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Production of antimicrobial metabolites active against *Burkholderia pseudomallei* from *Bacillus amyloliquefaciens* strains kku11 and kku14

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Abstract

Bacillus amyloliquefaciens is a Gram-positive, aerobic or facultative anaerobic, endospore-forming bacterium commonly found in soil. One of the main characteristics shared among *Bacillus* species is the ability to produce a wide range of antimicrobial compounds that active against bacteria and fungi. *B. amyloliquefaciens* KKU11 and KKU14 were isolated from soil. Their secondary metabolites showed antimicrobial activity against *Burkholderia pseudomallei*, a Gram-negative pathogen also found in soil in the endemic areas such as Southeast Asia and northern Australia. This pathogen is intrinsically resistant to several antibiotics and caused a severe infectious disease called melioidosis. In order to produce their metabolites for purification and characterization, the growth curve and active metabolites from strains KKU11 and KKU14 were observed in basal and complex media which are Luria Bertani (LB) and Brain Heart Infusion (BHI) broth. The antimicrobial activity active against *B. pseudomallei* as measured by agar well diffusion method were detected after the KKU11 and KKU14 entered stationary phase and gave the highest activity at 72 h of growth. The antimicrobial metabolites from the KKU11 produced in LB gave 14.47±0.6 mm inhibition zone while in BHI gave 20.0±0.6 mm. The activity from KKU14 was not detected when grew in LB but gave 18.0±1.5 mm clear zone using BHI. The secondary metabolites from the KKU11 and KKU14 when grew in BHI medium can also inhibit *B. pseudomallei* drug resistant strains, *Ralstonia solanacearum* (plant pathogen) and also *Salmonella* group D. Further formulation of production medium for each of them may lead to a better yield of their secondary metabolites and also might influence the spectrum of inhibition to obtain the best benefit from each isolate of *B. amyloliquefaciens*.

Introduction

Bacillus species are Gram-positive, rod-shaped, aerobe or facultative anaerobe bacteria. They have peritrichous flagella for their motility. *Bacillus* species have many advantages because they can produce antibiotics, enzymes, and other metabolites that can be useful in medical, pharmaceutical, agricultural, and industrial processes. However, the problem of spores that are resistant to sterilization and disinfection makes it difficult to eliminate them when contaminated in foods, medical supplies and laboratory[1]. In 1943, *B. amyloliquefaciens* was discovered in soil by Juichiro Fukumoto. Fukumoto gave this name to the bacterium because it produced (faciens) a liquifying (lique) amylase (amylo)[2]. *B. amyloliquefaciens* is similarly related to *B. subtilis* and the other two species which are *B. licheniformis* and *B. pumilus.* These bacteria shared common property of the ability to produce antimicrobial activity. *B. amyloliquefaciens* has the phenotype similar to *B. subtilis*. It's hard to separate these bacteria solely on basis of classical tests[3]. Optimal temperature for growth is 37˚C. *B.*

amyloliquefaciens has a very important role in the nature because they can produce secondary metabolites that can be used as the biological control against bacterial pathogen and plant pathogen[4]. Moreover, they can act as agents of symbiosis between microorganisms[5].

Melioidosis is an infectious disease caused by Gram-negative, aerobic bacterial named *Burkholderia pseudomallei*[6]. The bacterium is a soil-dwelling environmental saprophyte. The disease is endemic in Southeast Asia and northern Australia and some sporadic cases have been reported in a several continents[7]. In northeastern parts of Thailand, patients who are infected with *B. pseudomallei* were accounted as 20% of community-acquired septicemia and the mortality rate is 40%[6]. The bacterium is intrinsically resistant to several antibiotics and resistant to its drug of choice also has been reported[8]. The most common risk factors for infection are diabetes mellitus and the most common clinical manifestation is visceral abscesses and pneumonia. Route of infection are inoculation through skin abrasions, inhalation, and ingestion of contaminated soil and surface waters[6].

B. amyloliquefaciens KKU11 and KKU14 were isolated from soil and their culture supernatant was found to be active against *B. pseudomallei*. Therefore, this study aims to study growth condition to produce their secondary metabolites that are active against *B. pseudomallei.* Moreover, the antimicrobial metabolites against other pathogenic bacteria were also observed.

Materials and methods

Bacterial strains

B. amyloliquefaciens strains KKU11 and KKU14 were isolated from soil in Khon Kaen (Potisap C, under submission) and their secondary metabolites showed antimicrobial activity against *B. pseudomallei*. *B. pseudomallei* P37 was isolated from patient's blood that contained no inducible bacteriophage. *B. pseudomallei* drug resistant strains NU2 and NU3 were obtained from the Mahidol Oxford Tropical Medicine Research Unit (MORU), Faculty of Tropical Medicine, Mahidol University. *Ralstonia solanacearum* and *Salmonella* group D were obtained from Melioidosis Research Center (MRC), Khon Kaen University, Thailand.

Agar well diffusion method

The antimicrobial activity in the culture filtrates of *B. amyloliquefaciens* strains KKU11 and KKU14 either from LB or BHI medium were measured by agar well diffusion method.[9] Briefly, *B. pseudomallei* were grown in LB for 16-18 h and 1.0% was used to inoculate into 4 ml LB and incubated for 4 h until reached log phase. The culture then was swabbed on Mueller-Hinton agar (MHA) plates and then sterile plastic pipette tip was used to punch 6.6 mm diameter of wells. A hundred microliters of culture filtrate from *B. amyloliquefaciens* KKU11 and KKU14 that grew for 72 h was added into the well and incubated at 37 °C for 24 h. Antimicrobial activity was evaluated by measuring the inhibition zone against *B. pseudomallei.* The ceftazidime, which is the drug of choice, at 60 µg/ml was used as a positive control. All activities were evaluated in duplicate and reported as an average of diameter.

Bacterial growth curve and antimicrobial activity

B. amyloliquefaciens KKU11 and KKU14 from glycerol stock were inoculated into Luria Bertani (LB) broth and incubated at 37ºC with shaking for overnight. The culture then was streaked on LB agar plate to obtain single pure colonies and inoculated each of *B. amyloliquefaciens* KKU11 and KKU14 separately into 50 ml LB broth in 125 ml Erlenmeyer flasks and cultured with 200 rpm shaking at 37°C for overnight. Culture supernatant was adjusted and monitor by spectrophotometer to obtain OD_{600nm} ~ 0.1 and use 1% to inoculate into 300 ml of BHI broth. The culture was grown with shaking at 37°C and 7 ml were aliquot at 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 30, 36, 42, 48, 60, 72, 84 and 96 h. One milliliter of culture was used to measure OD_{600nm} and another 1 ml was diluted with PBS to plate for

colony count. Five milliliters of culture were centrifuged at 4°C, 16,000 g for 15 min, filtered through 0.45 µm membrane to remove the bacterial cells and kept the filtrate in sterile tubes until used to test for antimicrobial activity by agar well diffusion.

Antimicrobial activity of KKU11 and KKU14 against other pathogenic bacteria

B. pseudomallei drug resistant strains, *Ralstonia solanacearum* (plant pathogen) and also *Salmonella* group D were grown, spread on MHA and prepared agar wells as previously described. One hundred microliters of culture filtrate from *B. amyloliquefaciens* KKU11 and KKU14 that grew in BHI medium and LB medium were added into the wells and incubated at room temperature for 2 h for diffusion and then further incubated at 37 °C for overnight. Antimicrobial activity was evaluated as described earlier.

Results and Discussion

The inhibition zone of the antimicrobial activity against B. pseudomallei

The antimicrobial activity active against *B. pseudomallei* as measured by agar well diffusion method was detected after the KKU11 and KKU14 entered stationary phase and gave the highest activity at 72 h of growth. The antimicrobial metabolites from the KKU11 produced in LB medium gave 14.47±0.6 mm inhibition zone while in BHI medium gave 20.0±0.6 mm. The activity from KKU14 was not detected when grew in LB medium but gave 18.0±1.5 mm clear zone using BHI medium and gave 24.0±1.0 mm inhibition zone in the ceftazidime 60 µg/ml that is the drug of choice used as a positive control (Figure 1).

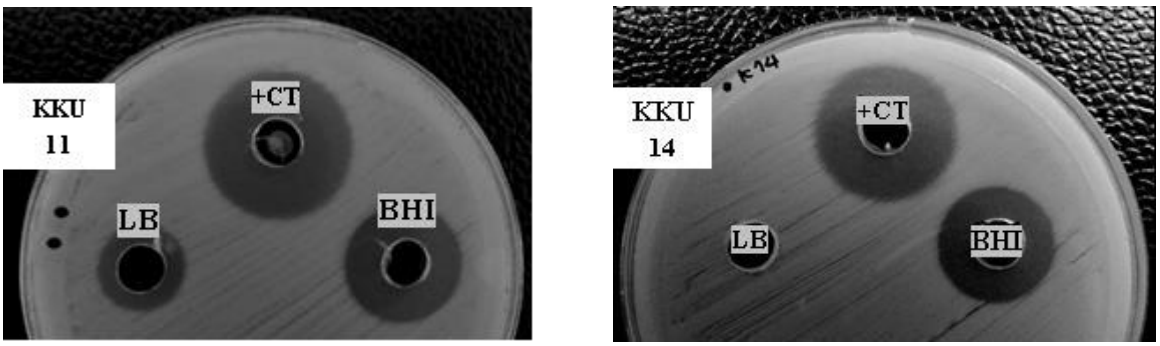

Figure 1. The antimicrobial activity against *B. pseudomallei* from the secondary metabolites of *B. amyloliquefaciens* strains KKU11 and KKU14. The antimicrobial activity of the culture supernatant of KKU11 (left) and KKU14 (right) that grew in either LB or BHI medium was shown as clear inhibition zone on *B. pseudomallei* lawn. Ceftazidime 60 µg/ml, the drug of choice for *B. pseudomallei,* that used as a positive control also showed a clear inhibition zone.

Bacterial growth requires both physical factors such as pH, temperature and oxygen and the chemical factors which are carbon and nitrogen sources, minerals and trace elements. Nutrients in growth media normally compose of all the elements necessary for the synthesis of new organisms and activity of microorganisms such as production of secondary metabolites to meet their requirement. Carbon source is the main energy source of microorganism for sustaining life and the synthesis of its organic molecules[9]. The nitrogen source is normally the next most abundant substance in the fermentation media that served as the energy source in bacteria. Nitrogen may be supplied as ammonia salts[10] or an organic compound like casein, peptone, beef extract, and yeast extract[11]. Organic nutrients such as amino acids and more complex protein degradation products such as peptones[12,13] may also supply nitrogen and sulfur in the reduced form as well as carbon and energy.

In this study, *B. amyloliquefaciens* KKU11 and KKU14 were cultured in commercial available medium that commonly used for culture bacteria which is LB that consists of tryptone, NaCl and yeast extract. BHI is a nutrient-enrich medium composed of calf brain-beef heart infusion, pancreatic digestion of gelatin, NaCl, disodium phosphate and dextrose generally used to grow fastidious organism. It was also selected as production medium to compare with LB and observed the growth and production of secondary metabolites that active against *B. pseudomallei*. Both *B. amyloliquefaciens* KKU11 and KKU14 can grow on these 2 media but better in BHI. However, the production of secondary metabolites to inhibit *B. pseudomallei* from KKU14 was lost when passage several times in LB. The antimicrobial activities from KKU11 was produced higher in BHI when compared with LB and BHI can retain the production of active compounds in KKU14. The result indicated that the components in BHI medium had some effect on antimicrobial metabolites production. The BHI is a complex medium with a wide variety of compounds that could promote the production of these secondary metabolites. This is similar to a report that indicated the production of secondary metabolites were depended on the environmental factors which are stress, starvation condition and also nutrients supplementation which are carbon, nitrogen, amino acids, phosphate and other mineral that have a plenty influence on the production of secondary metabolites. The development of specific formulation of media for bacterial culture therefore can enhance the production of secondary metabolites such as antimicrobial compounds[14].

Growth curve and antimicrobial activity of active metabolites from B. amyloliquefaciens KKU11 and KKU14

The KKU 11 and KKU14 grew better in BHI medium and only gave antimicrobial activity against *B. pseudomallei*, therefore, the growth curve was measured only in BHI. The growth of *B. amyloliquefaciens* KKU11 appeared as logarithmic phase during 6 h to 20 h and remained in the stationary phase between 20-72 h before declined (Figure 2). The antimicrobial activity produced from KKU11 can be detected starting from 48 h of growth and reached maximum at 72 h. KKU14 that grew in BHI as shown in Figure 3 reach logarithmic phase at 6 h to 18 h and enter the stationary phase after 18 h to 72 h and then declined. The antimicrobial activity active against *B. pseudomallei* can be detected at 60 h of growth and reached highest level at 72 h. The antimicrobial activity against *B. pseudomallei* from KKU11, as measured by agar well diffusion at 72 h of growth gave 20.0±0.6 mm of the inhibition zone while that of KKU14 gave the inhibition zone of 18.0±1.5 mm.

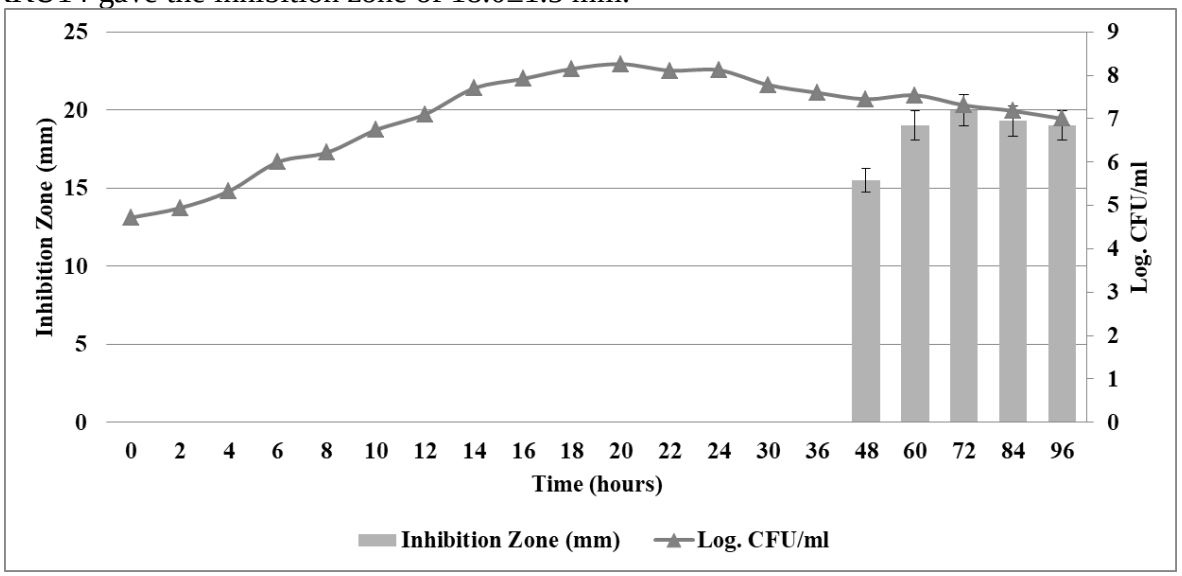

Figure 2. The growth curve and antimicrobial activity of *B. amyloliquefaciens* KKU11. The growth of KKU11 as CFU/ml was shown as line while the antimicrobial activity against *B. pseudomallei* as measured by clear zone diameter was shown as gray bars.

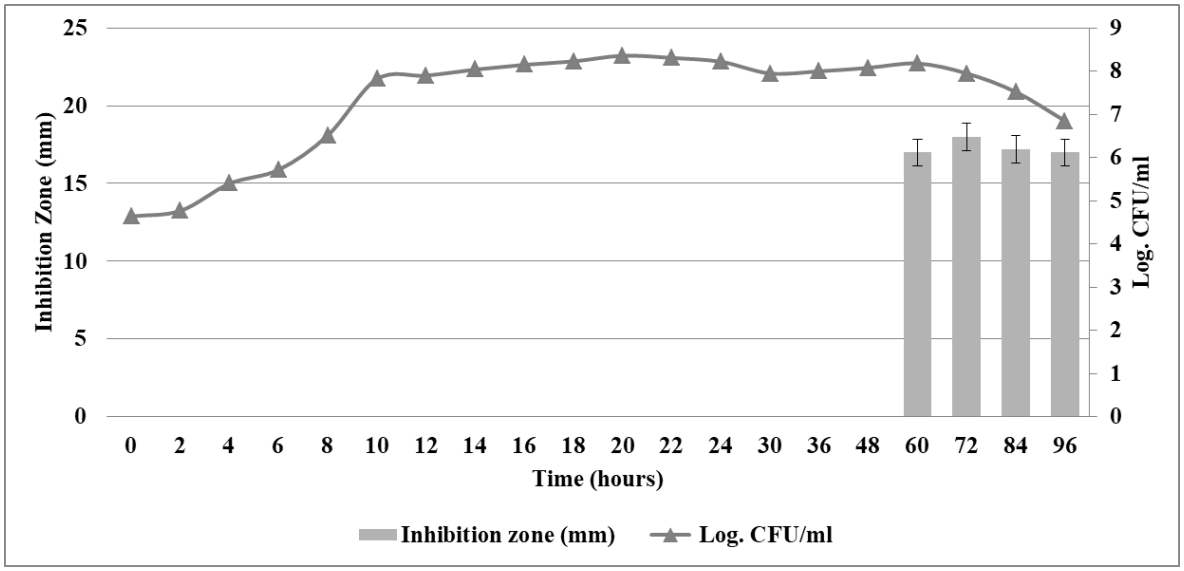

Figure 3. The growth curve and antimicrobial activity of *B. amyloliquefaciens* KKU14. The growth of KKU14 as CFU/ml was shown as line while the antimicrobial activity against *B. pseudomallei* as measured by clear zone diameter was shown as gray bars.

From the growth curve of both *B. amyloliquefaciens* KKU11 and KKU14 showed the production of their antimicrobial compounds when they grew in the stationary phase that confirmed the active compounds as a part of the bacterial secondary metabolites.

Antibacterial spectrum

The data of antimicrobial activity in culture filtrate from BHI broth from *B. amyloliquefaciens* KKU11 and KKU14 against drug resistant strains of *B. pseudomallei* (NU2 and NU3), *Ralstonia solanacearum* (plant pathogen) and also *Salmonella* group D were shown in table 1. The spectrum of the compounds not only active against *B. pseudomallei* but also its drug resistant isolates that showed strong benefit of these compounds that worth further characterization. Moreover, the crude secondary metabolites showed a broad spectrum of activity as they also can inhibit another Gram-negative pathogen; *Salmonella* group D and also *Ralstonia solanacearum* which is a plant pathogen. *B. amyloliquefaciens* KKU11 and KKU14 should be able to produce more than one active compound similar to what has been reported in soil bacteria such as *Streptomyces avermitilis* that twenty-five kinds of secondary metabolite gene clusters of various function including avermectin, of which is commercially important in human and veterinary medicine were found from genome analysis[15].

Table 1. Antimicrobial spectrum of culture supernatant from *B. amyloliquefaciens* KKU11 and KKU14 in complex medium (BHI).

Bacterial indicators	**Inhibition Zone (mm)**	
	***B. amyloliquefaciens* KKU11**	***B. amyloliquefaciens* KKU14**
B. pseudomallei p37	20	18
B. pseudomallei NU2 (drug resistant)	22.5	18
B. pseudomallei NU3 (drug resistant)	22.5	19.5
R. solanacearum	18.8	15.5
Salmonella group D	15	13

As mentioned about antibiotics resistant problem in *B. pseudomallei*, an urgent need for new antibiotics that could be used to treat melioidosis should be expected. *B. amyloliquefaciens* KKU11 and 14 were isolated from soil in Khon Kaen that is an important endemic area of the disease. They showed ability to kill the bacterium when co-cultured in liquid medium (data submitted for publication). This raised an exciting issue as *B. amyloliquefaciens* has never been reported to produce secondary metabolites that kill human pathogen. In order to facilitate the production, purification and characterization of the active compounds, growth condition of the bacterium to produce the metabolites should be investigated.

Bacillus species are commonly known to be a good source of various enzymes, antibiotics and other secondary metabolites that are beneficial biotechnology industries, pharmaceutical industries and also agricultural applications[16]. *B. amyloliquefaciens* is one of the *Bacillus* species that is well known for the production of secondary metabolites and has no report of causing any diseases in human and animal. During starvation at the end of log phase and the beginning of stationary phase, it was reported to produce antifungal and antibacterial metabolites. Recent report of secondary metabolites from *B. amyloliquefaciens* strains DA12 showed inhibition against several plant pathogens including mycotoxigenic *Fusarium* species (*F. asiaticum*, *F. graminearum*, *F. proliferatum*, and *F. verticillioides*) and other fungal pathogens such as *Colletotrichum coccodes*, *Botrytis cinerea*, *Rhizotonia solani*, *Raffaelea quercus-mongolicae* and *Endothia parasitica*[17]. *B. amyloliquefaciens* does not only synthesize antifungal against plant pathogens but also reported to produce antibacterial compounds that can inhibit bacterial plant pathogens including *Burkholderia glumae*, *P. syringae* pv. *actinidiae*, *Xanthomonas arboricola* pv. *Pruni* and *Ralstonia solanacearum*[9].

Conclusion

This study also used commercial media, LB and BHI which are among the richest media used for cultivation of fastidious pathogenic microorganisms and consists of the complex component with expensive cost. The antimicrobial activities resulted from using these media were compared. The KKU11 grown in LB provided 14.47±0.6 mm inhibition zone against *B. pseudomallei* and no activity was found from KKU14 that grow in LB. When BHI was used to compare the antimicrobial activity with the LB medium, the inhibition zone of the KKU11 was 20.0±0.6 mm and that of the KKU14 was 18.0±1.5 mm. The production of antimicrobial activities when grew in BHI medium was higher than in LB medium for both the KKU11 and the KKU14. Every bacterium requires the nutrients for growth and produced the secondary metabolites for survival. Carbon source, nitrogen source, mineral, pH, and temperature are necessary factors for bacterial growth. So, tthe production of secondary metabolites from *B. amyloliquefaciens* KKU11 and KKU14 can be promoted by BHI, a commercial nutrient-rich medium. Further study by formulating their production medium may lead to a better yield of their active secondary metabolites and also might influence the spectrum of inhibition to obtain the best benefit from each isolate of *B. amyloliquefaciens*.

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Acknowledgments

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