Purification of Non-Peptide Compounds from *Bacillus amyloliquefaciens* that Inhibit *Burkholderia pseudomallei*

การเตรียมสารที่ไม่ใช่เปปไทด์จาก *Bacillus amyloliquefaciens* ที่มีฤทธิ์ในการยับยั้งเชื้อ *Burkholderia pseudomallei* ให้บริสุทธิ์

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ABSTRACT

Bacillus amyloliquefaciens N2-4 and N3-8 were isolated from soil and their secondary metabolites showed inhibition activity against *Burkholderia pseudomallei* and other pathogens. In this study, the non-peptide compounds with antimicrobial activity were absorbed from bacterial culture supernatant using non-polar resins. The MICs of the crude compounds against *B. pseudomallei* was less than 0.01 mg/mL, 0.09-0.37 for *Escherichia coli* and 0.05-0.75 for *Staphylococcus aureus*. After purified by solid phase extraction, the factions eluted by 80 and 100% methanol from N2-4 were active against *B. pseudomallei*, *E. coli and S. aureus*. Compounds from N3-8 also gave similar result excepted that they had lost their activity against *S. aureus*. There should be at least two different compounds produced by strain N3-8 and N2-4 that can inhibit these pathogens. Further purification and characterization of these compounds may lead us to discover some new drugs against *B. pseudomallei* and other pathogens.

บทคัดย่อ

จากการแยก *Bacillus amyloliquefaciens* N2-4 และ N3-8 จากดิน พบว่าสามารถผลิตสารเมแทบอไลท์ทุติยภูมิที่ยับยั้งเชื้อ *Burkholderia pseudomallei* และเชื้อแบคทีเรียก่อโรคอื่น ๆ ในการศึกษานี้ได้แยกสารที่ไม่ใช่เปปไทด์ที่มีความสามารถในการต้านเชื้อจุลชีพโดยใช้เรซินที่ไม่มีขั้วดูดซับจากอาหารเลี้ยงเชื้อ สารที่ได้เมื่อหาค่าความเข้มข้นต่ำ สุดที่สามารถยับยั้งการเจริญของเชื้อ *B. pseudomallei* พบว่ามีค่าน้อยกว่าหรือเท่ากับ 0.01 มก./มล. และ 0.09-0.37 มก./มล. เมื่อทดสอบกับ *Escherichia coli* และ 0.05-0.75 มก./มล. เมื่อทดสอบกับ *Staphylococcus aureus* หลังจากทำให้สารบริสุทธิ์ต่อโดยใช้การชะออกจากตัวดูดซับที่เป็นของแข็ง พบว่าสารจาก N2-4 ที่ได้จากการสกัดด้วย เมทานอล

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80 และ 100% มีฤทธิ์ในการยับยั้งเชื้อ *B. pseudomallei, E. coli* และ *S. aureus* สารจาก N3-8 ให้ผลเช่นเดียวกัน ยกเว้นไม่พบความสามารถในการยับยั้ง *S. aureus* ดังนั้นจึงน่าจะมีสารที่ต่างกันอย่างน้อยสองชนิด ที่สร้างจาก *B. amyloliquefaciens* N3-8 และ N2-4 ที่มีคุณสมบัติยับยั้งเชื้อก่อโรคเหล่านี้ การทำให้บริสุทธิ์ และศึกษาคุณสมบัติของสารดังกล่าวอาจจะทำให้สามารถค้นพบยาตัวใหม่ที่ใช้ในการรักษา *B. pseudomallei* และเชื้อ ก่อโรคอื่น ๆ ได้ต่อไป

Keywords: Antimicrobial compound, Solid phase extraction, NRPSs
คำสำคัญ: สารต้านเชื้อจุลชีพ การสกัดสารจากตัวดูดซับของแข็ง สารที่ไม่ได้สร้างโดยไรโบโซม

Introduction

Bacillus spp. are a group of Gram-positive bacteria that can produce a large variety of secondary metabolites, some of which having antimicrobial activities. Since the amount of antibiotic resistant microbes is increasing dramatically, this source of compounds therefore becomes attractive to characterize and use as an alternative treatment. *Bacillus* spp. can produce both ribosomal and non-ribosomal synthesized peptide compounds as their secondary metabolites. Bacteriocins are predominant class of secondary metabolites that generated from ribosomal peptide synthesis and were used to compete with other related microorganisms for survival under limited nutrient conditions [1]. An example is the cyclic peptide, subtilosin from *Bacillus amyloliquefaciens* isolated from diary product, which inhibits *Listeria monocytogenes*, *Gardnerella vaginalis* and *Streptococcus agalactiae* but do not affected vaginal Lactobacilli [2]. Besides peptides, non-peptide compounds, lipopeptides and polyketides which produced from non-ribosomal peptide synthesis also become compounds of interest by their anti-pathogenic properties. Lipopeptides from *Bacillus* spp. were synthesized by non-ribosomal peptides synthetases (NRPSs) or hybrid polyketides synthetases and non-ribosomal peptides synthetase (PKSs/NRPSs), by modular multi-enzymatic templates. Lipopeptides compose of a lipid tail that linked to a short linear or cyclic oligopeptide [3, 4]. Several *Bacillus* spp. can produce lipopeptides [5] such as surfactin, iturin and fengycin, of which are three families of cyclic compounds well known as biosurfactant in biotechnology and pharmaceutical field. Moreover, they are stable over the extreme conditions of temperature, pH and salinity [6]. Each family contains variants with the same peptide length but with different residues at specific position. Polyketides are another dominant family of secondary metabolites that produced by NRPSs or PKSs/NRPSs. Their synthesis is modularly organized starting from acyl-CoA precursors by decarboxylative Claisen condensations. Polyketides also have a broad antimicrobial activity against pathogenic bacteria.

B. amyloliquefaciens FZB42 for example is a plant-associated bacteria, of which 8.5% of its genome were reported to encode genes responsible for the non-ribosomal peptide synthesis [7]. The antifungul lipopeptides from *B. amyloliquefaciens* exhibited significant inhibition activities against *Curvularia lutana* by having excellent bio-surfactant property to destroy the fungal cell wall. *B. subtilis* and *B. licheniformis* also produce bio-surfactant that can reduce surface tension of water and reduce the critical micelle concentration that can emulsify and forming on biological membrane. Moreover, it exert

the anti-adhesion activity by inhibit biofilm formation of *Escherichia coli* and *Staphylococcus aureus* [8]. These properties make the metabolites from *Bacillus* spp. suitable to be used for controlling plant, animal and also human pathogen.

Burkholderia pseudomallei is a Gram-negative pathogen that cause a disease called melioidosis, the third of death among the infectious disease in northeast Thailand and conscientious for 20% of community acquired septicemia with 40% mortality rate [9]. Moreover, the bacterium is intrinsically resistant to several antibiotics and vaccine is not yet available. As *B. pseudomalli* that resist to ceftazidime, the drug of choice, also have been reported, novel compounds or alternative treatment therefore may be required in the near future.

B. amyloliquefaciens N2-4 and N3-8 were isolated from soil in the agriculture field of Khon Kaen University. They showed inhibitory activity against *B. pseudomallei* and other pathogenic bacteria of both Gram-positive (*S. aureus*, *Clostridium defficile and Enterococcus faecium*) and Gram-negative (*E. coli, Acinetobacter baumannii* and *Pseudomonas aeruginosa*) bacteria [10]. As non-peptide compounds produced from *B. amyloliquefaciens* were reported to have a wild-range of activity against several pathogenic bacteria similar to what was observed from *B. amyloliquefaciens* N2-4 and N3-8, this work therefore aims to partially purify non-peptide compounds with inhibition activity against *B. pseudomallei*.

Material and methods

Bacterial strains

Bacterial strains used in this present study are *B. amyloliquefaciens* N2-4, *B. amyloliquefaciens* N3-8 and *B. pseudomallei* P37 obtained from melioidosis research center, faculty of medicine Khon Kaen University, Thailand. Test organism which are *S. aureus* newman, *S. aureus* JE2, *E. coli* K12 and *E. coli* Tolc were obtained from Assoc. Prof. Julian Hurdle's laboratory, Center for Infectious and Inflammatory diseases, Institute of Biosciences and Technology, Texas A&M University, USA.

Culture condition and non-peptide compounds extraction

For the extraction of non-peptide compounds, which are mostly non-polar, XAD-16 and Diaion HP-20 non-polar resins were used to absorb the metabolites [11]. In brief, *B. amyloliquefaciens* N2-4 and N3-8 strains were cultured in a minimal medium [12]. (One liter composted of 5.0 g L-glutamic acid, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot H_2O$, 0.01 g NaCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.01 $CuSO_4 \cdot 7H_2O$ g and 0.015 g $CaCl_2 \cdot 2H_2O$) with 1% w/v glucose and 2% w/v of either XAD-16 or Diaion HP-20 in 250 mL medium as modified from Lee, et al. After cultured at 37°C with agitation at 200 rpm for 72 hours, the resins were removed by filtered through Whatman paper No.4 and washed with sterile distilled water. To extract the non-peptide compounds, 100 mL of methanol was added into the resins, shake for 2 hours and filtered through 0.2 μm membrane. The methanol was removed using rotary evaporation and weighted the remaining mass for further use.

Agar well diffusion method

Antimicrobial activity of the crude methanol extracts from *B. amyloliquefaciens* N2-4 and N3-8 strains were investigated by agar well diffusion method [13]. In brief, overnight cultured of *B. pseudomallei* or other indicator bacteria in LB medium were

inoculated in fresh LB and incubated at 37°C, 200 rpm for 4 hours until log phase and used approximately 10^5-10^6 CFU/mL to swab on Mueller-Hinton agar plate. The plate was punched to obtain 6.6 mm of wells by sterile pipette tip and then 100 µL (48 mg/mL) of sterile extracted compounds were added into agar wells. Ceftazidime (Sigma-Aldrich, St. Loius, MO) at concentration of 50 µg/mL and methanol were used as positive and negative controls. The plate was left at room temperature to allow the solution to diffuse. Thereafter, the plate was incubated at 37°C, for 18-24 hours. Inhibitory activity was evaluated by measuring the diameter of inhibition zone against *B. pseudomallei*.

Solid phase extraction (SPE)

In order to purify the active compounds, the crude compounds extracted from non-polar resins were applied into the SPE cartridge reverse phase chromatography, Strata C18-E (55 µm, 70A) 5g/25 mL Giga Tubes (Resprep, Bellefonte, PA) using different concentration of methanol as an eluent. In brief, column was activated with 40 mL of methanol and equilibrated with 40 ml of 1% methanol before the samples were applied. The crude compounds extracted were dissolved in 1 mL of 20% methanol then applied onto the column. Then, 20 mL of 1, 20, 40, 60, 80 and 100% methanol were added to elude the sample into a collector and label as fractions 1-6. These fractions were used to test for antimicrobial activity by agar well diffusion against *B. pseudomallei*, *E. coli* strain K12 and *S. aureus* strain newman.

Minimum inhibitory concentration (MIC) of antimicrobial compounds

The crude compounds extracted by non-polar resins from culture supernatant of *B. amyloliquefaciens* N2-4 and N3-8 with concentration of 48 mg/mL in methanol was used to determine its MIC by micro-broth dilution [14]. In brief, the antimicrobial compounds were diluted by two-fold serial dilution using Mueller-Hinton broth (MHB) in 96-well plate; methanol was use as a negative control. Then bacterial indicators approximately 10^5-10^6 CFU/mL were added into each well, mixed gently and then incubated at 37°C for 18-24 hours. The last concentration that provided clear turbidity when compared to growth control was recorded as MIC.

Result

Non-peptide compounds extraction and measurement of antimicrobial activity

The amount of crude compounds obtained from using either XAD-16 or Diaion HP-20 resin absorption was not different as shown in table 1. Antimicrobial activities of these crude extract were measured via agar well diffusion method against Gram-negative bacteria; *B. pseudomallei*, *E. coli* strains K12 and Tolc and Gram-positive bacteria; *S. aureus* strains newman and JE2 (Figure 1). The compounds from N3-8 absorbed by both XAD-16 and Diaion HP-20 could inhibit the tested Gram-negative bacteria but not the Gram-positive *S. aureus* strains newman and JE 2. The resin-absorbed compounds from N2-4, however, can inhibit both Gram-positive and Gram-negative bacteria.

Minimum inhibitory concentration (MIC) of antimicrobial compounds

The MIC of 48 mg/mL hydrophobic compounds extracted by either XAD-16 or Diaion HP-20 resins can inhibit all pathogenic strains tested in this study (Table 2). The most effective inhibition result was observed with *B. pseudomallei* P37 that gave MIC ≤0.01 mg/mL.

Solid phase extraction (SPE)

The crude methanol extracted from batch culture of *B. amyloliquefaciens* N2-4 and N3-8 isolates were fractionated and test each fraction for the antimicrobial activity. The fractions eluted by 80% (fraction 5) and 100% methanol (fraction 6) were found to be active against tested bacteria as shown in Table 3. The 2 active fractions from N3-8 can inhibit both *B. pseudomallei* P37 and *E. coli* K12 but not the tested Gram-positive. For N2-4, the fraction 5 can inhibit *B. pseudomallei* P37 and *E. coli* K12, while fraction 6 showed inhibition activity against both Gram-positive and Gram-negative bacteria, which were *B. pseudomallei* P37, *E. coli* K12 and *S. aureus* newman.

Discussion

B. amyloliquefaciens can be found in soil and can produce various kinds of secondary metabolites. The bacterium was reported as a plant growth-promoting bacterium that associated with rhizosphere. When it consumes nutrients from plant, the bacterium produces secondary metabolites to suppress plant pathogens and promote plant growth. Koumoutsi et al. described that *B. amyloliquefaciens* FZB42 can produce several non-peptide products such as lipopeptides; surfactin, fengycin and bacillomycin D [15] which can inhibit *Fusarium oxysporum* and polyketides; bacillaene, difficidin and macrolactin also can inhibit this pathogen [16-17]. This bacterium therefore draws a lot of attention as an important source for screening of antimicrobial compounds

In this study, *B. amyloliquefaciens* N2-4 and N3-8 isolates with antimicrobial activity against *B. pseudomallei* were used to produce secondary metabolites and then purify the non-peptide compounds using solid phase extraction method. The secondary metabolites by cross streak method form both *B. amyloliquefaciens* N2-4 and N3-8 isolates were able to inhibit *S. aureus*, a representative of Gram-positive and *E. coli*, a representative of Gram-negative [10]. The non-polar resins were selected for further step in purification of the non-peptides from N2-4 and N3-8. The non-polar XAD-16 and Diaion HP-20 resins are materials that Diaion HP-20 can bind to small molecular weight hydrophobic compounds and XAD-16 can bind to small to medium molecular weight compounds. XAD-16 was used as a column media in chromatography for characterization of polyketides [18] and lipopeptides from FZB42 and NJN-6 strains respectively, that have their inhibition effect on *F. oxysporum* f. sp. Cubense, and *Ralstonia solanacearum* [19]. Moreover, XAD-16 and Diaion HP-20 were used as absorbent resin for the adsorption of teicoplanin in batch cultured of *Actinoplanes teicomyceticus* ATCC 31121 [11]. XAD-16 resin is mostly used to absorb small molecules of hydrophobic compounds from *B. amyloliquefaciens* and also used to purify some peptide such as subtilosin A, which is a cyclic peptide produced by *B. subtilis* 168 [20]. Therefore, it would be possible that the compounds from N2-4 and N3-8 extracts might be lipopeptides, polyketides or some hydrophobic peptide antibiotics. The non-peptides in the secondary metabolites from *B. amyloliquefaciens* were reported to target on bacterial cell membrane as they could form pores or emulsification on cell wall of the target organism.

The compounds extracted from culture supernatant of N2-4 and N3-8 isolates that absorbed to XAD-16 and Diaion HP-20 were able to inhibit *B. pseudomallei* and other Gram-negative pathogens as proved by MIC. After the crude compounds were

eluted from the solid phase column using methanol, the 80 and 100% methanol fractions showed inhibition activity against the test bacteria. The active compound should contain more of the non-polar structures. Thus, both XAD-16 and Diaion HP-20 resins showed similar capacity to purify the compounds from *B. amyloliquefaciens* N2-4 and N3-8 isolates. In addition, there should be at least two compounds in each crude extract that have inhibitory activity against other bacteria as there were two different fractions and have antimicrobial affected on different bacteria.

Previous result from cross streak method showed culture supernatant of N2-4 and N3-8 isolates can inhibit both Gram-negative and Gram-positive bacteria including *S. aureus* strains newman and JE strains [10]. After non-polar resins absorption, the compounds from N3-8 showed no inhibition against *S. aureus* strains newman and JE2. Therefore, the compound with inhibition activity against Gram-positive bacteria in N3-8 may be a polar compound. The secondary metabolites with inhibition activity may compose of both peptides and non-peptides both of which having strong inhibition potential against *B. pseudomallei*. The purification and characterization for the pure compounds, cytotoxicity test and other examination of these compounds will be further investigate. The pure compounds with antimicrobial activity against *B. pseudomallei* and some other pathogen might lead us to discover new compounds that can be used as a new drug in the near future.

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Table 1 The concentrations of compounds absorbed by XAD-16 or Diaion HP-20 resins.

Compounds	**Concentration (mg/mL)**
HP-20 extract	
N2-4	110.3
N3-8	91.5
XAD-16 extract	
N2-4	109.4
N3-8	92.2

Table 2 The MIC of antimicrobial compounds absorbed either by XAD-16 or Diaion HP-20 resins.

Bacterial indicators	**XAD-16 extracted MIC (mg/mL)**		**HP-20 extracted MIC (mg/mL)**	
	N2-4	**N3-8**	**N2-4**	**N3-8**
B. pseudomallei P37	≤0.01	≤0.01	≤0.01	≤0.01
E. coli K12	0.37	0.18	0.09	0.09
S. aureus newman	0.18	0.75	0.05	0.75

Table 3 Inhibition activity of compounds eluted from solid phase extraction as measured by agar well diffusion method.

Samples	Active* fractions	Antimicrobial activity inhibition zone (mm)		
		E. coli K12	*S. aureus* newman	*B. pseudomallei P37*
HP 20 absorption				
N2-4	crude	15	21	20
	1	0	0	0
	2	0	0	0
	3	0	0	0
	4	0	0	0
	5	22	0	20
	6	22	20	29
N3-8	crude	15	0	22
	1	0	0	0
	2	0	0	0
	3	0	0	0
	4	0	0	0
	5	18	0	20
	6	20	0	24
XAD16 absorption				
N2-4	crude	19	22	24
	1	0	0	0
	2	0	0	0
	3	0	0	0
	4	0	0	0
	5	20	0	20
	6	21	22	30
N3-8	crude	17	0	25
	1	0	0	0
	2	0	0	0
	3	0	0	0
	4	0	0	0
	5	17	0	21
	6	21	0	29

*Active fraction 5 was eluted by 80% methanol and fraction 6 by 100% methanol

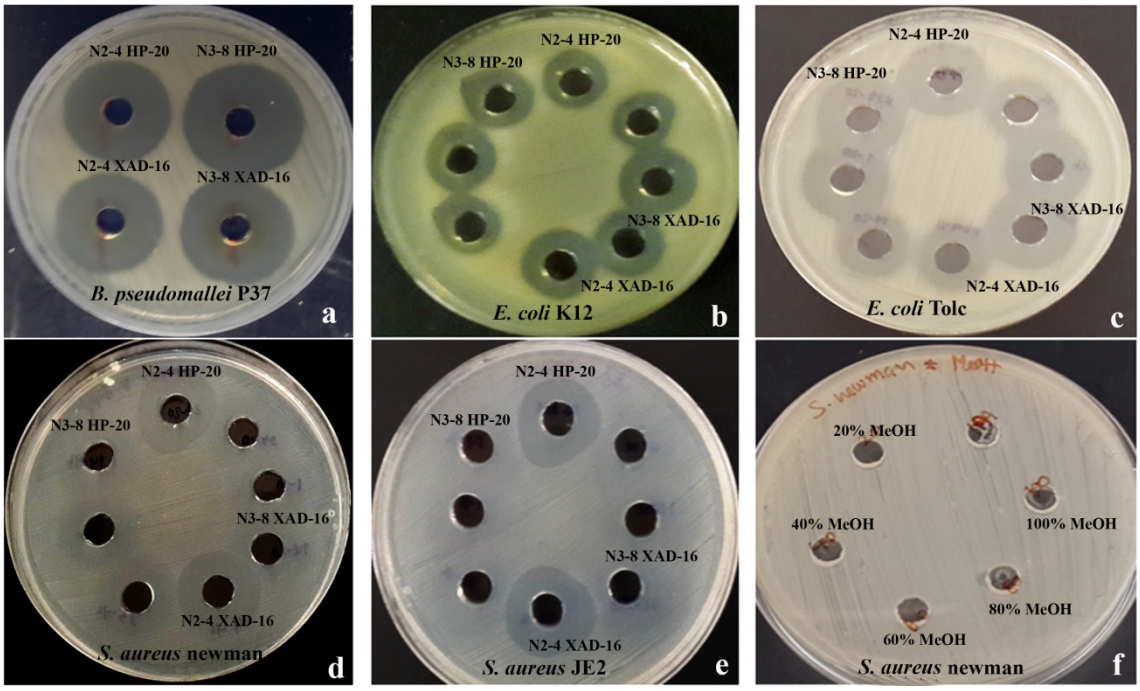

Figure 1 The agar well diffusion test of crude compounds obtained from non-polar resin absorption of secondary metabolites of *B. amyloliquefaciens* N-4 and N3-8. The inhibitory activity of compounds obtained by either XAD-16 or HP-20 resins from *B. amyloliquefaciens* N2-4 and N3-8 were test against *B. pseudomallei* P37 (a), *E. coli* K12 (b), *E.coli* Tolc (c), *S. aureus* newman (d) *S. aureus* JE2 (e) and methanol in different concentrations (f) was used as a negative control.