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Screening, Identification and Antibacterial Activities of Effective Thermotolerant *Bacillus* spp. Strains Isolated from Raw Milk

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ABSTRACT

Forty-one isolates of *Bacillus* species were isolated from raw milk, analyzed using the spot on lawn and agar diffusion method in terms of their general inhibition effects to test bacteria (*Escherichia coli* TISTR 887 and *Staphylococcus aureus* TISTR 517). The results demonstrated that most isolates are effective against Gram-positive and Gram-negative bacteria whereas their extensive inhibition effect is particularly against Gram-positive bacteria. Only 2 effective thermotolerant isolates, BA8 and BA16, exerted broad spectrum antibacterial activities against both test bacteria. Based on biochemical and physiological properties, they were classified as *Brevibacillus laterosporus* and *Geobacillus thermoglucosidasius*, respectively.

Keywords: *Bacillus* species, *Escherichia coli*, *Staphylococcus aureus*

INTRODUCTION

Polypeptide antibiotics produced by *Bacillus* that are used in medical treatments are bacitracin, gramicidin S, polymyxin, tyrotricidin which constitute the *Bacillus* bacteria have been gaining importance as a result of studies. The *Bacillus* species that produce antibiotics are *B. subtilis*, *B. polymyxa*, *B. brevis*, *B. licheniformis*, *B. circulans* and *B. cereus* [1-4]. In these studies, it is stated that antibiotics produced by the *Bacillus* species are more effective for Gram-positive bacteria; however, the production of large spectrum and anti-fungal antibiotics that are effective for Gram-negative is relatively low [1,3,5]. *Bacillus* species have a wide range of antimicrobial activities since they are used as antiviral agents [6], anti-fungal agents [7], anti-mycoplasma agents [8] and anti-ameobocytic agents [9].

This study attempts to screening and investigate the antibacterial activity of 41 *Bacillus* species isolated from raw milk against test bacteria.

MATERIALS AND METHODS

Isolation, identification and growth conditions

Raw milk samples were taken from the University farm office of Walailak University, Nakhon Si Thammarat in Thailand. Each sample was heated at 80 °C for 30 min in a water bath. Then the liquid was serially diluted in sterile distilled water, and the dilution from 10^{-1} - 10^{-10} was plated on a nutrient agar (NA) medium. Plates were incubated at 37 °C for 24 - 48 h. Then, the selected bacteria colonies were streaked on a Brain Heart Infusion (BHI) medium for antibacterial activities determination.

In the identification of isolated bacteria species, standard taxonomic descriptions from Sneath [10] were used. Also, API 50CH (API Laboratory Products Ltd., Biomerieux) was used to determine the biochemical and carbohydrate fermentation patterns. The experiment was performed at 37 °C. The results of biochemical tests and carbohydrate fermentation were determined after 24 and 48 h, and *Bacillus* species were identified.

Test bacteria

The test bacteria (*Staphylococcus aureus* TISTR 517 and *Escherichia coli* TISTR 887) used in this study were obtained from the Thailand Institute of Scientific and Technological Research, Bangkok, Thailand. Bacteria were incubated at 37 °C and were activated by incubating for a period of 24 h in a Mueller Hinton (M-H) broth medium (Oxoid). Both strains were maintained in the M-H broth medium containing 15 % glycerol at –80 °C. For routine work, they were kept on an M-H agar medium.

Detection inhibitory effect

Detection of antibacterial activity was carried out by the spot on lawn method [11]. Multiple plates of 10-fold serial dilution were overlaid with test bacteria and

incubated under aerobic conditions for 24 h at 37 °C and 45 °C. The colonies producing zones of growth inhibitor in the test bacteria lawn were selected and isolated from the agar using a sterile pasture pipette to remove a small plug containing the colony of interest. The agar plug was grown in a BHI broth medium overnight and streaked out on a BHI agar medium to get the pure culture. The culture fluid (CF) of each colony (in a BHI medium at 37 °C for 24 h) was prepared as a sample to determine the inhibitory effect using agar-well diffusion.

The determination of the inhibitory effect of isolates on test bacteria was carried out according to the agar-well diffusion method. All test bacteria were cultured on an M-H broth medium and incubated at 37 °C for 24 h. The M-H agar medium (20 ml) was poured into each sterile Petri dish (90 mm diameter). Suspensions (100 µl) of test bacteria cultured for 24 h were spread on the plates, and wells 6 mm in diameter were punched in the agar with a sterile tip. The *Bacillus* cultures were centrifuged at 6,000 g for 15 min to remove cell debris. After centrifugation, each CF (100 µl) was directly filled into the wells of agar plates inoculated with test bacteria. The plates were incubated for 24 h at 37 °C, and the diameter of the inhibition zone was measured with calipers in millimeters. The measurements were done basically from edge at the zone to the edge of the well [12-14].

Determination of growth at 50 °C

To determine effective thermotolerant *Bacillus* strains, 1 colony from a 1 day culture was inoculated into the BHI agar medium and incubated for 24 and 48 h at 50 °C. The growth was evaluated by observing the colony on the surface of the BHI agar medium.

RESULTS

Screening for *Bacillus* producing antibacterial substances

Forty-one *Bacillus* strains isolated from raw milk from the University farm office, Walailak University, Nakhon Si Thammarat, Thailand were primary examined for antibacterial activity by the spot on lawn method. The *S. aureus* TISTR 517 and *E. coli* TISTR 887 were used as test bacteria. They were divided into 2 groups according to inhibition activity. The group I, broad spectrum activity, showed inhibition activity to both test bacteria, they were BA26, BA27 and BA38. The isolates belonging to group II demonstrated narrow spectrum activity and could only inhibit growth of *S. aureus* TISTR 517, they were BA8, BA14, BA16 and BA29 as shown in **Table 1**. However, the CF of BA8 and BA16 inhibited growth of both test bacteria when examined by agar-well diffusion. Therefore only 7 isolates were further studied for effective thermotolerance and antibacterial activities against both test bacteria.

Table 1 Antibacterial activity of the *Bacillus* species isolated from raw milk.

Isolates	Inhibition zone (mm)			
	Spot on lawn		Agar well diffusion	
	E. coli	*S. aureus*	*E. coli*	*S. aureus*
BA8	0.00	15.00	11.25	16.00
BA14	0.00	15.25	ND	ND
BA16	0.00	16.54	12.00	15.00
BA26	11.25	13.00	0.00	20.00
BA27	12.25	15.00	ND	ND
BA29	0.00	12.00	ND	ND
BA38	13.52	14.00	ND	ND

ND, not determined.
E. coli, *Escherichia coli* TISTR 887; *S. aureus*, *Staphylococcus aureus* TISTR 517.

Identification of selected strains

Seven effective isolates from group I and group II were selected for further study. As shown in **Table 2**, all isolates were gram positive, sporeforming and catalase producing. All isolates were able to grow at high temperatures up to 45 - 50 °C, except the isolates BA14 and BA29. Therefore, the isolates BA8, BA16, BA26, BA27 and BA38 were categorized as thermotolerant strains. Since we are more interested in the thermotolerant strains, therefore only the isolates BA8, BA16, BA26, BA27 and BA38 were further studied for biochemical tests.

Table 2 Relevant properties of *Bacillus* species isolated from raw milk.

Isolates	Macroscopic examination				Microscopic examination			Catalase activity	Growth at 50 °C
	Form	Elevation	Surface	Edge	Shape	Gram	Spore		
BA8	circular	flat	smooth	entire	rod	positive	+	+	G
BA14	circular	flat	smooth	entire	rod	positive	+	+	NG
BA16	circular	flat	smooth	entire	rod	positive	+	+	G
BA26	circular	convex	smooth	entire	rod	positive	+	+	G
BA27	circular	convex	smooth	entire	rod	positive	+	+	G
BA29	circular	convex	smooth	entire	rod	positive	+	+	NG
BA38	circular	flat	smooth	entire	rod	positive	+	+	G

G, growth; NG, no growth.

The ability to utilize various carbon sources was determined using an API 50 CHB test strip. Initial identifications made by API database correlation indicated 59.1 %, 91.5 %, 93.6 %, 55.0 % and 99.9 % similarity to *Brevibacillus laterosporus* BA8, *Geobacillus thermoglucosidasius* BA16, *Brevibacillus* non reactive BA26, *Brevibacillus* non reactive BA27 and *Bacillus pumilus* BA38, respectively. Unfortunately, isolate BA27 and BA38 died during storage.

DISCUSSION AND CONCLUSIONS

The bacterial strains secreting antibacterial substances were screened from raw milk in Walailak University, Thailand. As many antibacterial producers belong to the *Bacillus* species, which can produce heat-resistant spores [10], the collected samples were heated at 80 °C to kill the vegetative cells and other bacteria. Then the surviving spores were harvested, spread on an NA medium and incubated at 37 °C for 24 h.

In this study, it was determined that effective thermotolerant *Bacillus* isolates (*Brevibacillus laterosporus* BA8, *Geobacillus thermoglucosidasius* BA16, *Brevibacillus* non reactive BA26, BA27 and *Bacillus pumilus* BA38) showed an inhibition zone diameter of 12.0 to 16.5 mm against *S. aureus* TISTR 517, and that only *Brevibacillus* non reactive BA26, BA27 and *Bacillus pumilus* BA38 showed an inhibition zone diameter of 11.2 to 13.5 against *E. coli* TISTR 887, when using the spot on lawn method. Various strains of *Bacillus* species displayed antimicrobial activity against *Pseudomonas aeruginosa*, *P. fluorescens* RSKK 380, *B. thuringiensis* RSKK 380, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *Micrococcus luteus*, *M. flavus*, *Yersinia enterocolitica*, *Bacillus subtilis*, *B. megaterium* [2,3,5,13,14].

The other significant findings of our study are as follows: both *Brevibacillus laterosporus* BA8 and *Geobacillus thermoglucosidasius* BA16 have inhibition zones of 11 - 12 mm against *E. coli* TISTR 887, and they have 15 - 16 mm inhibition zones against *S. aureus* TISTR 517, when using agar well diffusion. It is found that *S. aureus* which was resistant to methicillin (MRSA) was also sensitive to all effective thermotolerant *Bacillus* isolates (data not shown). Various strains of MRSA were also reported that are sensitive to the bioactive crude of *Brevibacillus laterosporus* SA14 [15]. The *Bacillus* strains were most active against Gram-positive but not Gram-negative bacteria [1,5]. The findings of the present study indicate that the effective thermotolerant *Bacillus* isolates have antibacterial activities particularly against Gram-positive test bacteria. However, *Brevibacillus laterosporus* BA8 and *Geobacillus thermoglucosidasius* BA16, have inhibitory affects on both Gram-positive and Gram-negative bacteria, and were kept at the School of Allied Health Sciences and Public Health, Walailak University, Thailand.

The benefits of our isolates and the chemical characterization of the antibacterial species determined are currently being subject to further studies.

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บทคัดย่อ

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การคัดกรอง วินิจฉัยแยกชนิดและฤทธิ์ต้านแบคทีเรียของบาซิลลัสทนร้อนที่แยกได้จากน้ำนมดิบ

บาซิลลัส สปีชีส์จำนวน 41 ไอโซเลตที่แยกได้จากน้ำนมดิบและนำไปวิเคราะห์หาฤทธิ์ต้านแบคทีเรีย *Escherichia coli* TISTR 887 และ *Staphylococcus aureus* TISTR 517 ด้วยวิธีจุดเชื้อและวิธีซึมแพร่เข้าเนื้อวุ้นของสาร พบว่าบาซิลลัสส่วนใหญ่ที่แยกออกมาได้สามารถออกฤทธิ์ต้านแบคทีเรียแกรมบวกได้ดีกว่าแบคทีเรียแกรมลบ และในการทดลองครั้งนี้สามารถแยกบาซิลลัสทนร้อนได้จำนวน 2 ไอโซเลต คือ BA8 และ BA16 ที่ออกฤทธิ์ต้านแบคทีเรียทั้งสองชนิดที่นำมาทดสอบและสามารถวินิจฉัยแยกชนิดได้เป็น *Brevibacillus laterosporus* และ *Geobacillus thermoglucosidasius* ด้วยการทดสอบทางชีวเคมีและศึกษาดูรูปร่างลักษณะ

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