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Heat Resistance Assay and Identification for *Bacillus* spp. spores Isolated from Raw Milk

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A B S T R A C T

Heat-resistant spore-forming *Bacillus* spp. can survive the heat treatment process of milk and lead to spoilage of the final product. The objective of the research is to assay heat-resistant ability (D values, minutes) for *Bacillus* spp. spores. A total of 7 *Bacillus* spp. spores from eight of raw milk samples were tested biochemical properties and measured heat resistance in 1% peptone solutions. The best heat-resistant spores were chosen to identify them by 16S rRNA genes sequencing. The results showed that all of them belonged to the species of genus *Bacillus*, survived at the heat treatment of 90 °C for 12 minutes with the D values ranging from 1.86 to 23.66 minutes. Among of them, the best heat-resistant spores, namely BT2 and OM1, survived at the heat treatment of 100 °C for 30 minutes with the D values of 8.78 and 4.89 minutes, respectively; and were identified as *Bacillus sporothermodurans* BT2 and *Bacillus cereus* OM1, respectively.

Introduction

The milk and its products are made from raw milk sources and contain a lot of the required nutrients for human. The bacterial composition of raw milk affects to the quality of milk and milk products because of their deterioration activities (Liang *et al.*, 2022).

In the raw milk, spore forming bacteria as *Bacillus* spp., *Bacillus cereus*… may affect to both the public health as well as the economy of the dairy industry. It is necessary to control the quality of received raw milk (Osama *et al.*, 2020). *Bacillus* spp. can form endospores and can thus survive during treat treatments of raw milk. Predominant spore-formers are *Bacillus pumilus*, *Bacillus licheniformis*,

Bacillus subtilis… Spores of the species were not affected by heat processes such as pasteurization (Marangoz *et al.,* 2018).

Bacillus spp. contaminated from raw milk was difficulty to eliminate them from the dairy chain because of their highly resistant spores forming. It may be eliminated by the powerful tools in the dairy processing plants as ultra-high temperature, high-pressure processing, ultrasound treatment... (Ledina *et al.,* 2021). Among of them, heat treatment is the most widely used processing technology in the dairy industry to reduce bacterial counts in milk. However, as spores can survive thermal treatments. (Ledina *et al.,* 2021).

Therefore, it is necessary to determine heat resistance of spores from raw cow's milk to prevent them by heat treatment in the future. The aim of the research is to assay heat-resistant ability of *Bacillus* spp. spores (determination of D values) isolated from raw cow's milk in 1% peptone solutions.

Materials and Methods

A total of 7 *Bacillus* spp. spores isolated from eight of raw milk samplesfrom cow-milk farms in Binh Thuy and O Mon districts, CanTho city, spores were prepared as described by the previously study (Dat *et al.,* 2022).

Determination of biochemical properties of *Bacillus* spp. spores

The 7 *Bacillus* spp. spores were sub-cultured onto Luria Bertani (LB) agar. The *Bacillus* spp. colonies were purified and identified via biochemical tests. The biochemical characteristics included Gram staining, spore forming staining, motility, catalase, oxidase, and Gelatin liquefaction tests (Luong *et al.,* 2006; Thuoc, 2006).

Morphological characterization

Color, shape, transparency, and margin were examined and recorded as colony morphological characteristics. Microscopic features were recorded for all isolates via Gram stain protocol.

Gram staining

The Gram staining process includes four basic steps, including: Applying a primary stain (crystal violet). Adding a mordant (Gram's iodine). Rapid decolorization with ethanol, acetone, or a mixture of both. Counter staining with safranin.

Staining of endospore forming

Endospores are formed within a vegetative bacterial cell when the environmental conditions no longer support cell growth. The presence of endospores in a bacterial culture can be detected by staining with Methylene blue dye. The *Bacillis* spp. were cultured on agar slant tubes for 2 weeks, prepared and air-dried heat fixed slide with the *Bacillis* spp. Fix the specimen on the flame of the alcohol lamp.

Methylene blue dye, heat the bottom and keep for 1 min. Wash the stain thoroughly until the color disappears.0.5% neutral red dye for 1 min. Wash, dry and observe on view slide under the microscope under the oil immersion objective (100X). The spores were blue. The Cytoplasm were red.

Motility

Motility is the ability of an organism to move by itself. Motility of *Bacillus* spp. were determined by upward motion in semi-liquid Luria broth (LB) agar supplemented with 0.7% agar. After 24 hours at 37°C, if the bacteria grew and gave a diffuse spreading growth that is motility. In contrast, non-motile bacteria will only grow in the LB agar tube and only the area where they are inoculated.

Biochemical characterization

Oxidase test

Tested *Bacillus* spp. colony was smeared on the filter paper previously saturated with freshly

prepared oxidase reagent (1% tetramethyl-p phenylenediamine dihydrochloride). Positive oxidase test was recorded as the development of a blue-purple color within several minutes.

Catalase test

Gas bubbles formation within 10 s after the *Bacillus* spp. colony was added to a drop (5 mL) of 3% hydrogen peroxide solution and considered as a positive catalase test.

Gelatin liquefaction test

Inoculated the *Bacillus* spp. on a nutrient medium containing gelatin on a petri dish and then incubated at room temperature for 3 days. Added 5-10 mL of trichloroacetic acid to the top of the medium to precipitate gelatin. Gelatin liquefaction was identified by forming a gelatinous ring and the cloudy medium.

Testing D values of *Bacillus* spp. spores

Before testing of heat resistance of *Bacillus* spp. spores, the *Bacillus* spp. spore solution must be 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).

Determination of Heat resistance (D value) of the *Bacillus* spp. spore

Thermal death is the death of a population of microorganisms due to exposure to an elevated temperature such as sterilization. The effectiveness of each sterilization method for a specific bacterial strain can be assessed by either bacterial death rate or a survival curve, expressed as a D value (decimal reduction time). The D value is the number of minutes exposure to a defined temperature to reduce viable bacteria by 90% (Garg, 2019).

The D value at a defined temperature is calculated by the formula:

$$D_T = -\frac{t}{\lg N - \lg N_o}$$

Where:

D_T : Decimal reduction time at temperature T (minutes)

N : The number of microorganisms in the product at time t (CFU/L)

N_0 : The initial number of microorganisms (CFU/mL)

t: Heating time (minutes)

To determine heat resistance (D values) of the *Bacillus* spp. spore, 2.5 mL of the prepared spore solution was transferred to a test tube (10 × 100 mm) and then treated it by heat conduction in a temperature - controlled water bath. The heat treatment regime was performed at 03 temperature levels of 80, 90, and 100°C for the holding time of each the temperature level of 12, 15, 20, 25, and 30 minutes, respectively.

The treated spore solution was cooled immediately in an ice bath. The number of spores in the spore solution before and after heat treatment were determined by the method of counting colony plates

with threefold repetitions to record average results (Baril *et al.*, 2012; Petersen and McLaughlin, 2016; Eijlander *et al.*, 2019).

Identification of the spores by 16S RNA sequencing

The high heat resistant spores forming *Bacillus* spp. were selected to sequence by 16S rRNA method. Universal 16S rDNA primers were used as 27F (5'AGAGTTTGATCCTGGCTCAG3') and 1540R (3'AAGGAGGTGATCCAACCGCA5'). Thermal cycling of PCR reaction was designed as: denaturation 95 °C for 15 minutes; after the denaturation for 30 s; annealing at 60 °C for 30 s; extending at 72 °C for 1 min. The number of cycles was 40 cycles. The results of bacterial 16S rRNA sequencing were compared with the sequences on the NCBI gene bank using the NCBI BLAST online tool (https://blast.ncbi.nlm.nih.gov).

Statistical analysis

All experiments were performed with three replicates and calculated by Statgraphics 15.2 and Excel Software 2010.

Results and Discussion

Biological characteristics of the *Bacillus* spp. spore

A total of the 7 spores belonged to genus *Bacillus* were isolated from the 8 samples of raw milk on Luria Bertani (LB) agar added 2 % skim milk powder (LB-SMP) at 37°C for 48 hours, namely BT1, BT2, BT3, BT4, OM1, OM2, and OM3, respectively. In the previous study (Dat *et al.*, 2022), the colonies of 7 isolates displayed different colors including opaque white and ivory white on the LB-SMP Petri dishes; and had lobate and entire margins; raised and flat elevations. The bacterial cells were short rod – shaped by the morphological examination; cell sizes ranged from 2.0 to 5.0 mm; and were gram-positive bacteria, able to form endospore in the cells.

The examination of morphological cell characteristics (Gram stain, mobility and endospore forming) and biochemical tests (catalase, oxidase, and Gelatin liquefaction tests) for the 7 bacterial isolates were performed to identify them as traditionally identification methods. Results of the testing were presented in Table 3.1 and showed that 7 isolated species were identified as *Bacillus* spp. according to the key Bergy's manual of determinative bacteriology and the studies of Navin Chandran *et al.*, (2014) and Orozova *et al.*, (2017), including the characteristics of short rod – shaped, Gram (+), Catalase (+), Motility, Endospore forming.

In the study of Al-Allaf and May (2011), the bacterial isolates belonged to the genus *Bacillus* spp., isolated from milk and cheese, were positive to proteolysis activity by forming clearing zones around the growth of the bacteria, because of its ability to degradation proteins to amino acids. All the strains formed spores, were Gram (+), rod - shaped, motile, positive for citrate utilization, oxidase, urease, gelatin hydrolysis, catalase, lecithenase, nitrate reduction, voges-proskover, and starch hydrolysis tests. They grew well in 5 % and 7 % NaCl at 42°C.

To identify the specific bacterial isolates, the comparison between the results obtained from the previous studies for chemical characteristics of *Bacillus* spp. were also performed. The results (in Table 3.1) showed that the BT1, OM2, and OM3 isolates were negative for Oxidase test and positive for Gelatin liquefaction test, identified as *Bacillus subtilis* according to Siu-Rodas *et al.*, (2017); *Bacillus subtilis* was isolated from the thermophilic phase from composting with coffee residues, had endocellulase and exocellulase activities, and was a cellulolytic potential based on the diameter of the hydrolysis halo.

The BT3 and BT4 were negative for Oxidase and Gelatin liquefaction tests, identified as *Bacillus coagulans* MA-13 isolated from bean processing waste, could ferment lignocellulose-derived sugars

to lactic acid at 55 °C and pH 5.5. The bacterium secreted soluble thermophilic cellulases at low temperature (37 °C), retained an optimal operational activity at 50 °C, and was a promising thermophilic and cellulolytic strain to produce lactic acid from lignocellulosic hydrolysate (Aulitto *et al.,* 2017).

The BT2 was positive for Oxidase test and negative for Gelatin liquefaction test, identified as *Bacillus sporothermodurans* when compared with the study of Pettersson *et al.,* (1996). *Bacillus sporothermodurans* was isolated from Ultra High Temperature (UHT) treated milk, formed high heat-resistant endospores, could survive UHT treatments of milk, and germinate in the final product (Pettersson *et al.,* 1996). In the study of Pettersson *et al.,* (1996) presented *Bacillus sporothermodurans* were strictly aerobic; catalase and oxidase positive, filamentous rods, produced a granular or string of-pearls appearance in the Gram reaction. The cells were motile by means of peritrichous flagella. The spores of them were ellipsoidal, located terminally and did not distend the sporangium. The colonies were small, smooth, and off-white. Some strains hydrolyzed esculin, and most produced weak hydrolysis of casein; grew at 20 to 45°C.

The OM1 was positive for Oxidase and for Gelatin liquefaction tests, identified as *Bacillus cereus* SW7-1 (Li *et al.,* 2015). This strain utilized glucose, sucrose, and maltose; was highly susceptible to gentamycin, streptomycin, erythromycin, norfloxacin, and ofloxacin and moderately susceptible to tetracycline and rifampicin. The SW7-1 had hemolytic activity and produced extracellular lipase, and amylase.

Heat resistance testing of *Bacillus* spp. spores

Before testing of heat resistance of *Bacillus* spp. spores, the *Bacillus* spp. spore solution must be prepared. The spore solutions of the 7 spores belonged to genus *Bacillus* isolated from the raw milk (namely BT1, BT2, BT3, BT4, OM1, OM2 and OM3, respectively) were tested heat resistance ability in the 1% peptone solution by determination of D values. The D values of the 7 spores were presented in Table 3.2.

The results in the Table 3.2 showed that:

At the same experimental temperature, the longer heat treatment time resulted in the lower heat resistance of *Bacillus* spp., means the D-value of the spores decreased for all the spores tested. For the bacterial spore BT2 was treated at 80°C, the heat treatment time increased from 12 minutes to 15, 20, 25, and 30 minutes respectively, the D values were decreased from 46.30 to 46.11, 45.14, 44.59, and 44.19 minutes, respectively.

For the same heat holding time, increasing the heat treatment temperature from 80°C to 90 and 100°C shifted the D values of 7 spores to lower values than one measured at the original temperature (80°C). The obtained D value were reduced gradually for all the spores tested. For example, at the same heat holding time of 30 minutes, the increasing the heat treatment temperature of the bacterial spore OM1 from 80°C to 90 and 100°C led to the D values of the spore decreased gradually. The obtained D values of the spore OM1 at the temperatures of 80°, 90°, and 100°C for 30 minutes were 15.10, 7.42, and 4.89 minutes respectively.

In the study, the total of 7 spores survived at 80 and 90°C for heat holding time of 12, 15, 20, 25 and 30 minutes, respectively. Until raising the temperature up to 100°C and hold the temperature for 12 minutes, the BT1, BT3, BT4, OM2 and OM3 spores could not survive, germinate, and grow on the medium. This means that these spores could be killed at temperatures of 100°C for 12 minutes. Among of them, the spores BT2 and OM1 could sustain the high heat of 100°C.

At the heat treatment regime with temperature of 100°C and of holding time of 12, 15, 20, 25, and 30 minutes, respectively, only the two spores named BT2 and OM1 could survive for all the holding times. The average D values of the spores BT2 and OM1 were 9.18 and 5.09 minutes, respectively.

Table.1 Biological characteristics of the 7 *Bacillus* spp.

S.No	Bacterial isolate	Gram (+/-)	Endospore forming	Motility	Catalase	Oxidase	Gelatin
1	BT1	+	+	+	+	-	+
2	BT2	+	+	+	+	+	-
3	BT3	+	+	+	+	-	-
4	BT4	+	+	+	+	-	-
5	OM1	+	+	+	+	+	+
6	OM2	+	+	+	+	-	+
7	**OM3**	+	+	+	+	-	+

Note:(-): negative; (+): positive; BT: Binh Thuy; OM: O Mon

Table.2 D values of the 7 spores belonged to genus *Bacillus*

Isolate	Temperature (^{o}C)	Holding time (minutes)					Average
		12	15	20	25	30	
BT1	80	8.35	8.13	7.74	7.62	7.59	7.89±0.34^{cd}
	90	3.62	3.54	3.49	0.00	0.00	2.13±1.95^{cd}
	100	0.00	0.00	0.00	0.00	0.00	0.00^{cd}
BT2	80	46.30	46.11	45.14	44.59	44.19	45.26±0.92^{a}
	90	23.66	23.15	22.18	21.86	21.27	22.43±0.97^{a}
	100	9.78	9.33	9.08	8.91	8.78	9.18±0.40^{a}
BT3	80	12.01	11.98	10.77	10.48	9.70	10.99±1.00^{c}
	90	5.66	5.60	5.45	5.33	5.24	5.46±0.18^{c}
	100	0.00	0.00	0.00	0.00	0.00	0.00^{c}
BT4	80	11.00	10.83	10.15	9.95	9.12	10.21±0.75^{c}
	90	4.98	4.87	4.80	4.73	4.54	4.78±0.17^{c}
	100	0.00	0.00	0.00	0.00	0.00	0.00^{c}
OM1	80	16.60	16.15	15.69	15.35	15.10	15.78±0.60^{b}
	90	7.81	7.77	7.64	7.49	7.42	7.63±0.17^{b}
	100	5.36	5.13	5.05	5.01	4.89	5.09±0.17^{b}
OM2	80	9.98	9.13	9.01	8.93	8.88	9.19±0.45^{cd}
	90	2.56	2.48	0.00	0.00	0.00	1.01±1.38^{cd}
	100	0.00	0.00	0.00	0.00	0.00	0.00^{cd}
OM3	80	4.34	4.18	4.07	3.98	0.00	3.31±1.86^{d}
	90	1.86	0.00	0.00	0.00	0.00	0.37±0.83^{d}
	100	0.00	0.00	0.00	0.00	0.00	0.00^{d}

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

Table.3 16S rRNA sequences of the bacterial isolate BT2

**CCCCAATCATCTGTCCCACCTTCGGCGGCTGGCTCCAAAAGGTTACCTCACCGACTTCGG
GTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACC
GCGGCATGCTGATCCGCGATTACTAGCGATTCCGGCTTCATGTAGGCGAGTTGCAGCCTA
CAATCCGAACTGAGAATGGTTTTATGGGATTGGCGTAACCTCGCGGTCTAGCAACCCTTT
GTACCATCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTC
ATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTG
GCAACTAAGGTCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAG
CTGACGACAACCATGCACCACCTGTCACTCTTGTCCCCGAAGGGAAATCCCTATCTCTAG
GGAGGGCAAGAGGATGTCAAGACCTGGTA**

Table.4 16S rRNA sequences of the bacterial isolate OM1

**CCCCAATCATCTGTCCCACCTTAGGCGGCTGGCTCCAAAAAGGTTACCCCACCGACTTCG
GGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCAC
CGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCATGTAGGCGAGTTGCAGCCT
ACAATCCGAACTGAGAACGGTTTTATGAGATTAGCTCCACCTCGCGGTCTTGCAGCTCTTT
GTACCGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTC
ATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTAAATGATG
GCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAG
CTGACGACAACCATGCACCACCTGTCACTCTGCTCCCGAAGGAGAAGCCCTATCTCTAGG
GTTGTCAGAGGATGTCAAGACCTGGTAAG**

Fig.1 The spore BT2 germinated and developed on the LB agar.

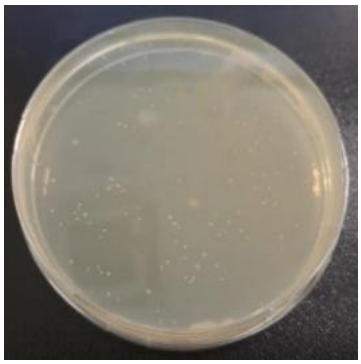
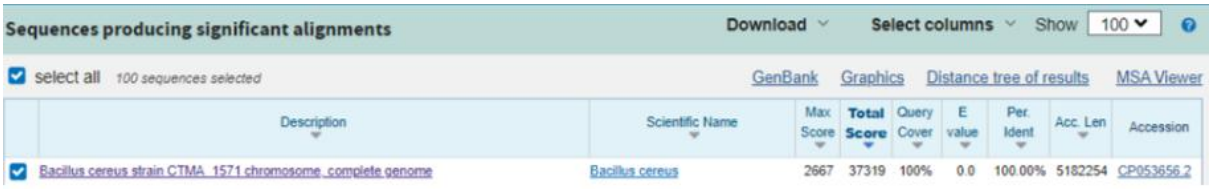


Fig.2 Similarity percentage of 16S rDNA sequences for the BT2 isolate compared to strains in Genbank

Fig.3 Similarity percentage of 16S rDNA sequences for the OM1 isolate compared to strains in Genbank



The BT2 spore exhibited the most heat-resistant ability, had the D value of 8.78 minutes at 100°C for 30 minutes, and was statistically different from the other spores. The spore OM1 was the next heat-resistant ability with the D value of 4.89 minutes.

Figure 3.1 showed the image of the bacterial spores BT2 germinated and developed on the LB agar after incubation of at 37°C for 48 hours, after the heat treatment regime of 100°C and 12 minutes.

In summary, only 2 the bacterial spores, namely the BT2 and OM1 could survive at the highest temperature of 100°C for treatment time of 30 minutes.

Identification of the highest heat resistance *Bacillus* spp. by molecular method

The two strains of *Bacillus* spp. with the highest heat resistance were chosen to sequence by the 16S rRNA sequencing. The sequences of the two strains were presented in Table 3.3, Figure 3.2, Table 3.4, and Figure 3.3.

The results showed that the similarity percentage of BT2 was 100 % compared to *Bacillus sporothermodurans* strain HBUM07101 16S rRNA gene (MF662527.1); the similarity percentage of OM1 was 100 % compared to *Bacillus cereus* strain CTMA 1571 chromosome (CP053656.2). *Bacillus* species present in soil, air, animal feed, and water. Therefore, they can be introduced into raw milk easily. The *Bacillus* spp. have been isolated from cracks in pipes, equipment, pipe gaps, valves and equipment joints used in dairy factories; were recognized as the main cause of milk spoilage after heat processing (Gopal *et al.*, 2015).

According to the study by Awad Aseed and El Zubeir (2020) showed that *Bacillus* species such as *Bacillus cereus, Bacillus pantothenticus, Bacillus licheniformis, Bacillus mycoides, Bacillus coagulans, Bacillus megaterium,* and *Bacillus subtilis* were detected in raw milk samples. In sterile milk, *Bacillus sporothermodurans* was the main cause of spoilage and shortening the shelf life of milk (Scheldeman *et al.*, 2006).

The studies of Huemer *et al.,* (1998); Janštová and Lukášová (2001); Sa Xu *et al.,* (2006) and Stoeckel *et al.*, (2016) were performed to evaluate the heat resistance of *Bacillus* spp. spores isolated from raw milk, ultra-high temperature (UHT) milk and other dairy products. The results showed that the spores survived at temperatures ranged from 95 to 120°C. Among of them, *Bacillus sporothermodurans* isolated from the UHT milk had the high heat resistance ability, with the D_{140} values ranging from 3.4 to 7.9 seconds. Although *Bacillus sporothermodurans* was not a pathogen, but the presence of them in pasteurized milk could become a quality problem and cause an economic loss (Scheldeman *et al.*, 2002).

A total of 103 raw milk samples were tested for the presence of *Bacillus cereus*. The results showed that the percentage of the samples contained *Bacillus cereus* was 38.8%, of which 60% of the samples were positive for *Bacillus cereus* with the bacteria load over the recommended limit (>10° CFU/mL) (Abraha *et al.*, 2017).

Bacillus cereus can grow at cold storage temperature of 4 - 7°C in raw milk before processing and could produce harmful toxins to consumers' health (Zeighami *et al.*, 2020) (Ehling-Schulz *et al.*, 2006).

The grow of most strains of *Bacillus* in milk often resulted in the production of thermostable extracellular enzymes such as proteases and lipases; the gelation of pasteurized milk; the milk with bitter or sour taste; and shorting of shelf life of dairy products (Topçu *et al.,* 2006; Samarzija *et al.,* 2012).

Bacillus spp. present in nature including soil, water and on animals. The bacteria can contaminate into milk form the surrounding environment. They can form biofilm. *Bacillus licheniformis*, *Bacillus coagulans*, *Bacillus cereus*, *Bacillus pumilus*, and *Geobacillus* sp. were identified from biofilms in dairy plants (Burgess *et al.,* 2010; Shaheen *et al.,* 2010; Yuan *et al.,* 2012). In the dairy factories, processing lines are usually made of stainless steel, had smoot surfaces. Sometimes, in places such as valves, joints the agglomeration, adhesion, contamination, and bacterial biofilm formation were detected (Bremer *et al.,* 2009).

In other studies, *Bacillus* spp. spores were easier to attach to stainless steel surfaces than vegetative cells because they were relatively hydrophobic, could resist heat and chemicals, and could adhere to higher solid surfaces (Rönner *et al.,* 1990; Hüsmark and Rönner, 1992). The presence of film-forming bacteria in milk such as *Bacillus* spp. need to prevent in milk sterilization equipment.

In this study, at 100°C, the D values of the BT2 and OM1 were higher than the D values of 8 *Bacillus cereus* isolated from 61 samples of rice in the Polymyxin Mannitol EggYolk Phenol Red Agar (PMYPA) medium (Sarrías *et al.,* 2002). The D values of the 8 strains ranged 0.43 to 1.09 minutes.

The previous study, the killing of the *Bacillus* spp. spores such as *Bacillus subtilis* and *Bacillus licheniformis* presented pineapple juice by the conducting heat method at different temperatures ranged from 85 to 100° C, brix ranged from 11 to 30% were performed (Evelyn *et al.,* 2021). The results showed that the D values decreased linearly with the increasing in temperature of heat-treatment. The D vales of *Bacillus subtilis* spores were 13.2; 6.8; and 2.1 minutes, respectively at 90, 95, and 100°C, respectively. The D vales of *Bacillus licheniformis* spores were 16.6; 7.6; and 3.6 minutes, respectively at 86, 90, and 95°C, respectively.

The investigation of the heat resistance of *Bacillus sporothermodurans*, *Bacillus subtilis*, and *Geobacillus stearothermophilus* at temperature ranged from 120 to 127.5ºC were performed. The results showed that the spores of the two strains as *Bacillus sporothermodurans* and *Bacillus subtilis* had the high heat resistance abilities and the D values ($D_{127.5}$) of 0.48 and 0.44 minute, respectively. The *Geobacillus stearothermophilus* spore had a lower D value ($D_{127.5}$) than the two strains, was 0.11 minute (Gómez and Chumillas, 2014).

The 7 spores belonged to the species of genus *Bacillus*, identified as *Bacillus subtilis* (BT1, OM2, and OM3), *Bacillus coagulans* (BT3 and BT4), *Bacillus sporothermodurans* (BT2), and *Bacillus cereus* (OM1). They survived at 80 and 90 °C for 12, 15, 20, 25, and 30 minutes, respectively. Among of them, two the BT2 and OM1 spores were the highest heat resistance spores; identified as the spores of *Bacillus sporothermodurans* BT2 and *Bacillus cereus* OM1, survived at 100 °C for 30 minutes. It is necessary to consider and apply the appropriate the heat treatment regime to kill the spores in raw milk pasteurization or sterilization in the future.

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