นิพนธ์ต้นฉบับ • Original Article

ฤทธิ์ต้านจุลชีพและการยับยั้งการเกาะของสารลดแรงตึงผิวชีวภาพแบบหยาบจากเชื้อบาซิลลัสต่อเชื้อ *Acinetobacter baumannii* ที่แยกได้จากผู้ป่วยโรงพยาบาลศรีนครินทร์

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Antimicrobial and Antiadhesive Activities of the Crude Biosurfactant from *Bacillus* sp. Against Clinical Isolates of *Acinetobacter baumannii* from Srinagarind Hospital

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หลักการและวัตถุประสงค์: *Acinetobacter baumannii* เป็นสาเหตุของการติดเชื้อในโรงพยาบาล เชื้อสามารถสร้างไบโอฟิล์มบนผิวอุปกรณ์ทางการแพทย์ซึ่งสัมพันธ์กับการดื้อยาปฏิชีวนะและการเกิดโรคติดเชื้อแบบเรื้อรัง สารลดแรงตึงผิวชีวภาพที่ผลิตจากเชื้อจุลชีพมีคุณสมบัติต้านแบคทีเรีย รา และไวรัส การศึกษาก่อนนี้พบว่าสารลดแรงตึงผิวจาก *Bacillus* sp. สามารถป้องกันการสร้างไบโอฟิล์มของเชื้อก่อโรคในมนุษย์ได้ วัตถุประสงค์ของการศึกษานี้เพื่อประเมินคุณสมบัติของสารลดแรงตึงผิวแบบหยาบจากบาซิลลัสต่อการยับยั้งการเจริญและการเกาะของ *A. baumannii* ที่แยกได้จากผู้ป่วยโรงพยาบาลศรีนครินทร์

วิธีการศึกษา: สารลดแรงตึงผิวชีวภาพแบบหยาบสกัดจาก *B. amyloliquefaciens* 2 สายพันธุ์ ได้แก่ KKU3 และ KKU14 และนำมาทดสอบคุณสมบัติต้านเชื้อ *A. baumannii* ใน 96-well plate และยับยั้งการเกาะบนสายน้ำเกลือ

ผลการศึกษา: สารลดแรงตึงผิวชีวภาพแบบหยาบจาก *B. amyloliquefaciens* สายพันธุ์ KKU14 ยับยั้งการเจริญของ *A. baumannii* ในช่วง 2-3%, 32-33%, 35-37%, 78-82% และ 81-84% ที่ความเข้มข้น 50, 80, 100, 150 และ 200 มิลลิกรัม/มิลลิลิตร ตามลำดับ และยับยั้งการเกาะของ *A. baumannii* บนสายน้ำเกลือโดยเทียบกับสายน้ำเกลือที่ไม่ได้เคลือบสารลดแรงตึงผิว

Background and Objectives: *Acinetobacter baumannii* is a cause of hospital-acquired infection. It can form biofilm on medical devices which is related to antibiotic resistance and the chronic infections. Biosurfactants (BS) were produced from microorganisms that have antibacterial, antifungal, and antiviral activities. Previous study showed BS from *Bacillus* sp. prevent biofilm formation of pathogens in human. The aims of this study were to determine the activities of crude BS from *Bacillus* sp. to inhibit the growth and adhesion of clinical *A. baumannii* isolates from Srinagarind hospital.

Methods: Two samples of crude BS were extracted from 2 isolates of *B. amyloliquefaciens* KKU3 and KKU14. Crude biosurfactants were determined antimicrobial activity on 96-well plates and antiadhesive activity on intravenous (IV) tubes.

Results: Crude BS sample from *B. amyloliquefaciens* KKU14 strain can inhibit the growth of five clinical isolates *A. baumannii* which were inhibited in range of 2-3%, 32-33%, 35-37%, 78-82% and 81-84% at 50, 80, 100, 150 and 200 mg/ml respectively, and inhibited adhesion of *A. baumannii* on IV tube when compare

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สรุป: สารลดแรงตึงผิวนี้อาจใช้เป็นสารทำความสะอาดอุปกรณ์ทางการแพทย์ในห้องปฏิบัติการหรือเคลือบผิวอุปกรณ์เพื่อป้องกันการปนเปื้อนของเชื้อก่อโรคในโรงพยาบาลและอาจเป็นทางเลือกของการรักษาเฉพาะที่ในอนาคต

คำสำคัญ: สารลดแรงตึงผิวชีวภาพ, บาซิลลัส, *Acinetobacter baumannii*

with untreated IV tube.

Conclusions: BS may be used as a new clean up agent for washing the medical-equipments in laboratory or a coating agent on medical devices to prevent the contamination of pathogens in hospital and may use as an alternative therapeutic agent for local infection in the future.

Keywords: Biosurfactant, *Bacillus* sp., *Acinetobacter baumannii*

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Introduction

The biofilm formation of *A. baumannii* on medical devices especially implanted catheters which related to urinary tract infection.[1] This bacteria is a causative agents of resistance among hospital-acquired infection and ventilator-associated pneumonia patients in Srinagarind hospital, Thailand.[2] The bacterial biofilm increase antibiotic resistance and contributes to the chronicity of infections[3] that is an important problem of worldwide public health.

Biosurfactants (BS) are new compound using in the medical field for protection and removal of bacterial biofilm have increased during the past decade.[4] The wide range of BS chemical structure are synthesized and secreted from microorganisms vary from low to high-molecular-mass.[5] These compounds have amphipathic property of hydrophilic and hydrophobic moieties within the same molecule that show the effect to bacterial membrane.[4] The various *Bacillus subtilis* strains produced the potent extracellular lipopeptide-surfactin BS.[6] In 2008, the first evidence reported that the uncharged and negatively charged of BS-lipopeptides from *B. subtilis* can alter the morphology of gram-negative bacteria.[7] *Bacillus amyloliquefaciens* produced lipopeptide-bamylocin biosurfactant which strongly inhibited the growth of plant-pathogenic fungi.[8] The cell-free supernatant of *B. amyloliquefaciens* inhibit the growth of *Candida albicans*.[9] BS-pseudofactin II can be used as a disinfectant or surface coating agent against colonization and biofilm formation of uropathogens on different surfaces e.g. implants or urethral catheters.[10] The crude BS produced by lactobacilli exhibit strong antimicrobial, antiadhesive and antibiofilms properties against multi-drug-resistant pathogens.[11] In this study focus on BS of *Bacillus* sp. which are isolated from soil in the absence *Burkholderia pseudomallei* area[12, 13], because we expected they may secreted the BS against *B. pseudomallei* and may have antimicrobial, antiadhesive and antibiofilms inhibit other pathogens which can inhibit *A. baumannii*.

Therefore, the BS is an alternative therapeutic approach in medical application for the prevention and/or treatment of hospital-acquired infections. Here is the first study about the activity of crude BS from *Bacillus* spp. can inhibit adhesion of *A. baumannii* on intravenous tube.

Materials and methods

Strains and growth conditions

Two isolates of *B. amyloliquefaciens* strains KKU3 and KKU14 were isolated from the soil samples by Miss Chotima Photisap[12] and Miss Patcharaporn Boottanun[13], meliodosis research center, Faculty of Medicine, Khon Kaen University, Thailand. Five clinical isolates of *A. baumannii* were provided from Srinagarind hospital laboratory, Faculty of Medicine, Khon Kaen University, Thailand. All bacteria were stored at -80 °C in Luria-Bertani

(LB) medium (Difco, Sparks, MD, USA) containing 20% (v/v) glycerol.

Inoculum preparation

Each strain of *Bacillus* sp. was cultivated in LB broth, incubation in shaker incubator at 37 °C, 200 rpm for 18 h.

Biosurfactant production

BS was produced from each stain of *Bacillus* sp. according to Phitnaree et al.[14] Production medium was Mckeen medium which consisted of (w/v) 0.5-2.5% glucose, 1.0% monosodium glutamate, 0.3% yeast extract, 0.1% $MgSO_4.7H_2O$, 0.1% K_2HPO_4, 0.05% KCl and 0.1%v/v of trace element (0.64 g of $MgSO_4.7H_2O$, 0.16 g of $CuSO_4.5H_2O$ in 100 mL of distilled water).[14] The inoculum size was 5% (v/v). Then, incubated conditions at 30 °C, 200 rpm for 36 h. The cultured medium was centrifuged at 13,000 *xg* at 4 °C for 15 min. The supernatant was filtered through 0.2 μm membrane filter. The BS was isolated from filtrated supernatant by precipitation with 6 N HCl solution and left at 4 °C overnight. The precipitate was dissolved in acid water, neutralized to pH 8 using 2 N NaOH and finally, the crude BS was lyophilized.[15] The crude BS powder was resuspended in phosphate-buffered saline (PBS) before testing.

Antimicrobial assay

Antimicrobial activity of BS was determined according to Janek et al.[10] Briefly, 50 μl volumes of LB medium were dispensed into each wells of a 96-well microplate. Then, 50 μl volumes of crude BS at various concentrations solution in PBS were added into the microplate wells and mixed with the medium. Then, 2 μl of each overnight clinical isolate *A. baumannii* culture (OD_{600} = 0.1) was inoculated to each well. Negative control was sterile medium with BS and growth control was medium with bacterial suspension (without BS) and positive control was 0.1% TritonX-100 with bacterial suspension. The microplates were incubated for 24 h at 37 °C. Measuring the growth using microplate reader at wavelength 600 nm. The percentages of growth inhibition for each clinical isolate at different crude BS concentrations was calculated as follow.

% growth inhibition = $[1 - (OD_T/OD_C)]x100$. Where OD_T represents the optical density of the well with a given crude BS concentration and OD_C is the optical density of the control well (growth without crude BS).

Antiadhesive assay on intravenous tubes (IV tubes)

Antiadhesive ability of BS to *A. baumannii* on intravenous tube was determined according to Janek et al.[10] Briefly, a sterile tube containing 4 cm of sterile IV tube[10] (Unomedical A/S ConvaTec Company, Denmark) was pretreated with 2 ml of 20 mg/ml of crude BS by adding in a tube (while untreated tube was treated with PBS), incubated for 2 h at 37 °C and subsequently washed twice with PBS. Overnight culture of *A. baumannii* (OD_{600} = 1.0) were added in IV tubes and incubated overnight at 37 °C. The *A. baumannii* culture was removed and IV tubes were washed with distilled water for 3 times. After washing, IV tubes were fixed with 3 ml of absolute ethanol for 10 min and stained with 3 ml of 0.1% crystal violet for 20 min. The stained biofilm was rinsed gently 3 times with distilled water, allowed to dry at room temperature for 15 min. The results were observed the purple of crystal violet compared between treated and untreated tubes which the purple indicated the concentration of *A. baumannii* on IV tubes. Assay was carried out three independent experiments.

Statistical analysis

A paired *t* test was used to compare mean-values of two population (treated and untreated with crude BS) by the statistical significance in *p-value*, significant different at $p<0.05$.

Results

Five clinical isolates *A. baumannii* (A1, A2, A3, A4, and A5) were treated with crude BS KKU14 from *B. amyloliquefaciens* at 50, 80, 100, 150 and 200 mg/ml. The antimicrobial activity of crude BS KKU14 against 5 clinical *A. baumannii* in dose-dependent manner as shown

in figure 1. The results showed that 5 clinical isolates of *A. baumannii* were inhibited with crude BS KKU14 in the ranges of 2-3%, 32-33%, 35-37%, 78-82% and 81-84% at 50, 80, 100, 150 and 200 mg/ml, respectively. Therefore, crude BS KKU14 may be used as an antimicrobial agents for treatment in the future. Two crude BS samples from *B. subtilis* couldn't kill *A. baumannii* (data not shown). However, this crude BS should be purified and characterized structure of effective compound. Characterization of secondary metabolites and other secreted compounds from *B. subtilis*[16] and also *B. amyloliquefaciens*[17] may have advantage for medical application.

The sterile IV tubes were treated with crude BS KKU14 at 20 mg/ml then inoculated with *A. baumannii* 18 h culture. The results revealed that crude BS KKU14 had antiadhesive activity against *A. baumannii* on IV tube by observed the intensity of blue color when compared with untreated tube as shown in figure 2. Crude BS KKU14 at 20 mg/ml couldn't kill *A. baumannii in vitro* from antimicrobial assay (not show data). However, low

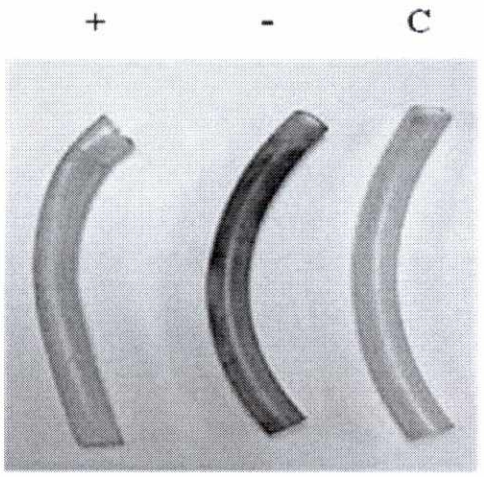

Figure 2 Crude BS KKU14 inhibits biofilm formation on intravenous tubes. Pretreatment of crude BS KKU14 on IV tubes before added with overnight cultures of *A. baumannii*. The sterile tubes were compared between treated (+) and untreated (-) tube with crude BS at 20 mg/ml and control tube (C). Assay was carried out three independent experiments.

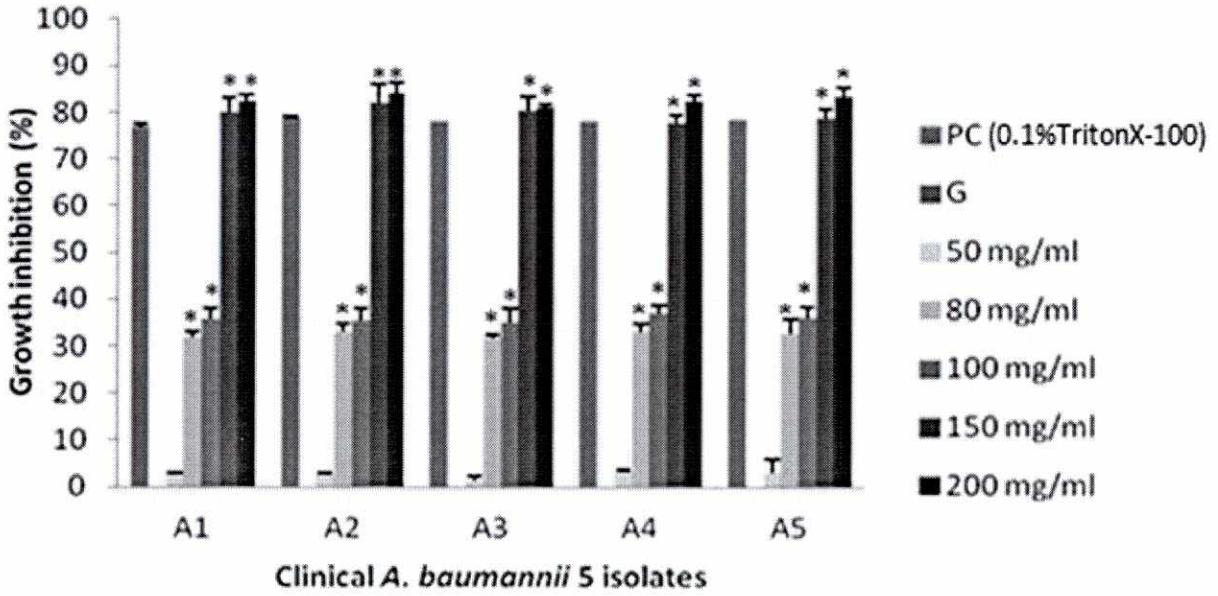

Figure 1 Antimicrobial activity of crude BS KKU14 against 5 clinical isolates of *A. baumannii*. Crude BS KKU14 was used at 50, 80, 100, 150 and 200 mg/ml. Used a commercial surfactant 0.1%TritonX-100 as a positive control (PC) and untreated by BS as a growth (G) control. The results represent the mean values. Paired *t* test was used for determine the statistical significance of the treated versus untreated conditions (*, $p<0.05$).

concentration (20 mg/ml) of crude BS may be used as a cleaning agents or antibiofilm forming agents on equipments or medical devices.

Discussions

Biosurfactants are the one group of secondary metabolites which are secreted from *Bacillus* sp., have a wide range of chemical structure and several biological activity such as antiadhesive, antimicrobial, antiviral, antifungal, antitumor, and immunosuppressive activities that allow the bacterium to survive in its natural environments.[4, 18] These compounds are amphiphilic property that show the effect to bacterial membrane.[4] The extracted-BS from lactobacilli showed strong inhibition the growth of multi-drug-resistant pathogens include *A. baumannii*, *E. coli*, and *S. aureus* which showed non-toxic to mammalian cell line at 25-200 mg/ml. The 50 mg/ml of extracted-BS could inhibit adhesion of *A. baumannii* and *E. coli* while *S. aureus* was used at 25 mg/ml.[11] Therefore, the ability of BS to inhibit pathogens was related with strain of biosurfactant-producing bacteria and dose-dependent manner. In this study, crude BS of *B. amyloliquefaciens* KKU14 strain at various concentrations were used to determine antiadhesive activity, we found that 20 mg/ml of crude BS KKU14 could prevent adhesion of *A. baumannii*.

The purified-surfactin-BS from *B. subtilis* inhibit biofilm formation of *Salmonella enterica* on urethral catheter.[19] The crude BS KKU14 showed antiadhesive activity on IV tube (as the pretreatment) as it inhibit initial step of biofilm forming that may use as the antimicrobial-coating agent. However, posttreatment of crude BS KKU14 on IV tube was needed to confirm antibiofilm-forming activity. Moreover, crude BS should be tested cytotoxicity. Although, high concentration of crude BS show non-toxic may have not to purify or classify BS. Finally, characterization of effective compound in crude BS is very important for applications in the future.

Conclusions

Crude BS KKU14 sample from *B. amyloliquefaciens* can inhibit the growth of 5 clinical isolates of *A. baumannii* which are inhibited in the ranges of 2-3%, 32-33%, 35-37%, 78-82% and 81-84% at 50, 80, 100, 150 and 200 mg/ml, respectively. Crude BS KKU14 also has antiadhesive activity against *A. baumannii* on intravenous (IV) tube when compare with untreated tube. Thus, BS may be used as a new clean up agent for washing the equipments in laboratory or a coating agent on medical devices for prevention the pathogens contamination particular implanted medical for long term and may be an alternative therapeutic agent in the future.

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