium-2 mixed with CMC. The results found that the best bacteria capable of degrading cellulose were found in sample 4 of termite nest. BGNACMC 4-3, BGNACMC 4-4 and BGNACMC 4-5 isolates gave cellulolytic index (CI) of 5.0, 4.0 and 3.0 mm, respectively (**Table 2** and **Figure 4**). The CI of the bacterial isolates from termite gut showed that isolates BTNACMC 5-3, BTNACMC 4-2 and BTNACMC 3-5 gave the highest CI of 2.38, 2.16 and 2.00, respectively. Cellulolytic activity test of bacteria isolated from termite nests gave higher CI value (5.0) than the research carried out by Ferbiyanto *et al.* [23] and Oktiarni *et al.* [18], yielding in the range of 2.5 - 4.0 CI values from *Paracoccus yeei* RA2, *Bacillus* spp., *Brevibacterium* spp., *Staphylococcus* spp. and *Microbacterium* spp. isolated from termite gut and similar to the study by Gupta *et al.* [22] also gave IC values in the range of 4.29 - 5.49 of bacterial isolates from termite. Furthermore, the results of this study found that the bacteria isolated from termite nests could be better able to digest cellulose than from termite guts. This is because termite mound nests have high organic matter because they consist of various components such as clay particles, organic carbon and the saliva or secretions of the termites themselves. Additionally, the pH of termite nests was slightly acidic to neutral [20], therefore it is suitable to become bacteria habitat [41].

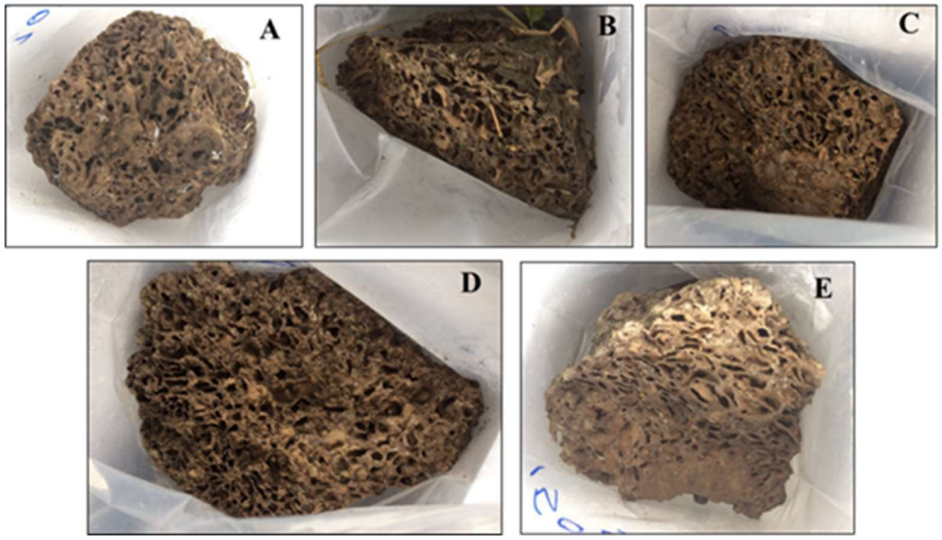

Figure 3 Characteristics of termite nests from 5 points from Don Sai Subdistrict, Photharam District, Ratchaburi Province from Point 1 GPS Number 592903/1505784 (A) Point 2 GPS Number 593060/1505671 (B) Point 3 GPS Number 592907/1505796 (C) Point 4 Number GPS592908/1505820 (D) and Point 5 GPS Number 592912/1505815 (E).

Table 2 Cellulolytic index (CI) of cellulose-degrading bacteria isolated from termite nest and guts.

Points	Termite nests				Termite guts			
	Isolates	**Diameter of colony (mm)**	**Diameter of a cellulolytic zone (mm)**	**CI**	**Isolates**	**Diameter of colony (mm)**	**Diameter of a cellulolytic zone (mm)**	**CI**
1	BGNACMC 1-1	6.25 ± 0.35	9.00 ± 0.00	0.44 ± 0.08^{op}	BTNACMC 1-1	6.00 ± 0.00	9.00 ± 0.00	0.50 ± 0.00^{no}
	BGNACMC 1-2	9.00 ± 0.00	9.00 ± 0.00	0.00 ± 0.00^{w}	BTNACMC 1-2	4.50 ± 0.71	4.50 ± 0.00	0.00 ± 0.00^{w}
	BGNACMC 1-3	20.50 ± 0.71	22 ± 0.00	0.07 ± 0.04^{uvw}	BTNACMC 1-3	16.00 ± 0.00	22.25 ± 0.35	0.39 ± 0.02^{opq}
	BGNACMC 1-4	6.50 ± 0.71	6.50 ± 0.71	0.00 ± 0.00^{w}				
	BGNACMC 1-5	13.50 ± 0.71	16.5 ± 0.71	0.21 ± 0.01^{rstu}				
2	BGNACMC 2-1	4.00 ± 0.00	5.00 ± 0.00	0.25 ± 0.00^{qrst}	BTNACMC 2-1	5.00 ± 0.00	8.75 ± 0.35	0.75 ± 0.07^{ijklm}
	BGNACMC 2-2	3.00 ± 0.00	8.75 ± 0.35	1.92 ± 0.12^{f}	BTNACMC 2-2	12.75 ± 0.35	22.00 ± 0.00	0.73 ± 0.05^{jklm}
	BGNACMC 2-3	3.00 ± 0.00	4.00 ± 0.00	0.33 ± 0.00^{pqrs}	BTNACMC 2-3	11.00 ± 0.00	13.00 ± 0.00	0.18 ± 0.00^{stuv}
					BTNACMC 2-4	7.25 ± 0.35	17.00 ± 0.00	1.35 ± 0.11^{g}
					BTNACMC 2-5	4.00 ± 0.00	11.25 ± 0.35	1.81 ± 0.00
					BTNACMC 2-6	12.00 ± 0.00	20.50 ± 0.71	0.71 ± 0.06^{jklm}
3	BGNACMC 3-1	5.00 ± 0.00	6.00 ± 0.00	0.20 ± 0.00^{stuv}	BTNACMC 3-2	20.05 ± 0.71	25.50 ± 0.71	0.24 ± 0.01^{qrst}
	BGNACMC 3-2	14.75 ± 0.35	15.00 ± 0.00	0.02 ± 0.02^{w}	BTNACMC 3-3	4.00 ± 0.00	7.00 ± 0.00	0.75 ± 0.00^{ijklm}
	BGNACMC 3-3	7.00 ± 0.00	7.75 ± 0.35	0.11 ± 0.05^{tuvw}	BTNACMC 3-4	5.00 ± 0.00	11.25 ± 0.35	1.25 ± 0.07^{g}
	BGNACMC 3-4	12.50 ± 0.71	14.00 ± 0.00	0.12 ± 0.06^{tuvw}	BTNACMC 3-5	5.00 ± 0.00	15.50 ± 0.71	2.15 ± 0.14^{e}
	BGNACMC 3-7	6.00 ± 0.00	6.00 ± 0.00	0.00 ± 0.00^{w}	BTNACMC 3-6	20.00 ± 0.00	34.75 ± 0.35	0.74 ± 0.02^{jklm}
	BGNACMC 3-8	6.00 ± 0.00	6.00 ± 0.00	0.00 ± 0.00^{w}				
4	BGNACMC 4-1	30.50 ± 0.71	57.50 ± 0.71	0.89 ± 0.02^{ji}	BTNACMC 4-1	5.00 ± 0.00	5.50 ± 0.71	0.10 ± 0.14^{vw}
	BGNACMC 4-2	4.00 ± 0.00	5.50 ± 0.71	0.38 ± 0.18^{opqr}	BTNACMC 4-2	6.00 ± 0.00	12.75 ± 0.35	1.13 ± 0.06^{h}
	BGNACMC 4-3	**3.00 ± 0.00**	**18.50 ± 0.71**	**5.17 ± 0.24^{a}**	BTNACMC 4-3	10.00 ± 0.00	18.00 ± 0.00	0.80 ± 0.00^{ijk}
	BGNACMC 4-4	3.00 ± 0.00	15.50 ± 0.71	4.17 ± 0.24^{b}	BTNACMC 4-4	19.00 ± 0.00	20.50 ± 0.71	0.08 ± 0.04^{tuvw}
	BGNACMC 4-5	7.00 ± 0.00	27.75 ± 0.35	2.96 ± 0.05^{c}	BTNACMC 4-8	11.00 ± 0.00	18.75 ± 0.00	0.70 ± 0.03^{lm}
	BGNACMC 4-7	15.00 ± 0.00	26.5 ± 0.71	0.77 ± 0.05^{ijk}				
5	BGNACMC 5-4	6.00 ± 0.00	9.00 ± 0.00	0.50 ± 0.00^{no}	BTNACMC 5-1	6.75 ± 0.35	7.00 ± 0.00	0.04 ± 0.05^{vw}
					BTNACMC 5-2	24.75 ± 0.35	39.50 ± 0.71	0.60 ± 0.01^{mn}
					BTNACMC 5-3	18.25 ± 0.35	61.50 ± 0.71	2.37 ± 0.03^{d}
					BTNACMC 5-4	15.00 ± 0.00	28.50 ± 0.71	0.90 ± 0.05^{i}

Superscript letters within a column indicate significant ($p < 0.05$) differences of means within CI.

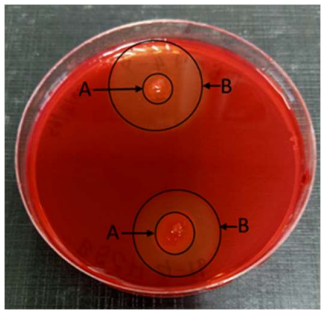

Figure 4 Cellulolytic activity of bacteria on Medium-2 added with CMC, (A) Diameter of cellulose-degrading clear zone and (B) Bacterial colony.

Siderophore-producing bacteria

Isolates of siderophore-producing bacteria in CAS agar were isolated from 5 points of termite nest and gut. The bacterial isolates indicating the siderophore production showed an orange zone (**Figure 5**). The BTNASP 5-2 isolated from the termite gut of point 5 gave the highest SPI was 4.16 ± 0.21 mm, followed by BTNASP 5-1 isolated from point 2 was 2.27 ± 0.09 mm (**Table 3**). The bacterial isolates from termite guts showed the higher siderophore production than those from termite nests. Isolate BTNASP 5-2 (SPI = 4.16 ± 0.21) isolated from termite gut has similarity with *B. subtilis* (**Figure 6**). The *B. subtilis* BTNASP 5-2 was similar to the previous finding of siderophores-producing bacteria found in mainly genus *Bacillus* but different source because both *B. cereus* TSH77 and *B. endophyticus* TSH42 are isolated from termitarium soil (termite nest) [8]. Microbes from termite nests can be used in bioremediation of metal-contaminated sites due to the presence of genes resistance to mercury, arsenic, chromium, vancomycin, cadmium, cobalt-zinc-cadmium and zinc [39]. Microbial siderophores possess low molecular weight (200 - 2,000 Da) metal chelating agents that binds with ferric irons, specifically, to the outside of the cell. Consequently, Fe- siderophore complex is then transported via a very specific receptor proteins into the outer cellular membrane [42,43], where Fe helps in the growth of microorganisms and is important in the catalyst enzymatic process, oxygen metabolism, electron transfer and DNA and RNA synthesis [44]. Moreover, the siderophore plays a role in the removal of Fe and can also remove other essential elements by forming complexes with other essential elements such as Mo, Mn, Co and Ni in the environment [39]. Rhizosphere microorganisms secrete siderophores to stimulate plant growth by providing iron and inhibit the proliferation of fungal pathogens. However, microbial siderophores inhibit iron uptake if plant roots are unable to use iron that binds to siderophores [42,45]. Moreover, siderophore can be used in plant growth promotions, biocontrol activity, bioremediation, biosensor and medicine [46].

Table 3 Siderophore production (SPI) in CAS medium of bacteria isolated from termite nests and guts.

Points	Termite nests				Termite guts			
	Isolates	**Average diameter of the colony (mm)**	**Average diameter of phosphate solubilization zone (mm)**	**SPI**	**Isolates**	**Average diameter of the colony (mm)**	**Average diameter of phosphate solubilization zone (mm)**	**SPI**
1	-	-	-	-	-	-	-	-
2	-	-	-	-	BTNASP 2-1	7.67 ± 0.00	11.33 ± 0.58	1.49 ± 0.19^{e}
					BTNASP 2-2	7.50 ± 0.35	21.67 ± 1.53	2.89 ± 0.24^{b}
3	-	-	-	-	-	-	-	-
4	BGNASP 4-3	9.33 ± 0.71	12.00 ± 1.00	1.29 ± 0.06^{e}	-	-	-	-
	BGNASP 4-7	7.67 ± 0.00	14.33 ± 0.58	1.88 ± 0.23^{d}				
5	-	-	-	-	BTNASP 5-1	8.67 ± 0.71	19.67 ± 0.58	2.27 ± 0.09^{c}
					BTNASP 5-2	**10.33 ± 0.00**	**43.00 ± 2.65**	**4.16 ± 0.21^{a}**

Superscript letters within a column indicate significant ($p < 0.05$) differences of means within SPI.

Phosphate solubilizing bacteria

A total of 4 isolates of phosphate-solubilizing bacteria were isolated from PVK agar. It was found that BTPK 5-3 isolated from the termite gut of point 5 gave the highest PSI (2.10 ± 0.14 mm), followed by a bacterial isolate from the termite nest of point 2 was BGPK 2-2 (2.00 ± 0.00 mm) as shown in **Table 4**. Phosphate-solubilizing bacteria have the ability to degrade mineral phosphate solubilization by both inorganic (such as sulphuric, nitric and carbonic acids) and organic acids (such as 2-Keto gluconic acid and gluconic acid), resulting in an increase soil fertilizer, plant P uptake and crop yield [47]. The 2-Keto gluconic acid and gluconic acid are major organic acids from mineral phosphate solubilization. It chelates cations to bind with phosphate, making phosphate available to plants [48].

Table 4 Phosphate solubilization index (PSI) of bacteria in isolated PKV agar medium from termite nests and guts.

Points	Termite nests				Termite guts			
	Isolates	**Diameter of colony (mm)**	**Diameter of phosphate solubilization zone (mm)**	**PSI**	**Isolates**	**Diameter of colony (mm)**	**Diameter of phosphate solubilization zone (mm)**	**PSI**
1	BGPK 1-2	3.25 ± 0.35	4.50 ± 0.71	1.38 ± 0.07^{bc}	BTPK 1-1	11.00 ± 1.41	16.25 ± 0.35	1.49 ± 0.22^{ab}
					BTPK 1-2	7.60 ± 0.71	12.50 ± 0.71	1.67 ± 0.06^{ab}
2	BGPK 2-2	3.75 ± 0.35	7.50 ± 0.00	2.00 ± 0.00^{a}	BTPK 2-1	6.50 ± 0.71	10.50 ± 0.71	1.62 ± 0.07^{ab}
3	-	-	-	-	-	-	-	-
4	BGPK 4-1	15.5 ± 0.71	18.75 ± 0.35	1.21 ± 0.03^{c}	-	-	-	-
	BGPK 4-3	7.50 ± 071	10.50 ± 0.71	1.40 ± 0.04^{ab}				
5	-	-	-	-	BTPK 5-1	15.00 ± 0.00	19.50 ± 0.71	1.30 ± 0.05^{bc}
					BTPK 5-2	5.50 ± 0.71	8.00 ± 0.00	1.47 ± 0.19^{ab}
					BTPK 5-3	**5.50 ± 0.71**	**11.50 ± 0.71**	**2.10 ± 0.14^{a}**

Superscript letters within a column indicate significant ($p < 0.05$) differences of means within PSI.

Antagonistic bacteria to inhibit the growth of P. parasitica

The efficacy of antagonistic bacteria against the growth of soil rot pathogens by the dual technique was determined according to Intana *et al.* [32]. The percentage of growth inhibition of soil rot pathogens was analyzed from the efficacy test of antagonistic bacteria to inhibit the growth of soil rot pathogens. The BTNA 5-1, BTNA 3-1 and BTNA 5-3 isolated from termite guts gave the highest percentage inhibition of radial growth (PIRG) of 67.07 ± 4.02, 66.67 ± 1.15 and 65.89 ± 0.57 %, respectively (**Table 5**). The isolate BTNA 5-3 was identified as *B. cereus* (NR_074540) (**Table 6**). It was found that *B. cereus* BTNA 5-3 isolated from termite guts could inhibit root rot fungi *P. parasitica*. This result was consistent with Devi *et al.* [13] found that in the genus *Bacillus* (*B. methylotrophicus* BTS16*)* and genus *Staphylococcus* (*S. saprophyticus* BTS14) isolated from termite nests were active against pathogenic fungi including *Fusarium oxysporum*, *Alternaria brassicae*, *Rhizoctonia solani*, *Sclerotium rolfsii* and *Colletotrichum truncatum*. Our results indicated that bacteria from termite guts are effective in inhibiting the growth of soil rot pathogenic fungi. Previous research demonstrated that the antifungal compounds isolated from termite guts were found in genus *Bacillus* produced Bacillaene (a polyene polyketide) inhibited the fungal mycelial growth of *Pseudoxylaria* spp*.*, *Trichoderma* spp. and *Fusarium* spp. [49] and in genus *Streptomyces* produced Natalamycin inhibited mold and yeast such as *Aspergillus* spp., *Cephalosporium* spp., *Fusarium* spp. and *Penicillium* spp. [50,51]. These bacteria make the enzymes chitinase, β1,3-glucanase, proteases, lipases and cellulase, whose enzymes can decompose the constituents in the fungal hyphae, thus inhibiting the growth of fungi [49]. Furthermore, *B. endophyticus* TSH42 and *B. cereus* TSH77 isolated from termite nests help to inhibit the growth of *F. solani* fungi, causing rot in crops such as potatoes, in which both *B. cereus* TSH77 and *B. endophyticus* TSH42 produces lipopeptide antibiotics such as fengycin and surfactin expect iturin produces only by *B. endophyticus* TSH42 [52].

Table 5 Percent inhibition of radial growth (PIRG) of *P. parasitica* causing soil rot disease of antagonistic bacteria isolated from termite nests and guts.

Points	Termite nests		Termite guts	
	Isolates	**PIRG (%)**	**Isolates**	**PIRG (%)**
1	BGNA1-4	4.88 ± 1.15^{efg}	-	-
	BGNA1-5	26.02 ± 5.75^{d}		
2	BGNA 2-1	0.00 ± 0.00^{g}	-	-
	BGNA 2-2	0.00 ± 0.00^{g}		
3	BGNA 3-3	4.07 ± 1.15^{efg}	BTNA 3-1	66.67 ± 1.15^{a}
	BGNA 3-4	13.01 ± 6.90^{ef}	BTNA 3-2	14.63 ± 1.15^{e}
	BGNA 3-5	3.66 ± 1.72^{efg}		
	BGNA 3-6	0.00 ± 0.00^{g}		
	BGNA 3-7	29.27 ± 6.90^{cd}		
	BGNA 3-8	1.22 ± 4.02^{g}		
4	BGNA 4-1	6.10 ± 2.87^{efg}	-	-
	BGNA 4-2	6.50 ± 5.75^{efg}		
	BGNA 4-3	0.00 ± 0.00^{g}		
	BGNA 4-4	2.85 ± 0.57^{fg}		
	BGNA 4-5	0.81 ± 2.30^{fg}		
	BGNA 4-6	35.77 ± 4.60^{cd}		
	BGNA 4-7	0.00 ± 0.00^{g}		
5	BGNA 5-1	0.00 ± 0.00^{g}	**BTNA 5-1**	**67.07 ± 4.02^{a}**
	BGNA 5-3	34.15 ± 1.15^{cd}	BTNA 5-2	56.50 ± 18.97^{b}
	BGNA 5-4	39.43 ± 1.72^{c}	BTNA 5-3	65.85 ± 2.30^{a}

Superscript letters within a column indicate significant ($p < 0.05$) differences of means within PIRG.

Bacterial identification

The bacterial isolates were identified by analyzing the 16s rRNA gene regions from the 16s rRNA sequencing analysis and comparing them with biological databases and evolutionary tree. In the classifying bacteria, it was found that isolate BGNACMC 4-3 produced cellulase, isolate BTNA 5-1 inhibited *P. parasitica* (**Figure 5(B)**) and isolate BTPK 5-3 solubilized phosphate, closely related to bacteria *B. cereus* ATCC 14579^{T} (AE016877), Isolate BTNASP 5-2, which produced siderophore (**Figure 5(A)**), was closely related to bacteria *B. subtilis* NCIB 3610^{T} (ABQL01000001), All isolates exhibited similarity (%) over 99 %, as shown in **Table 6**. The accession numbers of *B. cereus* BGNACMC 4-3, *B. cereus* BTNA 5-1, *B. subtilis* BTNASP 5-2 and *B. cereus* BTPK 5-3 deposited in GenBank were PP204049, PP204047, PP204050 and PP204048, respectively. *B. cereus* and *B. subtilis* are facultatively anaerobic that found in termite guts. The characteristics of bacteria that reside in termite guts are facultative anaerobe or microaerophile properties [53]. Furthermore, the termite nests are also suitable for the growth of the genus *Bacillus* due to the pH of termite nests of have approximately neutral (pH range of 6.88 - 7.38). Previous research also demonstrated that the pH values of from *Globitermes sulphureus*, *Microcerotermes crassus* and *M. distans* mound nests in Thailand were 6.99, 6.88 and 7.11, respectively [54]. Additionally, the pH 7.38 of *Coptotermes formosanus* mound nest was also found similarity in India [55].

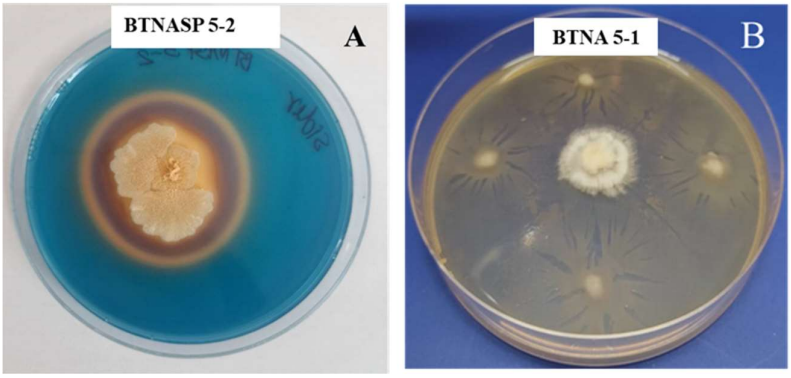

Figure 5 The orange zone of siderophore-producing bacterium in CAS agar plate assay (A) and antagonistic bacteria to inhibit the radial growth *P. parasitica* (B).

Table 6 16S rRNA sequences were compared with biological databases for the classification of bacteria.

Characteristics of plant growth promoting	Sources	Isolates	Closed homology	Similarity (%)	GenBank accession numbers
Cellulose degradation	Termite mound nest	BGNACMC 4-3	*Bacillus cereus* ATCC 14579^T (AE016877)	99.86	PP204049
P. parasitica inhibition	Termite gut	BTNA 5-1	*Bacillus cereus* ATCC 14579T (AE016877)	99.86	PP204047
Siderophore production	Termite gut	BTNASP 5-2	*Bacillus subtilis* NCIB 3610^T (ABQL01000001)	99.80	PP204050
Phosphate solubilization	Termite gut	BTPK 5-3	*Bacillus cereus* ATCC 14579^T (AE016877)	99.53	PP204048

In this study, cellulose degrading bacteria from termite mound nest, namely *B. cereus* BGNACMC 4-3 is consistent with the study of Ali *et al.* [19], found from termite gut. In addition, the genus *Bacillus* was also found in both termite nests and termite guts. Furthermore, cellulose-degrading bacteria are found in the other genera from termite nests, namely *Cellulomonas*, *Glycosyl* and *Cellulomonas* [56,57], including in phyla like *Acidobacteria*, *Firmicutes*, *Actinobacteria* and *Proteobacteria* [58]. In termite gut, cellulolytic bacteria *B. megaterium* RU4, *B. cereus*, *Paracoccus yeei* RA2, *Paenibacillus lactis*, *Lysinibacillus macrolides*, *L. fusiformis*, *Stenotrophomonas maltophilia* were found [23,19]. The study of bacteria from termite guts using a gene-specific bacterial primer by Mathew *et al.* [17] found *Lactobacillus*, *Peptococcus*, *Bacteriodetes*, *Clostridium*, *Peptostreptococcus*, *Bifidobacterium*, *Ruminococcus*, *Fusobacterium*, *Eubacterium* and *Termitomyces* species. In this study siderophore producing bacteria was *B. subtilis* BTNASP 5-2 found in the genus *Bacillus*, which are also found in other bacteria from termite nests such as *Fluorescent pseudomonads* aid in the phyto-extraction [59] and *Pseudomonas monteillii* aid in the Cd uptake [60]. Similarity, the results of phosphate-degrading bacteria found in the genus *Bacillus* (*B. cereus* BTNA 5-1) as these results reported by Chauhan *et al.* [8] and Chakdar *et al.* [48] in which *Bacillus* genus are capable of degrading phosphate, namely *B. cereus* TSH77 and *B. endophyticus* TSH42 were isolated from termitarium soil. The previous study reports are found in *Kosakonia*, *Bacillus* and *Pantoea* isolated from termite nests. The phosphate degradation by bacteria can release 2-Keto gluconic acid and gluconic acid to dissolve phosphate [48].

Our result showed that *B. cereus* BTNA 5-1 isolated from termite nest has the ability to inhibit *P. parasitica*. The other bacteria from termite nests are shown antimicrobial activities, such as: *Streptomyces* sp. against *Metarhizium anisopliae* [61]; *B. endophyticus* TSH42 and *B. cereus* TSH77 against *Fusarium solani* [62]; *S. saprophyticus* and *B. methylotrophicus against F. oxysporum, Alternaria brassicae,*

Rhizoctonia solani, Sclerotium rolfsii and Colletotrichum truncatum [13]; *Streptomyces padanus* CMU-NKS-3 strongest antagonist *Aspergillus flavus* [20]. In addition, the antibiotic-producing bacteria from termite nests found *Proteobacteria* and *Actinobacteria* [39] and antimicrobial actinobacteria from the 3 types of termite nests (mound, carton and subterranean nests) in Thailand found the genera *Streptomyces*, *Amycolatopsis*, *Pseudonocardia*, *Micromonospora* and *Nocardia* [20]. Furthermore, the metagenomic study of free-living bacteria in termite guts reported by Do *et al.* [63] found that the dominant genes for ansamycin synthesis in *Treponema*, *Lactococcus*, *Pseudomonas*, *Dysgonomonas*, *Enterobacter* and *Clostridium*. Previous studies have reported that the genus *Bacillus* isolated from termite nests has activities to promote plant growth and nutrient uptake. In addition, other genera of bacteria that have growth-promoting activity include *Achromobacter*, *Agrobacterium*, *Azotobacter*, *Burkholderia*, *Flavobacterium, Micrococcus*, *Pseudomonas* and *Rhizobium* [48,64].

Figure 6 A neighbor-joining phylogenetic tree of bacteria isolated from termite nests and guts based on 16S rRNA sequences. Bar = 0.02 substitutions per nucleotide position.

Conclusions

The best bacterial isolates from termite mounds and guts for phosphate solubilization, siderophore production, cellulose degradation and inhibition of *P. parasitica* were selected. Three isolates, namely BGNACMC 4-3, BTPK 5-3 and BTNA 5-1 were extremely close to *B. cereus*. Only isolate BTNASP 5-2 was similar to *B. subtilis*. The 4 isolates exhibited a high level (> 99 %) of sequence similarity with the genus *Bacillus*. The *B. cereus* BTNA 5-1 from termite gut inhibited *P. pythium*, which causes soil rot pathogenic fungi. The results indicated that only highest cellulose-degrading bacteria (isolate BGNACMC 4-3) found in termite mound nest, while in termite guts found the best bacteria that could to degrade phosphate (isolate BTPK 5-3), inhibit *P. parasitica* (isolate BTNA 5-1) and produce siderophore (isolate BTNASP 5-2). The *B. cereus* was a wide range of biological activities, showing the highest cellulase, phosphate-solubilizing and antifungal activities, while *B. subtilis* produced only a siderophore.

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