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Author: Saghar Safamanesh	………………………………………………………… (signature of author)

Program coordinator:

Mark van der Giezen

Faculty supervisor:

Heinz Peter Ruoff

External supervisors:

Tone Mari Rode, Jan Thomas Rosnes

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Control of psychrotrophic *Bacillus cereus* with high pressure processing and preservatives

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Abstract

Bacillus cereus are Gram-positive endospore-forming bacteria with a growth temperature range of 4-55 °C. The ability of psychrotrophic strains of *B. cereus* spores to survive pasteurization, grow at refrigerated conditions and cause food poisoning is a concern with regard to food microbiological safety. The main objective of this master thesis work was to obtain knowledge about *B. cereus* with relevance to inactivation spores with thermal, pressure-thermal treatment, and growth inhibition with preservatives.

Testing the psychrotrophic growth of the strain was the first experiment. Then, spores were heated in LB medium at 8 different combinations of temperatures (80 and 90 °C) and time in a water bath. The effect of thermal process on spores, as well as the decimal reduction times (D-values) were determined. The effect of Tween 20 on spore heat resistance and prevention of clump formation was studied. The growth of *B. cereus* was also tested in different types and concentrations of preservatives. Different concentrations of sodium chloride (NaCl), potassium chloride (KCl), sodium nitrite ($NaNO_2$), and nisin as well as different levels of pH were applied to *B. cereus* to investigate the growth process of vegetative cells and spores. Spores were then heated in food matrices (minced meat and minced fish) at 85, 90, and 95 °C, and spore reductions were investigated. Finally, the effect of 6 different pressure-thermal process conditions was studied on spores in minced meat and minced fish.

The lowest growth temperature for this strain was determined as 5 °C. Heating spores in LB medium at 80 °C resulted in 1.9, 1.8, 3.0 and 2.9 log reductions after 5, 10, 15 and 20 minutes respectively. Heating spores in LB medium at 90 °C reduced spores by 2.2, 4.0, 5.1 and 5.2 logs after 2, 5, 8, and 10 minutes. D-values were determined as 7.1 min at 80 °C and 1.9 min at 90 °C. Adding 0.1% of Tween 20 to the washing solution, prevented spore clumping to a great extent without effect on the spore's heat resistance. The growth experiment with preservatives revealed that the inhibitory effect of preservatives on both vegetative cells and spores was the same. In the presence of 25 µg/ml of nisin and pH 3 and 4, no growth was observed. The time to detection (TTD) was longer in 4% NaCl and KCl than in 1 and 2%. No change in growth was observed in the presence of all concentrations of $NaNO_2$ and also in lower concentrations of nisin (15 and 7.5 µg/ml). When spores were heated in food matrices, the resulted log reductions were very similar in minced meat and minced fish. Spore population was reduced by 3.3 log in minced meat and 3.5 log in minced fish after heating at 95 °C for 5 minutes. Comparing the log reductions of spores heated in LB and food matrices, revealed that

spores were more heat-stable in food matrices than in LB medium. Regarding the effect of pressure-thermal processing, in minced meat and minced fish, the highest log reductions of 3.2 and 3.3 were obtained respectively at processing conditions of 600 MPa, 55 °C, and holding time of 10 minutes.

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Abbreviations

a_w	Water activity
CFU	Colony forming units
D-value	Decimal reduction time
DPA	Dipicolinic acid
HPP	High pressure processing
IU	International Units
Milli-Q water	Ultrapure water (trademark)
MPa	Megapascal
OD	Optical density
PATS	Pressure-assisted thermal sterilization
rpm	Rotation per minute
TTD	Time to detection
z-value	Thermal resistance constant

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1.INTRODUCTION

According to the Centers for Disease Control and Prevention, one out of six people (48 million) in the US acquires a foodborne illness every year, resulting in 128,000 hospitalizations and 3,000 deaths. Bacteria and viruses were the two most common types of foodborne pathogens in the United States in 2017 (Choi & Kim, 2020). Bacterial pathogens, such as *Escherichia coli* O157:H7, *Salmonella*, *Vibrio parahaemolyticus*, *Campylobacter*, *Staphylococcus aureus*, *Clostridium perfringens*, and *Bacillus cereus*, were responsible for these outbreaks (Scallan et al., 2011; Tewari & Abdullah, 2015). Among these hazardous microorganisms, *Bacillus* species are significantly resistant to physical (heat, UV radiation, ϒ-Radiation) or chemical treatments such as acids, bases, phenols, aldehydes, alkylating agents, and oxidizing agents via formation of endospores (Nicholson, Munakata, Horneck, Melosh, & Setlow, 2000). *B. cereus* related food poisoning has generally mild, short duration and self-limiting symptoms, however, there have been reports of fatalities (Bintsis, 2017). Death was attributed to severe metabolic acidosis and liver failure (Choi & Kim, 2020).

B. cereus is a major concern to food safety because resistant spores can tolerate food processing and preservation methods, leading to food deterioration or foodborne diseases after spore germination, growth, and toxin production (R. P. Lopes, M. J. Mota, A. M. Gomes, I. Delgadillo, & J. A. Saraiva, 2018). The ability of the *B. cereus* to grow and survive at refrigeration temperature is another factor that makes it a potential threat to food processing (Tewari & Abdullah, 2015). According to European Food Safety Authority (2005), *B. cereus* can grow at temperatures from 4 to 55 °C.

Milk and rice perhaps are the two common contaminated food items. Meat and meat products are also reported to be contaminated by this organism. Spores of this bacterium have a great ability to adhere to surfaces and food industry instruments. Apart from that, spores are present in several spices and additives so there is a possibility of increasing *B. cereus* contamination of food products in each stage of food processing (Tewari & Abdullah, 2015). This bacterium is isolated from 28% of the meat product samples and among these food samples, heat-treated meat products alone showed 48% positivity for the *B. cereus* (Schlegelova, Brychta, Klimova, Napravnikova, & Babak, 2003). This is due to the resistance of spores to heat treatment (Tewari & Abdullah, 2015). *B. cereus* is also found in frozen food products. Kl Mira and MA Abuzied (2006) collected different types of ready-to-eat chicken food and frozen half-cooked chicken products from restaurants and supermarkets and found

that *B. cereus* was present in 100% of samples. *B. cereus* has been isolated from other kinds of food such as infant foods, ready-to-serve foods, seafood, cocoa/chocolate, cereals and cereal derivatives, and fresh vegetables (Tewari & Abdullah, 2015).

The application of heat is a common method used in the food industry to control microbial contamination. Pasteurization, involves a moderate heat treatment (< 75 °C), kill pathogenic vegetative cells. Sterilization which is a higher heat treatment (121°C) is able to inactivate spores of *Bacillus*. Although high temperature can inactivate spores, it can influence food's structure, taste, and nutritional values. Due to the negative effects of heat on food, the food industry is interested in using techniques that can guarantee safety while minimizing the impact on important food characteristics to retain more of their "fresh-like quality" (Soni, Oey, Silcock, & Bremer, 2016). Consumers demands for safe foods with high nutritional value and good taste are increasing, so recent attention has been focused on non-thermal inactivation methods (such as pressure processing) for inactivating of resistant spores (Cho & Chung, 2020). To choose and decide which technology should be applied in a particular case, it is important to assess the sensitivity of target spores to different treatment condition by taking into consideration the features of the products (R. P. Lopes et al., 2018). Pressure processing is an efficient preservation technology that can inactivate microorganisms and spores and retain many sensory and nutritional attributes of foods (Balakrishna, Wazed, & Farid, 2020). However, the resistance of microorganisms to pressure varies considerably depending on some factors such as type of the bacteria and content of the food. Therefore, application of pressure needs more investigations.

1.1.Aim of the study

The main objective of this study was evaluation the efficacy of thermal and pressure-thermal treatment for the inactivation of *Bacillus cereus* spores. For this purpose, spores were heated at 8 different temperature-time combinations in LB medium, at 3 different temperature-time combinations in minced meat, and at 3 different temperature-time combinations in minced fish. Spores also were treated in six different pressure-thermal conditions in both minced meat and minced fish. Besides, the effect of specific concentrations of preservatives (NaCl, KCl and $NaNO_2$, nisin) and pH were studied on the growth pattern of *B. cereus* spores and vegetative cells in LB medium. An overview of the different experiments performed in this thesis is presented in Figure 1.

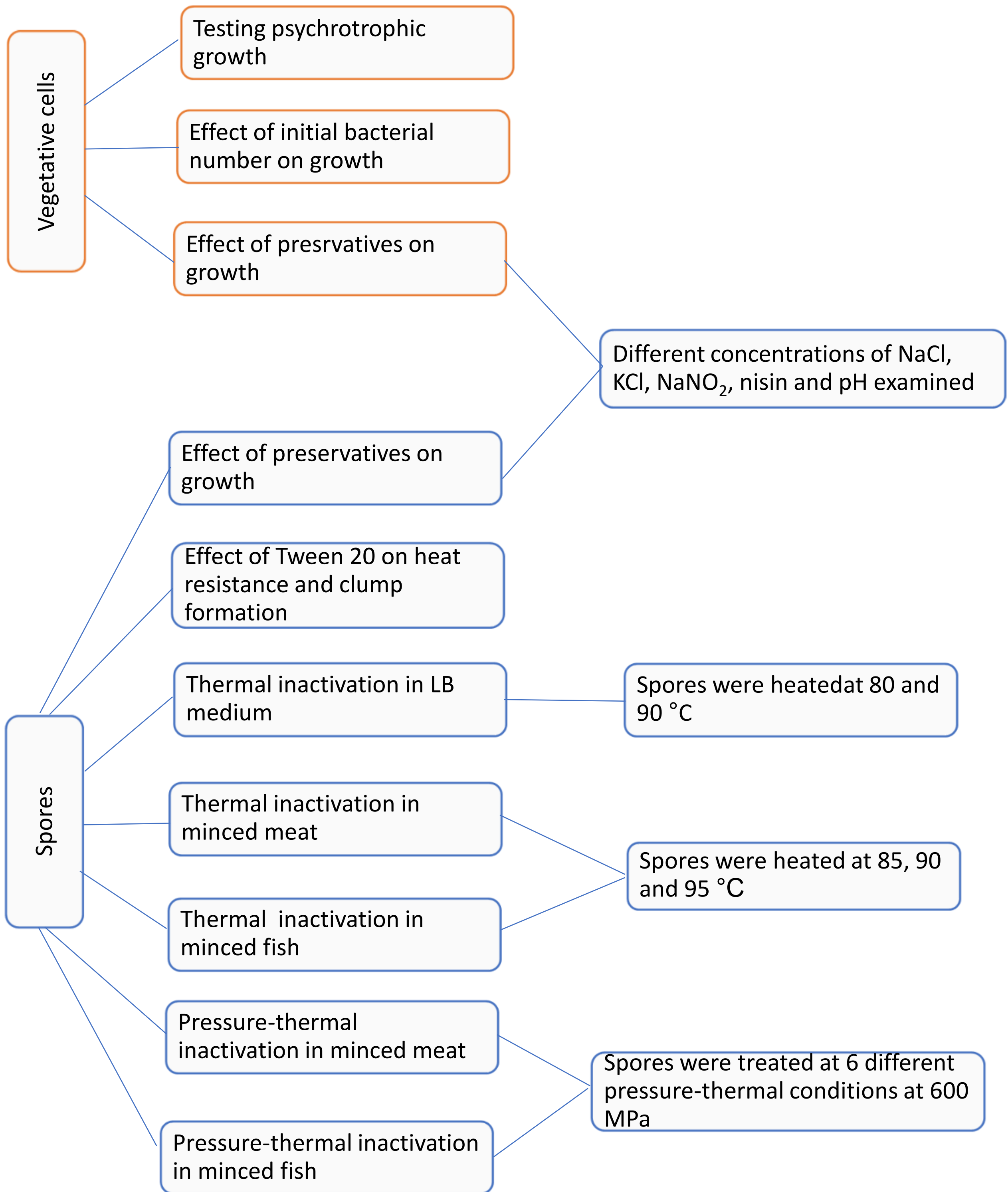


Figure 1. *An overview of the experiments conducted in this thesis.*

2.THEORY

2.1.The genus *Bacillus*

The genus *Bacillus* is distributed widely in nature and includes thermophilic, psychrophilic, acidophilic, alkalophilic, and halophilic bacteria (Cihan, Tekin, Ozcan, & Cokmus, 2012; Nazina et al., 2001). This genus belongs to the Bacillaceae family, which was founded by Ferdinand Cohn in 1872 (Logan & Halket, 2011). Members of the genus *Bacillus* are one of the predominant bacterial genera found in soil and they are known to have beneficial traits for decomposition of organic matters (Saxena, Kumar, Chakdar, Anuroopa, & Bagyaraj, 2020). *Bacillus* species can also be found in a variety of places, including the air, water, human and animal guts, as well as foods (Elshaghabee, Rokana, Gulhane, Sharma, & Panwar, 2017). Their role in foodborne illnesses and food spoilage is documented (Özdemir & Arslan, 2019). Figure 2 shows the taxonomy of the genus *Bacillus*.

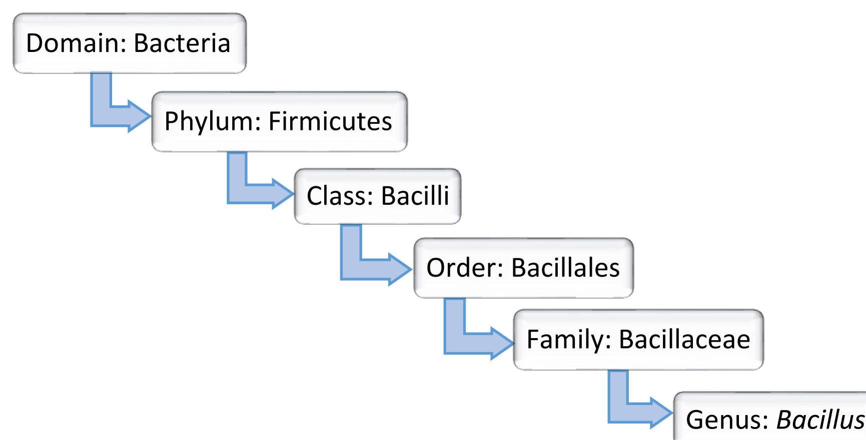


Figure 2. *Taxonomy of genus Bacillus (Elshaghabee et al., 2017).*

2.1.1.Properties of *Bacillus* species

The genus *Bacillus* is a large collection of Gram-positive or Gram-variable staining, endospore-forming, aerobic or facultatively anaerobic, rod-shaped bacteria that has undergone significant reclassification as molecular biology has shown phylogenetic heterogeneity (Cihan et al., 2012). The genus *Bacillus* is the most diversified genus in the Bacillaceae family. Over 200 species of *Bacillus* have been identified and differentiated by genetic approaches. Taxonomy based on the 16S rRNA gene is one approach of identifying bacteria. According to

this method, the genus *Bacillus* is divided into three major groups: The *Bacillus subtilis* group, *Bacillus cereus* group and *Bacillus pumilus* group (Shu & Yang, 2017).

One of the most distinct characteristics of the *Bacillus* species is their ability to produce heat-resistant spores in response to adverse growth conditions. Spores are resistant to wet heat, dry heat, radiation, desiccation, extreme pH, chemicals, enzymes, and high pressure (RYU & BEUCHAT, 2005). Dormant endospores are substantially more resistant to standard inactivation and disinfection treatments than vegetative cells and this high degree of resistance must be considered when designing processes to inactivate *Bacillus* spores in foods. Processing and preservation of foods should be designed in a way to ensure that the spores are either inactivated or prevented from germination and outgrowth (Cho & Chung, 2020). If spores are not inactivated completely during food preservation, they germinate to vegetative cells and produce enzymes that may change organoleptic properties, resulting in reduced shelf life or safety (Soni et al., 2016).

2.1.2.*Bacillus cereus*

Bacillus is a Latin term that means "small rod", and cereus means "wax-like." The name refers to *B. cereus's* easily recognizable morphology when viewed under a microscope or on blood agar plates. It is a large (1.0–1.2 µm by 3.0–5.0 µm) Gram-positive and motile rod-shaped bacterium. In growth media most strains form endospores within a few days. Characteristics such as motility, haemolysis, carbohydrate fermentation (*B. cereus* does not ferment mannitol) and lecithinase (phospholipase) production are commonly used for its identification (Bintsis, 2017; Stenfors Arnesen, Fagerlund, & Granum, 2008).

B. cereus may be isolated from grains, dairy products, spices, dried foods, and vegetables (Berthold-Pluta, Pluta, Garbowska, & Stefańska, 2019). *B. cereus* is commonly found in industrial food production equipment due to the formation of adhesive endospores, so cross-contamination can cause distribution of the bacteria to other foods, such as meat products (Bintsis, 2017; Majed, Faille, Kallassy, & Gohar, 2016). *B. cereus* spores are responsible for attachment and, their survive during heat treatment or other procedures which remove vegetative bacteria. Spores are hydrophobic and the hydrophobicity differs among strains (Stenfors Arnesen et al., 2008). Spores produced by *B. cereus* have a higher hydrophobicity than other *Bacillus* spores. This bacterium can grow at temperatures from 4 to 55 °C but prefers 30–40 °C, depending on the strain (Bintsis, 2017; European Food Safety Authority, 2005). Strains that are able to grow at a temperature of 8 °C or below are named psychrotrophic strains

(Martin D. Webb, Gary C. Barker, Kaarin E. Goodburn, & Michael W. Peck, 2019); therefore, during the storage of minimally processed chilled food, psychrotrophic bacterial cells can multiply and reach a concentration that causes illness (M. D. Webb, G. C. Barker, K. E. Goodburn, & M. W. Peck, 2019). This microorganism can grow at pH levels of 4.5–9.5 (Rodrigo, Rosell, & Martinez, 2021), however in meals, the inhibitory effect of pH may be reduced, as documented by limited growth of this bacteria in meat at pH 4.3 (Bintsis, 2017).

According to Stenfors Arnesen et al. (2008), *B. cereus* is an important cause of foodborne disease worldwide. Concentration of at least 10^6 CFU/g can cause illness in adults. Infants, children, and the immunosuppressed are most likely to be susceptible to the lower concentration of 10^3 CFU/g (Jaquette & Beuchat, 1998). *B. cereus* was the third most common source of the food-poisoning outbreaks in Hungary, followed by Finland, Netherlands and Canada between 1960 and 1968. In addition, many foodborn illnesses related to *B. cereus* were reported from USA, UK, Scandinavia, Japan and Norway (Tewari & Abdullah, 2015).

2.1.3.*Bacillus cereus* Toxins

B. cereus can cause two types of illnesses: emetic-type and diarrhoeal-type. Emetic illness is caused by the synthesis of the toxin cereulide (emetic toxin). This toxin is pre-formed in food at temperatures of 12 to 37 °C. The Emetic toxin is heat stable at 100 °C for >2 hours. (Thirkell, Sloan-Gardner, Kacmarek, & Polkinghorne, 2019). Diarrhoeal-type illness is caused by one or more associated toxins including enterotoxin FM which is not pathogenic but contributes to the severity of diarrhoeal illness; haemolysin BL (Hbl); cytotoxin K (CytK); and non-haemolytic enterotoxin (Nhe). The illness is caused when dormant spores are ingested which then germinate, proliferate, and produce toxins in the small intestine (Thirkell et al., 2019). Table 1 compares the characteristics of the Emetic and Diarrhoeal-type illnesses.

When spores are not inactivated during the processes designed for food production, their germination and outgrowth in the finished food products is possible when food is stored in a favourable condition. Although gentle heat treatment and pasteurization at temperatures of 65-75 °C (Amit, Uddin, Rahman, Islam, & Khan, 2017), eliminate background microbiota and vegetative cells, spore germination can be induced by this treatment. Similarly, pasteurization cannot inactivate the emetic toxin produced by *Bacillus* spp. Only heat treatments used for canning low acid foods (121°C for 3 minutes) can assure complete destruction of spores. However, this severe heat treatment can change both sensory and nutritional attributes of foods.

So, a combination of high pressure and temperature is required to inactivate spores (EFSA Panel on Biological Hazards, 2016).

Table 1. *Characteristics of the two types of B. cereus foodborne disease (Stenfors Arnesen et al., 2008).*

Characteristics	**Diarrhoeal disease**	**Emetic disease**
Type of toxin	Protein; enterotoxin(s): Hbl, Nhe, CytK	Cyclic peptide; emetic toxin (cereulide)
Location of toxin production	In the small intestine of the host	Preformed in foods
Symptoms	Abdominal pain, watery diarrhoea and occasionally nausea, Lethality has occurred	Nausea, vomiting and malaise. A few lethal cases (possibly due to liver damage)
Incubation time	8–16 h (occasionally >24 h)	0.5–6 h
Duration of illness	12–24 h (occasionally several days)	6–24 h
Foods most frequently implicated	Proteinaceous foods; meat products, soups, vegetables, puddings, sauces, milk and milk products	Starch-rich foods; Fried and cooked rice, pasta, pastry and noodles

Some factors can inhibit or delay the growth of *B. cereus,* for example, low pH (< 4.5) and reduction in a_w (below 0.92). Food storage at below 10 °C, results in prolonged lag time and generation times, particularly whenever other factors (i.e., pH, a_w) are not optimum for *B. cereus.* Refrigeration at temperatures lower than 4 °C can prevent growth of all types of *B. cereus*, including psychrotrophic strains (European Food Safety Authority, 2005). Anaerobic condition is another factor that causes longer lag time, slower growth rate and weak sporulation, especially when combined with chilled storage or increased acidity (M. D. Webb et al., 2019). Addition of some food preservatives such as propionic acid, calcium or potassium propionate, calcium acetate, potassium sorbate, and nisin can inhibit growth and toxin formation of *Bacillus* species in foods (Mohamed & Ghanyem, 2015; J. Smith, Daifas, El-Khoury, & Austin, 2002).

2.1.4.Vegetative cell growth

Species of the genus *Bacillus* can be found in two distinct states, vegetative and spore state. In the vegetative state, bacteria are metabolically active and use available nutrients to grow and replicate. Vegetative cells divide by binary fission, a process that generates two identical daughter cells from a single mother cell (Eichenberger, 2017). When a bacterial population grows in a culture, there is a pattern of growth known as a bacterial growth curve (Figure 3). This curve is depicted as the number of live cells in a population over time and consists of four phases: lag, exponential (logarithmic), stationary, and death (decline) (Książek, 2010).

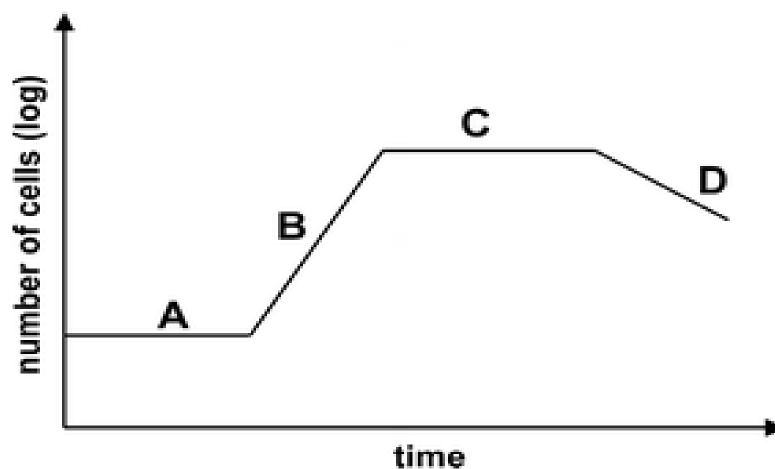


Figure 3. *Phases of the bacterial growth curve; a lag phase (A); an exponential growth phase (B); a stationary phase (C); a death phase (D) (Książek, 2010).*

The initial cell growth phase, the lag phase, is a period during which the bacteria adjust themselves to the new environment. The Lag phase is characterized by cellular activity but not growth. Briefly, bacteria increase in size, store nutrients, and synthesize materials necessary for replication, before the onset of exponential growth (Książek, 2010; Rolfe et al., 2012). In the exponential phase, cells are dividing by binary fission and doubling in numbers after each generation time. It involves multiple rounds of DNA synthesis, transcription, and translation, to synthesize necessary macromolecules. Bacteria reproduce at a constant rate and the logarithm of the bacterial population rises linearly with time. The growth rate is strain-specific and can happen with a doubling time as short as 20 min for E*scherichia. coli* (Książek, 2010; Rolfe et al., 2012).

As available nutrients diminish, and waste products start to accumulate, bacterial cell growth reaches a plateau or stationary phase. This is where the number of dividing cells equals the number of dying cells. During this period, the culture has the highest population density and endospore formation starts after cells enter the stationary phase. As a population overcrowding takes place, nutrients become less available, and toxic products increase, the number of dying cells exceeds cell multiplication. In the death phase, the number of living cells decreases exponentially, and population growth shows a decline (Książek, 2010; Pletnev, Osterman, Sergiev, Bogdanov, & Dontsova, 2015; Serra, Earl, Barbosa, Kolter, & Henriques, 2014).

2.1.5.Spore structure

A spore has a very different structure than that of a vegetative cell, as the spore has several layers and components that are not present in growing cells (Figure 4). The exosporium is the outer surface layer of spores and its size varies in different species. For example, it is relatively big in *B. cereus* while in *B. subtilis* spores, if present at all, it is extremely little (Elaine P. Black et al., 2007). The exosporium is composed of carbohydrate and protein and the specific function of this layer is unknown. The spore coat is below the exosporium and is composed of several protein layers. The coat layer is important in spore resistance to some chemicals and lytic (lysozyme-like) enzyme (Setlow, 2006). Spores with a defective coat retain their resistance to high hydrostatic pressure, implying that the coat plays no role in spore pressure resistance. The presence of the coat layer has a limit or no influence on spore resistance to heat and radiation (Elaine P. Black et al., 2007; Setlow, 2006).

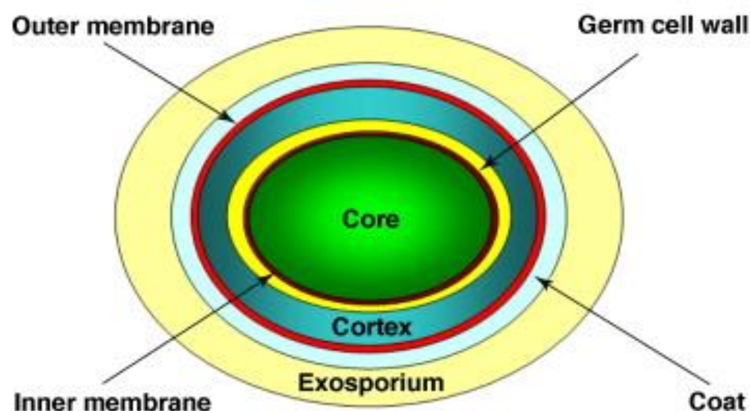


Figure 4. *Spore structure. Starting from the outside, the endospore layers include the exosporium, coat, outer membrane, cortex, germ cell wall, inner membrane and central core (Paredes-Sabja, Setlow, & Sarker, 2011).*

The outer membrane is located underneath the spore coat. Although this membrane is an important element in spore generation, its specific function is unknown (Piggot & Hilbert, 2004). Indeed, removal of this membrane has no notable effect on spore resistance to radiation, heat, and some chemicals (Setlow, 2006). The next layer is the spore cortex, which is mostly made of peptidoglycan. The cortex is required for establishing and maintaining spore dormancy by reducing the water content of the spore core. In spore germination, the cortex is degraded and this cortex hydrolysis is essential for spores to "return to life" (Setlow, 2003). The germ cell wall located under the cortex likely plays no role in spore resistance but becomes the cell wall of the outgrowing spore. The next spore layer is the inner membrane that plays a major role in spore resistance to many chemicals (Elaine P. Black et al., 2007; Setlow, 2006).

The final spore layer is the core that contains most spore enzymes as well as DNA, ribosomes and tRNAs. There are also two molecules whose concentrations in the core have a key role in spore resistance (Setlow, 2006). The first is the water molecule. Water comprises only 27–55% of the spore core wet weight (depending on the species), in contrast to the vegetative cells in which 75–80% of the wet weight of the protoplast is water. The low water content of the core is responsible for the spore's enzymatic dormancy as well as its heat resistance. Dipicolinic acid (DPA) chelated with divalent cations, mainly Ca^{2+}, is the second small molecule of the core with an important role in spore resistance (I. Smith & Slepeky, 1989). Another small molecule in the core that plays a role in spore resistance is small acid-soluble proteins (SASP). SASP bind to the spore DNA and protect it against a variety of agents, including moist and dry heat and oxidizing agents (Elaine P. Black et al., 2007).

2.1.6.Sporulation

Sporulation is an adaptive response of the vegetative cells to harsh environmental conditions, that allow cells to survive in a dormant state. During sporulation, the cells undergo different morphological changes as the spore develops and matures (Figure 5). The sporulation process is commonly described in seven steps (stages 0 - VII):

Stage 0 is where cells are still growing by binary fission. In the next stage, stage I, DNA replication and the creation of an axial filament occur. Bacterial cell elongates in this stage. Stage II is characterized by asymmetric division of the cell. At one end of the cell, an asymmetric septum forms, to produce a small double-membrane structure called a forespore. Engulfment is the third stage. The membranes generated in stage II develop around the smaller compartment and engulf it in a process like phagocytosis, resulting in the formation of a cell

inside a mother cell. Stage IV is cortex formation. The spore cortex now forms between the two membranes of the mother cell and the developing spore. Moreover, calcium-DPA accumulates in the core in stage IV. In stage V, the spore coat is synthesized, consisting of ~80 proteins deposited by the mother cell. During stage VI spore maturation occurs, many enzymes found in vegetative cells are destroyed and replaced by a distinct collection of spore components, and the spores become resistant. The mother cell undergoes lysis by lytic enzymes and releases a matured spore in stage VII (Cho & Chung, 2020; Sella, Vandenberghe, & Soccol, 2014).

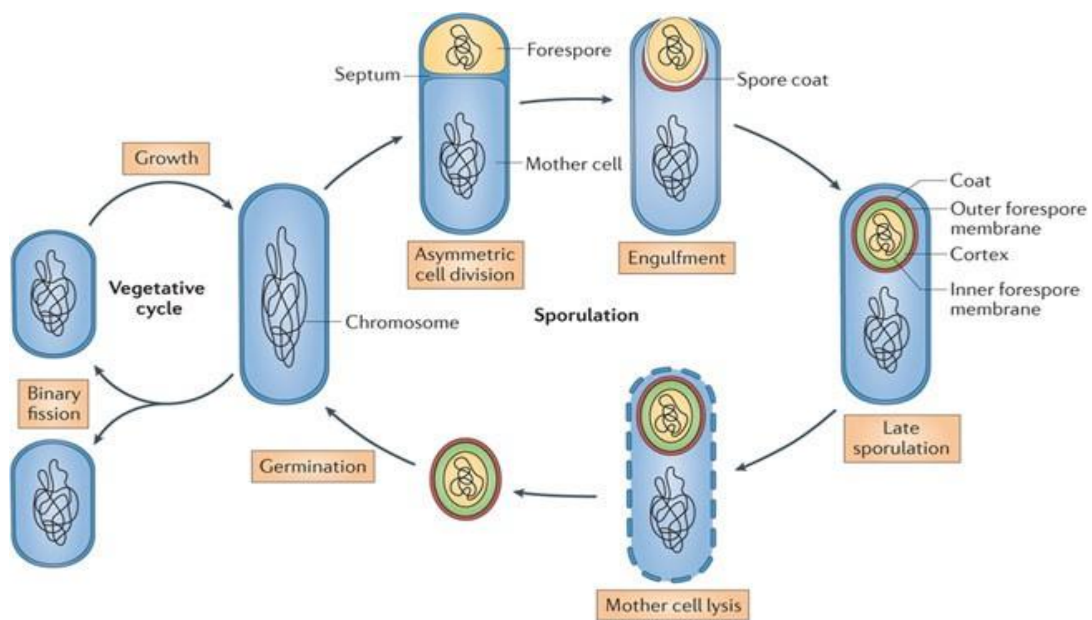


Figure 5. *The main stages of sporulation (McKenney, Driks, & Eichenberger, 2013).*

2.1.7.Germination and outgrowth

Spores may remain dormant for a long time and retain significant resistance to environmental stresses such as heat, radiation, chemicals, and pH extremes. The spore breaks dormancy and initiates growing under favourable conditions, a process that starts with spore activation, followed by germination and outgrowth. The presence of nutrients often triggers germination. The identity of these nutrients varies with the species and strain of spore-formers, but common nutrients are amino acids, sugar, and nucleosides (Elaine P. Black et al., 2007; Sella et al.,

2014). The processes involved in the germination of endospores (Figure 6) are the following stages:

Activation: Activation is the start of a process in response to nutritional replenishment that happens when germinating molecules (amino acids, sugar, and nucleosides) are recognized by germination receptors (GRs) in the spore's inner membrane. This interaction between receptors and nutrients changes the permeability of the membrane (Cho & Chung, 2020; Sella et al., 2014). Sublethal heat treatment is a common way to activate spores however, the exact mechanism of activation is not well understood yet (Setlow, 2003), hence the question marks in Figure 6.

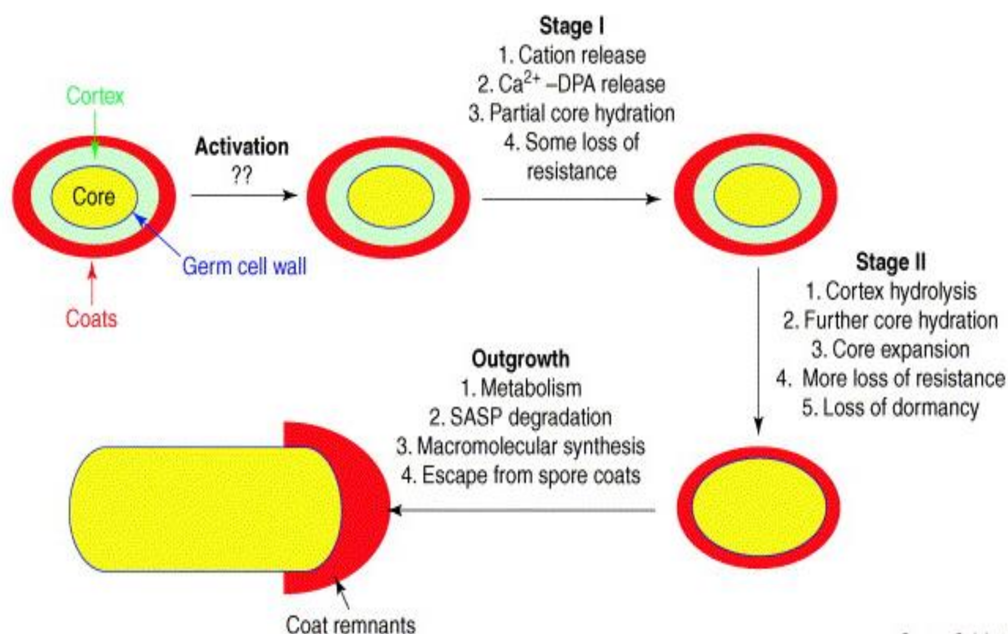


Figure 6. *Events in spore germination; Activation, Stage I, Stage II, and Outgrowth (Setlow, 2003).*

Stage I: The spore releases H^+, K^+, Na^+, and Ca^{2+}, causing the pH of the spore core to rise from 6.5 to 7.7. The cortex lytic enzymes are activated since cortex hydrolysis is necessary for the succeeding subsequent stages of germination, which include the start of spore metabolism and the growth of the germinated spore (Cho & Chung, 2020; Sella et al., 2014). DPA is degraded and released and rehydration of the spore core happens (Zhang et al., 2010).

Stage II: Hydrolysis of the bacterial spore peptidoglycan (cortex) happens in this stage. The water content of the core increases sufficiently to activate enzymes. The germ cell wall of the spore expands (Setlow, 2003).

Outgrowth: This is the step when DNA, RNA and proteins are synthesized (Sella et al., 2014; Tehri, Kumar, Raghu, & Vashishth, 2018). During the first stages of outgrowth, ATP is generated through the resources stored in the spore core. At a later stage, the outgrowing spores use extracellular nutrients. It is also documented that amino acids derived from SASP degradation, can support some metabolisms of the outgrowth stage (Elaine P. Black et al., 2007; Cho & Chung, 2020).

2.2.Inactivation kinetics

Inactivation kinetics of bacteria and their spores normally follow log-linear kinetics. The D and z-values are used as parameters to estimate bacterial cell or spore inactivation. The D-value (decimal reduction time) is the number of minutes required to decrease viable bacteria by 90% when exposed to a specific temperature. It can be used as a measure of the rate of microbial inactivation. The z-value is the temperature change required to achieve a 1-log reduction of the D-value. These kinetic parameters are used to ensure safety of food preservation processes. They also permit the comparison of different food process technologies in reduction of microbial populations (Food and Drug Administration, 2000; C. Kim et al., 2019; Rui Li, Kou, Zhang, & Wang, 2018).

Multiple variables impact inactivation kinetics such as bacterial strains, age of the culture, food composition (fat, NaCl, pH and a_w), and physiological state of the organisms (Rui Li et al., 2018). Spores are more heat resistant than vegetative cells and a significant difference in *B. cereus* vegetative cell and spore heat resistance was shown by Byrne, Dunne, and Bolton (2006): while 50 °C yielded a D-value of 33 min for vegetative cells, 85 °C was required for the same D-value with the microorganism's spore. Regarding the effect of food composition on inactivation kinetics, $D_{90\ °C}$-values of 3.15 min and 1.03 min were reported for *B. cereus* ATCC 11778 spores in skim milk and beef slurry respectively (E. Evelyn & F. V. M. Silva, 2015). D and z values also vary in different strains of bacteria. For instance, a z-value of 25 °C is found for spores of *B. cereus* CFR 1521 in saline while another strain of *B. cereus* (F 4810) showed a z-value of 16.7 °C at the same cultured conditions (Desai & Varadaraj, 2010). Table 2 shows how bacterial strain and heating substrates influence D-values of the *B. cereus* spores.

Different bacteria show different levels of heat resistance in food products, and those with higher levels of resistance will be able to grow in storage condition. *C. botulinum* Group I is the target microorganism in low acid canned foods since it produces the most thermal resistant spores (Brunt et al., 2020; Smelt & Brul, 2014). To obtain safe shelf-stable foods, the 12-D concept is used to ensure the product's commercial sterility and the concept is accepted by regulatory agencies and the food industry. This concept can be explained in the following way: if it is considered the worst possible condition, spore count is believed to be 10^{12} CFU/ml in a can full of *Clostridium botulinum* spores. Thus, putting the food through a cooking process that results in a 12 decimal reduction should destroy all the spores of *Clostridium botulinum* in this worst possible case. At 121 °C, it takes 0.21 minutes to reduce the spore population by 90% ($D_{121\ °C}$ = 0.21). So, a 12-D cook at 121 °C is equivalent to 2.52 minutes (12 × 0.21) (Sevenich, Rauh, & Knorr, 2016).

Table 2*. D-values of different strains of B. cereus spores in various heating substrates (Desai & Varadaraj, 2010).*

Heating medium	BHI broth		Skim milk		Whole milk	
	85 °C	**90 °C**	**85 °C**	**90 °C**	**85 °C**	**90 °C**
B. cereus **CFR1521**	12.5	7.8	11.9	6.2	9.2	7.5
B. cereus **CFR1532**	12.1	9.5	15.4	12.5	9.5	6.9
B. cereus **CFR1534**	14.3	8.3	16.4	7.7	9.1	8.7
B. cereus **F4810**	13.2	7.9	12.1	7.9	10	7.2

The decimal reduction time (D-value) can be calculated mathematically by using the following equation:

$$\frac{t}{\log N_0 - \log N}$$

Where 't' is the heating time of the samples (minute); 'N_0'is the initial number of microorganisms and 'N' is the final number of microorganisms after heating. The D-value can also be obtained by plotting the log concentration of microorganisms (CFU/ml) against the appropriate heat treatment time (minutes) and getting a linear curve, see Figure 7. This curve is known as a survivor curve. By using the slope of the line, D- value is calculated by using the Equation:

$$D = \frac{-1}{a}$$

Here 'a' represents the slope of the line (Liato, Labrie, Viel, Benali, & Aïder, 2015).

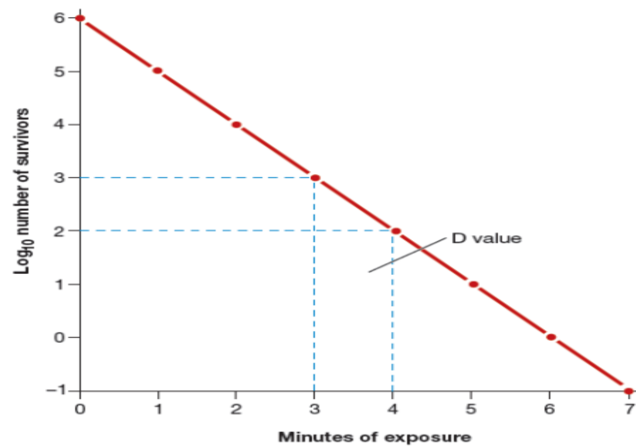


Figure 7. *Graphical presentation of D-value.*

For z-value calculation, several Log D-values is plotted against temperature resulting in a graph known as the heat resistance curve (Figure 8).

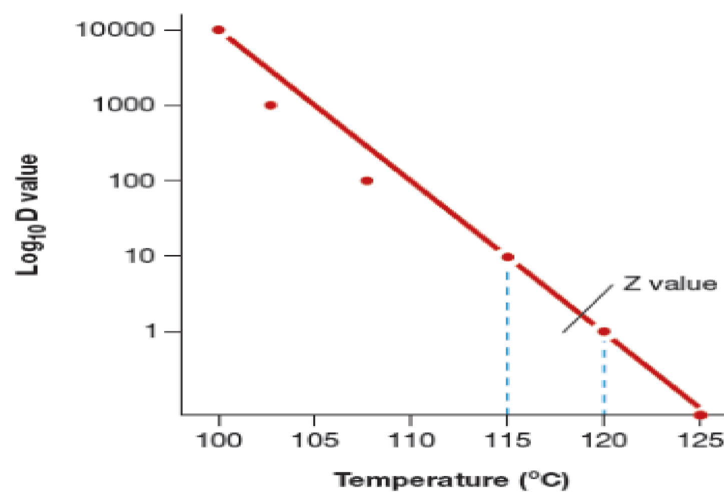


Figure 8. *Graphical presentation of z-value.*

These equations are used for calculating z-value:

$$Z = (T2 - T1) / (\text{Log } D1 - \text{Log } D2) \text{ and } D = \frac{-1}{a}$$

Where D_1 and D_2 are D-values at at T_1 and T_2, respectively (Liato et al., 2015).

2.3.Food processing

In food processing, foods are transformed through a series of unit operations into more palatable, portable, shelf-stable and useful value-added products that are safe to consume (Bhargava, Mor, Kumar, & Sharanagat, 2021). According to this definition, microbial safety and bacterial elimination or growth inhibition is a concern in food processing steps. Major issues arise in relation to resistant endospores of bacteria which pose safety and spoilage problems for the food industry (Setlow & Johnson, 2019). Food microbial spoilage can be influenced by both intrinsic and extrinsic factors. Intrinsic factors include pH, water activity, nutrient content, and oxidation-reduction potential. Relative humidity, temperature, presence and activities of other microorganisms are extrinsic factors. The growth of microorganisms can be prevented or limited by adjusting both intrinsic and extrinsic factors such as storage temperature, reducing water activity, lowering pH, using preservatives, and using proper packaging (Amit et al., 2017).

The concept of food processing has gradually changed throughout the years as food processing technologies have advanced and evolved. Initially, just obtaining safe food products with a long shelf-life was the aim (Chacha et al., 2021). Drying, frying, smoking, salting, and pickling are examples of traditional processing and preservation processes that are still widely and efficiently employed to prepare raw food products (Bhargava et al., 2021). The primary food processing methods, such as thermal treatments, ensure effective reduction of microorganisms but simultaneously adversely affect both the nutritional properties and sensory characteristics of foods such as flavor, color, and texture. (Chacha et al., 2021).

In recent years, due to consumer demands for foods that are fresh with fewer chemical antimicrobials, prevention of foodborne illness and spoilage by spore-formers has become more challenging. Food processing techniques are becoming more advanced and examples of these novel technologies are pulsed electric fields (PEF), high-pressure processing (HPP), ozone treatment, non-thermal plasma/cold plasma (NTP), and ultrasound technology. There is a great need for understanding the construction and operation of these methods, as well as their

impacts on food (Amit et al., 2017; Jadhav, Annapure, & Deshmukh, 2021; Setlow & Johnson, 2019).

2.3.1.Thermal process

The application of heat can inactivate microorganisms and extend the shelf life of heat-treated foods. However, different bacteria have different degrees of tolerance to high temperatures. Typically, vegetative cells are the most sensitive while in reaction to stress, certain bacteria produce resistant spores, which can cause food spoilage or human illness (Reineke & Mathys, 2020). Thermal processing is an operation in which foods are heated to a sufficiently high temperature for a certain time to eliminate vegetative microbial cells and spores. Pasteurization and sterilization are two categories of thermal processing.

Pasteurization is a method in which food is heated up to a specific temperature, >75 °C. This method eliminates non-spore forming bacteria, yeasts, and molds. As a result, the shelf life of food increases. Thermal sterilization is another method of thermal processing that is different from pasteurization as it destroys all the viable microorganisms (yeasts, molds, vegetative bacteria, and spore formers) resulting in a longer period of shelf life (Amit et al., 2017). In pasteurization, spores can survive as opposed to sterilization. FDA considers a 6D (meaning 6 log reduction of microorganisms) process (time/temperature combinations) suitable for pasteurized products (Evelyn & Silva, 2019; Peng et al., 2017; Wang et al., 2015), while in sterilization, due to the strong resistance of spores, a higher heat treatment is required to eliminate them from foods.

Based on the kinetic parameters (D- and z-values) a 12-D cook, commonly known as "botulinum cook," is used in the canning of low-acid foods (pH > 4.6) (Setlow & Johnson, 2019). The aim of this type of treatment is to attain a target sterilization temperature (121°C for 3 minutes) to reduce the number of spores by 12 $\log_{10}$. (Anderson et al., 2011; Liato et al., 2015). Although thermal treatments ensure food safety, they also affect the nutritional properties and sensory characteristics of the food. These changes occur in biochemically important components such as proteins, lipids, as well as vitamins, and minerals (Barbosa-Canovas, Medina-Meza, Candogan, & Bermudez-Aguirre, 2014; Ran Li, Wang, Zhou, Li, & Ye, 2020).

Today, due to consumer demands for fresh foods with more nutritional values and increased shelf life, researchers innovate and develop non-thermal processing methods.

(Chacha et al., 2021). So non-thermal processes technologies are designed to avoid the negative impacts of heat on the flavour, appearance, and nutritive value of foods. Non-thermal food processing means the inactivation of food pathogens without the direct application of heat (Hernández-Hernández, Moreno-Vilet, & Villanueva-Rodríguez, 2019).

2.3.2.High pressure processing (HPP)

HPP is a novel technology that has been proven successful in inactivation of numerous bacteria. This technology can ensure food safety, shelf-life stability and maintenance of the fresh-like attributes of foods, meeting consumer demands for fresh-like and minimally processed products (Huang, Hsu, & Wang, 2020). In HPP high pressure is applied at 100–600 MPa for a few seconds/minutes. Typically, the foods are vacuum packed and placed in a basket, which is then loaded into the HPP equipment where pressure is transmitted immediately and evenly throughout the product by using a pressure transmitting medium (PTM). To increase microbial inactivation, the temperature can be adjusted to 60–65 °C in addition to the pressure (pressure-thermal treatment) (Sehrawat, Kaur, Nema, Tewari, & Kumar, 2020).

In microbial cells, cellular structure and essential reactions are significantly affected by high pressure treatment. The lethal impact of HPP on microbial population is through simultaneous disruption of the microbial cell membrane and changes in cell membrane permeability, cell morphology, biochemical reactions as well as interference in the genetic mechanism. However, the specific process that causes cell death is unknown (Sehrawat et al., 2020). According to Sehrawat et al. (2020), different bacteria show different resistance to the pressure and structural variations may be the reason for differences in pressure resistance. Gram-positive bacteria are more resistant to pressurization than Gram-negative bacteria due to the presence of rigid teichoic acid in their cell walls.

Pressure alone at or near ambient temperature has very limited or no effect on spore destruction (Balasubramaniam, Martínez-Monteagudo, & Gupta, 2015). Spores can survive in severe conditions. However, when spores germinate to vegetative cells, their resistance decreases dramatically. As a result, the germination of spores is of high interest to researchers (Lv, Liu, & Zhou, 2021). That is why two-step exposure treatment is proven to be more effective for spore inactivation. In the first step, spore germination is triggered by low to moderate pressures (100 to 400 MPa). Germination increases the sensitivity of spores to HPP, and in the second step high pressures (>400 MPa) result in the inactivation of germinated spores (Pinto et al., 2020).

Depending on the strength of the treatment, pressure with or without heat can either result in food pasteurization or sterilization. For example, high pressure (400–600 MPa) at ambient or chilled temperatures is useful for pasteurizing food products (Balasubramaniam et al., 2015). The industrial HPP process is commonly carried out at pressures ranging from 200 to 600 MPa, with a holding time of up to 10 minutes. Compression increases the temperature of foods so, during pressure processing, there is a temperature increase rate of approximately 3 °C per 100 MPa. This increase in temperature, which is caused by compression and is product-dependent, is termed adiabatic heating (Schottroff et al., 2018). For instance, in foods containing high amounts of fat, such as butter or cream, the temperature rise is larger (8–9 °C/100 MPa) (Barbosa-Canovas et al., 2014).

Most of the studies carried out with *Clostridium* and *Bacillus* have shown that tailing happens in HPP processing. Tailing is defined as a decrease in inactivation levels toward the end of the treatment with increasing process intensities. The presence of microbial populations with different individual pressure resistances due to genetic diversity, as well as adaptation to external pressures, might explain this tailing behaviour (Evelyn & Silva, 2019; Schottroff et al., 2018). It is also verified that during pressure processing, clumps of spores can form. Formation of these clumps reduces the effectiveness of the process in spore inactivation and induces tailing phenomenon (R. P. Lopes et al., 2018). So it is critical to be ensured that preservation processes by use of HPP are designed in such a way that they are severe enough to completely inactivate the bacterial target population (Schottroff et al., 2018).

2.3.3.Pressure-assisted thermal sterilization (PATS)

HPP processing is insufficient to achieve sterilization, and thus much higher pressure and temperature levels are required to inactivate resistant bacterial endospores (Serment-Moreno, Barbosa-Cánovas, Torres, & Welti-Chanes, 2014). PATS is a processing technology that is utilized for the sterilization of shelf-stable low-acid foods (pH > 4.6). In PATS, pressures in the range of 600–800 MPa and initial chamber temperature of 60–90 °C are used to ensure the inactivation of bacterial spores. Due to adiabatic heating, the temperature can reach 90 to 130 °C during internal compression heating at pressures of 600 MPa or more. The temperature rapidly decreases after decompression throughout processing. Fast processing time, as well as low influence on nutrients and food quality are features of this method. Unlike conventional thermal processes, a shorter processing time results in minimum influence on food nutritional

and sensory quality (Al-Ghamdi et al., 2022; R. P. Lopes et al., 2018). Figure 9 compares sterilization condition in conventional thermal process (T Retort) and PATS.

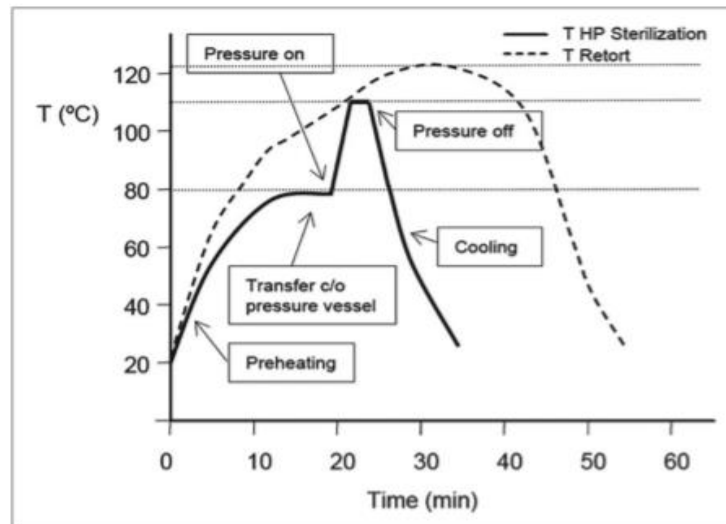


Figure 9. *Typical product temperature profiles in a retort (conventional thermal sterilization) and PATS (T HP Sterilization) processes (R. P. Lopes et al., 2018).*

There are six major steps in the PATS process: (i) vacuum packaging and product loading, (ii) preheating to the target temperature, (iii) product equilibration to initial temperature, (iv) product temperature increase to pressurization temperature by means of compression heating, (v) product temperature decrease during decompression, and (vi) product cooling to ambient temperature. Generally, PATS begins with removing air from the food and vacuum packing the product in a flexible pouch, or container. Prior to PATS, the prepacked product is preheated to the required temperature. To reduce heat loss to the environment and ensure equal heat treatment, the pressure chamber is also warmed. The packed food is placed in a load basket once it has been preheated, and loaded into the pressure chamber, which is then closed and sealed.

The pressure cycle begins and a pressure transferring fluid (with the proper initial temperature) is pumped into the chamber, letting the remaining air to exit through a vent valve. Then the vent valve is closed, and additional fluid is forced into the chamber using hydraulic pumps and pressure intensifiers to achieve the appropriate pressure. Depending on the power of the hydraulic pump, the ramp rates to the desired pressure might range from a few seconds to several minutes. At the end of the process after opening the chamber, the loaded basket is

removed, and the product is chilled to ambient temperature to prevent any further thermal degradation. (Barbosa-Canovas et al., 2014; Jeremy & Balasubramaniam, 2009; Serment-Moreno et al., 2014).

During the process, pressure is transmitted instantly and undiminished throughout the sample. Processing time is also independent of sample shape and size because heat transfers equally and uniformly through the sample. Most food products have high moisture content, and these products are not severely deformed under pressure. The reason is that water is compressed only by 10% at 300 MPa and by 17% at 600 MPa resulting in a good chance for shape retention at the macroscopic level. However, food products such as strawberries marshmallows, and leafy vegetables that are porous and contain large amounts of air will be deformed as a result of pressure treatment (Jeremy & Balasubramaniam, 2009). Although applying pressure processing has advantages, this method has some limitations which are summarised in Table 3.

Effect of pressure-thermal processing on spore inactivation

The exact mechanism of bacterial spore inactivation by a combination of high pressure and high temperature is yet unknown (Rita P Lopes, Maria J Mota, Ana M Gomes, Ivonne Delgadillo, & Jorge A Saraiva, 2018). Some investigators, however, have found that releasing DPA could result in loss of spore heat resistance and, spore germination (Reineke, Ellinger, et al., 2013). Higher pressures (500–600 MPa) promote the opening of Ca–DPA channels, resulting in the release of massive DPA depots. Then, spores rehydrate and germinate, resulting in a reduction in spore resistance. Two factors determine spore pressure resistance: the capacity of the spore to maintain DPA and the heat resistance of DPA-free spores. The higher efficacy of spore inactivation with PATS treatment compared to heat treatment is due to Ca^{2+}-DPA release and spore rehydration (R. P. Lopes et al., 2018; Margosch, Ehrmann, Gänzle, & Vogel, 2004; Reineke, Ellinger, et al., 2013; Reineke, Mathys, Heinz, & Knorr, 2013; Reineke, Schlumbach, Baier, Mathys, & Knorr, 2013).

In the literature, different inactivation methods have been proposed. For example, a 3-step process was suggested by Mathys, Chapman, Bull, Heinz, and Knorr (2007) and, Heinz and Knorr (1996), which includes germination, an unknown step, and an inactivation step. On the other hand, other investigators proposed a 2-step kinetic model, with the spore germination in the first step and the spore inactivation in the second step by a combination of pressure and

temperature due to higher stress sensitivity. As a result, further research is needed to better understand the methods by which PATS inactivates spores (Reineke, Mathys, et al., 2013).

Table 3. *Advantages and limitations of high pressure processing (Balasubramaniam et al., 2015).*

Description	Advantage	Limitations
Hydrostatic pressure	Fast, uniform distribution throughout the product	Semicontinuous operation
Thermal distribution	Minimal or reduced thermal exposure. Instant temperature increase and subsequent cooling upon depressurization	Preheating step for pressure-assisted thermal processing (PATS)
Physical compression	Suitable for dishes with a high moisture content	Not suited to foods consisting of dissimilar compressibility materials, such as marshmallows
Product handling	Suitable for both liquid and pumpable foods	Throughput limited owing to Semi-continuous operation
Process time	Independent of food shape and size	
Reaction rate	Pressure accelerates microbial inactivation in some pressure-thermal ranges.	pressure alone cannot inactivate all bacterial spores. Variable efficacy in enzyme inactivation.
Consumer acceptance	Consumer acceptance as a physical process	Higher processing costs and semi-continuous operations are obstacles for commodity product processing

Tailing phenomenon is reported in PATS processing. Spores of *Clostridium botulinum* and *Bacillus amyloliquefaciens* were subjected to PATS processing with the condition of 600 to 1400 MPa, 70 to 120 °C for 8 min by Margosch et al. (2006). Although the rate of

inactivation accelerated with increasing pressure and temperature, a noticeable pressure-dependent tailing was seen at 120 °C and 1400 MPa. In contrast, no detectable tailing was observed following the heat treatment alone (100 to 120 °C for 8 minutes). These authors observed that conditions of up to 120 °C and 1400 MPa can be tolerated by a small fraction of the spore populations and this pressure resistance is strain dependent. Due to this tolerance, a case-by-case study is necessary to prove spore inactivation by PATS processing (S Furukawa, Noma, Shimoda, & Hayakawa, 2002; R. P. Lopes et al., 2018; Margosch et al., 2006; Wuytack, Soons, Poschet, & Michiels, 2000).

Although the effectiveness of PATS on the inactivation of microorganisms directly increases with the magnitude of pressure, time, and temperature, bacterial species is another factor that can affect inactivation effectiveness (Sevenich et al., 2016). Table 4 compares inactivation of *B. cereus* spores by different pressure-thermal processing conditions.

***Table 4**. Log reductions of B. cereus spores by different pressure-thermal processing.*

Species of *B. cereus*	Substrate	Treatment condition	Log reduction	Reference
ATCC 11778	milk	600MPa ,70 °C, 15 min	3	(Evelyn & F. V. M. Silva, 2015)
		200MPa ,70 °C, 40 min	3.6	
ATCC 9139	Cheese	400 MPa, 30 °C, 15 min	<0.5	(López-Pedemonte, Roig-Sagués, Trujillo, Capellas, & Guamis, 2003)
ATCC 9818	Cooked Rice	600 MPa, 85 °C, 4 min	7	(H. Daryaei, Balasubramaniam, & Legan, 2013)
NZ 6	Skim milk	600 MPa, 85 °C, 1 min	3.8	(Robertson, Carroll, & Pearce, 2008)
NZ 7			4.5	

Inactivation rates are also dependent on the composition of suspension media or food, cell growth phase, pH, and water activity. For instance, acidic foods (pH values < 4.6) with a high water activity (a_w > 0.95), favour spore inactivation, whereas low acidic foods with low

water activity require a longer treatment time or higher temperature and pressure to achieve spore inactivation (Hossein Daryaei, Yousef, & Balasubramaniam, 2016; R. P. Lopes et al., 2018). Although PATS processing is an advantageous method due to its shorter time in comparison to thermal sterilization, it is necessary to identify optimal temperature/pressure/time combinations (Barbosa-Canovas et al., 2014).

2.3.4.Hurdle technology

Food processing technologies are mainly based on the inactivation of microorganisms or the delay or inhibition of microbial growth. Factors used for food preservation are called “hurdles”. Hurdles can be divided into different groups: physical, physical nonthermal, physicochemical, and microbiological hurdles (Erkmen & Bozoglu, 2016). Table 5 shows examples of hurdles.

Table 5. *Types of hurdles for food preservation (Erkmen & Bozoglu, 2016).*

Type of hurdles	Examples
Physical hurdles	Electromagnetic energy (microwave, radiation), high temperature (blanching, pasteurization, baking), low temperature (chilling, freezing), and so on
Physical nonthermal hurdles	High hydrostatic pressure, pulsed electric field, pulsed light, and so on
Physicochemical hurdles	CO_2, O_2, lactic acid, lactoperoxidase, low pH, low a_w, smoking, nitrite/nitrate, sulfite, spices, and so on
Microbiological hurdles	Competitive flora, microbial products

The hurdle technology is combining several mitigating approaches (hurdles). Each of individual hurdle is not sufficient by itself, but together they can reduce or eliminate microbial hazards. The main aims of the hurdle technology are microbial safety, food stability, organoleptic and nutritional quality of food products. Choice of hurdle and the magnitude of microbial reduction achieved through the use of hurdles depends on the food type and expected pathogen burden (Mogren et al., 2018). In milk, for example, key combination treatment includes pasteurization, cool storage, and protection against cross-contamination. In jams, heating, a high solid content (low a_w), and high acidity are used as hurdles (Erkmen & Bozoglu,

2016; Mogren et al., 2018). The use of any single hurdle with high strength or high concentration can have a negative impact on food quality, such as loss of nutrients, texture, and color. Therefore, the intelligent combination of several hurdles is important. They need to be combined in a way that work in synergy and result in multitarget reliable preservation effects on food products (Erkmen & Bozoglu, 2016).

When microorganisms are exposed to several stresses and jump over hurdles their energy will be sharply reduced. A synergistic effect of hurdles disturbs different targets in the bacterial cell including cell membrane, DNA, and enzyme systems. This simultaneous antimicrobial activity of hurdles is known as “multitarget preservation.” In general, a combination of hurdles has a greater inhibitory effect than any single one (Erkmen & Bozoglu, 2016). In conclusion, hurdle technology is an effective and simple method in food processing and preservation, but it needs strategic processes. Hurdles such as acidification or the addition of preservatives can combine with pressure processing to inhibit or slow down the growth of resistant spore-formers which may have survived the HPP process. It can extend the shelf life, keep the foods safe and healthy to eat, and fulfill the consumer demands for fresh and natural food products (Evelyn & Silva, 2019; Pal, Ravi, Kumari, & Singh, 2021).

3.MATERIAL AND METHODS

3.1.Bacterial strain and preparation of frozen stock culture

The psychrotrophic *B. cereus* ATCC 9139 was provided from the Nofima culture collection. Microbank cryovials were used for storage and retrieval of bacterial cultures (Figure 10). Microbank™ is a sterile vial containing porous beads and a cryopreservative solution (glycerol). Under aseptic conditions, a single colony from an overnight culture (grown on LB agar at 37 °C) was inoculated into two Microbank cryovials (Prolab Diagnostic, Wirral, U.K.) using a loop. Vials were inverted 4-5 times to emulsify microorganisms. At this point, microorganisms will be bound to the porous beads. Excess cryopreservative was then aspirated using a sterile Pasteur pipette leaving the inoculated beads as free of liquid as possible. Bacteria were stored at – 80 °C until use.

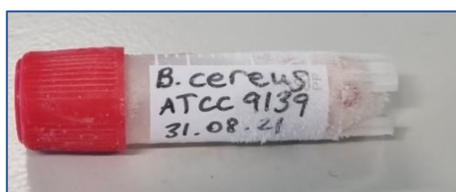


Figure 10. *Microbank cryovial used for bacterial storage.*

In this study, several experiments were carried out on vegetative cells and spores of *B. cereus*. Table 6 and Table 7 show an overview of the experiments conducted in this study.

Table 6*. An overview of the experiments carried out with B. cereus vegetative cells.*

Experiment	Description
Testing psychrotrophic growth	Growth was investigated in 4, 5, 6, 8, 10 °C
Investigating the effect of initial bacterial number on growth	A turbidometer (Bioscreen) was used
Effect of preservatives on growth	Different concentrations of: NaCl, KCl, $NaNo_2$, pH, nisin were tested

Table 7. An overview of the experiments carried out with B. cereus spores.

<table>
<tr><th>Experiment</th><th colspan="3">Description</th></tr>
<tr><td>Effect of preservatives on growth</td><td colspan="3">Different concentrations of: NaCl, KCl, $NaNo_2$, pH, nisin were tested</td></tr>
<tr><td>Effect of Tween on spore heat resistance</td><td colspan="3">Heating spores at 80 °C for 10 min, 90 and 95 °C for 2 min</td></tr>
<tr><td>Thermal inactivation in LB medium</td><td colspan="3">Heating spores at 80 °C for 5, 10, 15, 20 min and 90 °C for 2, 5, 8, 10 min</td></tr>
<tr><td>Thermal inactivation in minced meat</td><td colspan="3">Heating spores at 85, 90, 95 °C for 5 min</td></tr>
<tr><td>Thermal inactivation in minced fish</td><td colspan="3">Heating spores at 85, 90, 95 °C for 5 min</td></tr>
<tr><td rowspan="8">Pressure-thermal inactivation in minced meat</td><td colspan="3">6 different treatment conditions:</td></tr>
<tr><td>pressure</td><td>temperature</td><td>Holding time</td></tr>
<tr><td>600 MPa</td><td>20</td><td>2</td></tr>
<tr><td>600 MPa</td><td>20</td><td>10</td></tr>
<tr><td>600 MPa</td><td>40</td><td>2</td></tr>
<tr><td>600 MPa</td><td>40</td><td>10</td></tr>
<tr><td>600 MPa</td><td>55</td><td>2</td></tr>
<tr><td>600 MPa</td><td>55</td><td>10</td></tr>
<tr><td>Pressure-thermal inactivation in minced fish</td><td colspan="3">Treatment condition similar to the minced meat</td></tr>
</table>

3.2.Psychrotrophic growth

Psychrotrophic behaviour of the strain was tested by inoculation of bacterial cells in LB media and incubation at low temperatures. First, an overnight culture of the strain was prepared by the following procedure. A bead from the Microbank cryovial tube was placed into a sterile 100 ml Erlenmeyer flask containing 50 ml LB medium. The flask was then incubated at optimal temperature (37 °C) with continuous orbital shaking (150 rpm) in a shaker incubator (New Brunswick Scientific, Innova40, country) for 18-20 hours. Counting cells in the overnight culture showed a concentration of 10^8 CFU/ml which then was diluted to obtain the desired concentration (10^5 CFU/ml) using LB medium. Falcon tubes containing diluted bacteria were

incubated at five different temperatures (4, 5, 6, 8 and 10 °C) and growth at all temperatures was monitored by counting cells using a Thoma counting chamber once a week. An increase of ≥ 1 log unit for each of the inoculated tubes at the different temperatures was defined as growth.

3.3.Spore production

In this study, different batches of spores were prepared to find the optimal conditions for spore production and purification. The strain was sporulated on 2x Schaeffer's glucose (SG) liquid medium, SG, LB, and Trypticase Soy Agar (TSA) agars.

3.3.1.Spore production in SG liquid medium

SG liquid medium was selected for sporulation as its efficacy in spore production for other strains of *Bacillus* was approved previously in the microbiology laboratory at Nofima. To produce spores on SG medium (batch A), one drop from the overnight bacterial cell suspension was added into each of three 250 ml Erlenmeyer flasks containing 40 ml of SG liquid medium each. All flasks were incubated at 37 °C while shaking at 200 rpm. The degree of conversion of vegetative cells to spores was inspected every day using a differential phase contrast microscope (Leica DM2000) at PH3 and 1000x magnifications to estimate the approximate ratio of spores to vegetative cells. After 10 days of incubation, spore production was not successful in SG medium, so, sporulation on agars was examined for spore generation as a further stage.

3.3.2.Spore production using three different agars

Three different batches of spores were produced on three types of agars to compare sporulation quality. For this purpose, 150 µl of the overnight culture were spread on 3 TSA agar (batch B), 3 SG agar (batch C) and 3 LB agar (batch D) plates using a sterile L-shaped spreader. All plates were incubated at 37 °C. Spores were checked each day to estimate the approximate ratio of spores to vegetative cells and to determine the optimal time for harvesting. After 3-4 days when more than 90% sporulation was obtained, spores were harvested from agars by adding approximately 6 ml cold sterile Milli-Q water and gently rubbing the surface of the plates with a sterile L-shape spreader to dislodge the spores. Spores from LB, TSA and SG agars were aspirated and pooled separately in 3 sterile 50-mL Falcon tubes. During all steps of harvesting

and washing spores, the Falcon tubes were kept on ice and all steps were performed in a Biological Safety Cabinet using aseptic techniques.

In the washing step, three tubes containing three different spore batches were washed three times on day one by centrifugation at 10000 x g for 10 minutes at 4 °C. In each washing step, the supernatant was removed, and the pellet was resuspended in 18 ml cold sterile Milli-Q water. Pellets were homogenized with vortexing and then incubated for 24 hours at 4 °C while rotating. The next 2 days, the spore suspensions were centrifuged at 20000 x g for 20 minutes at 4 °C and then washed. Finally, to prepare and compare working spore solutions, pellets were suspended in 18 ml Milli-Q water. A microscopic slide from the supernatant was a confirmation of presence the large spore clumps and losing spores through the supernatant, so in the next step (section 3.3.3) some modifications were applied to tackle this problem.

3.3.3.Spore washing modifications

Changing the centrifugation speed

Due to challenges related to losing spores in the supernatant, an alternative washing procedure was tested, and a new spore batch was produced (batch E). Spores were produced on 3 LB agars. All steps of harvesting and washing were similar to the previous section (3.3.2) with a change in the centrifugation speed. Centrifugation speed was reduced to 4000 x g. (Two steps of washing were performed on the first day and the third step on the next day). Samples from the supernatant and the final suspended pellet were investigated under the microscope and indicated that this modification was successful to prevent losing spores from the supernatant. However, the suspended pellet contained small spore clumps. To investigate if it is possible to obtain single spores in the pellet, adding Tween 20 was the further modification (see the next part).

Adding Tween 20 in the washing procedure

Here a new spore batch (batch F) was produced to investigate if adding Tween 20 can prevent spore clumps in the pellet as spores present as single spores were desirable. Spores were produced on 3 LB agar plates by the same method described before (section 3.3.2). A washing solution added 0.1% Tween 20 in Milli-Q water was prepared and sterile filtrated (0.2-micron pore size). The resulting washing solution was subsequently added to plates for the purpose of harvesting and dispersing spores. This spore batch was washed and centrifuged three times at a lower speed, 4000 x g, for 10 minutes at 4 °C (Two steps of washing were performed on the

first day and the third step on the next day). The existence of spore clumps in the pellet was checked and compared with batch E. Table 8 shows spore batches produced in this study.

3.3.4.Enumeration of spores

To determine the number of spores per ml of batch E and F, spore suspensions were diluted serially in microcentrifuge tubes, (10^{-1}, 10^{-2}, 10^{-3}, etc). One ml of spore dilutions was spread on LB agars. After 24 hours of incubation at 37 °C, spreading colonies with irregular edges made it difficult to calculate the number of colonies. So, in the next step, to have individual colonies, spore concentrations were enumerated on LB agar using pour plate method instead of the spread plate technique.

The pour plate method was done as follows: In the pour plate method, 1 ml of spore solution was placed in the center of a sterile Petri dish using a sterile pipette. Cooled, molten agar was then poured into the Petri dish containing the inoculum. The Petri dish was swirled gently to mix the culture and the medium thoroughly. After the solidification of the agar, the plates were incubated at 37 °C. After 24 hours of incubation, counting colonies was difficult because of migration of colonies to the surface and spreading (see Figure 18 in the result part). To tackle this problem and count colonies in an accurate way, some modifications were made to the pour plating method:

- After agar solidification, extra agars were added to the plates to delay migration of colonies to the surface.
- Plates were incubated at 30 °C instead of 37 °C
- Incubation time reduced to 18-20 hours instead of 24 hours

After counting the colonies on the different plates, the number of colony forming units (CFUs) was determined by the Equation:

$$\frac{CFU}{ml} = \frac{number\ of\ colonies}{dilution\ factor \times volume\ plated\ (ml)}$$

Spore concentration was 10^7 spores/ml in both E and F stock solutions.

Table 8. *Spore batches produced in this study.*

Batch	Production media	Washing solution	Centrifugation speed	Quality of spores (confirmed by microscopic slides)
A	SG broth	*	*	≈60% sporulation after 10 days of incubation
B	3 TSA agar	Milli-Q water	10,000 x g 20,000 x g	More cell debris, less free spores; large clumps in the supernatant; reduced number of spores in the pellet ($<10^7$ CFU/ml)
C	3 SG agar	Milli-Q water	10,000 x g 20,000 x g	More cell debris, less free spores; large clumps in the supernatant; reduced number of spores in the pellet ($<10^7$ CFU/ml)
D	3 LB agar	Milli-Q water	10,000 x g 20,000 x g	Large clumps in the supernatant; reduced number of spores in the pellet ($<10^7$ CFU/ml)
E	3 LB agar	Milli-Q water	4000 x g	Small clumps in the supernatant; pellet containing 10^7 CFU/ml of spores with small clumps
F	3 LB agar	Milli-Q water added 0.1% Tween 20	4000 x g	Less individual spores in the supernatant; pellet containing individual spores (rare clumps) with concentration of 10^7 CFU/ml.
G	6 LB agar	Milli-Q water added 0.1% Tween 20	4000 x g	Less individual spores in the supernatant; pellet containing individual spores (rare clumps) with concentration of 10^8 CFU/ml (production procedure is provided in section 3.8.1)

* Due to low sporulation, the spores were not harvested.

3.4. Investigating the possible effect of Tween 20 on heat resistance of spores

This experiment was aimed at the comparison of spore survivors in two different spore batches (batch E and F from section 3.3.3) after heating. To investigate if Tween 20 can affect heat resistance of spores, spore batches were heat treated. Batch E (without Tween) was used as a control and each sample was evaluated in triplicate in two experiments. First 0.5 ml of both spore suspensions (batch E and F) were transferred into 2 ml Eppendorf tubes separately. All tubes were kept on ice prior to inactivation to prevent spore germination. Heat experiment was carried out at three different temperatures and times using a water bath. Both samples were heated at 80 °C for 10 minutes, and at 90 and 95 °C for 2 minutes. For each experiment, tubes were placed on a floating element and put into a closed water bath (Figure 11). In addition to the digitally displayed temperature on the water bath, an external thermometer was also used for confirmation of the correct temperature close to the tube's tips. After heating, tubes were removed, and cooled in ice slurry immediately to avoid further inactivation. Heat resistance was determined and compared by counting the number of colonies after 18-20 h incubation at 30 °C.

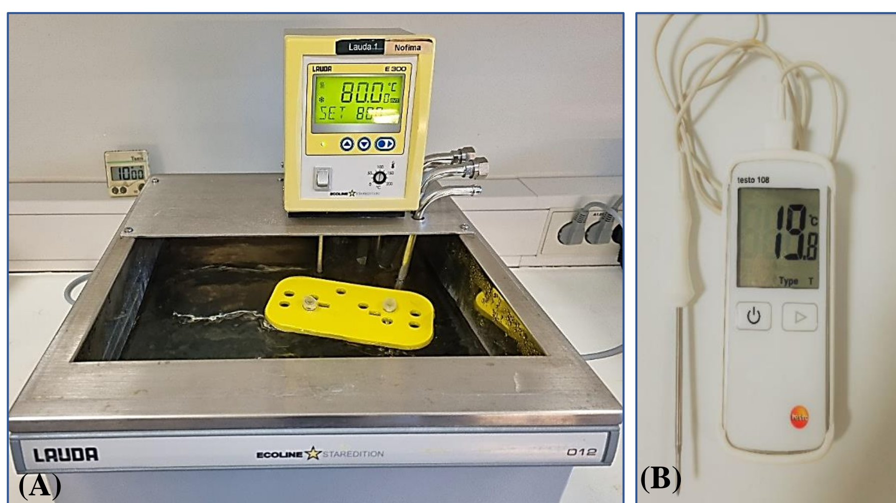


Figure 11*. Water bath used for heat treatment experiment. A floating element holds the tubes in the water bath for heat treatment (A); External temperature detector (B).*

3.5. Heat treatment of spores in LB medium

This test was made to assess the inactivation of spores by thermal treatment and calculation of D-values.

3.5.1.Heating process

Experiment was conducted by pipetting 0.5 ml of spore suspension (batch E) into 2 ml Eppendorf tubes. The tubes were submerged in the water bath at 80 and 90 °C constant temperatures for various times (5, 10, 15 and 20 minutes at 80 °C and 2, 5, 8 and 10 minutes at 90 °C). Heat treatments were performed in two experiments each containing three parallels. Heated spores were cooled, diluted serially, and allowed to germinate by pour plating method in LB agar. Lastly, after 18-20 hours incubation at 30 °C, by counting colonies in the control and heat-treated samples the concentration (CFU/ml) was calculated. Log reductions were compared in heat-treated samples.

3.5.2.D-values determination

The D-values were determined as follows: The data for each individual temperature (5 data points per graph) were used to plot a survivor curve (the $\log_{10}$ of the surviving cells against time). The line of the best fit for each curve was found and D-values were calculated using the slopes of the lines (D= -1/slope) for each survival experiment (80 and 90 °C).

3.6.Investigating the effect of initial bacterial number on growth rate

Bioscreen was used to compare Time to detection (TTD) in different dilutions (with different cell numbers) of microorganisms. Bioscreen C (Bioscreen C MBR automated turbidometric analyser, Growth Curves Ltd, Finland) is an instrument that can measure turbidity (optical density) of 200 bacterial solution samples per test (Figure 12). Bacterial solutions are pipetted into two 100-Well Honeycomb plates and the instrument can be adjusted for OD measurement at a specific temperature and shaken at different time intervals.

Figure 12*. Bioscreen C used for monitoring growth.*

3.6.1.Preparation of bacterial suspensions

B. cereus cells were grown to stationary phase in a 100 ml Erlenmeyer flask containing 50 ml LB medium (growth conditions: 18 h / 37 ºC /150 rpm). Following calculation of bacterial cell concentration, 10-fold serial dilutions (10^{-1} to 10^{-6}) of the inoculum were prepared in LB medium.

3.6.2.Bioscreen growth test and data analysis

To evaluate the growth, 100 ml of each microbial dilution were transferred to microtiter plate wells (in three replicates) and were mounted in the Bioscreen programmed to measure absorbance at 600 nm. The plate was run at 25 °C and the machine was programmed to read the OD of test wells every 30 minutes, while shaking the plate for 10 s before each reading. The experiment was repeated two times. The mean of the six sets of data (ODs from Bioscreen) was calculated and used to create growth curves (OD plotted against time). Finally, detection time that is related to cell number, was compared for each dilution, and used to construct a calibration graph.

3.7.Effect of preservatives and pH on growth pattern of spores and vegetative cells

The effect of preservatives was investigated on vegetative cells and spores using Bioscreen C. Stock solutions of the preservatives NaCl, KCl, $NaNO_2$ and nisin as well as different pH values were prepared. Then stock solutions were further diluted to obtain other desired concentrations (Table 9). The OD600 data obtained from the Bioscreen were processed and used for generating curves.

3.7.1.Preparation of nisin solution

According to the EFSA (European Food Safety Authority) panel, 1 IU would correspond to 0.025 µg of nisin and therefore µ1 g of nisin is equivalent to 40 IU (Maged Younes et al., 2017). In this study, nisin was used as its commercial product, 2.5% (wt/wt) (Sigma). A stock solution of nisin containing 4,000 IU/ml, equivalent to 100 µg/ml pure nisin was prepared, by dissolving 0.04 gr of nisin in 0.02 M HCl (1 ml) and adding 9 ml of distilled water (Lee et al., 2015; Prado-Acosta, Ruzal, Allievi, Palomino, & Sanchez Rivas, 2010). The stock solution was further diluted with LB medium to prepare three different concentrations of nisin, 50, 30 and 15 µg/ml.

Table 9. Different concentrations of preservatives used in Bioscreen growth test.

Preservatives	Concentration in stock solutions	Preparation	Further dilutions	Final concentration after mixing with bacterial solution (50:50)
NaCl	8%	16,89 g NaCl in 200 ml LB medium	8% 4% 2%	4% 2% 1%
KCl	8%	16,80 g KCl in 200 ml LB medium	8% 4% 2%	4% 2% 1%
$NaNo_2$	1000*ppm (1 mg/ml)	0.2 g $NaNo_2$ in 200 ml LB medium (*sterile filtration)	1000 ppm 250 ppm 125 ppm	500,0 ppm 125,0 ppm 62,5 ppm
pH	4.0 2.8 2.3		- - -	5 4 3
Nisin	100 µg/ml		50 µg/ml 30 µg/ml 15 µg/ml	25 µg/ml 15 µg/ml 7.5 µg/ml

***ppm:** "parts per million" and it also can be expressed as milligrams per liter (mg/L)

***Sterile filtration:** Filtration through a membrane with 0.2-micron pore size

3.7.1.Bioscreen C experiment

Overnight bacterial culture in LB medium was prepared. The initial concentration was 10^8 CFU/ml, and further dilution using LB medium provided final concentrations of 10^4 CFU/ml.Bacterial concentration was confirmed by both direct microscopic counts using Thoma counting chamber and pour plating serial dilutions of the bacterial suspension in LB agar. In the next step wells were filled with 50 µl from each concentration of preservatives and pH values and 50 µl from bacterial cells (final volume of 100 µl/well). Bacterial cells (10^4

CFU/ml) and pure LB medium (without bacteria) were added as a standard growth and negative control respectively. See Table 10 for the Bioscreen C mircotiter plate setup.

Table 10. *Bioscreen C microtiter plate setup for B. cereus vegetative cells, a representative figure of the microtiter plate.*

	01	**11**	**21**	**31**	**41**	**51**
1	4% NaCl	4% KCl	500 ppm $NaNo_2$	pH 5	nisin 25 µg/ml	*Overnight bacterial cells
2	4% NaCl	4% KCl	500 ppm $NaNo_2$	pH 5	nisin 25 µg/ml	Overnight bacterial cells
3	4% NaCl	4% KCl	500 ppm $NaNo_2$	pH 5	nisin 25 µg/ml	Overnight bacterial cells
4	2% NaCl	2% KCl	125 ppm $NaNo_2$	pH 4	nisin 15 µg/ml	LB
5	2% NaCl	2% KCl	125 ppm $NaNo_2$	pH 4	nisin 15 µg/ml	
6	2% NaCl	2% KCl	125 ppm $NaNo_2$	pH 4	nisin 15 µg/ml	
7	1% NaCl	1% KCl	62.5 ppm $NaNo_2$	pH 3	nisin 7.5 µg/ml	
8	1% NaCl	1% KCl	62.5 ppm $NaNo_2$	pH 3	nisin 7.5 µg/ml	
9	1% NaCl	1% KCl	62.5 ppm $NaNo_2$	pH 3	nisin 7.5 µg/ml	
10	*LB	LB	LB	LB	LB	

***LB;** LB medium without bacterial cells as a negative control

***Overnight bacterial cells;** *B. cereus* growths in LB medium for 18 hours (37 °C) with a final concentration of 10^4 cfu/ml

The microtiter plate was mounted in Bioscreen and absorbance values of the cell suspensions were measured at 600 nm at regular intervals of 30 minutes for up to 5 days. The experiments were performed at 25 °C and before each OD reading, the cell cultures were automatically shaken for 10 seconds. For monitoring the effect of preservatives on spores, spore stock solution (batch E) (10^7CFU/ml,) was diluted to obtain 10^4 CFU/ml and all steps in Bioscreen setup were repeated.

3.7.2.Data analysis

The OD values from Bioscreen were analyzed and the averages of the six replicates (three replicates in two separate experiments) were calculated for each type of preservative in various concentrations. These averages were used to generate growth curves (OD $_{600}$ nm of the cultures versus incubation time). Effect of preservatives was investigated by determination of the time (h) until the OD of a well reaches 0.2 (TTD).

3.8.Inactivation of *B. cereus* spores in food matrices by heat treatment and pressure processing

Spore inactivation by heat treatment and combination of high pressure processing with moderate heat was investigated in two different food matrices, minced meat, and minced fish.

3.8.1.Spore production

For this experiment, higher concentration of spores was needed. To obtain spores with concentration of 10^8 CFU/ml, spores were harvested from more agar plates (6, 8 and15 agar plates) in three separate experiments. Steps of harvesting and washing of spores were performed with Milli-Q water (without Tween) as described previously for the batch E (in section 3.3). But there were challenges and despite harvesting more spores, spore enumeration showed 10^7 CFU/ml. Challenges are documented with pictures (section 4.8.1, in Result and Discussion). Finally, *B. cereus* was sporulated on 6 LB agar plates as described previously (section 3.3), but with the following modifications:

- Milli-Q water containing 0.1% Tween 20 was used for harvesting and washing spores.
- Centrifugation time was increased from 10 to 15 minutes.
- After the last step of washing and discarding the supernatant, the spore pellet was resuspended in 10 ml of the Milli-Q water.

3.8.2.Inactivation of spores by heat treatment in minced meat

The minced meat was purchased as a commercial product, and its composition is shown in Table 11. Heating was done at 85, 90 and 95 °C for 5 minutes.

Table 11. *Composition of minced meat used for heating and pressure processing experiments.*

Compound	Protein	Fat	Salt
Amount/100 g	20	5	1

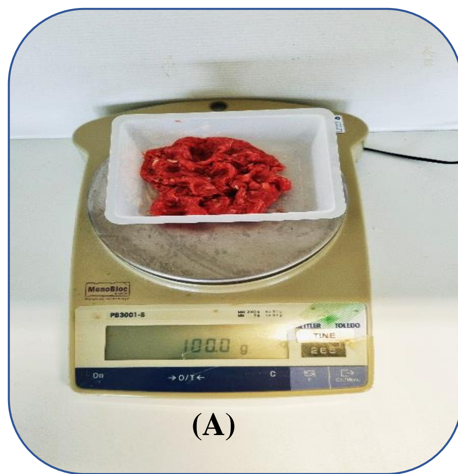
Food inoculation and packaging

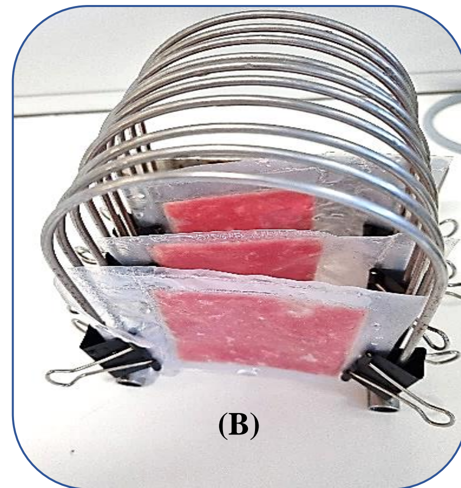
Minced meat was portioned in samples of 100 g and stored frozen (-20 °C). The first step of sample preparation was thawing 100 g of minced meat overnight in the refrigerator (+4 ˚C). Then 1.2 ml of the spore suspension was mixed with 20 ml of cold Milli-Q water in a Falcon tube and added to 100 g of minced meat. The spore suspension had an initial concentration of 10^8 CFU/ml. After homogenous mixing of spores with Milli-Q water and minced meat, the concentration of spores was diluted to 10^6 CFU/ml. Then 6 g of minced meat containing spores were transferred to plastic bags and sealed as a squared package (5×6 cm). To ensure consistent heat distribution throughout the product, minced meat was rolled flat inside the plastic bags. The experiment was performed for three temperature-time combinations at 85, 90 and 95 °C for 5 minutes. Samples were tested in two separate experiments and each experiment consisted of three replicates for each of the three temperature/time combinations. Non-heated samples with spores were analysed as control (three control samples in each experiment).

Heating process

For the heating processes, three parallel samples were immersed in the water bath simultaneously. Plastic bags were attached to a metal rack to prevent them from floating. Each bag was maintained separately from the others in the water bath, to allow water to circulate freely between bags and to achieve a uniform heat distribution in all bags, (Figure 13). To minimize a high temperature deviation, the metal rack without the samples was prewarmed in the water bath before starting the experiment. At the end of heat treatment, samples were placed immediately in ice slurry to prevent further inactivation. To analyse the concentration of surviving spores after different heat treatments, 4 g of the heated sample were combined with 36 ml of 1% peptone water (1:10) in a stomacher bag for 2 minutes using the stomacher

machine. Then, 10 ml of the homogenized sample were transferred from stomacher bag into a 15 ml sterilized Falcon tube. This tube was considered the 10^{-1}dilution and further 10-fold dilutions were made using sterile Milli-Q water. In the next step, LB agar was used for culturing (pour plate method) followed by incubation at 30 °C. After 18 hours the number of colonies was counted, and the log reductions were determined.





(C)

***Figure 13**. Figures of the procedure for thermal inactivation of spores in minced meat sample. 100 gr of the minced meat was mixed with spores (A). 6 g of minced meat containing spores was packaged, sealed, and attached to a metal rack for the heating process (B). After heat treatment, 4 g of the heated sample were transferred to a stomacher bag (C) and mixed with 36 ml of 1% peptone water using a stomacher machine.*

3.8.3.Inactivation of spores by heat treatment in minced fish

The minced fish raw material was prepared by one of the master students (Ingvild Gundersen) in the research laboratory at Nofima. All steps of food inoculation, packaging and heating were performed similar to the minced meat. Compositions of minced fish sample can be seen in Table 12.

Table 12. *Composition of minced fish used for heating and pressure processing experiments*

Compound	Haddock fille	Silver smelt	Milk 3.5% fat	Potato starch	Oil	WPC*	Casinat	Salt
Amount/100g	30	30	25	3	8	2	1	1

***WPC**: Whey protein concentrates

3.8.4.Inactivation of spores by pressure processing in minced meat

In this experiment the effect of high pressure (600 MPa) for 2 and 10 minutes at three different temperatures (20, 40 and 55 °C) on *B. cereus* spores was investigated.

Food inoculation and packaging

Sample preparation steps were the same as in the previous experiment (section 3.8.2). The inoculated meat samples were packed in 5×6 cm plastic bags as described before to allow for rapid heat and pressure transmission.

High pressure processing

A high hydrostatic pressure machine QFP 2L-700 (Avure Technologies Inc., Columbus, USA) was used for pressure and pressure combined heat treatment of *B. cereus* spores (Figure 14). Prior to the combined pressure‑heat treatment applications, plastic bags containing minced meat were preheated in a water bath at desired temperatures (40 and 55 °C) for 2 minutes to achieve the target temperature. Six different HPP treatments were employed to evaluate the inactivation of spores. All samples were treated at pressure of 600 MPa at 20, 40 and 55 °C and pressure holding times of 2 and 10 minutes.

After processing, treated samples were immediately immersed in ice slurry prior to enumeration of surviving spores. Samples were serially diluted in sterile Milli-Q water, plated

on LB agar (pour plating method), and incubated at 30 °C for 18 to 20 h. All samples were analysed in 3 replicates in two separate experiments (totally 6 replicates for each sample) and average counts were obtained. In addition, three non-treated samples were analysed as control in each experiment to determine the initial number of spores. Table 13 shows a summary of the pressure and temperature used during the HPP process cycle in this study.

***Table 13**. A summary of applied condition for pressure and pressure-thermal treatment of B. cereus spores in one experiment.*

Preheating Temperature/time	Pressure Temperature	Pressure holding time	Number of Samples
(control)	-	-	3
20 °C	600 MPa/20 °C	2 minutes	3
20 °C	600 MPa /20 °C	10 minutes	3
40 °C /2 minutes	600 MPa /40 °C	2 minutes	3
40 °C /2 minutes	600 MPa /40 °C	10 minutes	3
55 °C /2 minutes	600 MPa /55 °C	2 minutes	3
55 °C /2 minutes	600 MPa /55 °C	10 minutes	3

3.8.5.Inactivation of spores by pressure processing in minced fish

All steps of food inoculation, packaging, pressure processing and log reduction determination were performed similar to the minced meat.

(A)

(B)

(C)

***Figure 14**. HPP processing. Each 3 meat samples were sealed in a plastic bag (A) and placed inside the pressure chamber (B) of the the HPP equipment (C). ((B): adopted from thesis of Aklilu Ghebray)*

4.RESULTS AND DISCUSSION

Several experiments with *B. cereus* vegetative cells and spores have been carried out in this thesis. Table 6 and Table 7 (in the section 3) show an overview of the experiments.

4.1.Psychrotrophic growth

The psychrotrophic characteristic of the *B. cereus* was examined at different temperatures. An increase in bacterial numbers by ≥1-log unit in LB medium incubated at 5, 6, 8, 10 °C was considered as growth. No change was observed in bacterial cell number at 4 °C after 3 weeks. According to the literature studies, the growth temperature range for *B. cereus* is broad (4–50 °C) (Tewari & Abdullah, 2015; Vidic, Chaix, Manzano, & Heyndrickx, 2020). Strains that can grow below 10 °C, are known as psychrotrophic strains and some strains are able to multiply at temperatures as low as 4 °C (European Food Safety Authority, 2005). According to European Food Safety Authority (2005), due to the growth ability at low temperatures, food storage temperature is an important factor in keeping the *B. cereus* number low. Only storage at temperatures below 4 °C would guarantee that no *B. ce*reus growth is possible. Storage at cold temperatures slows the growth of *B. cereus* by increasing the generation time.

4.2.Spore production

Sporulation of *B. cereus* was first carried out in SG liquid medium (batch A). After 10 days of incubation, the ratio of spores to vegetative cells was as low as 60%. Without progress to the desired > 90% sporulation. Sporulation on agar medium was therefore examined. Spore production of the strain was compared in three different types of sporulation media, TSA agar (batch B), SG agar (batch C) and LB agar (batch D). The spores were examined under the microscope and results showed that although the sporulation of bacterial cells in all three types of agars was > 90% after 3-4 days, there were differences in spore quality. Sporulation of *B. cereus* on LB agar yielded more pure spores (free spores with less cell debris) than those of TSA and SG agar. Therefore, in this study all the experiments were performed using spores produced on LB agar. Spores were bright and ovular while vegetative cells appeared dark and rod-shaped, as monitored by phase-contrast microscopy, see Figure 15.

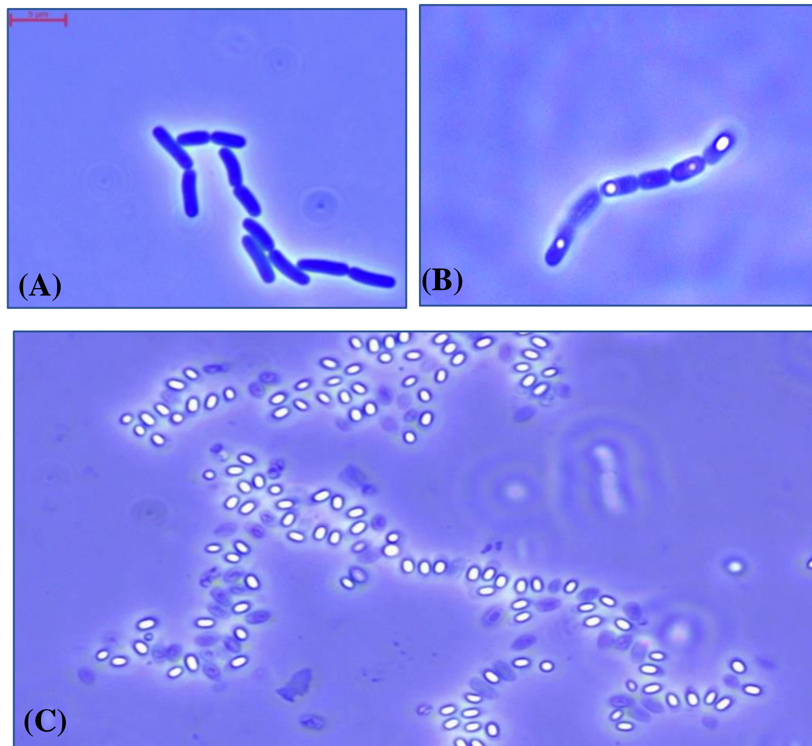


Figure 15. *Microscopic images of B. cereus. Vegetative cells aggregating in stationary phase (A), Spores inside mother cells (B), free spores (C). Spores are bright and ovular while vegetative cells are dark and rod-shaped.*

During the washing process and using high centrifugation speed (10000 and 20000 x g), spores stuck together, leading to clump formation and loss of spores through the supernatant. Examination of microscopic slides from the supernatant confirmed loss of spore clumps in the supernatant (Figure 16). This caused a reduction in spore concentration in the resuspended pellet.

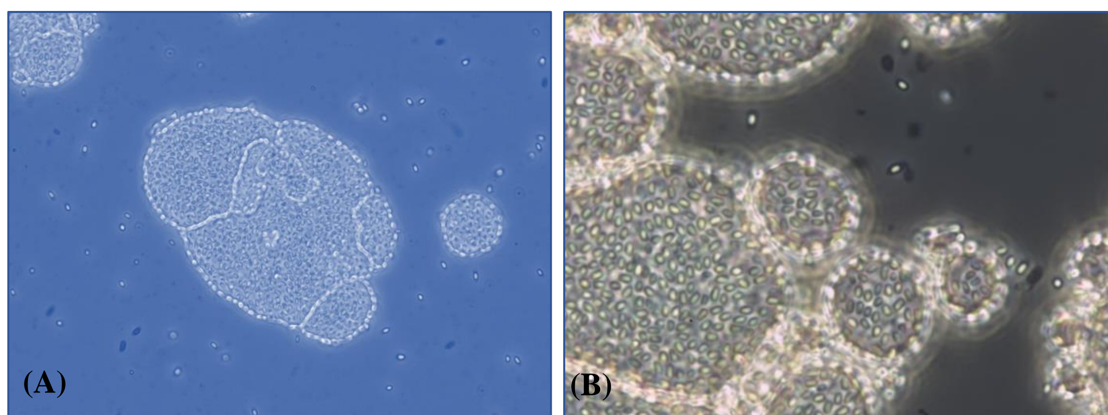


Figure 16*. Large clumps of spores in supernatant after washing and centrifugation at 20000 × g. 400x (A); 1000x (B) magnification.*

4.2.1.Spore washing modifications

Changing the centrifugation speed

To prevent spores from floating in the supernatant and losing spores in the washing process, changes were done in the washing procedure. Centrifugation speed was reduced to 4000 x g. This procedure gave fewer and smaller clumps in the supernatant thus increasing the number of spores in the pellet. Microscopic observation from suspended pellets showed a mixture of dispersed and clumps of spores (batch E).

Adding Tween 20 in the washing procedure

To investigate whether Tween 20 can prevent formation of spore clumps in the suspended pellet, 0.1% of Tween 20 was added to the washing solution. Microscopic slides (Figure 17) confirmed that Tween 20 can prevent clump formation and provide individual spores. Although as it can be seen (in Figure 17-B) spores are in clusters. Small clumps could be observed rarely (in batch F).

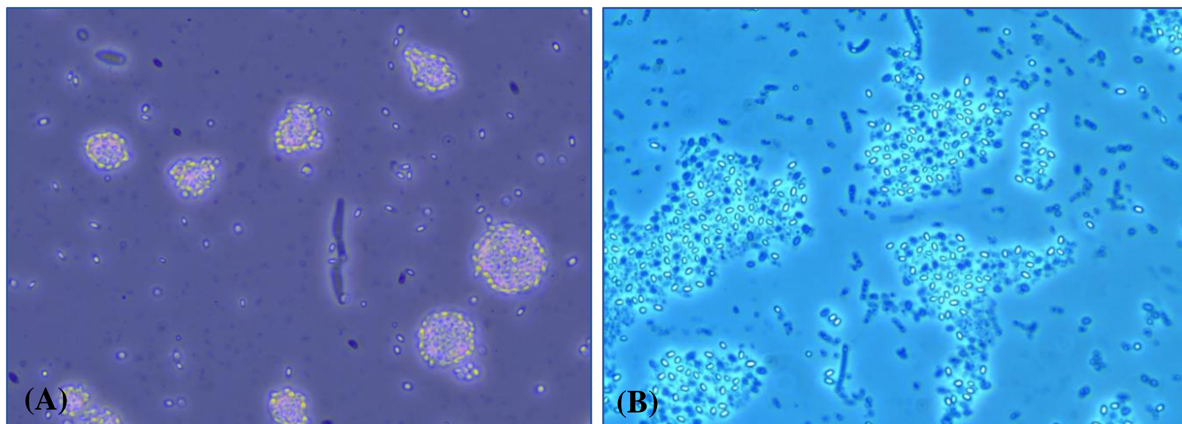


Figure 17. *Comparison of spores washed with Milli-Q water in batch E (A) and Milli-Q water containing 0.1% Tween 20 in batch F (B) in the final resuspended pellet. In the resuspended pellet washed with Milli-Q water (batch E), small clumps of spores can be seen. Adding Tween 20 to the washing solution (in batch F) prevents spore clumping.*

According to the other literature studies, 4000 x g is the most frequent centrifugation speed used for this bacterial species (E. Evelyn & F. V. M. Silva, 2015; López-Pedemonte et al., 2003). Based on the findings of this study, centrifugation at a higher speed (20000 x g) can cause floating of more spores in the supernatant. It is documented that spores of *B. anthracis* and its relatives such as *B. cereus* and *B. thuringiensis* have a very hydrophobic exterior exosporium layer. This hydrophobic spore surface causes these spores to clump together in an

aqueous solution. These clumps can trap air. This makes it difficult to sediment these aggregates with centrifugation since they float in the supernatant. Sonication, addition of Tween and centrifugation over Histodenz, a non-ionic density gradient medium (Sigma-Aldrich), are methods that can be used to deal with spore clumping (Setlow, 2019). In the previous studies performed in the microbiology laboratory at Nofima, applying a higher centrifugation speed (20000 x g) was efficient in the washing procedure for other strains of *Bacillus* (*B. pumilus, B. subtilis*). In the case of *B. cereus*, spores of different strains have been shown to be more hydrophobic than other *Bacillus* species (Hong, Duc, & Cutting, 2005).

4.3.Enumeration of spores

Determining the spore concentration was challenging since colonies of this strain migrated through the agar in pour plate method and spread on the surface (Figure 18). This behaviour is due to *B. cereus* swarming motility (Bottone, 2010). Due to a facultatively anaerobic growth (Cihan et al., 2012) bacterial migration to the surface and presence of oxygen may enhance the growth rate. A modified pour plate technique with an extra surface agar layer on top showed to be a good alternative to avoid migration of colonies.

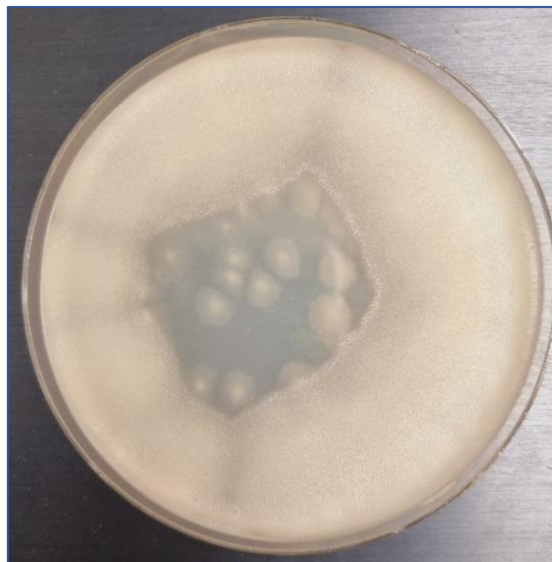


Figure 18. *B. cereus colonies in LB agar, pour plating method. Colonies migrated to the surface and spread on the surface.*

4.4.Investigating the possible effect of Tween 20 on heat resistance of spores

The hydrophobic nature of *Bacillus* spores is documented and the role of this hydrophobicity in the adhesion of these spores to surfaces and making clumps is approved (Cote et al., 2018; Setlow, 2019; Wiencek, Klapes, & Foegeding, 1990). Some treatments such as sonication are recommended to obtain individual spores without clumps. Tween, a non-ionic surfactant, is an agent that can improve the dispersion of bacterial endospores (Krawczyk et al., 2017; Krishna, Zhao, Pumprueg, Koopman, & Moudgil, 2016; Setlow, 2019). In this study, it was investigated whether the level of the heat resistance of spores can be affected by the presence of Tween 20. The viability of spores after heat treatment was compared in two spore batches.

Both spore batches, batch E (washed with Milli-Q water) and batch F (washed with Milli-Q water containing Tween 20) were heated for 10 minutes at 80 °C, 2 minutes at 90 °C and 2 minutes at 95 °C. Log reductions were evaluated and compared in two batches (Figure **19**Figure **19**). According to the results, log reductions were similar in two spore batches. After 10 minutes of heating at 80 °C, 1.3 and 1.1 log reduction was observed in batch E and batch F respectively. Heating spores for 2 minutes at 90 °C resulted in 1.9 log reduction in batch E, and this reduction was 1.8 in batch F. Heating spores at 95 °C for 2 minutes decreased the spore population by 3.1 log in batch E and 2.8 log in batch F. Results demonstrated that adding Tween 20 at a very low concentration, here 0.1%, could not alter the heat resistance of this strain.

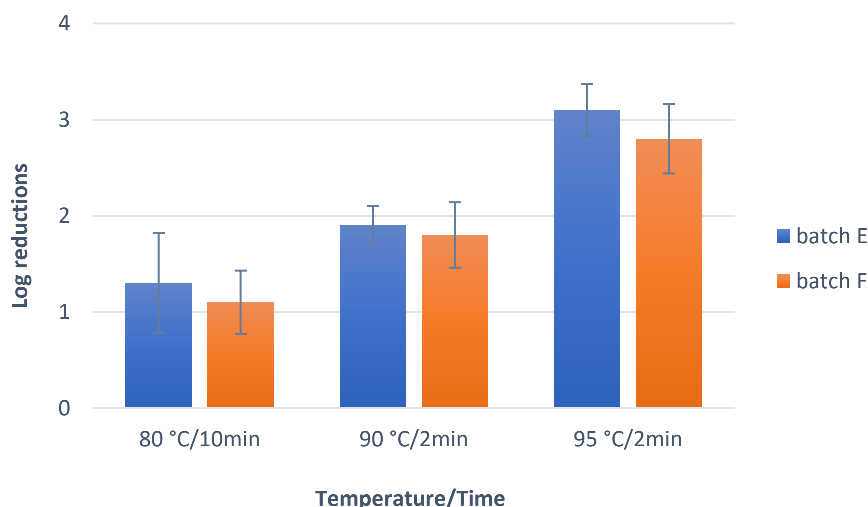


Figure 19. *Log reductions of B. cereus in batch E (washed with Milli-Q water) and F (washed with Milli-Q water containing 0.1% Tween 20) after heating in a water bath. Adding Tween 20 to the washing solution did not affect heat resistance of spores.*

This result is consistent with de Vries, Atmadja, Hornstra, de Vos, and Abee (2005). They showed that addition of Tween 80 had no effect on the spore heat resistance of *B. cereus* strain ATCC 14579.

4.5.Heat treatment of spores in LB medium

Thermal inactivation of *B. cereus* spores was carried out at 80 °C for 5, 10, 15 and 20 minutes and at 90 °C for 2, 5, 8 and 10 minutes. Figure 20 shows the log reductions of the spores heated at selected temperatures and times. The highest spore inactivation of 5.2 log was registered at 90 °C after 10 minutes, and the lowest effect on the spores was observed at 80 °C for 5 minutes (1.9 log). The log reductions at 80 °C were 1.8, 3.0 and 2.9 after 10, 15 and 20 minutes respectively. A 2.2 log reduction was obtained after 2 minutes and a 4 log-reduction after 5 minutes at 90 °C. Heat treatment at 90 °C for 8 minutes resulted in 5.1 log reduction. Increasing the temperature resulted in more spore inactivation and highlights the importance of temperature in spore inactivation. For example, log reduction increased by more than two times when the temperature was increased from 80 to 90 °C for 5 and 10 minutes; from 1.9 to 4 and from 1.8 to 5.2 log reduction respectively.

According to Figure 20, there were unexpected log reductions in some treatments. For example, there was no difference in log reductions when spores were heated at 90 °C for 8 and 10 minutes. Spores used for this experiment were from batch E (washed with Milli-Q water) and the presence of spore clumps may be a reason for this phenomenon. Addition of Tween might result in more reasonable outcomes by preventing clumps, but Tween was not used in this study to keep the assay as simple as possible. In some literature studies inactivation curves exhibited tailing and this behaviour is attributed to the creation of spore aggregates after heat treatment due to the spores' increased hydrophobicity (Soichi Furukawa et al., 2005; Stoeckel, Westermann, Atamer, & Hinrichs, 2013). This has been demonstrated that spores in suspension form clumps, which reduce the rate of spore inactivation during heat treatment. There is also correlation between spore concentration and clumping. It was believed that spore clumps would not develop at lower spore concentrations, thus the survival curve would not show tailing. For *B. licheniformis* in concentration below 4.9 log CFU/ml, the survival curves showed no tailing (Soichi Furukawa et al., 2005; Pflug, Holcomb, & Gomez, 2001). According to Soichi Furukawa et al. (2005), addition of Tween 80 to the bacterial suspensions inhibited the development of spore clumps and improved the rate of inactivation.

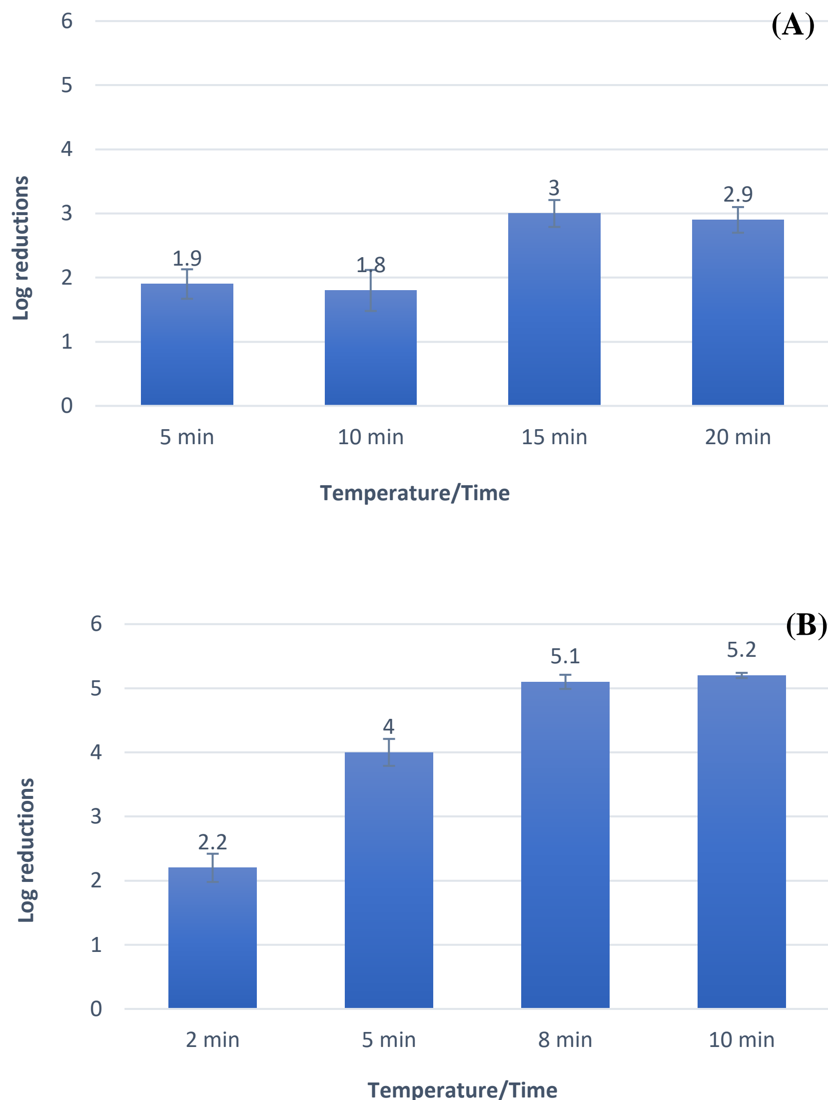


Figure 20. *Log reductions of B. cereus spores after heat treatment at 80 (A) and 90 °C (B) in LB medium.*

4.6. D-values determination

By using the slope of the curves in Figure 21, the D-values obtained for *B. cereus* spores in LB medium were 1.9 min at 90 °C (R^2 = 0.91), and 7.1 min at 80 °C (R^2 = 0.82). The D-value obtained in our work with *B. cereus* spores at 90 °C is comparable to the values obtained by E. Evelyn and F. V. M. Silva (2015) when they used the same strain for heat treatments in skim milk. They reported a D-value of 2 min at 90 °C. However, a higher D-value at 80 °C (8.5 min) was found by them. Regarding log reduction after heat treatment, they reported spore

inactivation of 2.5 log after 20 minutes heat treatment at 80 °C, very similar to our result of 2.9 log.

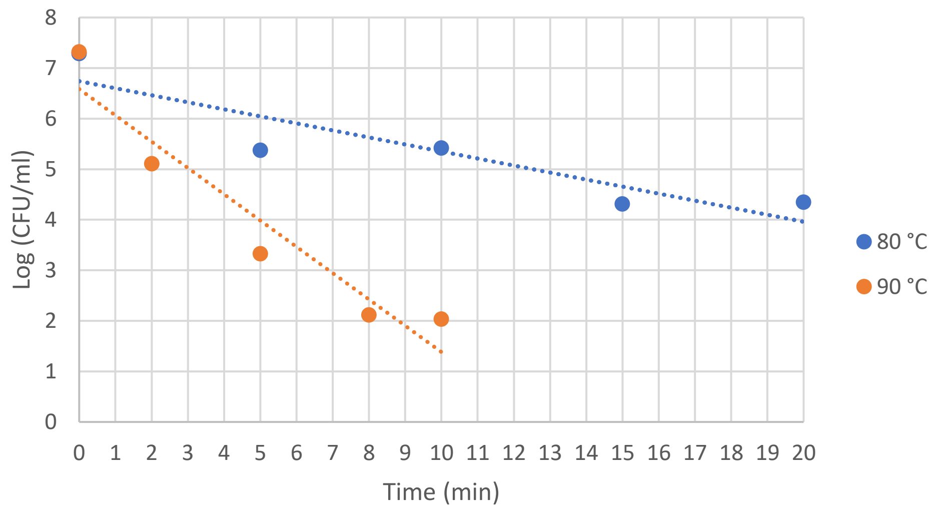


Figure 21. *Thermal survival curves of B. cereus spores in LB medium. Slopes of the curves were used for D-values determination.*

In the literature, a wide range of *B. cereus* spore heat resistance is reported, and it is highly dependent on the strain, sporulation temperature and sporulation medium (Evelyn & F. V. M. Silva, 2015; Mazas, Gonzalez, Lopez, Gonzalez, & Sarmiento, 1995). In another study by E. Evelyn and F. V. M. Silva (2015), D-values at 90 ˚C were 3.1 min in skim milk and 1.0 min in beef slurry for *B. cereus* ATCC 11778. According to these authors, D-value for the 80 °C thermal process was 4.2 in skim milk and 1.3 in beef slurry.

4.7.Bioscreen C experiments

In this step, before studying the effect of preservatives on bacterial growth, the effect of initial bacterial number on growth was investigated as a quality control test of the Bioscreen C equipment.

4.7.1.Investigating the effect of initial bacterial number on growth rate

Turbidity data were obtained from Bioscreen C, by which absorbance readings at 600 nm were measured every 30 minutes. Growth curves for each dilution was plotted (Figure 22).

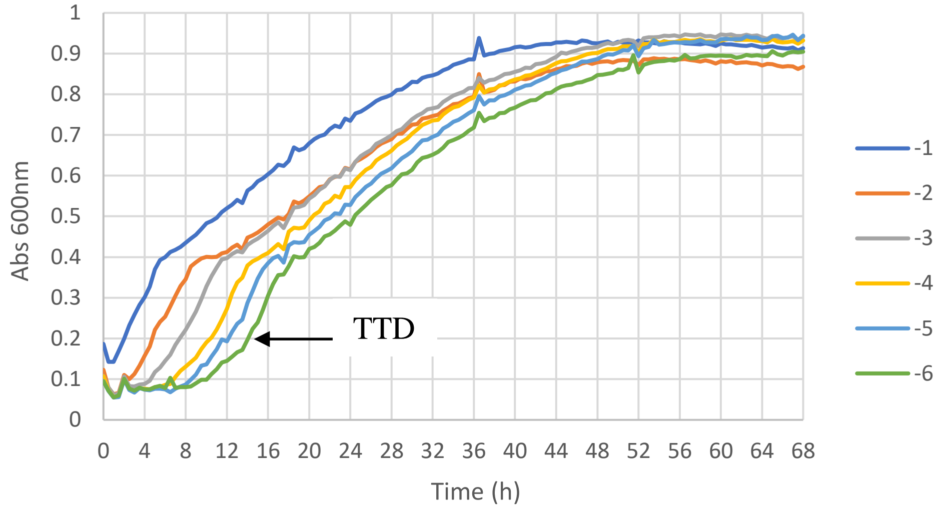


***Figure 22**. Turbidity growth curves of B. cereus in LB medium at 25 °C, obtained at absorbance = 600 nm. The Time to Detection (TTD) is indicated for each of 6 decimal dilutions.*

It can be seen from the results that the higher the cell number, the faster the change in OD, so the faster the detection time. For the high concentrated cell suspension, it took 2 hours to reach the OD = 0.2 while this time is 7 times more (14 hours) for the most diluted suspension. After determining the detection time for each cell number, these values were utilized to create a calibration curve (Figure 23).

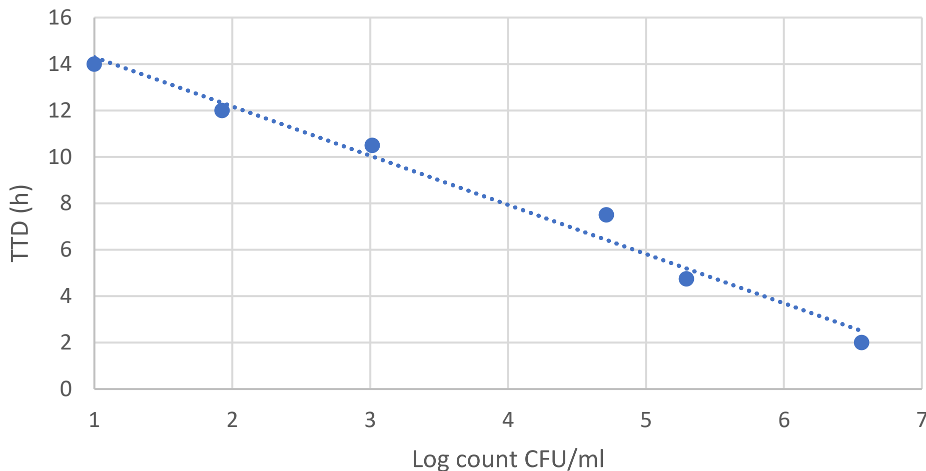


***Figure 23.** Calibration curve for B. cereus ATCC 9139 in LB medium at 25 ℃: TTD (detection times) plotted against initial cell numbers of B. cereus.*

It was revealed by the results of the calibration curve that there is good correlation between OD data and cell number, R^2 =0.98. This calibration curve can be used to convert Bioscreen data into cell number for this strain.

4.7.2.Effect of preservatives and pH on growth pattern of spores and vegetative cells

Controlling environmental factors (temperature, pH, salinity, atmosphere, presence of additives) can affect *B. cereus* growth in foods (Vidic et al., 2020). In this study Bioscreen C were used to study the growth of *B. cereus* in the presence of different concentrations of preservatives.

Effect of NaCl on growth

Growth of *B. cereus* is shown in Figure 24. At the highest salt concentration, 4%, *B. cereus* had the longest TTD value, and the absorbance was around 0.3 the entire experimental period. TTD for vegetative cells in LB containing 4% NaCl (18 h) was 3 times longer than in LB without salt (6 h). Similarly for spores, an extended lag phase with 22 h delayed TTD (compare to 8 h in control) was induced by 4% NaCl concentration. No major changes were observed in growth pattern in 1 and 2% NaCl. Antimicrobial effect of NaCl was confirmed by M. Kim, Young Park, Jung Park, and Ha (2017). According to this author, NaCl reduces the moisture content of food by reducing water activity. The capacity of sodium and chloride ions to bind with water molecules is responsible for salt's ability to reduce water activity and reduce the rate of growth (Rysová & Šmídová, 2021).

Salt can cause osmotic shock in microbial cells, resulting in the loss of water from the cell, cell death, or retarded growth (M. Kim et al., 2017). M. Kim et al. (2017) investigated how different NaCl concentrations affected *B. cereus* in salted shrimp. After 7 days of storage at 10 °C they observed 1.7, 2.7, and 3.4 log reductions with 5, 10, and 15% NaCl treatment, respectively. After 14 days there were no survivors in any of the samples, independent of NaCl concentrations. Stecchini, Del Torre, Donda, and Maltini (2000) found that an increase in the NaCl concentrations (0.5, 2.5 and 4.5%) resulted in increased times for *B. cereus* colonies to grow on a solid medium. It is reported that *B. cereus* is able to grow in the presence of up to 7% NaCl (M. Kim et al., 2017).

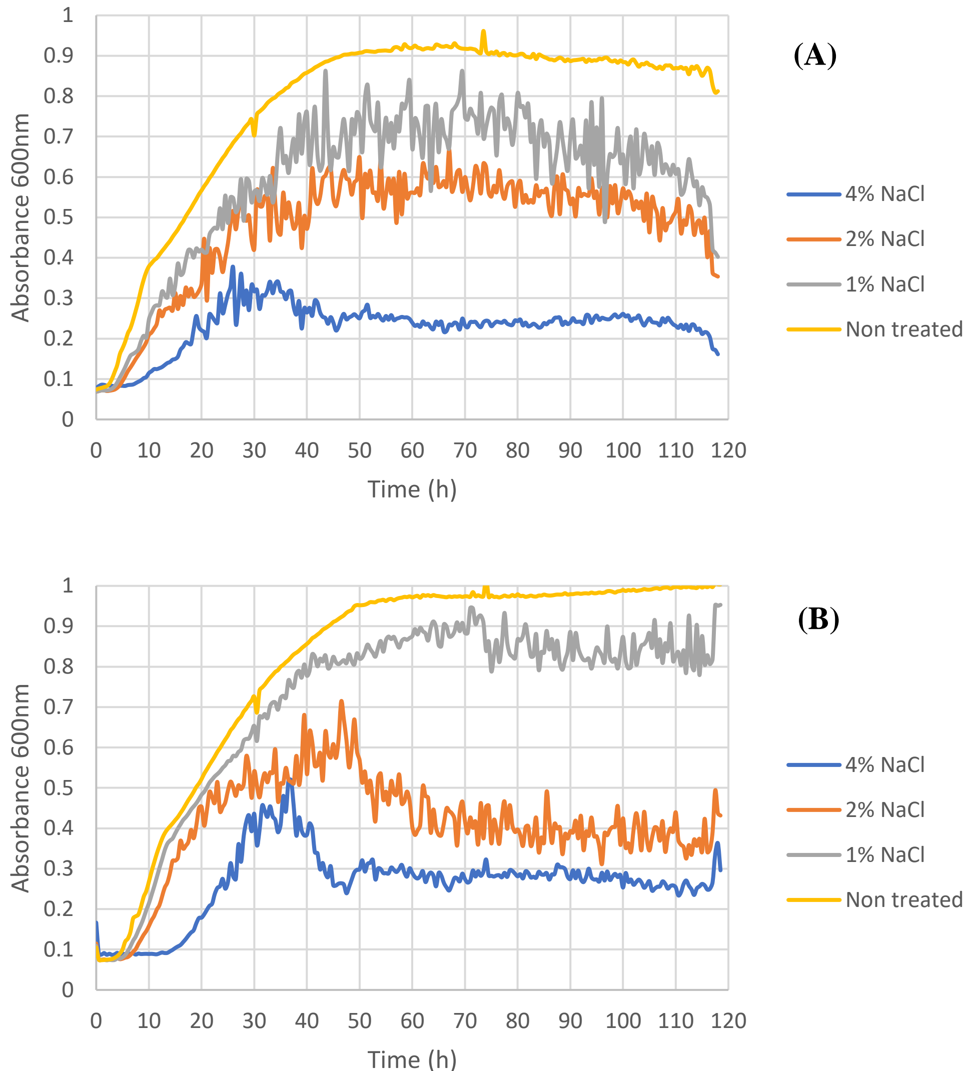


Figure 24*. Effect of different NaCl concentrations (in LB medium at 25 °C) on vegetative cells (A) and spores (B) of B. cereus. TTD was longer in 4% NaCl. The data is mean values of 6 parallels collected from Bioscreen.*

Despite lack of bactericidal effect in examined concentrations, it is not recommended to increase salt concentration in food products. There is a current trend to consume foods with reduced salt content since high blood pressure, renal disease, stroke, osteoporosis, and cirrhosis are caused by excessive salt consumption. (M. Kim et al., 2017; Mok & Song, 2000).

Effect of KCl on growth

Figure 25 shows the effect of KCl. The TTD was longer (18 h) at 4% KCl in both vegetative cells (A) and spores (B). In 1 and 2% KCl TTD was shorter and both spores and vegetative grew faster (TTD of around 8 h) at theses concentrations. At the highest concentration of KCl,

the absorbance was around 0.3 the entire experimental period. The antimicrobial effect of KCl was approved previously (Bidlas & Lambert, 2008).

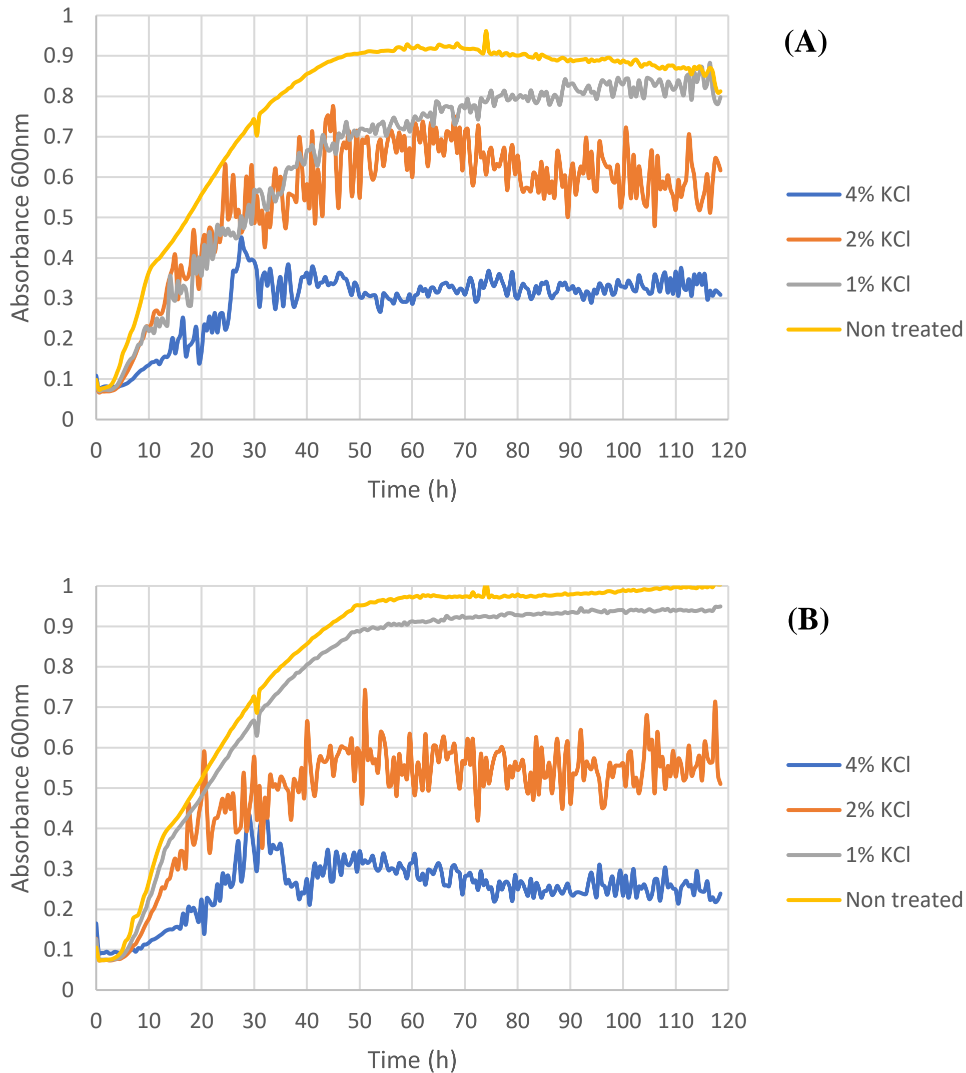


Figure 25. *Effect of different concentrations of KCl (in LB medium at 25 °C) on vegetative cells (A) and spores (B) of B. cereus. TTD was longer in 4% KCl. The data is mean values of 6 parallels collected from Bioscreen.*

In the Bioscreen experiments there were some challenges related to pellet formation in the wells. In the wells where bacterial cells and spores were mixed with NaCl and KCl, growth was not dispersed and there were aggregations causing noise in the OD measurements (Figure 26).

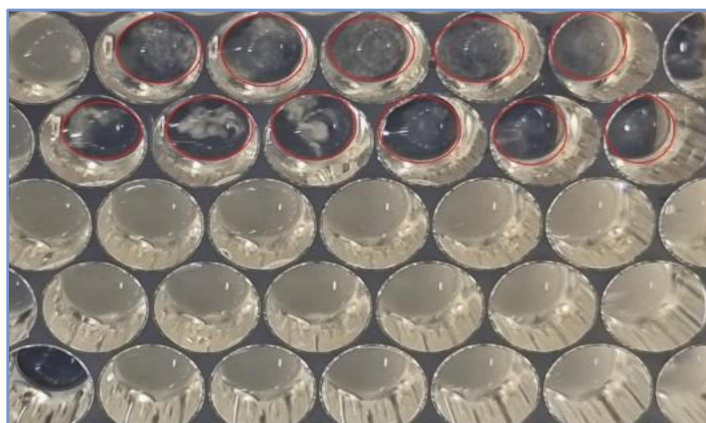


Figure 26. *The presence of some preservatives (NaCl and KCl) caused aggregates in wells that are marked with red circles. These aggregations caused noise in the OD measurements.*

Effect of $NaNO_2$ on growth

$NaNO_2$ (500, 125 and 62.5 ppm) did not affect the growth and TTD of vegetative cells and spores (Figure 27) of this strain. So, the strain of *B. cereus* was considered tolerant to all tested $NaNO_2$ concentrations at 25 °C and pH 7 in LB medium. Sodium nitrite has been employed as a coloring and antibacterial agent in food since the early 1900s. It is used as a food preservative at the maximum allowed concentration of 125 mg per 1 kg of meat in a final product (Cvetković, Živković, Lukić, & Nikolić, 2019) that is well known to suppress the germination of *Clostridium* spores in cured and processed meats (Bhusal, Nelson, Pletcher, & Muriana, 2021; Karwowska & Kononiuk, 2020).

The relative sensitivity of bacteria to the preservatives is related to the characteristics of the species and to the conditions utilized in the study. Nitrite exhibits an enhanced antibacterial activity in acidic environments, although some strains of *Bacillus* are resistant to nitrite (Abriouel, Maqueda, Gálvez, Martínez-Bueno, & Valdivia, 2002). Some investigators have examined the effects of sodium nitrite on the germination and growth of *Bacillus* species. In the presence of 300 µg/g nitrite at pH 6, all species germinated, but increasing nitrite concentrations to 750 and 2,500 µg/g inhibited germination, and the effects were stronger at the lower pH than at pH 7. In addition to the pH, temperature is another factor influencing bacterial sensitivity to nitrite. Nitrite was more inhibitory at 20 °C than 35 °C (Sofos, Busta, & Allen, 1979). Our experiment was performed at 25 °C and pH 7, resulting in bacterial resistance to the selected level of nitrite.

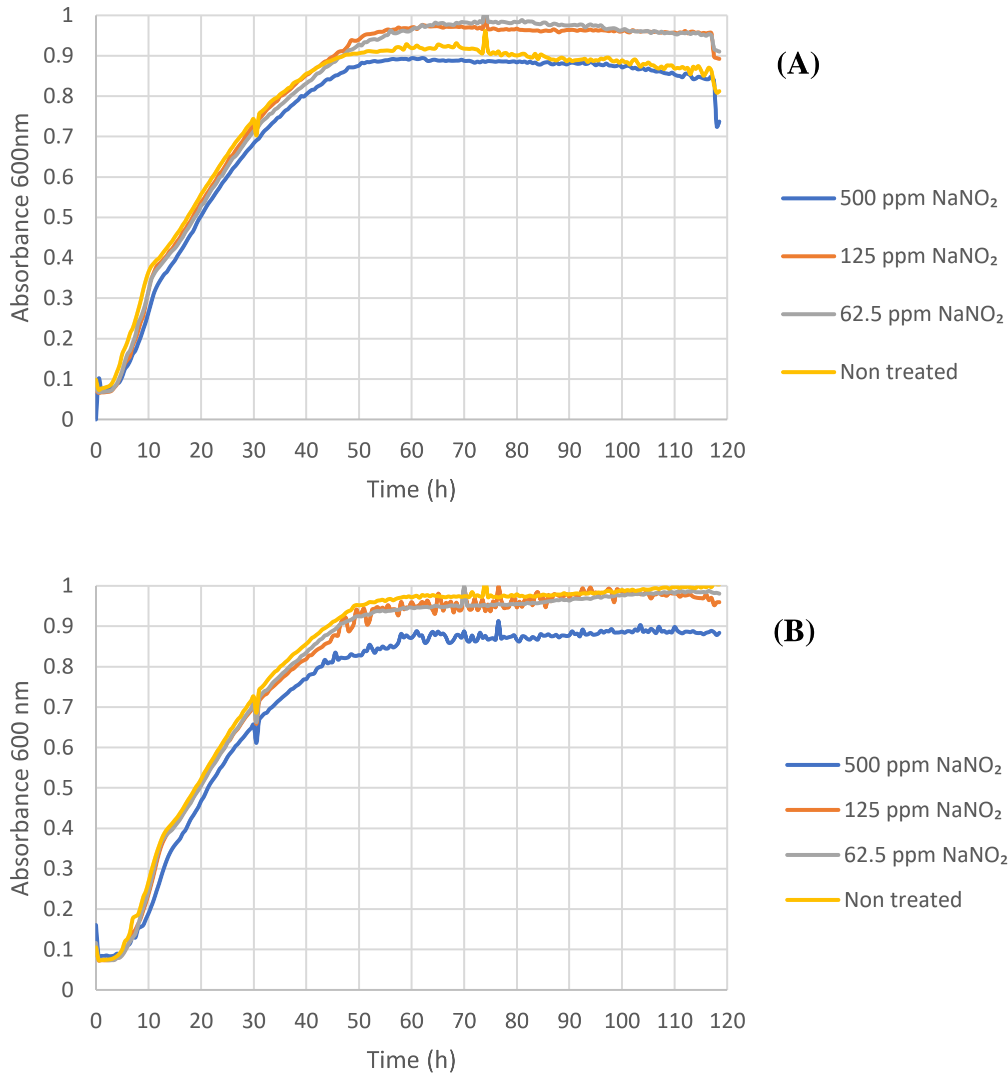


Figure 27. *Effect of NaNo$_2$ (in LB medium at 25 °C) on vegetative cells (A) and spores (B) of B. cereus. No difference in growth was observed in different concentrations of NaNo$_2$. The data is mean values of 6 parallels collected from Bioscreen.*

Effect of pH on growth

Effect of pH on growth was the same for vegetative cells and spores. Exploring the effect of pH on growth showed that pH 5 had the least inhibitory effect on *B. cereus* (Figure 28). TTD was 15-19 h at this pH. Concerning pH 3 and 4, there was an increase in OD that was not expected. Because according to literature, *B. cereus* can grow in a pH range between 4.5 to 9.5 (Rodrigo et al., 2021) so in this study, the observed increased OD at pHs lower than 4.5 was not expected.

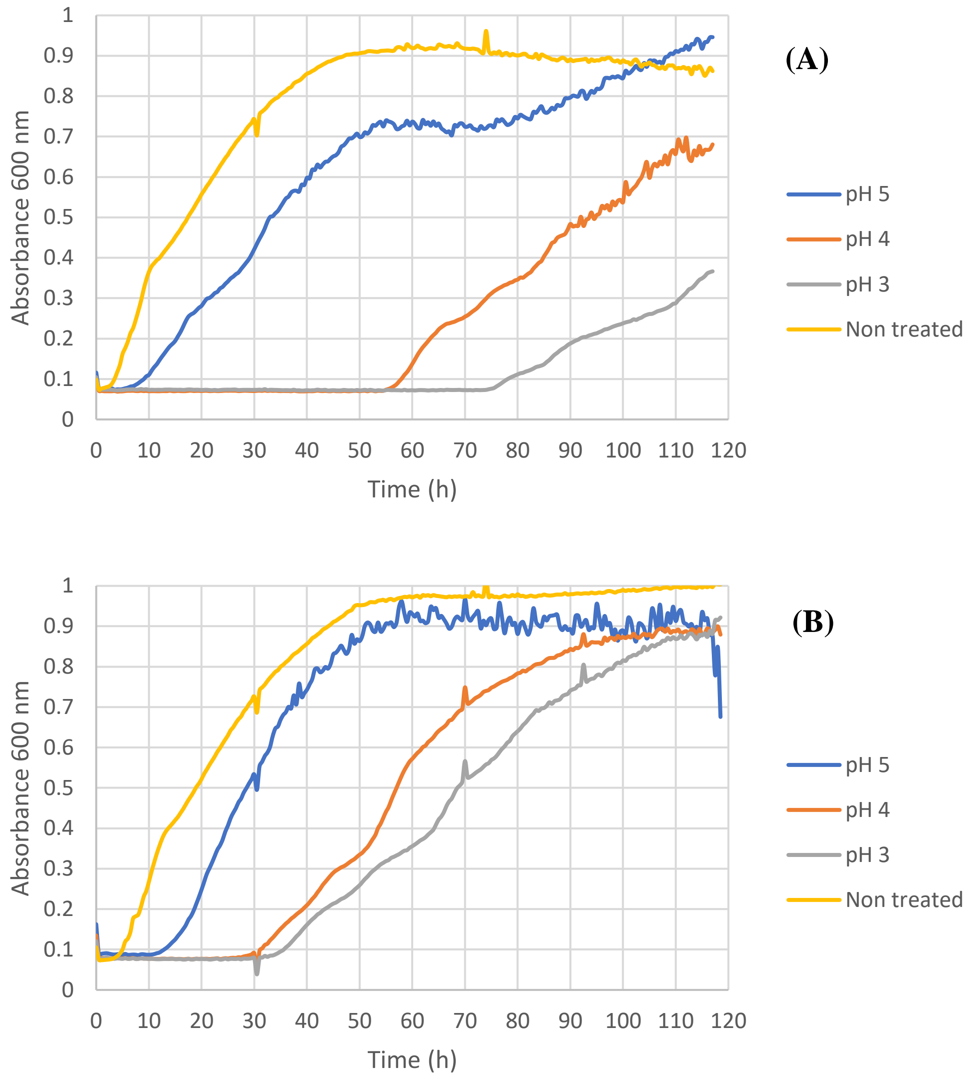


Figure 28. *Effect of pH (in LB medium at 25 °C) on vegetative cells (A) and spores (B) of B. cereus. pH 5 can delay growth (TTD 16-18). There was no growth at pH 3 and 4. An increase in OD at pH 3 and 4 was related to cell elongation. The data is mean values of 6 parallels collected from Bioscreen.*

To verify whether the observed turbidity was linked to vegetative growth, the Bioscreen test was repeated and also another experiment in an Erlenmeyer flask with the same conditions was conducted. Following 72 hours of contact time at low pHs (3 and 4), bacterial concentration was determined by plate counting the content of Erlenmeyer flasks. Shape and morphology of bacterial cells in bioscreen plate and flasks content was also assessed by making microscopic slides. The results of the verification test were as follow: After 18 hours incubation at 30 °C, colonies were counted, and bacterial concentration was determined 10^4 CFU/ml in both pHs indicating that the increase in OD is not related to the bacterial growth. Regarding the morphology, long rods were observed under the microscope that was documented with

pictures (Figure 29). The presence of these filaments was the reason for OD increase and observed turbidity.

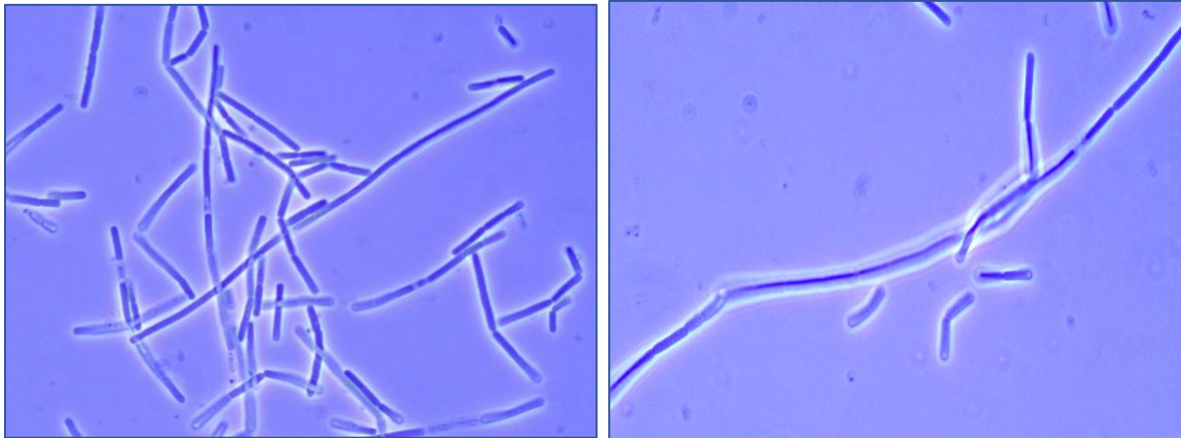


***Figure 29**. Microscopic slide of B. cereus cells in pH 4 and 3. Bacterial cells formed long filaments. This attitude contributes to the increase in culture turbidity and OD measurement.*

Results of this experiment are in agreement with Everis and Betts (2001). They confirmed the presence of elongated cells or chains of rods in the lower pH broths (pH = 4.3) which did not exists in the higher pH. According to these authors, subjecting spore-formers to the environmental stress of low pH, resulted in an increase in the length of the cells and elongated cells disappeared when they were returned to optimum pH conditions. In another study, increase the length of the *B. cereus* TZ415 from 2-5 to 15-40 µm was revealed after exposing to pH 5 (Jobin, Clavel, Carlin, & Schmitt, 2002).

These observations are related to acid tolerance response (ATR). ATR is an adaptive response upon exposure to acid conditions to maintain bacterial internal pH (Senouci-Rezkallah, Jobin, & Schmitt, 2015). Some strains of *B. cereus* such as ATCC14579, TZ415 strain, NCIMB11796 and ATCC14579 are able to survive low pH environment (Browne & Dowds, 2002; Jobin et al., 2002; Thomassin, Jobin, & Schmitt, 2006). It is stablished that spores of *Bacillus cereus* have a high capacity to survive under adverse environments, such as low pH (from 1 to 5.2) (Vidic et al., 2020).

Effect of nisin on growth

The antimicrobial effect of nisin against *B. cereus* vegetative cells shown in Figure 30. In the control sample (without preservative), the number of bacteria increased steadily, and the OD

reached to 0.2 after 6 hours. Vegetative cells tolerated 7.5 µg /ml and 15 µg /ml of nisin. Although prolonged lag phases were observed when exposing to 15 µg /ml of nisin (TTD = 25 h).

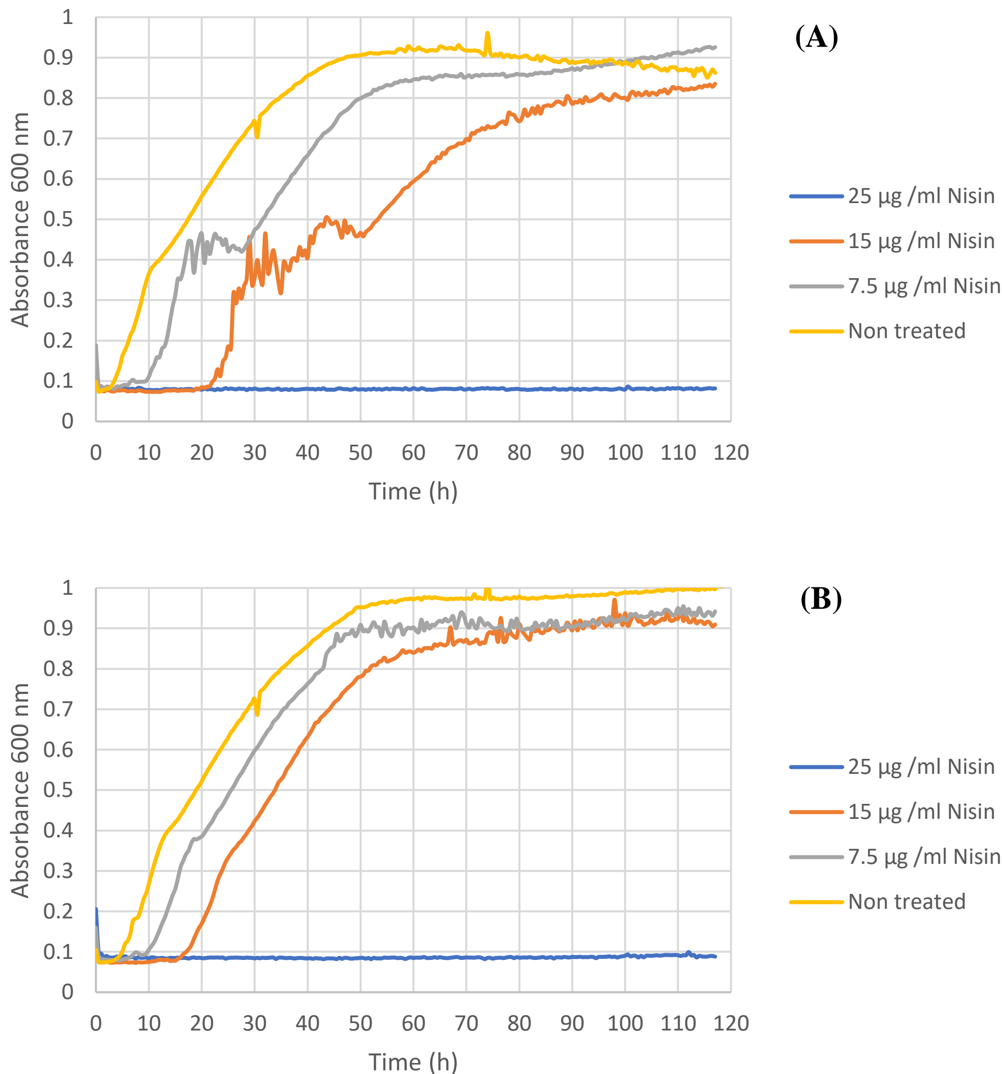


Figure 30. *Effect of different concentrations of nisin (in LB medium at 25 °C) on vegetative cells (A) and spores (B)of B. cereus. Growth was inhibited at 25 µg /ml of nisin. The data is mean values of 6 parallels collected from Bioscreen.*

At 7.5 µg /ml nisin, TTD was 13 h. At 25 µg /ml nisin, this strain was unable to initiate growth after 5 days. Regarding the effect of nisin on spores, lag phase increased at 7.5 and 15 µg /ml of nisin. OD reached 0.2 after 13 and 21 hours (at 7.5 and 15 µg /ml of nisin respectively). The outgrowth of spores was inhibited, and OD did not show an increase after 5

days in the presence of the highest concentration of nisin indicating the antimicrobial effect of this preservative in concentration equal to 25 µg /ml.

Nisin, a bacteriocin produced by Lactococcus lactis, exhibits antimicrobial activity against a wide range of Gram-positive vegetative cells (due to its inability to penetrate the outer membrane in Gram-negatives) and spores (Lee et al., 2015; Prado-Acosta et al., 2010). European Food Safety Authority (EFSA) concluded that the use of nisin as a food additive in unripened cheese (at maximum level of 12 mg/kg) and in heat-treated meat products (at maximum level of 25 mg/kg) would be safe (Maged Younes et al., 2017). The growth and increase bacterial number in the presence of nisin is dependent on storage temperature and initial number of bacterial cells (Paik et al., 2006). For instance, 1 µg/ml of nisin inhibited enterotoxin production of *B. cereus* in gravy at 8 °C, while 5 µg of nisin/ml was required for inhibition at 15 °C (Beuchat, Clavero, & Jaquette, 1997).

Another factor that can increase bacterial sensitivity to nisin, is low pHs (pH 5) (Faille, Membre, Kubaczka, & Gavini, 2002). In our experiment, nisin effectiveness was evaluated at pH 7 at 25 °C. So according to the documents, decreasing both factors (temperature and pH) may result in inhibitory effect or more growth delay at 7.5 and 15 µg /ml of this preservative. In another study, two different concentrations of nisin (100 and 500 IU) were used as a preservative in beef jerky. *B. cereus* started to grow after 3 days in 100 IU nisin/g treatment, and after 21 days in 500 IU nisin/g treatment. (Lee et al., 2015). For *Bacillus cereus* 6A1, 595 IU/ml was determined as minimum inhibitory concentration (MIC) (Prado-Acosta et al., 2010).

4.8.Inactivation of *B. cereus* spores in food matrices by heat treatment and pressure processing

4.8.1.Spore production

To obtain spores with concentration of 10^8 CFU/ml, more spores were harvested and washed. The final working spore solution was a 2-phase solution. A white dense layer was created at the top that stuck to the surface of the plastic tube (Figure 31).

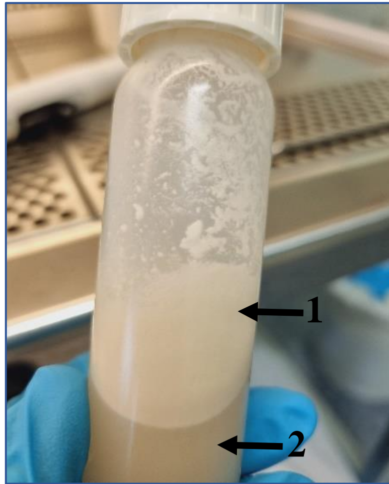


Figure 31. *A non-homogenous spore solution after harvesting and washing more spores. Arrow labelled 1 in the tube indicates a white layer on the surface and the arrow labelled 2 shows a more diluted solution.*

Despite harvesting more spores, spore enumeration in sample taken from the spore solution (Labelled 2 in Figure 31), showed a concentration of 10^7CFU/ml. Providing a microscopic slide from the white dense layer revealed high concentrated spores in aggregations (Figure 32).

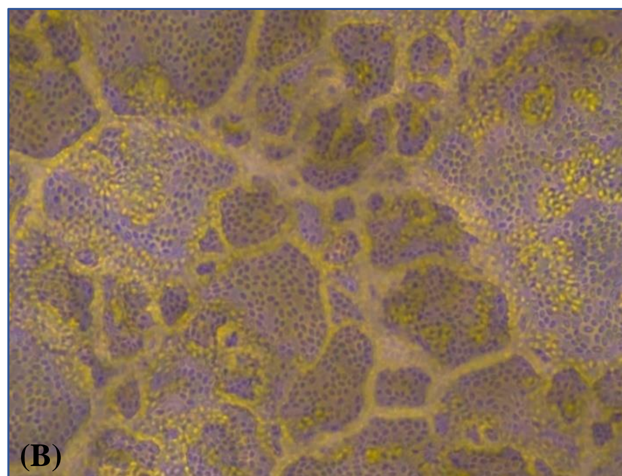


Figure 32. *After increasing harvested spores, a microscopic slide from the dense layer (A) determined thick layers of spores (B).*

As discussed in hydrophobic spores of *Bacillus* strains previously (in section 4.2), it is difficult to pellet spores due to clump formation. It is also assumed that there is a correlation between spore aggregation and its concentration as Soichi Furukawa et al. (2005) showed that spore clumps would not develop below a concentration of 4.9 log CFU/ml of *B. licheniformis* spores. So, according to this author, increasing spore aggregates at higher spore concentration is expected. Based on findings of this experiment and related challenges in spore production, to obtain a homogenous spore solution with higher concentrations than 10^7 CFU/ml it is necessary to replace Milli-Q water with Milli-Q water containing 0.1% Tween 20 in all steps of harvesting and washing.

4.8.2.Inactivation of spores by heat treatment

The effectiveness of *B. cereus* spore reduction under thermal processing was investigated in food. Figure 33 shows the log reductions of *B. cereus* in minced meat (A) and minced fish (B) after heating. Minced meat and minced fish were inoculated with spores and heated at three different temperatures (85, 90 and 95 °C) for 5 minutes. In the minced meat sample, the highest spore inactivation of 3.3 log was obtained at 95 °C, and the lowest reduction, less than 1 log (0.8), was at 85 °C. A 1.5-log reduction was observed when heated at 90 °C. Regarding minced fish sample, after 5 minutes of heating at 95 °C, the amount of *B. cereus* spores reduced by 3.5 log, while a 1.6 log reduction in spores was detected after heating at 90 °C. Heating minced fish at 85 °C resulted in a less than 1 log decrease (0.3) of spores.

Thermal inactivation of the same strain (*B. cereus* ATCC 9139) was examined in skim milk by Evelyn and F. V. M. Silva (2015). They heated spores at 70 and 80 °C for 20 min resulted in log reductions of 0.4 and 2.5 respectively. In their study, heating spores for 10 minutes at 90 °C caused 5 log reduction. Another investigator reported < 0.4 log reductions for *B. cereus* ATCC 9818 heated in cooked rice at 85 °C for 4 minutes (H. Daryaei et al., 2013). Comparison of spore elimination by heating in minced meat and LB broth (heating spores in LB is described in section 4.5) was performed and the result indicates that substrate composition has influenced on spore heat tolerance (Table14). Our findings revealed that heating spores (in the same heating condition: at 90 °C for 5 minutes) suspended in LB medium culminated in more than double log reduction compared to that was found in the minced meat (4 compared to 1.5 log reduction), pointing the protective effect of food matrices on spore resistance. Complex food systems could decrease spore inactivation. This complexity is included oil, salt, sugar and reduced a_w (Sevenich et al., 2016).

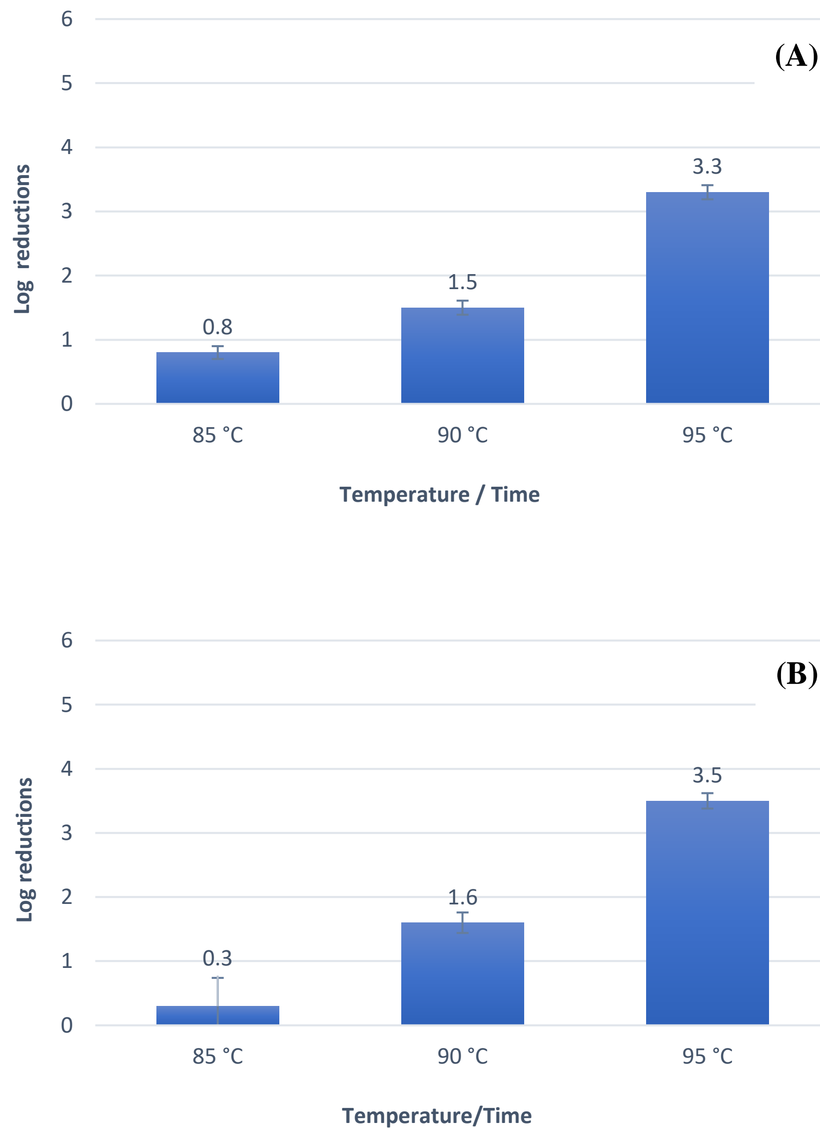


Figure 33. *Inactivation of B. cereus spores in minced meat(A) and minced fish (B) after heat treatment at 85, 90 and 95 °C for 5 minutes. The data is mean values of six parallels and the standard deviations are shown for each treatment.*

According to Coroller, Leguérinel, and Mafart (2001) the heat resistance of bacterial spores is affected by the media in which they are heated. They observed that increasing the thermal resistance of spores was more pronounced when decreasing of the water activity (Coroller et al., 2001). The stabilizing effect of lipids on spores was also documented by Molin and Snygg (1967) when they compared spore's heat resistance in phosphate buffer solution and oil.

Table 14. A comparison of log reductions of B. cereus spores after heat treatment in minced meat and LB medium. Similar treatment condition and related results are underlined.

	90 °C				95 °C
	2 min	5 min	8 min	10 min	5 min
LB	2.2	4	5.1	5.2	
Minced meat		1.5			3.3

In this study, with regard to the variation in heating substrates content (LB and minced meat), such as the presence of fat and lower a_w in minced meat and minced fish, higher heat tolerance for *B. cereus* in food products can be expected than in LB.

4.8.3.Inactivation of spores by pressure processing

The influence of HPP at three different temperatures on the *B. cereus* spore inactivation was studied and compared. Pouches containing 6 g inoculated minced meat and minced fish were subjected to pressure of 600 MPa at 20, 40 and 55 °C. All three pressure-temperature combinations were studied at two different holding times of 2 and 10 minutes (Totally 6 treatment condition for each food sample). Each experiment was performed in triplicate and repeated two times. Spore log reductions (log N_0/N) were plotted against treatment condition (Temperature/Time) (Figure 34). N_0 was the spore concentration in the untreated sample (control) and N was the spore concentration after a certain processing condition. Spore logarithmic reductions were also compared with the thermal results.

Log reductions showed differences when spores were treated at the same pressure but at different temperatures. The highest spore inactivation of 3.2 and 3.3 log in minced meat and minced fish respectively was achieved after 10 min at 55 °C, while at 20 °C (no additional heat), 0.3 log reduction was observed in both minced meat and minced fish after 10 min processing. The higher the HPP process temperature, the higher was the *B. cereus* spore inactivation, indicating the important role of temperature in inactivating spores. An explanation for this finding is that increasing the temperature can trigger spore germination, making the spore population more susceptible to high pressure (E. P. Black et al., 2005).

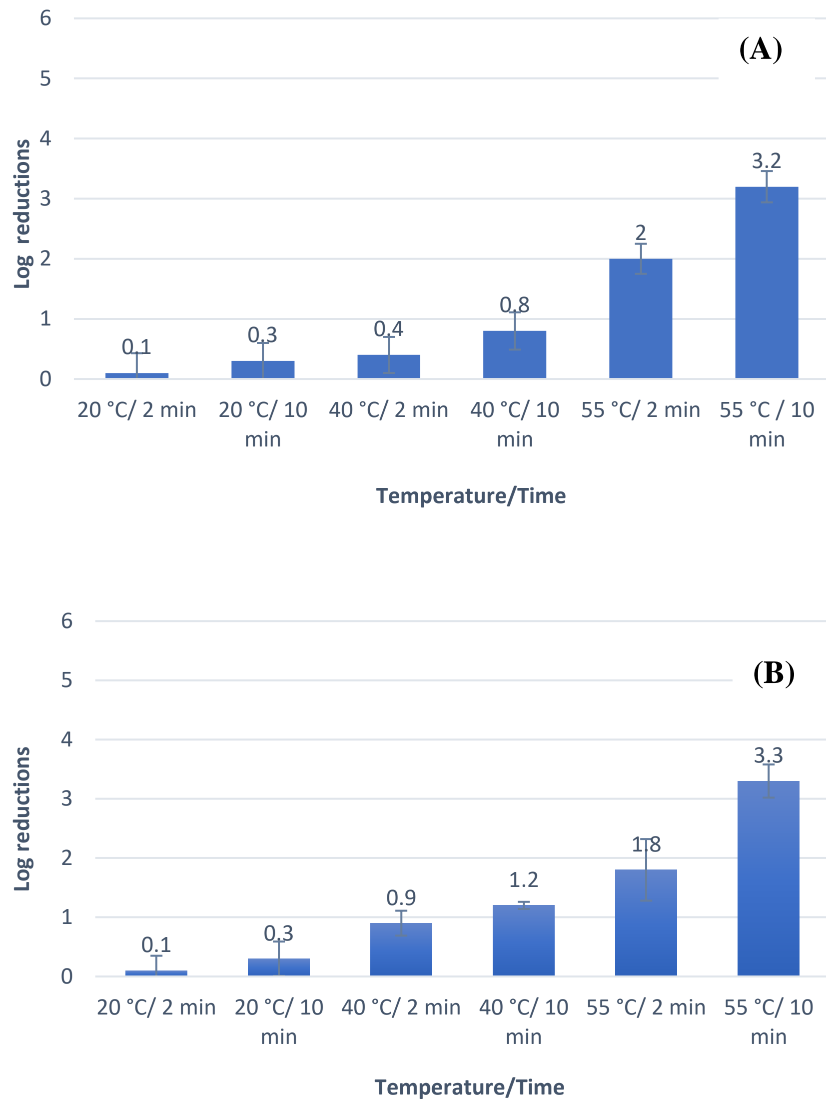


***Figure 34**. Inactivation of B. cereus spores in minced meat (A) and minced fish (B) after HPP processing at 600 MPa at different temperatures.*

The effect of processing time is also obvious. Increasing the time from 2 to 10 minutes at 55 °C, reduced spore population by 1.2-1.5 log in both food stuff. Comparison of log reductions was performed between the thermal treatments alone and pressure–thermal combination (Table 15). Based on the table, neither 85 °C nor 600 MPa alone (at 20°C) could achieve the level of highest obtained inactivation (>3 log reduction) and pressure-thermal process was found more effective. In both food matrices combination of 600 MPa pressure with a temperature 40 °C lower than thermal process alone (55 °C compared to 95 °C) resulted in the same spore inactivation. More than 3 log reduction was observed for the pressure–thermal process (600 MPa-55 °C) and thermal process (95 °C), indicating a remarkable advantage of using HPP technology.

Table 15. Comparison of log reductions of B. cereus spores after thermal processing (A) and pressure processing (B) in minced meat and minced fish.

(A)		**85 °C/ 5 min**	**90 °C/ 5 min**	**95 °C/ 5 min**
Log reduction	**Minced meat**	0.8	1.5	3.3
	Minced fish	0.3	1.6	3.5

(B)		**20 °C/ 2 min**	**20 °C/ 10 min**	**40 °C/ 2 min**	**40 °C/ 10 min**	**55 °C/ 2 min**	**55 °C/ 10 min**
Log reduction	**Minced meat**	0.1	0.3	0.4	0.8	2	3.2
	Minced fish	0.1	0.3	0.9	1.2	1.8	3.3

Similar observations were reported by Evelyn and F. V. M. Silva (2015) when they investigated inactivation of the same strain spores (*B. cereus* ATCC 9139) in reconstituted milk treated by high pressure combined with a thermal process, and compared it with thermal inactivation. In agreement with our findings, they found that the pressure–thermal process could enhance *B. cereus* spore inactivation. According to them, in a condition of 600 MPa at 70 °C for 20 minutes, spores showed a 4-log decrease, compared to only 0.4 log for the thermal process (70 °C for 20 min). Moreover, they studied the influence of temperature at 600 MPa on spore inactivation for up to 40 minutes. According to their results, spore inactivation in milk was increased by 3.5 log when raising the HPP temperature from 38 to 70 °C. In another study, Van Opstal, Bagamboula, Vanmuysen, Wuytack, and Michiels (2004) investigated the combination of mild temperatures with HPP treatment in relation to *B. cereus* spores. He discovered that inactivation levels of > 5 log were attained when utilizing 500 MPa pressure combined with a temperature of 60 °C.

Difference in spore sensitivity to HPP may be attributed to the kind of substrate and the content of the meal that have a significant impact on spore response during pressure treatment. Carbohydrates, proteins, lipids, and other nutritional elements can all provide protection. It is

also documented that spores are better protected in salt and sucrose content (Considine, Kelly, Fitzgerald, Hill, & Sleator, 2008).

Some authors also observed that pressure resistance is strain-dependent when subjecting different bacterial spore strains to HPP treatment (R. P. Lopes et al., 2018). Water activity of the food is another factor influencing spores' resistance to pressure. In a study when bacterial spores were treated by pressure-thermal processing in different food products such as cooked ground beef, egg patty mince, whole milk, and mashed potatoes, different spore resistances were observed in the different food matrices. Cooked beef and egg patties were the foods that resulted in higher spore resistance. This is due to the reduced water activity of these foods (Rajan, Ahn, Balasubramaniam, & Yousef, 2006; Rajan, Pandrangi, Balasubramaniam, & Yousef, 2006). More recently, Xu, Janahar, Park, Balasubramaniam, and Yousef (2021) verified that not only reduced a_w can decrease *B. cereus* spores inactivation but also acidic condition enhances the spore lethality during combined pressure-thermal treatment.

5.CONCLUSION

One important fact in this study is the synergetic effect of combined mild heat and high pressure which can dramatically enhance inactivation level. When spores were treated with pressure-thermal process, the increase of two parameters, temperature and time, enhanced spore inactivation, pointing to the influence of these two factors on spore inactivation. Pressure-thermal treatment resulted in spore inactivation to some degree (3 log reduction). Therefore, complete efficacy and higher levels of spore elimination might be achieved by the combination of approved effective preservatives in this study, like reduced pH (pH 3 and 4) and nisin (25 µg /ml) with pressure-thermal technology (hurdle technology).

Spores were heated in LB medium and D-values of 1.9 minutes at 90 °C and 7.1 minutes at 80 °C were obtained. Increasing the temperature during the experiment resulted in more spore inactivation, emphasizing the significance of temperature in spore inactivation. During the heating experiment, inactivation was not accompanied by increasing the time of heating at both temperatures (80 and 90 °C). Adding Tween may enhance the correctness of the curve.

Spores were also added to minced meat and minced fish and heat treated. The highest log reduction of ≈3.5 log was obtained at 95 °C in both food matrices. When spore log reductions were compared in LB and food, spore lethality was reduced in food compared to LB medium indicating the protective effect of food content on spore heat resistance. The reason for the lower log reduction in food products can be assumed that spore inactivation was greatly influenced by the intrinsic factors (fat and a_w) of the food products.

The growth experiment of *B. cereus* at different preservatives showed very similar results for vegetative cells and spores. Based on these results, nisin (25 µg /ml) and reduced pH (3 and 4) were the most effective inhibitors, and the OD did not increase during the experiment period in the presence of these preservatives. In contrast, $NaNo_2$ in concentrations of 62.5 to 500 ppm as well as lower concentrations of nisin (7.5 and 15 µg/ml) was unable to affect the growth. pH 5 increased the TTD to 15-18 hours. This research clearly illustrates that with increasing the concentration of NaCl and KCl from 1 to 4%, the TTD was longer. However, the effectiveness of preservatives was tested at 25 °C, and pH 7. But it also raises the question of whether decreased temperature or pH can boost their antimicrobial effect.

This study contributes to future works concerning the conditions of spore production and the modification of spore counting. Since information concerning challenges in these

subjects is limited. The presence of spore clumps was a big challenge in this work. So, adding the low concentration of Tween 20 (0.1%) to the washing solution is recommended since, using Tween 20 takes advantage of more individual spores without affecting the heat resistance of spores based on this study results.

5.1.Future works

There are several aspects in this project that require further study both in pressure-thermal treatment and preservatives:

- Expand the testing with different concentrations of NaCl, KCl and $NaNO_2$ and nisin or test the antimicrobial effect of other types of preservatives.
- Synergistic effect of preservatives is also promising and needs further investigation.
- Investigate the synergistic effect of preservatives with lower temperature or lower pH (hurdle technology).
- Investigate the effect of pressure-thermal processing in combination with preservatives and low pH (hurdle technology).
- Examine the effect of pressure-thermal processing with higher temperatures or pressures for longer times.
- Study the effect of pressure-thermal processing with other food matrices.

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Appendices

Appendix 1

All media used in this thesis were autoclaved at 121 °C for 15 minutes and stored at 4 °C. Below are an overview of ingredients, recipes, and preparation.

Recipe for LB medium

Reagent	Amount
Nacl	10 g
Tryptone	10 g
Yeast extract	5 g
Distilled water	1000 ml

Note: LB agar was made by adding 15 g agar into the LB medium.

Recipe for 2× Schaeffer´s-glucose (SG) medium

Reagent	Amount
Difco nutrient broth	16 g
KCl	2 g
Mg SO_4.7H2O	0.5 g
Distilled water	1 L

Note: pH of the medium was adjusted to 7.0 and autoclaved. Then the following components were separately sterilized by Millipore 0.2 mm filters and added to the cooled (approximately 55 °C) autoclaved medium.

Reagent	Amount
1 M $Ca(NO_3)_2$	1 ml
0.1 M $MnCl_2.4H_2O$	1 ml
1 mM $FeSO_4$	1 ml
50 % (w/v) glucose	2 ml

Note: SG agar was made by adding 17 g agar into the SG medium.

Recipe for 1% Peptone water

Reagent	Amount
NaCl	8.5 g
Bactopeptone	1 g
Distilled water	1 litre

Appendix 2

Counting bacterial cells with Thoma cell counting chamber

The frame of the counting chamber contains a large central square (with a 1 mm^2 which can be seen in its entirety with the 10x objective. This large central square is divided into 16 medium squares (with the 40x objective the medium squares can see completely), each with 25 small squares inside.

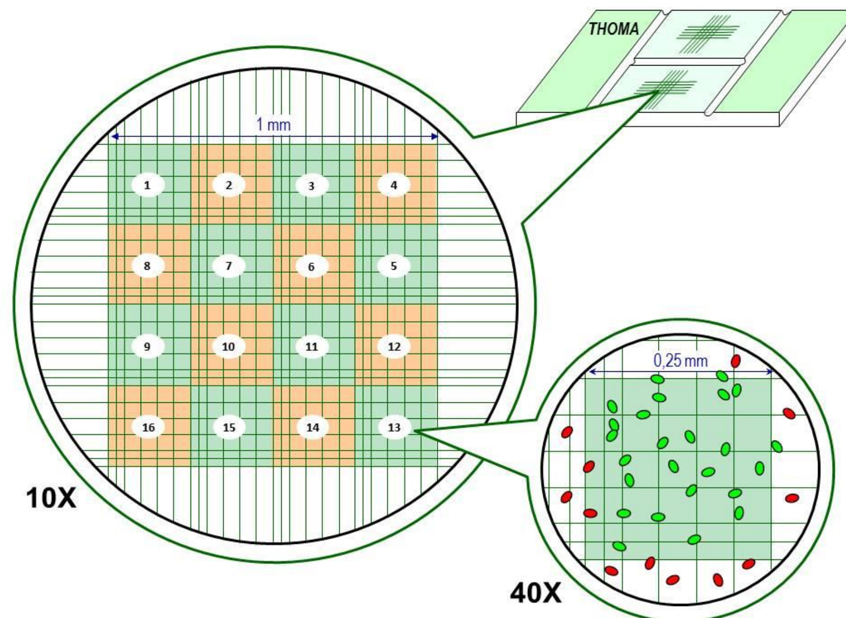

Figure. *Thoma cell counting chamber.*

When we put the sample under the coverslip, the cell suspension reaches a height of 0.1 mm. Taking these data into account, and considering one of the large squares, the volume will be:

$$1 \text{ x } 1 \text{ x } 0{,}1 = 0{,}1 \text{ mm}^3 = 10^{-4} \text{ ml}$$

With the 10 x objective of the microscope the counting area must be located. To count the cells the microscope must be switched to 40 x objective. All the cells in the 16 medium squares must be counted according to the following criteria:

All the cells within each medium square and those that are over the top and right sides of the square (even when they are partially out) are counted. Following this approach, in the figure the cells in green will be counted, but not the cells in red.

If we have counted N cells in one of the large squares, the concentration of our sample will be:

$$N \times 10^4 \text{ (cell/ml)}$$

When prior to counting we concentrated or diluted the initial sample, the count can be calculated by multiplying with dilution factor (f):

$$N \times 10^4 \times f \text{ (cell/ml)}$$