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Studies on the heat and disinfectant resistance of a spore-forming spoilage bacterium

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Abstract. Heat resistant thermophilic spore-forming bacteria, such as *Aeribacillus* (*A.*) *pallidus*, may contaminate the surfaces in food facilities resulting food spoilage of the products. The aim of this work was to determine the heat and disinfectant resistance of an *A. pallidus* strain that was isolated from a canning factory environment. Compared to other heat-resistant spore-forming bacteria, it did not prove to be very resistant to heat with a D_{10}-values of *A. pallidus* from 12.2 min to 2.4 min (at 102 °C and at 110 °C), with a calculated z-value of 11.6 °C. Not only spores but vegetative cells showed resistance against all investigated disinfectants.

1 Introduction

Thermophilic, endospore-forming bacteria can cause serious problems in different fields of food industry by their ability to form resistant spores and

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biofilms (*Flint et al.*, 2001; *Kilic et al.*, 2017). Endospores of bacteria are able to survive extreme environmental conditions. They are highly resistant to preservation techniques, e.g. to heat treatment, drying, irradiation, pressure treatment, or chemicals. Thermophilic bacteria are able to grow at 70 °C. The first research about their characterization was performed by *Miquel* in 1888. Since then, many strains of thermophilic bacteria belonging to the *Bacillus* and *Clostridium* genera have been characterized (*Maugeri et al.*, 2001; *Belduz et al.*, 2003). Recently, several bacteria of Bacillus genus have been reclassified, such as *Alicyclobacillus*, *Paenibacillus*, *Brevibacillus*, *Aneuribacillus*, *Virgibacillus*, *Salibacillus*, *Gracilibacillus*, *Ureibacillus*, and finally *Geobacillus*. Thermophilic bacteria in the 1. and 5. genetic groups were classified into the *Geobacillus genus* (*Iida et al.*, 2005).

Thermophilic bacteria have an optimal growth temperature range between 45 and 70 °C, wherefore they are isolated from hot environments such as hot springs, geothermally heated soils, shallow marine hot springs, petroleum reservoirs, deep-sea hydrothermal vents, the leachate of a waste pile from a canning factory, hot water pipelines, heat exchangers, waste treatment plants, etc. (*Rahman et al.*, 2004; *Bae et al.*, 2005). Microbial spoilage contributes to the vast amount of food that is wasted and the associated financial losses. Spoilage of heat-treated food products are usually provoked by spore-forming bacteria as a result of the resistance of spores to high temperatures commonly used to preserve foods. Canned food products generally undergo spoilage by thermophilic bacilli. *Denny* (1981) had demonstrated that thermophilic bacteria were the prime cause of spoilage of canned corn. Spoilage of heat-treated dairy products is often caused by thermophilic spore-formers. It was demonstrated by *Scheldeman* and co-workers (2006) that spoilage spore-formers, e.g. *Bacillus licheniformis* and *Aeribacillus pallidus*, were most frequently isolated from farms and may cause spoilage of treated milk.

In recent years, there has been evidence of contamination of canned food (especially canned corn) products by new spore-forming, thermophilic bacteria from the genus *Aeribacillus* (previously *Geobacillus*). Members of the genus *Aeribacillus* are aerobic, thermophilic, alkalitolerant, motile, and Gram-positive rods (0.8–0.962–5 nm) that occur singly, in pairs, or in chains. The reason for the spoilage of ready canned corn by *A. pallidus* can be the improper performance of thermal processes due to the incorrect relation between heating time and temperature. For that reason, investigation of the heat resistance of *A. pallidus* spores and determination of decimal reduction time (D-value) are high concerns for the canning industry. In comparison with other thermophilic bacteria from the genus *Geobacillus*, there are no reported D-value data for

A. pallidus in canned corn products.

Therefore, the aim of this study was to determine the influence of heat treatment and sanitizers on the survival of spores of *Aeribacillus pallidus*.

2 Materials and methods

2.1 Bacterial strain

In this study, *Aeribacillus pallidus* T6 3 (NCAIM B.01143) culture was used. The strain was isolated from spoiled canned corn products. Isolation and storage of *A. pallidus* culture was done using Casein-peptone Soymeal-peptone Agar (CASO agar, Merck).

2.2 Preparation of spore suspensions

Agar slants of *A. pallidus* cells were flooded with sterile distilled water, and then 2 ml of suspension was transferred on the surface of CASO agar in 200-mm-diameter Petri dishes.

Inoculated Petri plates were incubated at 55 °C for 48 hours and then were transferred to a refrigerator and stored at 15 °C for 72 hours. Spores then were collected by scrapping the surface of the agar with sterile metal spatula, suspended in sterile distilled water, and washed three times by centrifugation (4,000 × g for 10 minutes). Spore suspensions were stored at 4 °C until they were used. The number of spores in a suspension was determined by the pour-plate method, and it was 8×10^9 CFU/ml. Suspensions were diluted to obtain approximately 8×10^7 CFU/ml. Activation of spores was done by heating spore suspension in water bath at 80 °C for 10 minutes.

2.3 Heat treatment analysis

Canned corn brine (pH 6.11 ± 0.18) was used as a heating medium. Heating experiments were carried out in small glass vials. After filling them with 2.5 ml of spore suspension, vials were sealed with gas burner flame. Thermal inactivation was performed in temperature-controlled oil bath (Memmert, Model ONE 7, Germany). The samples were heated in the oil bath at temperatures of 102 °C, 104 °C, and 110 °C. The sample temperature was monitored continuously using Testo 110-1 channel NTC Thermometer with needle-type sensor. Triplicate samples were removed from the bath every 2 minutes, at 0, 2, 4, 6, and 8 minutes. After removal, the samples were immediately immersed into

cold water. The viable spores were counted by triplicate plating on CASO agar and incubated at 50 °C for 2 days.

2.4 Calculation of D10-values

D_{10}-values were calculated using the average slope ($D_{10} = -1/\text{slope}$) for each temperature treatment.

2.5 Disinfection efficacy test

2.5.1 Disinfection test against vegetative cells

A. pallidus was inoculated on CASO agar and incubated at 50 °C for 24 hours. The turbidity of the culture was set to OD 1 (the initial cell count was 3.2×107 CFU/ml). 400 μl of corn brine was applied to clean, pre-sterilized stainless steel coupon surfaces, and be distributed evenly throughout the coupons (8×6 cm). The brine was dried on the surface in a laminar box, and 400 μl of cell suspension (10^7 cell/ml) was uniformly dispersed on the surfaces and dried in a laminar box. 400 μl of disinfectant suspension was applied to the surfaces, and cells were removed from the surface with a swab soaked in an inactivating solution after the time of exposure (*Table 1*).

Table 1: Recommended parameters for the industrial application of disinfectants

Name	**Concentration**	**Contact time (min)**	**Temperature (°C)**
Apesin DSR	0.5%	30	25
Chlor-sept	1:10	1	25
Descosal	1%	30	25–30
Idro 86	3–5%	rinse as desired	
Innofluid-MF-M	2% (generally)	5–30	35–50
	8% (egg-contaminated surfaces)		
Megabrite	1–2% (generally)	10–30	30–80
	5–20% (heavy contamination)	15–60	30–80
Rimadet-SR-310	3–5%	20	25
Wunder	1:10	10	25–30

The surviving cells recovered by the swab were placed in an inactivating solution (1.56 g sodium thiosulfate ($Na_2S_2O_2{\cdot}5H_2O$), 0.07 g of lecithin, 0.1 mL Tween 80/100 mL PBS) and vortexed. After serial dilution, the number of surviving cells was determined by plate counting on CASO agar. Incubation

was carried out at 55 °C for 24–48 hours. As a control, clean brine-free surfaces were applied with the same procedure.

2.5.2 Disinfection test against spores

A. pallidus spores were heat-activated before disinfection test (10 minutes at 80 °C). Inoculation and treatment of spore suspension was carried out similarly as described in 2.5.1.

3 Results and discussion

3.1 The heat resistance of *Aeribacillus pallidus* spores

Thermal inactivation tests of *A. pallidus* spores at three different temperatures (102 °C, 104 °C, and 110 °C) in corn brine have been performed. Decimal reduction times (D_{10}-values) were calculated by linear regression analysis ($D_{10} = -1/\text{slope}$ of a plot of log surviving cells versus time). Survival curves of *A. pallidus* spores at different temperatures are shown in *Figure 1.*

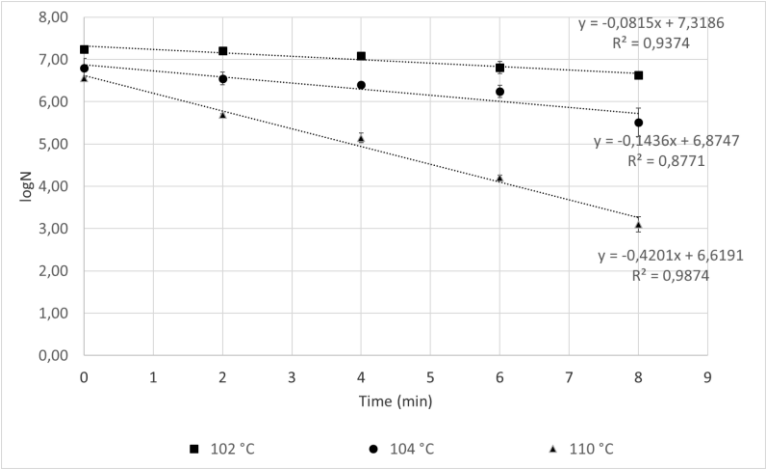

Figure 1: Survival curves of *Aeribacillus pallidus* spores in corn brine at different temperatures

Table 2 summarizes the D-values obtained at different heating temperatures in corn brine.

The D-values of *A. pallidus* ranged from 12.2 to 2.4 min (at 102 °C and at 110 °C), and the calculated z-value of the test strain was 11.6 °C.

Table 2: D_{10}-values of *Aeribacillus pallidus* spores at different temperatures in corn brine

Heating temperature (°C)	D10-values (min)
102	12.2
104	7.1
110	2.4

In comparison with literature data, the *A. pallidus* strain proved to be not extreme heat resistant in corn brine. *Geobacillus stearothermophilus*, a thermophilic spore-forming bacterium, has a D-value of 62.04 min at 112.8 °C, 18.0 min at 115.6 °C, and 3.33 min at 121.1 °C in aqueous suspension, with a z-value of 8.3 °C (*Feeherry et al.*, 1987).

Warth (1978), when examining the heat tolerance and optimal growth temperature of *Bacillus* species, found that there is a relationship between optimal growth temperature and heat resistance. The composition of the culture medium has no effect on maximum growth temperature. However, the composition of the heat treatment medium (pH, water activity, etc.) greatly influences bacterial heat tolerance. *Lopez et al.* (1996) showed that the heat tolerance of *B. stearothermophilus* spores was reduced by 7 to 23 times when the pH of the heat treatment medium was lowered from 7 to 4.

3.2 Disinfection efficacy tests

3.2.1 Efficacy of different disinfectants against *A. pallidus* vegetative cells

The metal coupons used in surface tests were designed to model the surface of industrial production lines, both in the form of clean and organic soil-contaminated surfaces.

Table 3 shows the extent of disinfectant-induced decrease in the number of cells between the initial cell count and the number of cells recovered after plating.

In general, disinfection treatment resulted in a mean 2.9 log reduction.

Against vegetative *Aeribacillus pallidus* cells, the most effective agents were Chlor-sept, Innofluid-MF-M, and Megabrite.

Chlor-sept disinfectant was examined at the specified concentration and at two end contact times (1 min, 30 min) as per the manufacturer's instruction.

Table 3: Cell count reduction of *A. pallidus* vegetative cells during disinfectant treatments

Disinfectant			Reduction (Log N)	
Name	**Used concentration (%)**	**Contact time (min)**	**Clean surface**	**Surface with brine**
Apesin	0.5	30	2.37	2.37
Chlor-sept	10	1	2.32	2.01
		30	3.73	3.36
Descosal	1	30	2.43	2.20
Idro-86	5	15	2.47	2.74
Innofluid-MF-M	2	10	2.47	2.32
	8	5	3.51	3.3
Megabrite	2	10	3.46	3.39
Rimadet	3	20	2.7	0.54
Wunder	10	10	3.01	2.84

For Innofluid, using the two concentrations (2%, 8%) given by the manufacturer, 8% treatment with only 5 minutes exposure time was more effective than 10 minutes contact time with 2% concentration. Megabrite has been shown to be effective at 2% concentrations for 10 minutes. The acidic detergent, Wunder, caused a reduction of 3 orders of magnitude in 10% concentration. The Rimadet chemical (3%) on clean surface resulted a considerable cell count reduction in contrast with the organic-loaded surface, which showed the lowest efficiency. In the case of Apesin, the agent showed very low activity against the test strain, with no difference between the clean and soiled surfaces. Descosal (1%) also had low efficacy, similarly to the previous agent, after 30 minutes of exposure. In the case of Idro-86, however, unexpected results were observed. After disinfection treatment, the number of surviving cells was higher on the clean surface than on the brine-containing surface.

Overall, the number of cells decreased several orders of magnitude; however, the lethal effect of the chemicals on the vegetative form of bacteria was not remarkable. The high survival rate is partly due to the presence of spores in the cell mass produced during vegetative cell culturing (the initial cell suspension showed the presence of spores after spore stain; results are not shown).

3.2.2 Efficacy of different disinfectants against *A. pallidus* spores

The results of the disinfectant test against spores are summarized in *Table 4*.

In the number of spores, 2–3 log reduction was observed generally after treatment. The greatest reduction was caused by the chlorine-containing Chlor-sept

as well as by Innofluid and Megabrite. Among them, the most effective one was the application of 10% Chlor-sept solution for 30 minutes – both on clean and organic soiled surfaces. Surprisingly, despite the efficacy of Innofluid, increasing the exposure time did not affect further the decrease in spore count. Megabrite was as effective against spores as against vegetative cells after only 2% treatment for 20 minutes.

In the case of Idro-86, a small but measurable difference could be observed between the results of the two surfaces. At 10% concentration, Wunder resulted a moderate reduction in cell number on a clean surface, while the surface exposed to organic matter showed less efficiency. Rimadet was the least efficient at 3% concentration on both clean and dirty surfaces. In the applied concentration, Descosal and Apesin were moderately effective against spores.

Table 4: Spore count reduction of *A. pallidus* during disinfectant treatments

Disinfectant			**Reduction (Log N)**	
Name	**Used concentration (%)**	**Contact time (min)**	**Clean surface**	**Surface with brine**
Apesin	0.5	30	3.06	2.68
Chlor-sept	10	1	2.44	1.51
		10	2.62	2.25
		30	3.52	3.3
Descosal	1	30	2.40	2.28
Idro-86	5	15	2.65	2.73
Innofluid-MF-M	8	5	2.89	2.87
		10	2.98	2.93
Megabrite	2	20	3.20	3.10
Rimadet	5	20	1.78	1.59
Wunder	10	10	3.15	2.62

Compared with other literature data, a similarly low spore count reduction was observed by *Guan* and co-workers (2013) for the heat resistant spore-forming bacterium *Geobacillus stearothermophilus*. Disinfectants reduced the spore counts on heavy-organic-load-containing surfaces by only less than 2 $\log_{10}$ within 2 hours of exposure.

In most cases, resistance decreased with increasing concentration, but increasing exposure time did not cause significant changes in either vegetative cells or spores. As expected, endospores proved to be more resistant to the vegetative form although the degree of resistance was not considerable.

4 Conclusions

Because of the adhesive characteristics of spore-forming bacteria, they are ubiquitous in the food industry, in raw materials, ingredients, packaging materials, environment, and processing lines. Therefore, the contamination of end-products can easily occur. Due to their heat resistance, commonly used pasteurization processes fail to kill spores. Moreover, heat resistance may vary within the strains of a species and may differ according to the physiological state of cells and the composition of the food product (e.g. composition, a_w, pH). Therefore, further research is needed to determine the resistance properties of spoilage spore-formers.

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