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การควบคุม *Burkholderia pseudomallei* ในดินด้วยวิธีทางชีวภาพโดย *Bacillus amyloliquefaciens*

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Bio-Control of *Burkholderia pseudomallei* in Soil by *Bacillus amyloliquefaciens*

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หลักการและวัตถุประสงค์: *Burkholderia pseudomallei* เป็นแบคทีเรียแกรมลบที่ก่อให้เกิดโรคเมลิออยด์ พบได้ในดินและน้ำของบริเวณที่เป็นแหล่งระบาด ส่วน *Bacillus amyloliquefacients* N3-8 ที่แยกจากดินผลิตสารทุติยภูมิที่มีผลฆ่า *B. pseudomallei*ได้ในอาหารเลี้ยงเชื้อ จึงนำมาใช้ควบคุมปริมาณ *B. pseudomallei*ในดิน

วิธีการศึกษา: เลี้ยงเชื้อ *B. pseudomallei* p37 และ *B. amyloliquefaciens* N3-8 ให้ได้ $1x10^6$ และ $1x10^9$ CFU.mL^{-1} ในอาหารเหลว นำมาผสมในอัตราส่วนเซลล์*B. pseudomallei*: *B. amyloliquefaciens* เท่ากับ 1:50, 1:100 และ 1:300 ในดิน 10 กรัม pH ดินเริ่มต้นเท่ากับ 6.98 บ่มไว้ที่อุณหภูมิห้อง 4 สัปดาห์

ผลการศึกษา: พบว่าอัตราส่วน 1:300 ของ *B. amyloliquefaciens* N3-8 สามารถลดจำนวนของ *B. pseudomallei* p37 ในดินอย่างชัดเจน ในสัปดาห์ที่ 2 โดย pH ของดินที่มี *B. amyloliquefaciens* N3-8 เมื่อเปรียบเทียบระหว่างสัปดาห์ที่ 0 กับ 4 มีค่าเพิ่มขึ้นอย่างมีนัยสำคัญ ($p < 0.01$) ในสัปดาห์ที่ 4 ดินที่มี *B. pseudomallei* p37 ค่า pH = 7.35 ± 0.05 ดินที่มี *B. amyloliquefaciens* N3-8 มี pH = 8.26 ± 0.06 ดินที่มี *B. pseudomallei*p 37 ร่วมกับ *B. amyloliquefaciens* N3-8 อัตราส่วนเซลล์ 1:50, 1:100 และ 1:300 มีค่า pH =8.08 ± 0.05, 8.03 ± 0.05 และ 8.81 ± 0.05

Background and Objectives: *Burkholderia pseudomallei*, a Gram-negative bacterium causes a disease called Melioidosis. It is mostly found in soil and stagnant water in endemic areas. *B. amyloliquefaciens* N3-8 was isolated from soil and its secondary metabolites can kill *B. pseudomallei* that may be used to control *B. pseudomallei* in soil.

Methods: *B. pseudomallei* p37 and *B. amyloliquefaciens* N3-8 were separately cultured to obtain $1x\ 10^6$ and $1X10^9$ CFU.mL^{-1}. Then mixed *B. pseudomallei* p37: *B. amyloliquefaciens* N3-8 with 1:50, 1:100 and 1:300 ratios of cell into 10 g of soil and incubated at room temperature for 4 weeks. The soil has pH=6.98.

Results: The ratio of 1:300 can reduce the number of *B. pseudomallei* p37 in soil starting from the second week. The pH of soil in the presence of *B. amyloliquefaciens* N3-8 was significantly increased when compared at the beginning with the 4th week ($p< 0.01$). At the end, soil with *B. pseudomallei* p37 had pH = 7.35 ± 0.05, soil with *B. amyloliquefaciens* N3-8 had pH = 8.26 ± 0.06, soil with ratios of 1:50, 1:100 and 1:300 had pH = 8.08 ± 0.05, 8.03 ± 0.05 and 8.81 ± 0.05.

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สรุป: *B. amyloliquefaciens* N3-8 ในสัดส่วน 1:300 สามารถลดปริมาณ *B. pseudomallei* p37ในดินได้ชัดเจน การทดสอบเพิ่มในดินธรรมชาติ จะช่วยประเมินศักยภาพการนำไปประยุกต์ใช้ลดปริมาณ *B. pseudomallei*ในพื้นที่ระบาดได้
คำสำคัญ: การควบคุมทางชีวภาพ สารทุติยภูมิ *B. amyloliquefaciens, B. pseudomallei*, ดิน

Conclusions: *B. amyloliquefaciens* N3-8 at 1:300 ratio can clearly reduce the number of *B. pseudomallei* p37 in soil. Further investigation in natural soil environment could support the potential use of *B. amyloliquefaciens* N3-8 as a bio-control to reduce *B. pseudomallei* in endemic areas.
Keywords: Bio-control, secondary metabolites, *B. amyloliquefaciens, B. pseudomallei*, soil

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Introduction

B. pseudomallei can be isolated from soil and water in endemic areas such as Southeast Asia and northern Australia[1]. The bacterium causes the disease called melioidosis that occurred in both human and animal[2]. Routes of *B. pseudomallei* infection are direct contact of soil and water that contaminated with *B. pseudomallei* to open wound or by inhalation and ingestion. It is an important cause of septicemia and approximately 20% of community-acquired bacteremia reported in Ubon Ratchathani, northeastern Thailand[3]. Moreover, melioidosis is the third most common cause of death in the northeast, Thailand from infectious disease inferior to HIV and Tuberculosis[4]. Soil is the most important reservoir of the bacterium and also the source of infection.

Biological control is a bio-effector-method for controlling one organism by its antagonistic microorganism. The mechanisms can be competition, parasitism or antibiosis[5]. As it does not use chemicals, the method will not cause environmental pollution. *Bacillus* spp. is a group of Gram-positive spore-forming bacterium commonly found in nature. They were known to produce a variety of secondary metabolites that can compete against other organisms in the same environment[6, 7]. From previous study in our laboratory, *Bacillus amyloliquefaciens* isolates N3-8 were isolated from soil in Khon Kaen that found negative for *B. pseudomallei*. The culture supernatant of *B. amyloliquefaciens* N3-8 can inhibit several isolates of *B. pseudomallei* including drug resistant isolates and co-culture of *B. amyloliquefaciens* N3-8 with *B. pseudomallei* in liquid medium clearly showed dramatically decrease of *B. pseudomallei*[8]. In this study, we therefore investigated if *B. amyloliquefaciens* N3-8 can be used as a bio-control for controlling *B. pseudomallei* in soil in laboratory setting condition.

Materials and methods

Soil samples

A batch of 10 kg of soil was taken from a grass field belonging to the Faculty of Agriculture. The soil was grinded and filtered through 2 mm sieve to get rid of roots, pebbles and others. The water holding capacity (WHC) of the soil was culculated and adjusted by sterile distilled water to have 100% WHC[9]. Then, the whole lot of soil was autoclaved at 121 C^{o} for 30 min and check by culture on nutrient agar (NA) to confirm sterility.

Strains and growth conditions

B. amyloliquefaciens strains N3-8 and *B. pseudomallei* p37 were obtained from the melioidosis research center, Faculty of Medicine, Khon Kaen university, Thailand. *B. amyloliquefaciens* strains N3-8 was isolated from soil and can produce secondary metabolites that can kill *B. pseudomallei*. *B. pseudomallei* p37 was isolated from blood of a patient in Khon Kaen province. It was proved to contain no inducible phages. For propagation, *B. amyloliquefaciens* N3-8 and *B. pseudomallei* p37 from the stock were cultured separately on nutrient agar and Ashdown's agar plates, respectively and incubated at 37 °C to obtain pure single colony.

The propagation of *B. amyloliquefaciens* N3-8 was performed by inoculated a single colony into LB broth and incubated in shaking incubator of 200 rpm at 37 °C for 18 h. One percent inoculum of the culture was used to inoculate into LB broth and culture for 24 h to obtain $1x10^{9}$ $CFU.mL^{-1}$ and used in the bio-control experiment. For *B. pseudomallei* p37, the bacterium was grown in LB broth and culture for 4 h to obtain $1x\ 10^{6}$ $CFU.mL^{-1}$.

Agar well diffusion

To confirm the production of metabolites from *B. amyloliquefaciens* strains N3-8 that can inhibit *B. pseudomallei,* agar well diffusion method was used to determine the antimicrobial activity[10]. In brief, *B. pseudomallei* were grown in Luria Bertani (LB) broth for 16-18 h and 10% inoculum were used to inoculate into 3 ml LB and incubated for 4 h until log phase. A hundred microliters of the culture were spread on Mueller Hinton agar (MHA) plates, dried in a biohazard laminar flow cabinet (Esco Technologies, Horsham, PA) and then punched 5-6 wells/plate using a sterile micropipette tip. A hundred microliters of supernatant from *B. amyloliquefaciens* strains N3-8 were then added into each well and incubated at 37 °C for 24 hours. Ceftazidime, the drug of choice for *B. pseudomallei* was used as a positive control and the production medium was used as a negative control.

Bio-control in soil conditions

All experiments were operated in triplicate in sterile 50 ml self-standing conical plastic tubes. *B. amyloliquefaciens* N3-8 and *B. pseudomallei* p37 were mixed into 10 g, 100% WHC soil to obtain 3 conditions 1.) Control *B. amyloliquefaciens* N3-8,1x10^8 CFU.g^{-1}soil, 2.) Control *B.pseudomallei* p37, 1x10^5 CFU.g^{-1}soil and 3.) Co-culture between *B. pseudomallei* p37 and *B. amyloliquefaciens* N3-8 with the CFU ratios of 1:50, 1:100 and 1:300. Each condition had 4 tubes to be sampled each week. The tubes with loosely closed cap were incubated at room temperature for 4 weeks.

On the 2nd week, 5x10^7 CFU.g^{-1} (1:50 ratio), 1x10^8 CFU.g^{-1} (1:100) and 3x10^8 CFU.g^{-1} (1:300) of *B. amyloliquefaciens* N3-8 were added into the co-culture soil, mixed well and incubate further for another 2 weeks. To determine the number of *B. pseudomallei* p37 in the control and co-culture at 0, 1, 2, 3 and 4 weeks, 10 ml of sterile DW was added, shake vigorously and stand for 30 minutes to let soil particles settle. The supernatant was collected to measure pH and also 10-fold serially diluted and then drop 10 µl on Ashdown's agar for *B. pseudomallei* p37 colony count. For *B. amyloliquefaciens* N3-8 colony count, the process was similar and performed only in *B. amyloliquefaciens* N3-8 control and Phenylethyl alcohol agar (PAA) was used for *B. amyloliquefaciens* N3-8 colony count (Figure 1).

Statistical analysis

Mann-Whitney U Test was used to compare mean-values of pH of soil in bio-control experiment and the statistic considered to be significance when $p<0.01$.

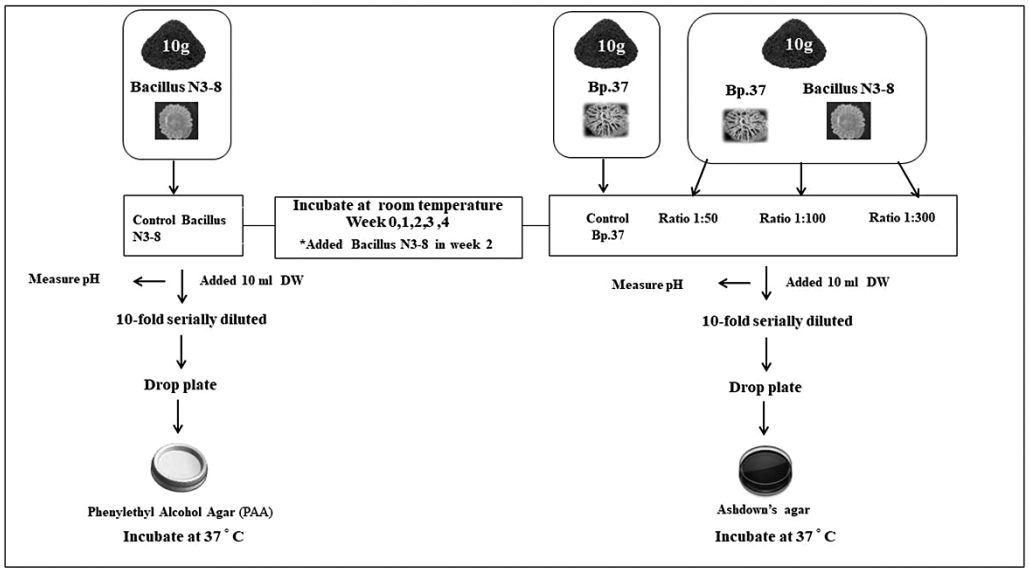

Figure 1 The schematic diagram of steps in the bio-control experiment.

Results

B. amyloliquefaciens N3-8 and its antimicrobial activity

The morphologies of *B. amyloliquefaciens* N3-8 are being white and large with wavy, lobed margins were observed on nutrient agar plates (Figure 2A) that confirmed by Gram's stain to be Gram-positive rods (Figure 2B) and confirmed for the production of antimicrobial activity as seen by clear zone on *B. pseudomallei* lawn (Figure 2C).

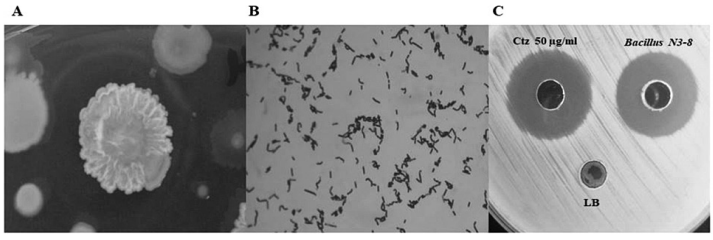

Figure 2 Morphology and antimicrobial activity of *B. amyloliquefaciens* N3-8. A) colonies of *B. amyloliquefaciens* N3-8 on nutrient agar plate, B) Gram's stain and C) clear zone from action of antimicrobial activity on *B. pseudomallei* p37 lawn. Ceftazidime (50 µg.mL^{-1}) was served as a positive control and LB as a negative control.

Bio-control in soil

The soil pH before the experiments was 6.89 and changed to be 7.26 when adjusted to have 100% WHC. The colony count of the control *B. pseudomallei* p37 in 10 g soil was 1x 10^5 CFU.g^{-1} at the beginning and increased to 1x 10^8 CFU.g^{-1} on the 1st week before slightly decreased on the second week and increased again until the 4th week (Figure 3). The number of *B. amyloliquefaciens* N3-8 in the control soil was 1x 10^8 CFU.g^{-1} at the beginning and slightly increased until the 4th week.

For the bio-control experiments, the pattern of CFU.g^{-1} of *B. pseudomallei* p37 in the 1:50 and 1:100 ratios were similar to what observed in the *B. pseudomallei* p37 control soil but the number of *B. pseudomallei* p37 was decreased about 0.5-1 $\log_{10}$ on the 2nd week onward when compared to the control. For the 1:300 ratio, the number of *B. pseudomallei* p37 was abruptly decreased from the 1st week and about 4 $\log_{10}$ on the 3rd week. The inoculation of *B. amyloliquefaciens* N3-8 into the bio-control experiments on the 2nd week could not decrease the pathogen in 1:50 and 1:100 ratios but can drop it down when1:300 ratio was used.

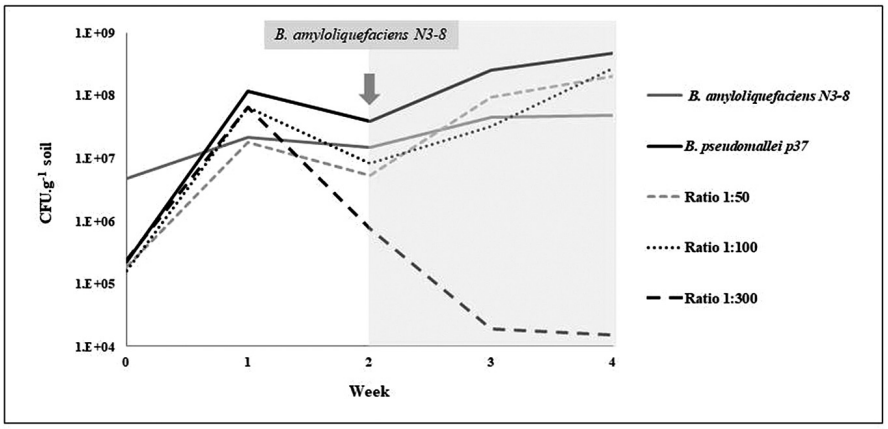

Figure 3 The colony count of *B. amyloliquefaciens* N3-8 and *B. pseudomallei* p37 in soil. The Y axis showed CFU.g^{-1}soil and the X axis showed time in week. The black line showed the CFU.g^{-1} soil of *B. pseudomallei* p37 control, dark brown line was *B. amyloliquefaciens* N3-8 control and green dashed, black dot and black dashed line were CFU.g^{-1}soil of *B. pseudomallei* p37 when co-culture with *B. amyloliquefaciens* N3-8 at the ratios of 1:50, 1:100 and 1:300. The arrow indicated the time when *B. amyloliquefaciens* N3-8 was added.

The pH of soil in all tubes were significantly increased when incubate for 4 weeks. The average pH of soil in the presence of *B. amyloliquefaciens* N3-8 control ($1x10^8$ CFU.g^{-1}) and all co-culture tubes on the 4th week were much higher than the *B. pseudomallei* p37 control and pH of soil before the experiments. (Figure 4).

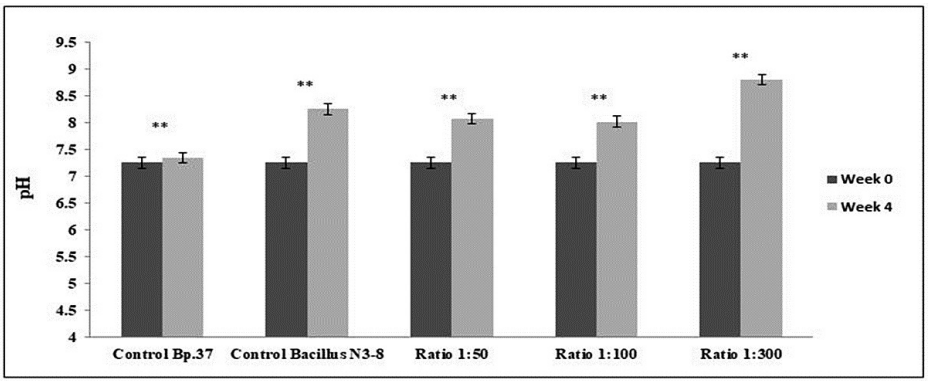

Figure 4 The pH of soil in each experiment. The Y axis is pH and the X axis indicated each experiment. The dark gray bars were pH at the beginning and light gray showed pH of soil on the 4th week. The asterisk (**) indicated significant between week 0 and week 4 ($p<0.01$).

Discussion

Soil is considered to be a complex environment and is a major reservoir of living organisms either in competitive or symbiotic microbial communities[11]. *Bacillus* species can produce a large number of metabolites such as peptides and non-peptides with biological activities, some of which can be used as bio-controls, mostly for plant diseases[12]. *Bacillus amyloliquefaciens* has been recognized as a good plant growth promoter and root colonizer[13]. Several strains of *B. amyloliquefaciens* were investigated as a bio-control agent against plant pathogens such as *B. amyloliquefaciens* SQR9 that controls cucumber and water melon wilt disease[14]. *B. amyloliquefaciens* FZB42 was also reported as a bio-fertilizer and bio-control agent in agriculture[15]. To our knowledge, none of the bio-control concept have been applied to control human pathogen is soil. The difficulties are mostly come from the complexity of soil that compost of a huge number of living organisms and the large area of soil to handle. However, when soil is the most important reservoir of a pathogen, controlling them in some certain areas such as in the zoo where melioidosis is endemic is therefore can be a clear benefit to protect centennial exotic animals that susceptible to *B. pseudomallei* infection.

B. pseudomallei is unevenly present in soil and the number of the bacterium varies for example from a few to 10^4 CFU.g^{-1} of soil as reported from Laos[16] or 10^3-10^6 CFU.g^{-1} in Khon Kaen (unpublished data). The most common route of infection is the exposure of skin abrasion to contaminated soil and water[17]. Therefore, the reduction of the bacterial number in soil might decrease the risk of infection. The amount of *B. pseudomallei* p37 used to spike into the soil was $1x10^5$ CFU.g^{-1} that relatively high comparing to what generally found. The 1:300 ratio of *B. pseudomallei* p37: *B. amyloliquefaciens* N3-8 can decrease the amount of *B. pseudomallei* p37 down to 10^6 within 2 weeks and lower the bacterial count further totally by 4 $\log_{10}$ at the 4th week after *B. amyloliquefaciens* N3-8 was added. Without adding the bacterium for the second time, the bacterium did not show a sharp decrease (data not shown). After the battle between these organisms was over and soil condition was suitable, *B. amyloliquefaciens* N3-8 can form spore and persist in soil waiting to germinate again similar to nature of other *Bacillus* spp. in soil[18]. The longer observation may help prove and predict if this situation will occur and could prolong controlling of *B. pseudomallei.*

Soil in the northeast of Thailand showed a few abiotic factors that were significantly different when compared between soil that positive and negative for *B. pseudomallei*[19, 20]. Acidic pH range was found to be related to the present of *B. pseudomallei*. The soil in this experiment was obtained from the area where *B. pseudomallei* were found, however, the pH was not in the acidic condition. When *B. pseudomallei* p37 was inoculated into the soil and incubated for 4 weeks, the bacterium showed ability to grow in this soil condition. The soil in *B. amyloliquefaciens* N3-8 control tube and co-culture in all ratios showed the increased in pH value in alkali range and more than soil with *B. pseudomallei* alone. This range of pH may not suitable for *B. pseudomallei,* however, the alkaline pH alone was not enough to decrease a large number of *B. pseudomallei* as seen in 1:50 and 1:100 ratios. The changes of pH if apply in the soil that found to be acidic may help improve the quality of soil. Nevertheless, more experiments have to be set-up in order to prove this hypothesis. The secondary metabolites from *B. amyloliquefaciens* N3-8 were reported to inhibit a range of pathogen but not non-pathogenic bacteria in soil even a closely related *B. thailandensis*[8]. Moreover, the bacterium was isolated from soil in the northeast of Thailand that unlikely to do harm to the ecosystem. However, investigation of bacterial diversity in soil after treatment with *B. amyloliquefaciens* N3-8 should be perform to confirm.

Conclusion

The 1:300 ratio of *B. pseudomallei*: *B. amyloliquefaciens* N3-8 bio-control can clearly decrease *B. pseudomallei* in laboratory soil condition. The pH changed may come from the secondary metabolites of *B. amyloliquefaciens* N3-8. Further investigation of the bio-control in a field experiment should provide more information if *B. amyloliquefaciens* N3-8 can be used as a bio-control of *B. pseudomallei* in nature.

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