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Screening and Isolation of *Bacillus* sp. Producing Thermotolerant Protease from Raw Milk

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ABSTRACT

Screening and isolation of *Bacillus* sp. were carried out from raw milk samples using Luria Bertani (LB) supplemented with 2 % skimmed milk as a selective media. Forty-one isolates with a clear zone surrounding a colony were primary selected and identified by using biochemical characteristics, Gram stain, and growth of bacteria at 50 °C. Ten out of 41 isolates showing a clear zone diameter of more than 10 mm were selected and evaluated for the presence of protease activity. BA26 and BA27 gave high levels of protease activity with 12 U/mg protein towards 1.5 % casein at 50 °C for 10 min. Based on the biochemical and physiological characteristics, BA26 and BA27 were classified as *Brevibacillus* non reactive. However, their 16S rRNA gene sequence showed 99 % identity to that of *Bacillus subtilis*. The enzymes were more specific to 1 % casein than 1 % gelatin. Moreover, the selected bacteria secreted extracellular protease upon incubation at 50 °C and the enzymes were stable even at high temperatures up to 100 °C and 121 °C. This confirmed that the enzyme protease produced by *Bacillus* sp. are thermotolerant proteases.

Keywords: *Bacillus* sp., thermotolerant protease, raw milk

INTRODUCTION

Enzymes have extremely high catalytic activity and unique substrate-specificity. In nature, the extracellular proteolytic enzymes are secreted by microorganisms with several biological functions and also have a range of biotechnological applications, e.g., as additives to laundry detergents, pharmaceutical, leather, laundry, food and waste processing industries [1,2]. To further improve these products, considerable effort has been devoted to the selection of microorganisms with new physical properties and tolerance of extreme conditions used in industrial processes (e.g. temperature, salts, and pH). The thermostable proteases are advantageous in some applications, due to employing higher processing temperatures, thus yielding faster reaction rates, increasing solubility of nongaseous reactants and products and reducing incidence of microbial contamination by mesophilic organisms. Proteases secreted from thermophilic bacteria are unique and have become increasingly useful in a wide range of commercial applications [3-7].

Among the various proteases, bacterial proteases are the most significant, compared with animal and fungal proteases. Additionally among bacteria, *Bacillus* species are specific producers of extracellular protease. Proteases produced by the *Bacillus* species are the most important group of secondary metabolites that are widely exploited [8]. The exploration of the protease enzyme produced by bacteria from raw milk was undertaken in this paper.

MATERIALS AND METHODS

Screening and Isolation of *Bacillus* sp. Producing Protease

Forty samples of raw milk were obtained from a farm at Walailak University, Nakhon Si Thammarat, Thailand. Samples were suspended and serial diluted with sterile distilled water. Each sample was heated at 80 °C for 30 min in a water bath, spread onto Luria Bertani (LB) agar and subsequently incubated at 37 °C for 24 h. Colonies producing a clear zone were selected from LB supplemented with 2 % skimmed milk agar and transferred onto a new agar by the point inoculation technique following incubation at 50 °C for 24 h. Bacteria having a clear zone diameter of more than 10 mm were selected for protease activity assay and were used throughout the study.

Determination of Protease Activity

The bacteria having a clear zone diameter of more than 10 mm were selected and cultivated in LB supplemented with 2 % skimmed milk broth. The culture broth was incubated at 50 °C in a rotary shaker operated at 200 rpm for 24 h. Afterwards the bacterial cell cultures were centrifuged at 10,000 rpm for 10 min. The supernatant was collected and assayed for protease activity. The reaction mixtures containing 1 ml enzyme solution and 1.5 % casein were incubated in a water bath at 50 °C for 10 min.

The supernatant was obtained by centrifugation at 10,000 rpm for 10 min. Next, 0.4 M Na_2CO_3 and Folin's reagent were added to terminate the reaction, and the reaction mixture was left to stand at room temperature for 10 min. The protease activity was determined spectrophotometrically at 660 nm. One unit of enzyme activity was defined as the amount of enzyme which produced 1 U/ml of tyrosine per min at 660 nm under control conditions. Specific activity was expressed as units per mg of protein of the enzyme extract. Additionally, the gelatinolytic activity and caseinolytic activity were performed according to the above protocol using 1 % gelatin and 1 % casein, respectively [9].

Effect of Temperature on Protease Stability

The effect of temperature on protease stability was evaluated by using various temperatures tested including 37 °C, 50 °C, 100 °C and 121 °C for 3 h, 1 h, 30 min and 15 min, respectively [9].

Protein Assay

Protein content was estimated by the Bradford method (Bio-Rad protein dye reagent). Bovine serum albumin (BSA) was used as a standard protein. The protein concentration was measured spectophotometrically at 595 nm [10].

Identification of *Bacillus* sp. Producing Protease

The isolates containing maximum proteolytic activity were identified according to physiological and Gram stain characteristics outlined in Bergey's Manual [11]. The morphology of the bacteria was observed with a light microscope. Gram staining was performed using crystal violet, I_2 in KI and safranin as reagents. The biochemical characteristics were tested using API 50 CHB medium (bioMérieux) according to the manufacturer's instructions, incubating at 55 °C. The 16S rRNA gene was amplified from genomic DNA obtained from the bacterial culture by the PCR method with the universal primers FC27 (5′-AGAGTT TGATCCTGGCTCAG-3′) and RC1492 (5′-TACGGCTACCTTGTTACGACTT-3′) [12]. PCR conditions consisted of an initial denaturation at 94 °C for 5 min; 30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min; and a final extension at 72 °C for 10 min. The amplification products were cloned into pGEM-TEasy (Promega) and sequenced using an ABI prism 377 apparatus. The 16S rRNA sequences were analyzed by the National Center for Biotechnology Information using the BLAST network service.

RESULTS AND DISCUSSION

Screening and Isolation of *Bacillus* sp. Producing Protease

Bacillus species are ubiquitous aerobic endospore forming gram-positive rod shaped bacteria. The morphology of *Bacillus* species that produce proteases are

displayed in **Figure 1a** and **Figure 1b**. Forty-one isolates exhibited a clear zone. Among these, 10 isolates showed clear zones with a diameter larger than 10 mm (**Figure 2** and **Table 1**). It has been reported that the most important *Bacillus* species contaminating raw milk are *B. licheniformis, B. cereus, B. pumilis, B. shaericus, B. amyloliquefaciens, B. subtilis* and *B. circulans* [13,14]. The results of this study also showed that *Bacillus* genera are wide-spread among bacteria in raw milk.

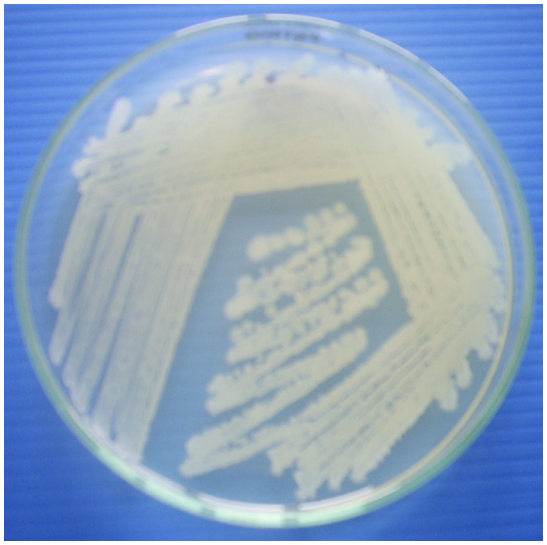

Figure 1a Colony of *Bacillus* species (BA26) on LB agar.

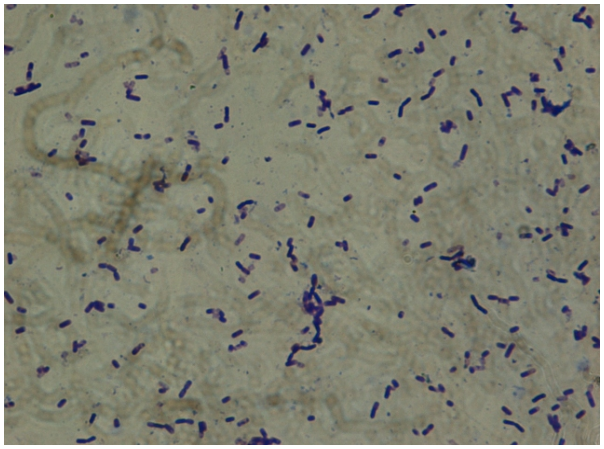

Figure 1b Gram-positive rod shaped bacteria observed with a light microscope (1,000x).

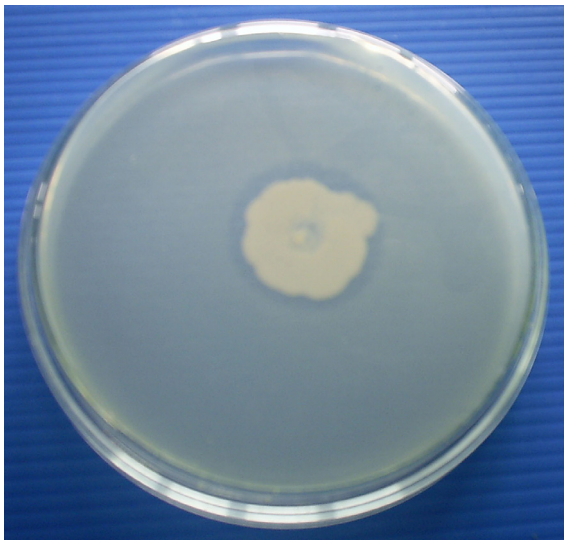

Figure 2 A clear zone of *Bacillus* sp. in LB + 2 % skimmed milk agar at 50 °C for 24 h.

Table 1 A clear zone diameter of more than 10 mm isolated from *Bacillus* sp. in LB + 2 % skimmed milk agar at 50 °C for 24 h.

Isolates	Colony/Clear zone (mm)	Clear zone (mm)
BA26	25.00/35.00	10.00
BA27	17.00/30.00	13.00
BA31	20.00/36.00	16.00
BA32	19.00/30.00	11.00
BA33	30.00/45.00	15.00
BA36	30.00/40.00	10.00
BA38	10.00/26.00	16.00
BA39	7.00/25.00	18.00
BA40	8.00/24.00	16.00
BA41	7.00/25.00	18.00

Determination of Protease Activity

Ten out of 41 isolates were selected in order to determine their protease activity. The BA26 and BA27 exhibited a maximum specific activity towards 1.5 % casein at 50 °C for 10 min with maximum levels of approximately 12 U/mg protein (**Table 2**). These 2 isolates were identified by biochemical and physiological characteristics as *Brevibacillus* non reactive, yet their 16S rRNA gene sequence showed 99 % homology to *B. subtilis*.

As *Bacillus* species grow in milk, they secrete heat-resistant extracellular protease that was thought to deteriorate the quality of the milk. Moreover, the proteases isolated from raw milk were specific to casein rather than gelatin (**Table 3**). According to Phutrakul and Kanasawud [9], the highest proteolytic activity of the thermostable proteases on substrates was casein followed by haemoglobin, gelatin and soybean, respectively [9].

Table 2 Protease activity produced by *Bacillus* sp.

Isolates	Protease activity (U/ml)	Specific protease activity (U/mg protein)
BA26	0.736	12.278
BA27	0.733	12.058
BA31	0.734	6.626
BA32	0.740	7.398
BA33	0.741	9.831
BA36	0.746	8.251
BA38	0.735	7.365
BA39	0.802	7.470
BA40	0.792	5.758
BA41	0.783	6.931

Table 3 Proteolytic activity on 1 % casein and 1 % gelatin.

Isolates	Proteolytic activity (U/ml)		Specific proteolytic activity (U/mg protein)	
	1 % casein	1 % gelatin	1 % casein	1 % gelatin
BA26	0.854	0.449	12.272	6.602
BA27	1.029	0.674	14.744	9.005
BA31	1.051	0.577	10.227	5.948
BA32	1.029	0.630	11.635	7.119
BA33	1.058	0.408	12.634	4.212
BA36	1.065	0.658	10.027	6.041
BA38	1.098	0.581	10.224	5.239
BA39	0.985	0.732	8.208	6.043
BA40	1.158	0.542	10.221	4.857
BA41	1.137	0.576	10.169	4.865

Effect of Temperature on Protease Stability

The protease activity was determined at various temperatures ranging from 37 °C to 121 °C and various times (15 min to 3 h). Enzyme activity increased when the temperature was increased from 37 °C to 50 °C. A reduction in enzyme activity was observed at 100 °C to 121 °C (**Table 4**). The optimum temperature for the protease activity was 50 °C which was similar to that described for other *Bacillus* proteases [15,16]. Matta and Punj [17] isolated and partially characterized a thermostable extracellular protease from *B. polymyxa* B-17. This protease was classified as a neutral metalloproteinase and was discovered to be active over a pH range from 5.5 to 10.0 with a maximum activity at pH 7.5 and 50 °C. The enzyme retained 35 % activity even after treatment at 70 °C for 10 min [17]. The study on the extracellular protease from thermophilic *Bacillus* sp. revealed that the optimum temperature of the enzyme was 60 °C [18]. This enzyme was stable for 2 h at 30 °C, while 14 % and 84 % of the original activities were lost at 40 °C and 80 °C, respectively [18].

Table 4 The effect of temperature on specific thermotolerant protease activity.

Isolates	Specific thermotolerant protease activity (U/mg protein)			
	37 °C (3 h)	50 °C (1 h)	100 °C (30 min)	121 °C (15 min)
BA26	10.729	11.501	7.659	6.232
BA27	10.079	11.069	7.097	6.262
BA31	7.425	7.026	5.485	4.812
BA32	8.367	7.883	5.530	4.769
BA33	8.564	9.198	5.808	5.334
BA36	6.979	7.615	4.626	4.038
BA38	6.742	7.034	4.987	4.539
BA39	6.684	6.951	4.042	3.767
BA40	7.008	6.383	4.392	4.310
BA41	6.937	6.934	4.599	4.082

CONCLUSION

The results of this study demonstrated that *Bacillus subtilis* BA26 and BA27 were excellent producers of extracellular protease at high temperatures. The 2 *Bacillus* species had maximum protease activity at 50 °C (approximately 12 U/mg protein). The proteolytic activity of these enzymes decreased when incubated at 100 °C for 30 min and 121 °C for 15 min. Finally, they were more specific for casein than gelatin.

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บทคัดย่อ

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การคัดเลือกและแยก *Bacillus* sp. ที่ผลิตเอนไซม์โปรติเอสทนร้อนจากน้ำนมดิบ

การศึกษาครั้งนี้ได้ทำการคัดเลือกและแยกเชื้อ *Bacillus* sp. จากตัวอย่างน้ำนมดิบ โดยเชื้อ *Bacillus* sp. ที่ผลิตเอนไซม์โปรติเอสจะสร้างวงใสรอบโคโลนีบนอาหารเลี้ยงเชื้อแข็ง LB ที่ประกอบไปด้วยความเข้มข้นร้อยละ 2 ของนมซึ่งสกัดเอาไขมันออกเพื่อเป็นแหล่งโปรตีน และจำแนกสายพันธุ์ของ *Bacillus* sp. ที่ผลิตเอนไซม์โปรติเอสโดยใช้ลักษณะทางชีวเคมี การติดสีย้อมและที่สามารถเจริญเติบโตได้ที่อุณหภูมิ 50 องศาเซลเซียส ผลการคัดเลือกพบแบคทีเรียที่สร้างวงใสรอบโคโลนีได้จำนวนทั้งสิ้น 41 ไอโซเลท และมีเพียง 10 ไอโซเลทเท่านั้นที่สร้างวงใสเส้นผ่านศูนย์กลางมากกว่า 10 มิลลิเมตร ซึ่งจะถูกคัดเลือกเพื่อนำมาศึกษาระดับฤทธิ์ของเอนไซม์ โดยไอโซเลท BA26 และ BA27 สามารถผลิตเอนไซม์โปรติเอสซึ่งมีฤทธิ์จำเพาะสูงที่สุดประมาณ 12 ยูนิตต่อมิลลิกรัมโปรตีน เมื่อทดสอบต่อสารตั้งต้นเคซีนที่ความเข้มข้นร้อยละ 1.5 ที่อุณหภูมิ 50 องศาเซลเซียส เป็นเวลา 10 นาที จากการทดสอบทางชีวเคมีและการศึกษารูปร่างลักษณะของทั้ง 2 ไอโซเลท พบว่าจัดอยู่ในกลุ่มของ *Brevibacillus* non reactive อย่างไรก็ตามจากการศึกษาลำดับนิวคลีโอไทด์ของยีน 16S rRNA พบว่ามีความเหมือนกันกับ *Bacillus subtilis* ร้อยละ 99 โดยพบว่าเอนไซม์โปรติเอสที่ผลิตได้มีความจำเพาะต่อเคซีนความเข้มข้นร้อยละ 1 มากกว่าเจลาตินความเข้มข้นร้อยละ 1 นอกจากนั้นยังพบว่า *Bacillus* sp. สายพันธุ์ที่คัดเลือกได้สามารถผลิตเอนไซม์โปรติเอสแบบหลั่งออกนอกเซลล์ได้ และเอนไซม์ยังคงมีฤทธิ์ในสภาวะที่อุณหภูมิเพิ่มสูงขึ้น ดังนั้นอาจบ่งชี้ได้ว่าเอนไซม์โปรติเอสที่ผลิตได้นั้นจัดเป็น thermotolerant protease

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