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Determination of Extracellular Enzyme Activities of *Bacillus* spp. spores Isolated from Raw Milk

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A B S T R A C T

Heat resistance spore-forming *Bacillus* spp. can survive the heat treatment process of milk, leading to spoilage of the final milk products. The aim of the study was to determine activities of extracellular protease and lipase enzymes from *Bacillus* spp. spores isolated from raw milk. A total of 7 spores belonged to genus *Bacillus* isolated from the raw milk, namely BT1, BT2, BT3, BT4, OM1, OM2 and OM3, respectively. Among of them, the OM1 isolate indicated the strongest extracellular protease enzyme activity after culturing at 37°C for 4 days, and the BT2 isolate indicated the strongest extracellular lipase enzyme activity after culturing at 37°C for 7 days.

Introduction

Milk and dairy products are at risk due to existence of spores survive in pasteurized and other heat-treated milk products, such as raw milk, milk powder, and cheese (Vidic *et al.*, 2020). Spore-formers are a primary cause of concern for milk products manufacturers and can be sub-categorized as being thermophilic, mesophilic or psychrotolerant in nature, with thermophilic spore-formers being more prevalent in the product.

Aerobic spore-forming bacteria such as *Bacillus* spp., *Bacillus cereus* are a major concern to the dairy industry, less for their pathogenicity but more for their spoilage-causing capabilities. They produce spoilage enzymes such as proteases and lipases that extensively influence the value and quality of milk and milk products by impacting on sensory qualities such as texture, taste, aroma, and nutritional value.

The *Bacillus* genus, part of the Bacillaceae family, is probably the oldest and most diverse genus of bacteria. *Bacillus* sp. distribute a remarkable range of physiological characteristics. Their main habitats are from soil in environment (Gopal *et al.*, 2015).

Therefore, it is necessary to isolate and determine activity of extracellular protease and lipase enzymes from *Bacillus* spp. spores isolated from raw milk to find out solutions for controlling raw milk quality collected from cow milk farms in future.

Materials and Methods

Raw milk samples

The eight samples of raw milk were collected randomly from the cow-milk farms in Binh Thuy (BT) and O Mon (OM) districts, CanTho city, Vietnam, including 04 samples from the Binh Thuy district and 04 samples from the O Mon district. The samples were kept in sterilized plastic bag, labelled before transfer to laboratory, stored in refrigerator at 4 -10°C for 24 hours for isolation.

Isolation of putative *Bacillus* spp. spores from raw milk

A total of 150 mL of the raw milk sample was transferred into a 250 mL sterile bottle with screw cap. The raw milk sample was heated at 80°C for 12 minutes in a temperature-controlled water bath to kill vegetative cells. After the heat treatment, the raw milk sample was cooled down immediately in an ice bath (Martinez *et al.*, 2017). The *Bacillus* spp. spores isolation was performed by Spread plate technique in Luria Bertani (LB) agar added 2 % skim milk powder (LB-SMP) and incubated at 37°C for 48 hours. The cultures were streaked on the media to obtain single colonies as described by Santong *et al.*, (2008) and Luong *et al.*, (2006).

Determination of extracellular enzyme activities of *Bacillus* spp. spores

Preparation of *Bacillus spp*. spore solution

Before determination of extracellular enzyme activity of *Bacillus* spp. spores, the *Bacillus* spp. spore solution was prepared. The preparation of *Bacillus* spp. spore solution was performed as follows: The obtained *Bacillus* spp. single colonies from the above isolation were spread on the LB agar plates and incubated at 37°C for 24 hours. After the incubation, the colonies obtained from the LB agar plate were transferred to 50 mL of 1% peptone solution in 250 mL sterile bottle with screw cap, and then incubated at 37°C for 14 days. After 14 days, then the peptone solution contained *Bacillus* spp. was heated to 80°C for 10 minutes to kill vegetative cells to obtain *Bacillus* spp. spore solution. Checking of spore-forming of *Bacillus* spp. was examined by the microscope OLYPIA 383A Microscope (100x). The *Bacillus* spp. spore solution was stored in refrigerator at 4°C for further use (Janštová and Lukášová, 2001; Petersen and McLaughlin, 2016; Eijlander *et al.*, 2019).

Lipase enzyme

To determine lipolytic activity of *Bacillus* spp. spores, the spores in the *Bacillus* spp. spore solution were plated on tributyrin agar, prepared with plate count agar supplemented with 0.5 % tributyrin. The plates were incubated at 37 °C for 7 days. Lipolytic activity was determined by measuring clear zone around each colony (Montanhini *et al.*, 2013; Duy *et al.*, 2020).

Protease enzyme

To determine proteolytic activity of *Bacillus* spp. spores, the spores in the *Bacillus* spp. spore solution were plated on LB agar supplemented with sterile milk (LB-SMagar) on Petri dishes. In the LB-SM agar, ratio of the LB agar to the sterile milk was 1:1. The Petri dishes were incubated at 37°C for 4 days. The presence of transparent zones around the colonies was recorded as positive strains referring to protease production (Luong *et al.*, 2006; Montanhini *et al.*, 2013).

Results and Discussion

Isolation of *Bacillus* spp. spores

Before isolation, eight raw milk samples collected from Binh Thuy (BT) and O Mon (OM) districts, CanTho city, were treated in water batch at 80°C for 12 minutes to kill vegetative bacterial cells.The results of the isolation of *Bacillus* spp. spores were presented in Table 1. A total of 7 putative *Bacillus* spp. spores were isolated on the LB-SMP mediaat 37°C for 48 hours, namely BT1, BT2, BT3, BT4,

OM1, OM2, and OM3, respectively. On the LB-SMPPetri dishes, the colonies of 7 isolates showed different colors: opaque whiteand ivory white. The bacterial colonies had lobate and entire margins; raised and flatelevations; sizes ranged from 2.0 to 5.0 mm. The morphology of all the bacterial isolates were short rod - shaped (Figure 1, Figure 2 and Table 1). All the bacterial isolates were gram-positve, able to form endospore in the cells (Figure 3).

Bacillus sp. species are ubiquitous aerobic endospore forming gram-positive rod-shaped bacteria (Santong *et al*., 2008). In the present stuc*y,* the characteristics of the *Bacillus* spp. colonies and cells were similar to previous studies by Tam and Thinh (2020); Han *et al.,* (2021); Aprilia *et al.,* (2021) including short rod – shaped bacteria with size of bacterial cell ranged from 2.0 to 5.0 mm, Gram - positive bacteria, and forming endospore. Based on the characteristics of the colonies and cells, the isolated isolates were identified as *Bacillus* spp..

Extracellular enzyme activities of *Bacillus* spp.

For considering that spores are present in raw milk, then they will be present in the whole production process with many sources of contamination as dead ends, pockets, valves, shafts, and gaskets. Milking equipment can also be a contamination source, with spores that may adhere to and germinate in tanks, pasteurizers, packaging machines, causing the post-treatment contamination of milk. The spore adherence to food contact surfaces such as stainless steel. Suitable temperatures, humidity and the presence of a liquid or air interface are favoring factors for the germination of the spores to form vegetative cells and for the consequent production of extracellular enzymes and biofilms (Tirloni *et al.*, 2022).

Protease activites

A total of the 7 spores belonged to genus *Bacillus* were evaluated the extracellular protease and lipase enzyme activity on the LB-SM agarafter culturing at 37°C.

Resuts of testing of extracellular protease enzyme activity on the LB-SM agar plates at 37°C for 4 days were presented in Table 2. The proteolytic activity (D/d) was detected in 54.17% (4/7) of the total of isolates, displayed by the isolates, namely BT2, BT3, OM1, and OM3, respectively. The 3 isolates that did not proceduce extracellular protease enzymes, namely BT1, BT4, and OM2, respectively.

The results in the table 2 showed that among of them, the proteolytic activity(D/d) of the 4 isolates increased over time fom day 1 to day 4, and the reached peaks on day 4 after the culturingat 37°C. The OM1 isolate obtained a peak on day 4 (D/d = 7.37), had the strongest extracellular protease enzyme activity, and was statistically different ($p > 0,05$) from other the isolates.

Means followed by different letters in a column are significantly different at $p < 0.05$

Proteolytic activity (D/d): d: diameter of colony (mm); D (mm): diameter of zone of around the colony; incubated at 37 °C for 4 days

As *Bacillus* spp. grow in milk and secrete heat-resistant extracellular such as protease. Proteolytic activity of extracellular protease from *Bacillus* spp. led to deteriorate the quality of the milk. Moreover, the proteases isolated from raw milk were specific to casein rather than gelatin. The highest proteolytic activity of the thermostable proteases on substrates was casein followed by hemoglobin, gelatin, and soybean, respectively (Santong *et al*., 2008).

Table.1 Colonies and cells morphology of isolated bacteria

Name of bacterial isolates	Cell morphology	Colonies morphology				
		Form	Color	Margin	Elevation	Dimension (mm)
BT1	Short rod-shaped	Circular	Opaque white	Lobate	Raised	4.0- 5.0
BT2	Short rod-shaped	Circular	Ivory white	Entire	Flat	2.0 - 3.0
BT3	Short rod-shaped	Circular	Opaque white	Entire	Flat	2.0 - 3.0
BT4	Short rod-shaped	Circular	Ivory white	Entire	Flat	4.0 - 5.0
OM1	Short rod-shaped	Circular	Opaque white	Entire	Flat	4.0 - 5,0
OM2	Short rod-shaped	Circular	Opaque white	Entire	Flat	2.0- 3.0
OM3	Short rod-shaped	Circular	Opaque white	Entire	Flat	2.0 - 3.0

Note: BT: Binh Thuy; OM: O Mon

Table.2 Proteolytic activity (D/d)of the bacterial isolates

Name of bacterial isolates	D/d			
	1 day	2 days	3 days	**4 days**
BT1	0.00^{d}	0.00^{d}	0.00^{d}	$\mathbf{0.00^{d}}$
BT2	2.00^{b}	1.94^{b}	2.89^{b}	$\mathbf{3.78^{b}}$
BT3	2.08^{bc}	2.43^{bc}	2.44^{bc}	$\mathbf{2.94^{bc}}$
BT4	0.00^{d}	0.00^{d}	0.00^{d}	$\mathbf{0.00^{d}}$
OM1	$\mathbf{2.89^{a}}$	$\mathbf{5.11^{a}}$	$\mathbf{5.96^{a}}$	$\mathbf{7.37^{a}}$
OM2	0.00^{d}	0.00^{d}	0.00^{d}	$\mathbf{0.00^{d}}$
OM3	$\mathbf{2.42^{c}}$	$\mathbf{1.94^{c}}$	$\mathbf{2.00^{c}}$	$\mathbf{2.00^{c}}$

Table.3 Lypolytic activities of the bacterial isolates

Name of bacterial isolates	Incubation time (day)						
	1	2	3	4	5	6	**7**
BT1	-	-	-	-	-	-	-
BT2	-	+	+	++	++	++	+++
BT3	-	-	+	+	+	+	+
BT4	-	+	+	+	+	+	++
OM1	-	+	+	+	+	++	++
OM2	-	-	-	-	-	-	-
OM3	-	+	+	+	+	+	++

"+" Zone of 0-1 mm around the colony, "++" zone of 1-2 mm; "+++" zone of >2 mm;incubated at 37 °C for 7 days

Fig.1 Shapes of bacterial colonies on the LB - SMP at 37°C for 48 hours, (a) BT1 isolate, (b) BT2 isolate,(c) BT3 isolate, (d) BT4 isolate, (e) OM1 isolate, (f) OM2 isolate and (g) OM3 isolate.

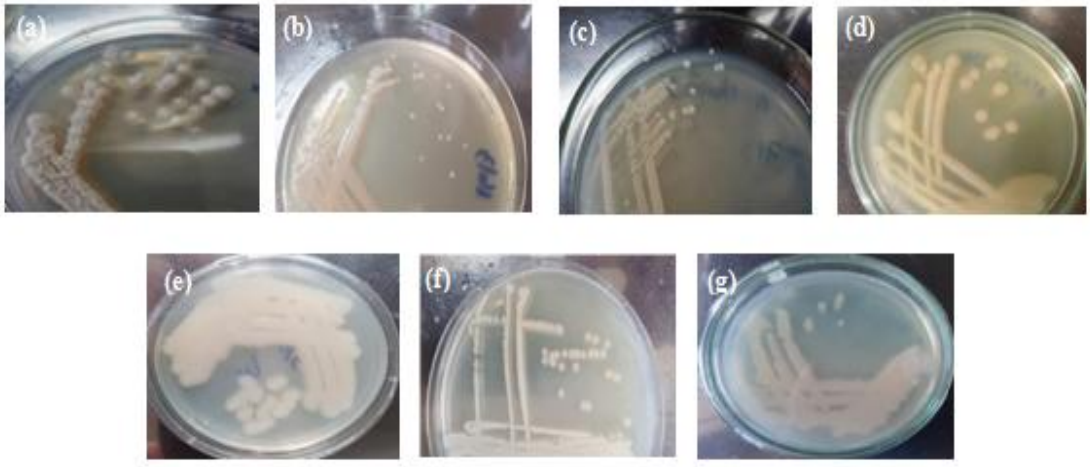

Fig.2 (a) BT1 isolate,(b) BT2 isolate, (c) BT3 isolate, (d) BT4 isolate, (e) OM1 isolate, (f) OM2 isolate and (g) OM3 isolate under the microscope OLYPIA 383A Microscope (100x).

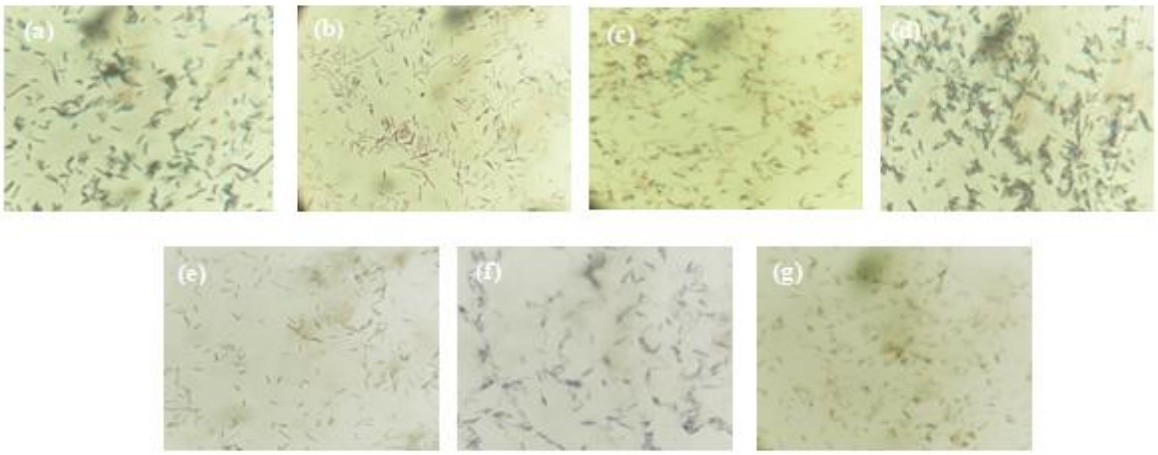

Fig.3 Gram staining (a) and Spore staining of the BT2 isolate(b)

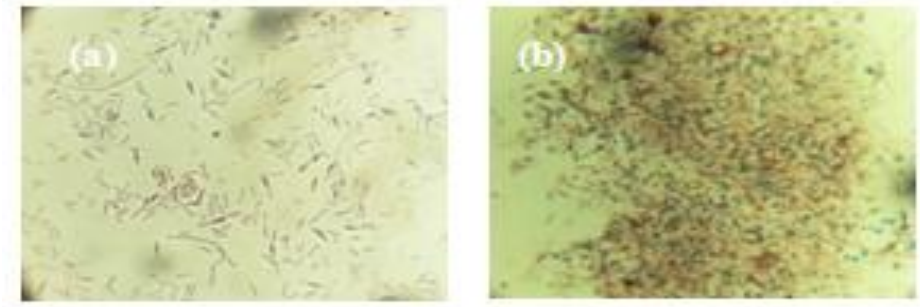

Fig.4 Image of Proteolysis of the OM1 isolate in the LB-SM agar

Fig.5 Lipolytic activity of the BT2 isolate in the tributyrin agar

Lipase activities

Lypolytic activity of the 7 bacterial isolates on the tributyrin agarwere presented in the Table 3. The results showed that the 2 isolates (BT1 and OM2) did not produce extracellular lipase enzymes.

The 5 isolates, namely BT2, BT3, BT4, OM1, and OM3, respectively, were able to produce extracellular lipase enzymes by creating clear zones around the colonies after culturing at 37°C for more than two days.

Among of the 5 isolates, 1, 3 and 1 of them could be classified as strong, moderate, and weak extracellular lipase enzymes producers. The BT2 isolate was the strongest extracellular lipase producer. In the previous studies, the present of *Bacillus* spp. in raw milk and milk powder could be a cause of milk fat degradation by extracellular lipase production such as triglyceride in the milk fats led to destroy the emusion system of milk (Chopra and Mathur, 1984; Chen *et al.,* 2003). Lipase activities could detected through the milk pastuerization and enven after UHT treatment of milk (Chen *et al.,* 2003).

A total of 7 spores belonged to genus *Bacillus* were isolated from the 8 samples of raw milk. The OM1 isolate had the strongest extracellular protease enzyme activity after culturing at 37°C for 4 days. The BT2 isolate had the strongest extracellular lipase enzyme activity after culturing at 37°C for 7 days.

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