The Potential use of Secondary Metabolites from *Bacillus Amyloliquefaciens* to Control *Acinetobacter Baumannii*

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Background and Objective : *Acinetobacter baumannii* is an important cause of hospital-derived infection or nosocomial infections in many hospitals. The bacterium has an ability to survive for a long time on common surfaces including medical equipments and drug resistant isolates have been increasingly reported. *Bacillusamyloliquefaciens* N3-8 was isolated from soil. The secondary metabolites that were produced from this organism showed inhibition againstseveral pathogens including *A. baumannii*. This study aimed toobserve the range of inhibition and to set a model investigating the ability of crude proteins produced from *B. amyloliquefaciens* in killing *A. baumanniion* plastic surface.

Material and Methods: The inhibition activity of crude proteins from *B. amyloliquefaciens* N3-8 was observed against 28 isolates of *A. baumannii* by agar well diffusion. Imipenem, the drug of choice, was used as a positive control. The efficiency of the crude proteins from the N3-8 to kill or prevent *A. baumannii* contamination was done on plastic surface and observed by colony count and streak plate methods using *A. baumannii* N5, which is a drug resistant isolate. Chlorhexidine of 1.5% was used as a positive control.

Results: The crude proteins could inhibit 71% (20/28) while imipenem could inhibit only 21% (6/28) of total *A. baumannii* isolates. Seven isolates showed no inhibition by both compounds, 15 isolates were inhibited by crude proteins but not imipenem, 5 were inhibited by both compounds and 1 isolate was inhibited by imipenem but not the crude proteins. Crude protein at concentrations of 1, 2, 4, 8, 16 and 32 mg/ml that spread over the $5x10^6$ CFU/ml of N5 or the other way round could completely kill the bacterium. When N5 was applied before and after the crude proteins, the concentrations of 2, 4, 8, 16 and 32 mg/ml could completely kill the N5 similar to 1.5% chlorhexidine.

Conclusion: These may be no advantage to use the crude proteins from *B. amyloliquefaciens* N3-8 for antiseptic purpose but they were superior to imipenem in killing the majority of *A. baumannii* isolates. Further purification and characterization may lead to the discovery of a new drug for treatment of *A. baumannii*.

Key Words: antimicrobial compound, nosocomial infection,secondary metabolites

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Introduction

Several bacteria can cause serious diseases in human and are also difficult to be cured due to their multidrug-resistance[1]. *Acinetobacter baumannii* is a saprophytic gram-negative bacterium that cause serious infectious diseases in human. The bacterium is resistant to several antibiotics by having beta-lactamase enzyme, efflux pumps to excrete antibiotics and also can produce biofilm to prevent the penetration of drugs[2,3]. *A. baumannii* is an important cause of hospital-derived infection or nosocomial infections in many hospitals, affecting people with compromised immune systems[15]. The bacterium has an ability to survive for a long time on common surfaces in the hospital that caused serious

problem when occurred with medical equipments in intensive care unit (ICU)[4].

Bacillus spp. is a group of Gram-positive spore forming bacteria 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, we could isolate *Bacillus amyloliquefaciens* that can produce secondary metabolites to inhibit various kinds of bacteria[8]. When crude proteins from *B. amyloliquefaciens* N3-8 were purified by precipitation with ammonium sulfate, can inhibited *B. pseudomallei* and other pathogens such as *Streptococcus pneumoniae, Corynebacterium diphtheria, Proteus vulgalis, Klebsiella pneumoniae and Acinetobacter baumannii*[8]. Therefore, the aim of this study was to test the ability of bioactive compounds from *B. amyloliquefaciens* N3-8 to inhibit and control *A. baumannii* by focusing on the reduction of these bacteria on plastic surface which is one of their problematic surface.

The objective of this study was to observe the range of inhibition and set a model to investigate the ability of crude proteins produced from *B. amyloliquefaciens*to kill *A. baumannii* on plastic surface.

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Material and Methods

1. Bacterial strains

B. amyloliquefaciens N3-8 was isolated from soil samples by Patcharaporn[8]. Twenty-eight *A. baumannii* isolateswere obtained from Srinagarind hospital, Faculty of Medicine, Khon Kaen University, Thailand.

2. The production of secondary metabolites form *B. amyloliquefaciens* N3-8 preparation

B. amyloliquefaciens N3-8 that produced secondary metabolites with inhibition activity against *A. baumannii* was cultured in 500 ml minimal medium at 37 degrees Celsius, 200 revolutions per minute (rpm) and collected the culture at 72 hours. Then, 60 % ammonium sulfate ($(NH_4)_2SO_4$) was used to precipitate proteins in the supernatant. The protein was harvested by centrifugation (Avanti® J-E, Beckman Coulter) at 17,600x *g* for 15 minutes, and then resuspended the protein by TE buffer pH 8.0 and filtered through a 0.2 µm membrane to get rid of bacteria. The protein concentration of the resuspended solution was measured by NanoDrop (Thermo scientific, US).

3. Crude protein inhibition against *A. baumannii* by agar well diffusion

The inhibitory activity of the crude proteins against *A. baumannii* was observed using agar well diffusion method[16] with some modification. Four milliliter of freshly cultured of *A. baumannii* N1 to N28 strains were inoculated in 96 ml of LB medium with shaking at 200 rpm and incubated at 37°C for 4 hours (log phase). Approximately 10^8 CFU/ml of each *A. baumannii* cells were swabbed on Mueller Hinton agar (MHA) plates in three directions and then punched 3 holes. Then, 100 µl of 27 mg/ml of crude proteins from *B. amyloliquefaciens* N3-8 was pipetted into a well. Imipenem (JW Pharmaceutical Corporation, Korea) 60 µg/ml, the drug of choice was used as a positive control that loaded into another well and production medium was used as a negative control. The plates was incubated at 37°C for 24 hours. The antimicrobial activity was measured by observing the clear zone of inhibition against the pathogenic bacteria.

4. Killing of *A. baumannii* N5 on plastic surfacebycrude proteins from N3-8

4.1 The efficiency to prevent *A. baumannii* N5 contamination

4.1.1 Detection of the number of bacterium by drop plate method

One hundred microliters of 0.5, 1, 2, 4, 8, 16 and 32 mg/ml of crude proteins from *B. amyloliquefaciens* N3-8 were spreaded in duplicate on sterile plastic plates and let them dry. Four milliliter of fresh cultured *A. baumannii* N5 was inoculated into 96 ml of LB medium and grew at 37°C with shaking at 200 rpm for 4 hours to reach log phase[9]. Then, one hundred microliters were spreaded on the same plastic plate and let it dry. Three microliters of phosphate-buffered saline (*PBS*), pH 7.2 were added on the plate and washed the surface. PBS then was 10 fold serially diluted and each dilution was drop onto MacConkey agar plate for the colony count (Figure1). The experiments used 1.5 % chlorhexidine that commonly used as antiseptic chemical in the hospitals

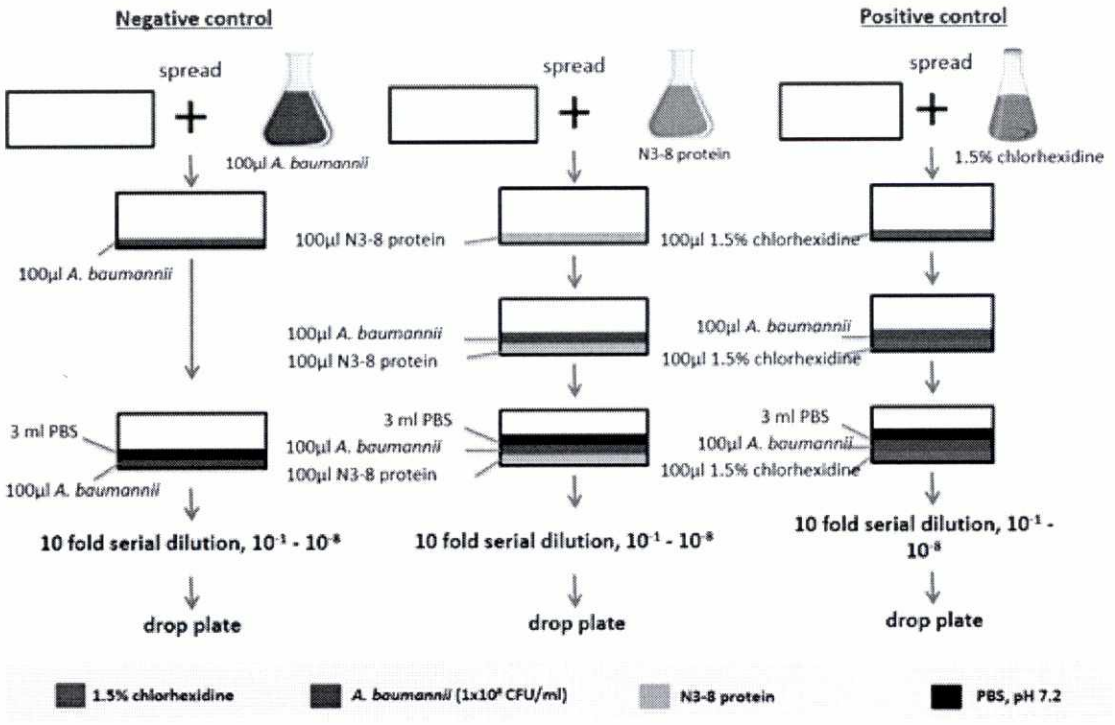

Figure 1 Schematic diagram shows steps to evaluate the efficiency of crude proteinsfrom *B. amyloliquefaciens* N3-8 to prevent contamination of *A. baumannii* N5 on a plastic surface. On the left was the negative control of *A. baumannii* on the plastic plate. In the middle was the experiment using crude proteins from *B. amyloliquefaciens* and on the right was the positive control using 1.5% chlorhexidine. Evaluation of the bacterial number left on the surface was done by drop plate method.

as the positive controls and the application of the *A. baumannii* N5 on plastic plate was used as a negative control.

4.1.2 Detection of the number of bacterium by streak plate

The concentrations of crude proteins from *B. amyloliquefaciens* N3-8 and the application of N5 was done similar to 4.1.1 excepted that the determination of *A. baumannii* N5 at 6 and 12 hrs were done by streak plate method. A sterile cotton swab was used to swab on the plastic plate surface at the end of the experiment and streak on one corner of the MacConkey agar plate and then streak out to obtain single colonies by sterile loop and incubated at 37°C for 24 hrs.

4.2 The efficiency against *A. baumannii* N5 that contaminated on the surface

4.2.1 Detection of the number of bacterium by drop plate method

One hundred microliters of the culture prepared as mentioned in 4.1.1 were spreaded induplicate on the surface of sterile plastic plates. After air dry, take 100 µl of1, 2, 4, 8, 16 and 32 mg/ml of crude proteins from *B. amyloliquefaciens* N3-8 and spread on the surface and allowed to dry before 3 ml of PBS pH 7.2 was added to the plate and mixed to wash the content. The samples were 10-fold serial diluted and drop onto MacConkey agar plate to obtain the colony count. The experiment was compared with the use of 1.5 % chlorhexidine (Figure 2). The negative control was 100 µl *A. baumannii* N5 that coating on plastic plate.

4.2.2 Detection of the number of bacterium by streak plate method

The concentrations of crude proteinsfrom *B. amyloliquefaciens* N3-8 and the application of N5 was done similar to 4.2.1 excepted that the determination of *A. baumannii* N5 at 6 and 12 hrs was done by using sterile cotton swab and streak plate to count the number of bacterium.

4.3 The efficiency against re-contamination of *A. baumannii* N5 on the surface

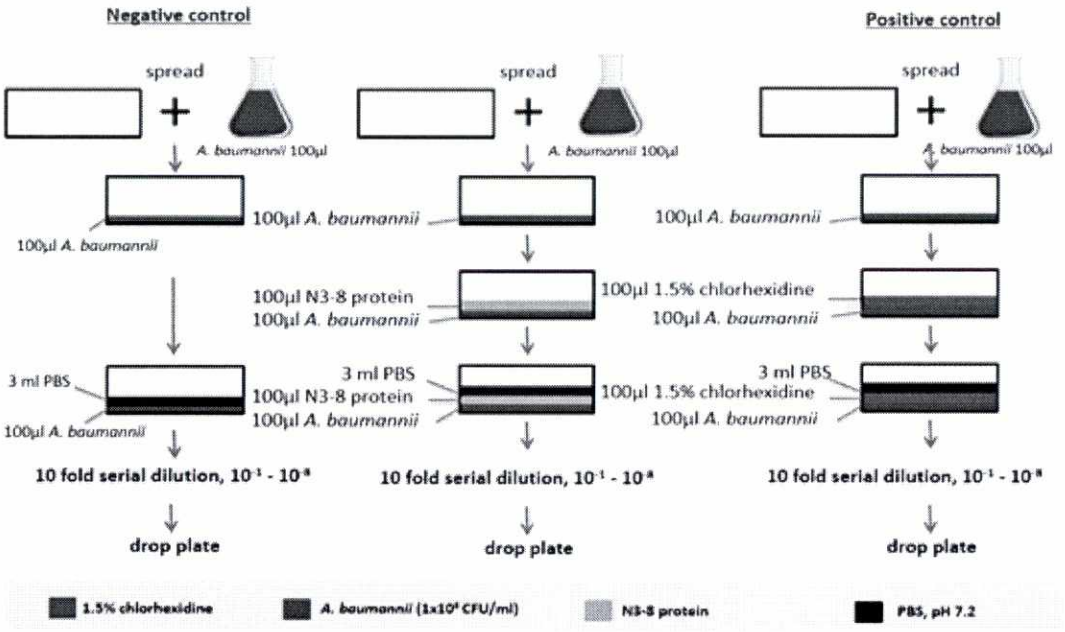

Figure 2 The schematic diagrams to observe the efficiency of crude proteinsfrom *B. amyloliquefaciens* N3-8 to reduce *A. baumannii* N5 on plastic plate. On the left was the negative control of *A. baumannii* on the plastic plate. In the middle was the experiment using crude proteins from *B. amyloliquefaciens* and on the right was the positive control using 1.5% chlorhexidine. Evaluation of bactericidal effect was done by counting the bacterium survival with drop plate technique.

4.3.1 Detection of the number of bacterium by drop plate and streak plate

One hundred microliters of the culture prepared as mention earlier were spread on the surface of plastic plate. After air dry, 100 µl of crude proteins from *B. amyloliquefaciens* N3-8 was spread on the surface and let it dry. Then, 100 µl of *A. baumannii* N5 culture were spread on top and let it dry. To determine the efficiency of proteins from *B. amyloliquefaciens* N3-8 to reduce the two-time application of *A. baumannii* N5, a streak plate methods was used to determine the number of bacteria left on the plate at 6 and 12 hrs.

Results

1. Antimicrobial inhibition test by agar well diffusion

The concentration of crude proteins that prepared by ammonium sulfate precipitation was 27 mg/ml. When 100 microliters of 27 mg/ml crude proteins from *B. amyloliquefaciens* N3-8 was used to test against 28 *A. baumannii*isolates (N1 to N28), the results were shown in Table 1. The crude proteins from N3-8 could inhibit 71% (20/28) of the 28 *A. baumannii* isolates while imipenem could inhibit only 21% (6/28). There were 7 isolates that showed no inhibition by both compounds. Interestingly, 15 isolates that resist to imipenem can be inhibited by crude proteins. There were 5 isolates that inhibited by antibiotic and the crude proteins and 1 isolate was inhibited by imipenem but not the crude proteins.

The example of clear zone of inhibition when the crude proteins were test against *A. baumannii* N4 that sensitive to imipenem and N5 that resist gave 15 and 17 mm clear zones around N3-8 crude proteins was shown in Figure 4. Imipenemthat used as a positive control gave a 22 mm clear zone and minimal media serve as a negative control did not give clear zone on N4 (drug susceptible). On the other hand, imipenem and minimal media showed no clear zone against*A. baumannii* N5. The *A. baumannii* N5 was chosen to test for the efficiency of crude proteins from *B. amyloliquefaciens* N3-8 to prevent *A. baumannii* contamination on plastic surface.

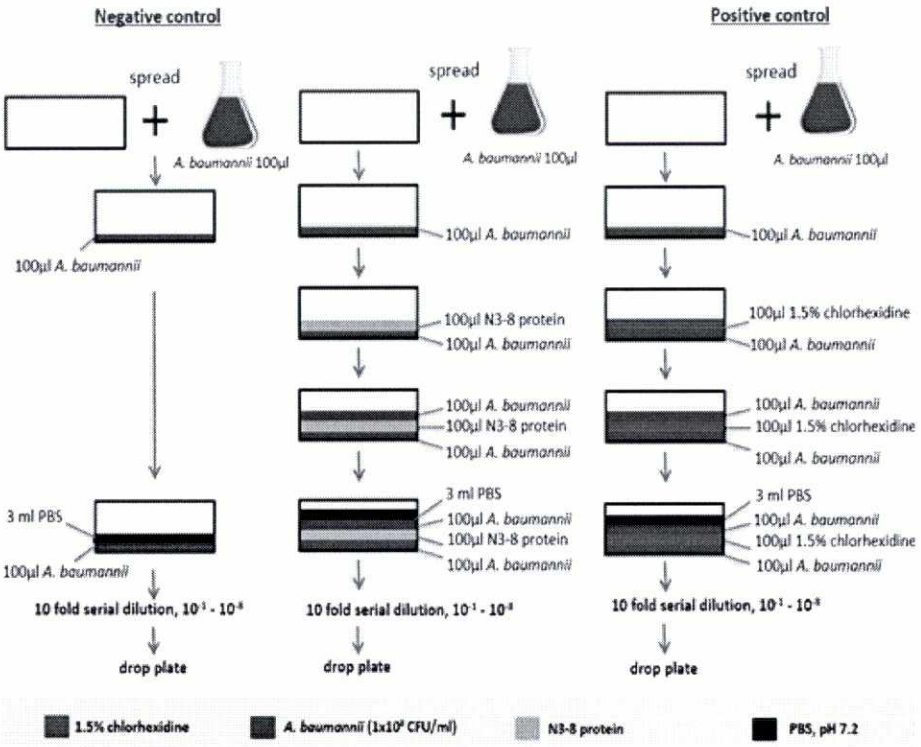

Figure 3 The schematic diagrams demonstrated the investigation of the crude proteins efficiencyfrom *B. amyloliquefaciens* N3-8 in preventing *A. baumannii* N5 re-contamination. On the left was the negative control of *A. baumannii* on the plastic plate. In the middle was the experiment using crude proteins from *B. amyloliquefaciens* and on the right was the positive control using 1.5% chlorhexidine. The survived bacterium was investigated by drop plate technique.

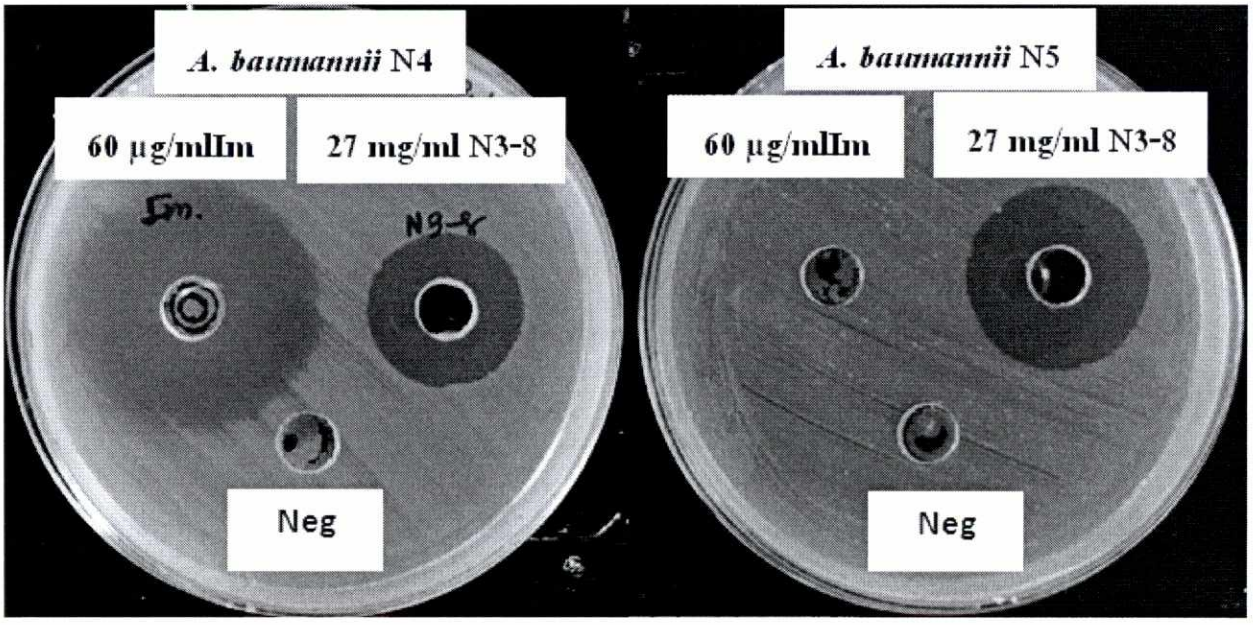

Figure 4 The antimicrobial inhibition test by agar well diffusion on *A. baumannii* lawn. *B. amyloliquefaciens* N3-8 crude proteins showed clear zone of inhibitory activity on both *A. baumannii* N4 and N5 lawn. **Neg** represents the 100 µl minimal medium serve as a negative control, **Im** represents 100 µl of 60 µg/ml imipenem, a drug of choice that N5 was resist to the antibiotic while N4 was susceptible.

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2. Killing of *A. baumannii* N5 on plastic surface by crude proteins from N3-8

2.1 The efficiency to prevent *A. baumannii* N5 contamination

2.1.1 Detection of the number of bacterium by drop plate method

When spreaded *A. baumannii* N5 on the plate that spread with 1, 2, 4, 8, 16 and 32 mg/ml of the crude proteins from *B. amyloliquefaciens* N3-8 compared with 1.5% chlorhexidine, the 10^6 CFU/ml bacterium was completely killed by both of them when compared to the control. The plate that treat with 0.5 mg/ml of the crude proteins cannot completely kill *A. baumannii* but can reduce the number of the bacterium by 2 $\log_{10}$ (5x 10^4 CFU/ml from 5x 10^6 CFU/ml). The positive control of 1.5% chlorhexidine also could completely kill all the bacterium (Table 2).

2.1.2 Detection of the number of bacterium by streak plate

When the number of bacteria was measured by streak plate, the outcome is similar to what obtained by drop plate method. At 6 and 12 hrs, most of the concentrations of crude proteinscan kill all *A. baumannii* N5 on the agar plate except by 0.5 mg/ml that a few of colony of *A. baumannii* N5 were appeared. The result in Figure 5 was the colonies appeared at 12 hrs of incubation.

2.2 The efficiency to kill *A. baumannii* N5 that contaminate and re-contaminate on the surface

2.2.1 Detection of the number of bacterium by drop plate method

Most of the concentrations of the crude proteins except 0.5 mg/ml can completely kill *A. baumanii* N5 the same way as when crude proteins were applied before re-contaminate of the bacterium. The 1.5% chlorhexidine also could kill all of the bacterium (Table 3). When *A. baumanii* N5 was re-applied onto the surface again, the condition that treated with 1.5% chlorhexidine and 2, 4, 8, 16 and 32 mg/ml of the crude proteins could kill all of the *A. baumanii* N5 but the condition that treat with 1 mg/ml decreased the bacterium for 2 $\log_{10}$ to be 1.2x10^3 CFU/ml (Table 3).

2.2.2 Detection of the number of bacterium by streak plate

The detection of *A. baumannii* N5 that applied before the crude proteins and re-applied twice by streak plate gave similar result as drop plate method (Table 3, Figure 6). The concentration of 1 mg/ml of the crude proteins cannot completely killed the bacterium after treated for both 6 and 12 hrs (Figure 6).

Table 1 The agar well diffusion method that tests the antimicrobial activity of 27 mg/ml crude proteins from *B. amyloliquefaciens* N3-8 against *A. baumannii* N1 to N28 strains. The results shown average of the inhibition zone ∅ (mm).

Strains	Inhibition zone ∅ (mm)		Strains	Inhibition zone∅ (mm)	
	Imipenem	N3-8		Imipenem	N3-8
N1	0	0	N15	0	0
N2	0	0	N16	26	14
N3	0	14	N17	18	13
N4	22	15	N18	0	12
N5	0	17	N19	0	0
N6	0	13	N20	0	13
N7	0	17	N21	0	15
N8	23	0	N22	0	15
N9	0	0	N23	0	0
N10	0	15	N24	0	12
N11	24	17	N25	0	13
N12	0	11	N26	0	0
N13	0	12	N27	35	12
N14	0	15	N28	0	12

Table 2 The efficiency of crude proteins from *B. amyloliquefaciens* N3-8 to prevent *A. baumannii* N5 contamination on a plastic surface

Conditions	Colony count (CFU/ml)
A. baumannii N5	5x 10^6
1.5% chlorhexidine + *A. baumannii* N5	0
0.5 mg/ml the crude proteins N3-8 + *A. baumannii* N5	5x 10^4
1 mg/ml the crude proteins N3-8 + *A. baumannii* N5	0
2 mg/ml the crude proteins N3-8 + *A. baumannii* N5	0
4 mg/ml the crude proteins N3-8 + *A. baumannii* N5	0
8 mg/ml the crude proteins N3-8 + *A. baumannii* N5	0
16 mg/ml the crude proteins N3-8 + *A. baumannii* N5	0
32 mg/ml the crude proteins N3-8 + *A. baumannii* N5	0

Discussion

Bacillus spp. can produce secondary metabolites that like antimicrobial, antiviral, antitumor activities and immunosuppressive metabolites to compete for their survival in the environment. The bacterium also produce secondary metabolites that benefit for symbiosis between microbe and other organism[10]. These compounds attracted the interest of pharmaceutical companies to develop the biological active compounds for new drug or agricultural company used them as biological control agent against plant pathogen[17]. Several strains of *B. amyloliquefaciens* can produce multiple antimicrobial compounds to suppress fungal and bacterial growth *in vitro*[12-14]. In previous work, *B. amyloliquefaciens* N3-8 was isolated from soil and showed broad spectrum to inhibit several pathogens including *A. baumannii*.

In this study, the crude proteins from *B. amyloliquefaciens* N3-8 showed antimicrobial activity against most the *A. baumannii* isolates including those that resist to imipenem, the drug of choice. As 28 isolates of *A. baumannii* were obtained from Srinagarind hospital without identification of their drug susceptibility, the drug sensitivity of these isolates were confirmed by dish diffusion that showed similar outcome (data not shown). It is quite surprised that the majority of isolates from the hospital were resist to the antibiotic. Among these, 15 of them can be inhibited by the crude proteins. However, 7 isolates showed no inhibition by both crude proteins and imipenem. The antimicrobial compounds from *B. amyloliquefaciens* have been reported to target the bacterial cell wall and cause lysis[18]while imipenem kill bacteria by inhibit cell wall synthesis. There similar target with different action may possibly synergist to get a better killing activity. Therefore, after purification and characterization of the crude proteins, the compound may be tested as a new drug candidate. Moreover, combination of the active compound with the drug of choice may be able to kill all drug resistan tisolates of *A. baumanii*.

When crude proteins were tested for their activity to

Table 3 The efficiency of crude proteins from *B. amyloliquefaciens* N3-8 against *A. baumannii* N5 that contaminate and re-contaminateon plastic surface

Conditions	Colony count (CFU/ml)
A. baumannii N5	5 x 10^5
A. baumannii N5 + 1.5% chlorhexidine	0
A. baumannii N5 + 1 mg/ml the crude proteins N3-8	0
A. baumannii N5 + 2 mg/ml the crude proteins N3-8	0
A. baumannii N5 + 4 mg/ml the crude proteins N3-8	0
A. baumannii N5 + 8 mg/ml the crude proteins N3-8	0
A. baumannii N5 + 16 mg/ml the crude proteins N3-8	0
A. baumannii N5 + 32 mg/ml the crude proteins N3-8	0
A. baumannii N5 + 1.5% chlorhexidine + *A. baumannii* N5	0
A. baumannii N5 + 1 mg/ml the crude proteins N3-8 + *A. baumannii* N5	1.2 x 10^3
A. baumannii N5 + 2 mg/ml the crude proteins N3-8 + *A. baumannii* N5	0
A. baumannii N5 + 4 mg/ml the crude proteins N3-8 + *A. baumannii* N5	0
A. baumannii N5 + 8 mg/ml the crude proteins N3-8 + *A. baumannii* N5	0
A. baumannii N5 + 16 mg/ml the crude proteins N3-8 + *A. baumannii* N5	0
A. baumannii N5 + 32 mg/ml the crude proteins N3-8 + *A. baumannii* N5	0

Proceeding

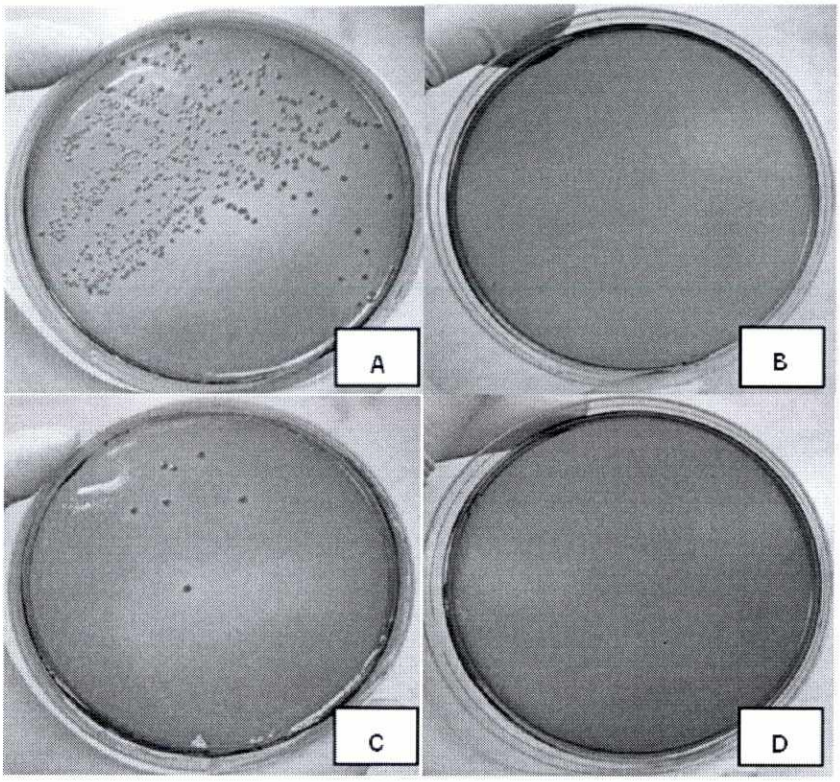

Figure 5 The efficiency of crude proteins from *B. amyloliquefaciens* N3-8 to reduce *A. baumannii* N5 as observed by streak plate method. The figure showed result after 12 hrs of incubation and streaking on MacConkey agar plate. **A**) *A. baumannii* N5 negative control, **B**) *A. baumannii* N5 treated with 1.5 % chlorhexidine served as a positive control, **C**) *A. baumannii* N5 with 0.5 mg/ml of the crude proteins, **D**) *A. baumannii* N5 treated with 1 mg/ml of the crude proteins.

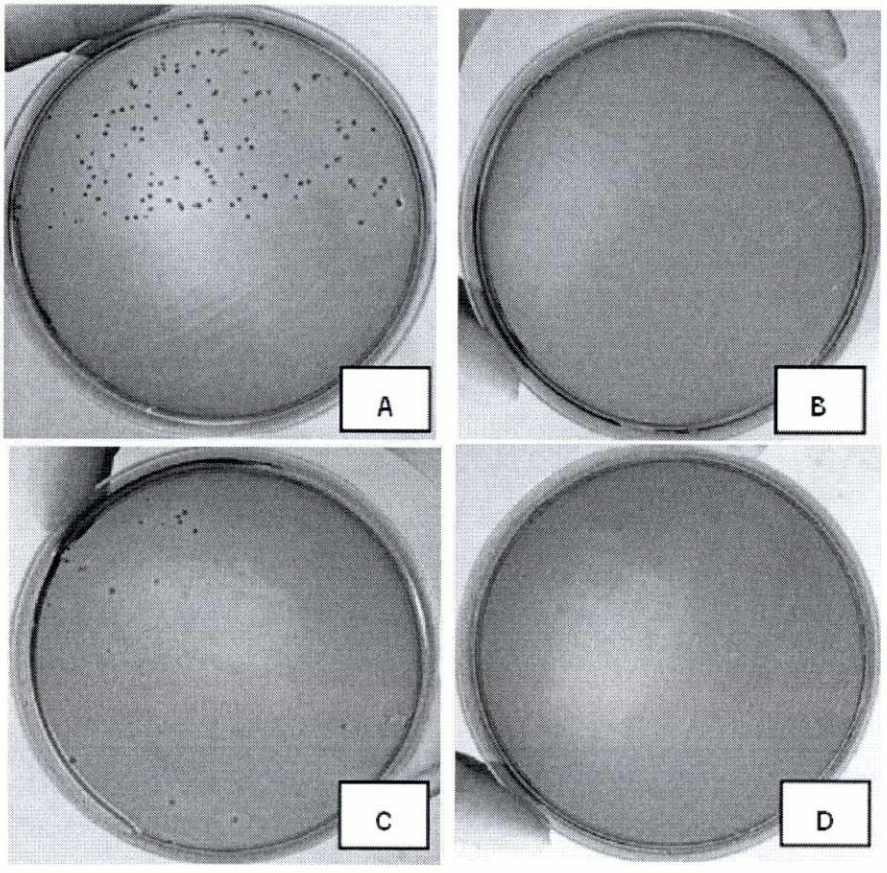

Figure 6 The efficiency of crude proteinsfrom *B. amyloliquefaciens* N3-8 against *A. baumannii* N5 that re-contaminated on a plastic surface. **A**; *A. baumannii* N5 without treatment that served as a negative control, **B**) *A. baumannii* N5 treat with 1.5 % chlorhexidine served as a positive control, **C**) *A. baumannii* N5 treated with 1 mg/ml of the crude proteins, **D**) *A. baumannii* N5 treated with 2 mg/ml of the crude proteins.

prevent contamination or kill the contaminated and re-contaminated *A. baumanii* N5, they gave similar outcome when compared with 1.5% chlorhexidine. The crude proteins may at this point concluded to have no advantage over chemical compound when applied on general surface. If the compound was proved to cause no allergic effect to human skin, it may show more advantage on bathing the patients especially during *A. baumanii* cause nosocomial infection problem. Lastly, the activity of crude proteins reported here may contributed by several metabolites produced by *B. amyloliquefaciens* N3-8. Again, purification and characterization of them may lead us to make use of the compounds better than at present time.

Conclusion

The crude proteins from*B. amyloliquefaciens* N3-8 can inhibit severali solates of *A. baumannii* including the drug resistant isolates. After purification and characterization, they may be able to develop as a new drug in the future.

Acknowledgements

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