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MASTER'S THESIS

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Abstract

This master thesis is a part of the projects Salt Process and KonSpor (control of spore-forming bacteria in food with medium and long durability) and financed by the foundation "NORCONSERV". This study is an integrated part of these projects where different techniques have been applied and documented to know how spores of pathogenic bacteria can be killed and how to develop new safe foods when reduced amount of salt is used during processing. This study has a special focus on thermal inactivation of spores by capillary tube methods and the effect of salt concentration on spore inactivation during heat treatment. Autoclave was also done by single and double heat treatment (Tyndallization) for further documentation of food product processing. As a target for the heat treatment, proteolytic *Clostridium botulinum* was selected for this study. The laboratory is not certified for working with *C. botulinum*. *Clostridium sporogenes* was selected as a surrogate of *C. botulinum*. *C. sporogenes* is a non-pathogenic spore forming bacteria often regarded as more heat resistant than *C. botulinum*.

The objective of this study is to determine the effect of salt concentrations used in foods on inactivation kinetics of *C. sporogenes* strain CCUG 7489 and compare the heat resistance among different strains of *C. sporogenes* (PA 3679, CCUG 24143 and CCUG 7489) by using classical heat treatment in capillary tubes. The second objective is to study the *C. sporogenes* spore inactivation using heat by autoclave on meat product.

In the first experimental design, three strains of *C. sporogenes* (PA 3679, CCUG 7489 and CCUG 24143) were used for heat treatment in capillary tube to determine the heat resistance of the spores. Capillary tube experiment was also used to document the effect in the survival of spores (CCUG 7489) with various salt concentrations. The second experimental design was done by autoclave to study the effect of single and double heat treatment and the inactivation process of spores in a minced meat 'model-product'.

With the capillary tube method, D-values were calculated to determine the inactivation kinetics of *C. sporogenes*. This method follows the linear first order kinetics and all three strains were studied to identify the most heat stable strains at 90 °C. PA 3679 was found less heat resistant among them. The D-values were calculated at 85 °C for strain CCUG 24143 (since no survival was found at 90 °C) and at 85 °C and 90 °C for CCUG 7489 to select for the salt experiment.

Different salt concentrations (0%, 3% and 6% NaCl w/v) were used to study the difference in killing of the spores during heat treatment.

The thermal inactivation for *C. sporogenes* by autoclave was done to study the difference between single and double heat treatment. It was also an objective to study the inoculated food product (minced meat) by thermal inactivation process. The inactivation in meat products was slower compared to the obtained D-values from the capillary tubes. Single heat treatment at 90 °C for 30 min reported in 1 log reduction of spores. After double heat treatment at 80 °C for 60 min and 90 °C for 30 min with holding time at 25 °C for 75 min in between, 1.3 log reductions were found. However, no difference was observed for spore reduction between single and double heat treatment.

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Acknowledgments

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1. Introduction

Van Ermengem (1897) was the first researcher who documented that botulism can be caused by consuming toxic foods and he isolated a microorganism from canned meat product (Frank J Erbguth, 2004). Although he named the organism *Bacillus botulinus*, the organism was strictly anaerobic and spore forming so it was later named under the genus *Clostridium* whereas *Bacillus* was replaced to aerobic spore-forming rods (Bengtson, 1924). Then *Clostridium botulinum* was one of the most studied pathogenic organisms when established by Prévot (1953). *C. botulinum* produces botulinum neurotoxin which is the cause of botulism in human and animals. The control of botulism is a challenge. Toxin produced by *C. botulinum* is known as 'the poisonous poison' which is capable of causing death to human in doses as small as 0.05-0.1µg (Kumar et al., 2013).

There are four different types of botulism i.e., food botulism, child botulism, wound botulism and adult botulism. *C. botulinum* are divided into four groups (I-IV) and among those; group I (proteolytic and mesophilic) and group II (non-proteolytic and psychrotrophic) are responsible to cause botulism in human. Seven types (A-G) of serologically distinct toxins are found which are responsible for different types of botulism (Hambleton, 1992). In this master's thesis, group I (proteolytic) was focused for inactivation and pasteurization experiments.

Proteolytic group I *C. botulinum* is more heat resistant than group II non-proteolytic. As the group I are mesophilic and do not grow below 10 °C (Ohye & Scott, 1953; Stringer et al., 2013), refrigerated food preservation can overcome the risk of proteolytic botulism. On the other hand group II (non-proteolytic) strains are psychrotrophic, so anaerobic packaging and low temperature will favor *C. botulinum* germination and toxin production. As the use of salt and other preservatives in some foods are limited, refrigeration is the only reliable condition regarding microbial safety. Growth and inhibition of *C. botulinum* (type B, E and F) in packaged foods require exact information about heat resistance of the spores. Using low temperature for short time is focused to achieve acceptable texture and sensory quality, for instance, consumers want food products with fresh color and good sensory characteristics. Food processing at high temperature and long time can give a safe meal but at the same time cause reduced flavor and dry structure. A lower temperature is preferred for fish products; however, this could reduce the shelf life. On the other hand, processing temperature of 60-75 °C cannot kill most of the vegetative

microorganisms and spores whereas, relatively high thermal treatment (>90 °C for 10 min) of these products may lead to survival of the spore forming bacteria and the condition (vacuumed-packed) is favorable for facultative and obligate anaerobes including *Bacillus* and *Clostridium* (Nissen, Rosnes, Brendehaug, & Kleiberg, 2002). Clostridia can spread from different contaminated food products; e.g., raw meat, cured meat and vacuum-packed. In Europe, *C. botulinum* type E was more common whereas primarily type A, B and C cause problems in North America (Table 1.1).

Table 1.1 Incidence of *C. botulinum* spores in meat and meat products (Hauschild and Doyle 1989)

Product	Origin	% Positive sample	Types identified
Raw meat	Europe	36	E
Raw meat	North America	<1	C
Cured meat	North America	2	A, B
Raw pork	United Kingdom	0	A
Vacuum-packed	United Kingdom	4	A, B
Bacon	United Kingdom	5	A, B
Cured meat	Canada	2	A
Liver sausage	Canada	2	A

According to ECDC (European Centre for Disease Prevention and Control) botulism cases was slightly higher (8%) in 2007 than 2006 (ECDC, 2010). Botulism cases in different countries in Europe are given in Table 1.2.

Table 1.2 Reports of botulism in different countries by ECDC (ECDC, 2010). A: Aggregated data report; C: Case based report; U: Unspecified

Country	Report confirmed	Type cases	Confirmed cases	Notification rate per 100 000 population
Austria	C	0	0	0.0
Belgium	U	0	0	0.0
Bulgaria	A	1	0	0.0
Cyprus	U	0	0	0.0
Denmark	U	0	0	0.0
Estonia	U	0	0	0.0
Finland	U	0	0	0.0
France	C	11	10	< 0.1
Germany	C	9	9	< 0.1
Greece	C	1	1	< 0.1
Hungary	C	5	5	< 0.1
Ireland	U	0	0	0.0
Italy	C	16	16	< 0.1
Latvia	U	0	0	0.0
Lithuania	A	4	4	0.12
Luxembourg	U	0	0	0.0
Malta	U	0	0	0.0
Netherlands	C	1	1	< 0.1
Poland	C	49	24	< 0.1
Portugal	C	10	10	< 0.1
Romania	C	38	31	0.14
Slovakia	U	0	0	0.0
Slovenia	U	0	0	0.0
Spain	C	4	4	< 0.1
Sweden	U	0	0	0.0
United Kingdom	C	22	14	< 0.1
EU total		171	129	0.03
Iceland	U	0	0	0.0
Norway	U	0	0	0.0
Total		171	129	0.03

Aim of study: The purpose of this work was to observe the inactivation of proteolytic spores (*C. sporogenes* as a surrogate) in different types of media including deionized distilled water, different salt (NaCl) concentrations (0%, 3% and 6% w/v). Documentation of inactivation in pasteurized foods with extended shelf life was another objective for this research work. *C. sporogenes* (CCUG 7489) was inoculated in a meat product and autoclaved using two different heat processing designs (single and double heat treatment) to differentiate the survival characteristics of the spores.

2. Theory

2.1 *Clostridium botulinum*

Clostridium botulinum belongs to the genus *Clostridium*. The five main species responsible for disease in humans are *C. botulinum, C. difficile, C. perfringens, C. tetani and C. sordellii* (Hambleton, 1992).

C. botulinum is a Gram-positive, rod-shaped, anaerobic, spore-forming and motile bacterium with the ability to produce the neurotoxin botulinum (M. W. Peck, 2009). Bacterial cells are found in single, 0.3-0.7 x 3.4-7.5 µm, motile with peritrichous flagellae (Roberts, Tompkin, & Baird-Parker, 1996). *C. botulinum* spores are normally found in soil and very easy to contaminate in non-processed food products (Costa, Dekker, Beumer, Rombouts, & Jongen, 1999; Smith & Young, 1980).

The biochemical properties separate *C. botulinum* into proteolytic and non-proteolytic strains. The neurotoxins (A-G) are produced from various organisms and differentiated serologically. Bacteria that produce proteolytic toxins can digest meat and casein. It can coagulate egg albumin while the non-proteolytic does not have this ability. According to Bergey's Manual (Cato, George, & Finegold, 1986), neurotoxigenic organisms that are designated as *C. botulinum* are divided into four groups which are listed in Table 2.1.

Table 2.1 Characteristics of organism capable of producing botulinum neurotoxin (Cato et al., 1986).

Factors	Group I	Group II	Group III	Group IV
Toxin types	A, B, F	B, E, F	C, D	G
Proteolysis	+	_	_	+
Optimal growth temperature	35-40 °C	18-25 °C	40 °C	37 °C
Minimum growth temperature	10+ °C	3.3 °C	15 °C	Not specified
Spore heat resistance (temp/D-value)	121 °C /0.21	80 °C /0.6-1.25	104 °C /0.1-0.9	104 °C /0.8-1.12
Apathogenic model organism	*C. sporogenes*	No specific organism	*C. novyi*	*C. subterminale*

The growth factors of *C. botulinum* according to Graham, Mason, Maxwell, and Peck (1997) are illustrated in Table 2.2.

Table 2.2 Growth characteristics and thermal inactivation values for spores of proteolytic and nonproteolytic *C. botulinum* (Graham et al., 1997)

Toxin type	Minimum (GT °C)	Maximum (GT °C)	Optimum (GT °C)	pH minimum for growth	a_w Minimum for growth	D values (min)
A (p)	10	50	35-40	4.6	0.94	D_{110}=2.27-2.89
B (p)	10	50	35-40	4.6	0.94	D_{110}=1.34-1.37
B (np)	3.3	45	35-40	5.0	0.97	$D_{82.2}$=1.49-73.61
E (np)	4.0	45	28-30	5.0	0.97	D_{80}=0.8
F (p)	10	50	35-40	4.8	0.94	D_{110}=1.47-1.82
F (np)	3.3	45	28-30	5.0	0.97	$D_{82.2}$=0.25-0.84

GT = growth temperature; p = proteolytic; np = nonproteolytic

The minimum growth factors as well as other environmental conditions (pH and a_w etc.) reported with slightly differences according to different researchers. For example nonproteolytic (group II) toxin type B and E can form less heat-resistant spores (Lindström, Kiviniemi, & Korkeala, 2006) and can grow at lower temperature (3.3 °C) (Solomon, Kautter, & Lynt, 1982). According to Stringer et al. (2013) the minimum growth temperature for group II can be 2.5-3.0 °C. Proteolytic (group I) type A and B strains of *C. botulinum* can produce more heat resistant spores (J. Smelt & Haas, 1978). Proteolytic type *Clostridium* A and B can grow at minimum pH 4.6 with lower a_w between 0.93-0.94 (McClure, Cole, & Smelt, 1994) and at minimum growth temperature of 10-12 °C (Stringer et al., 2013). Studies have shown that a_w of 0.94 corresponds to a salt (brine) concentration of approximately 10% (Hauschild & Doyle, 1989). The optimum growth temperature for *Clostridium botulinum* (Group I and II) is between 30 °C and 37 °C (Stringer et al., 2013). Studies have shown that some strains of both *C. botulinum* type A and B can germinate and grow in the range between 10 °C and 47.5 °C (Ohye & Scott, 1953).

Endospore formation of *Clostridium*

Sporulation is a response to environmental stress. The mechanism of sporulation (Figure 2.1) in *Bacillus* and *Clostridium* appear to be identical by several studies (Bahl & Dürre, 2008; Mayer, 1986; Piggot & Losick, 2002). During sporulation, the cytoplasmic membrane starts engulfing the forespore (a structure of bacteria in which the endospore develops) so that the segregated chromosome is trapped inside. Then the forespore is surrounded by two cytoplasmic membranes (inner and outer forespore membrane). Two layers of peptidoglycan, the cortex (mixture of protein) and a germ cell wall are formed between these two cytoplasmic membranes (Driks, Setlow, & Setlow, 2000). A spore coat consisting of several protein layers is formed outside of the outer forespore membrane.

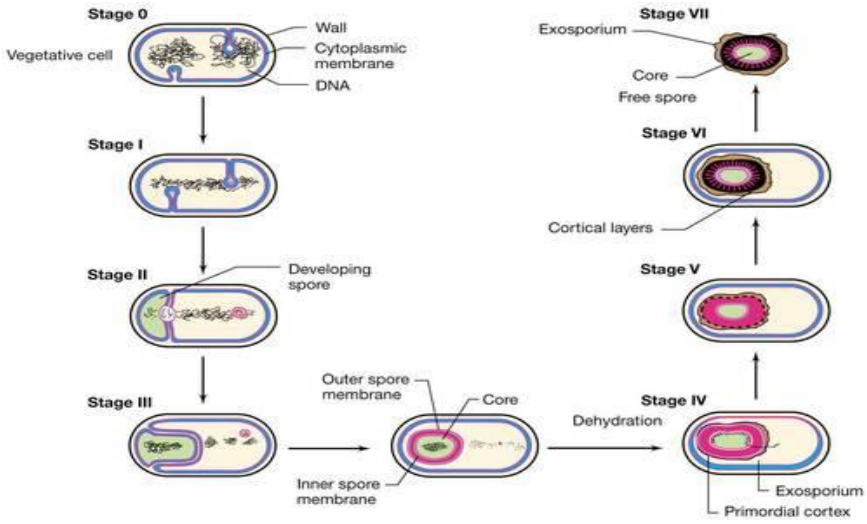

Figure 2.1 Cycle of spore formation. (https://www.tumblr.com/search/spore%20formation)

2.2 Factors affecting survival and growth

C. botulinum needs specific physical and chemical conditions for growth and survival. These factors are divided into intrinsic and extrinsic characteristics. Intrinsic factors are pH, salt concentration, fat content and a_w, whereas extrinsic factors include relative humidity and storage temperature. All these factors may have impact on growth and survival of bacteria.

2.2.1 Atmosphere

C. botulinum is strictly anaerobic and cannot grow in the presence of oxygen. A High growth rate was found at 100% CO_2 and lower at less CO_2 concentrations (Gibson et al., 2000). Studies have shown that toxin production by *Clostridium* in food was higher when it was initially packaged with 15-30% CO_2, whereas, toxin production was delayed with 45-75% CO_2 in combination with pH (5.5 and 6.5) and different NaCl concentration (0.5-2.5%) concentration (Lambert, Smith, & Dodds, 1991). Lövenklev et al. (2004), reported that growth and toxin production by nonproteolytic strains will increase if replacing the air in the headspace by CO_2 and can result in a hazard rather than protection. It has been shown that some *Clostridium* spp. can produce H_2 which inhibits the growth and the removal of H_2 favored the growth of *Clostridium* (Chung, 1976).

2.2.2 Temperature

Growth temperature

According to the type variance, both mesophilic and psychrotrophic *Clostridium* spp. are found. Several studies have shown that psychrotrophic *C. botulinum* can grow within a broad temperature range from 2.5 to 48 °C (Solomon et al., 1982; Stringer et al., 2013), with optimal growth levels between 35 °C to 37 °C (Ohye & Scott, 1953). Growth is decreased above 47.5 °C and ultimately no growth is found at 50 °C (Ohye & Scott, 1953). Ohye and Scott (1953), also reported that spores can germinate at 37 °C after 6 hr incubation. Heat activation can increase the range of incubation temperature for nonproteolytic *C. botulinum* type E and germination can occur at the highest level (40 °C). However, no germination was detected at 50 °C and above by Plowman and Peck (2002).

Inactivation temperature

According to J.-H. Mah, Kang, and Tang (2008), the Inactivation temperature for proteolytic surrogate *C. sporogenes* strain PA 3679 are reported to be 34 min at 90 °C while the z is 13 °C. Nygaard and Lie (2011), have found the D-value at 85 °C for *C. sporogenestic* (ATCC 19404) in phosphate buffer pH 7.0 was to be 253 min. This data shows that inactivation of spores may vary according to wide range of time-temperature profile.

2.2.3 pH

C. botulinum cannot germinate and grow under pH 4.8 (Wong, Young-Perkins, & Merson, 1988), and, for industrial processing; pH 4.6 (a ‘safety factor’) has been preferred as border line for high-acid and low-acid food (Code of Federal Regulation 1987, Title 21, part 114). However, a different statement has been found for the pH span of the minimum and optimal growth of Clostridia. The pH span for growth of nonproteolytic *C. botulinum* is 4.7 to 7 (Cameron, Leonard, & Barrett, 1980), and, by Plowman and Peck (2002), the optimum pH range is 5.5-8.0. However, spores of *C. botulinum* were found to germinate even at pH 4.2 in aqueous suspensions of soya protein and at pH 4.3 in skimmed milk (J. Smelt, Raatjes, Crowther, & Verrips, 1982). Other studies also agree that *C. botulinum* can grow and produce toxin below pH 4.6 if the food contains more than 3% soya or milk protein (Raatjes & Smelt, 1979).

2.2.4 Water activity (a_w)

Growth and inhibition of *C. botulinum* in certain a_w is closely connected to the NaCl content of that environment. Investigations have shown that inhibition of *C. botulinum* increases as the a_w decreases (Baird-Parker & Freame, 1967). The minimum a_w for proteolytic type A and B is 0.94 whereas for nonproteolytic type B, E and F is 0.97 (Graham et al., 1997). The optimal a_w for growth and survival of *C. botulinum* vegetative cells is 0.97 and the lower limit is 0.93 to 0.95 for toxin production (McClure et al., 1994). However, previous studies by Baird-Parker and Freame (1967), reported that toxin can be produced at pH $\leq$ 4.75 when a_w was $\geq$ 0.970 and at pH $\leq$ 5.75 when a_w was 0.960.

2.3 Risk of Group II *Clostridium botulinum* related to food processing

It is now well established that uncontrolled food supply and homemade storage foods are among the main potential sources for botulism. Contamination of food with *C. botulinum* may happen while the products are produced in the area or environment where the presence of spores is possible. So is it easy to contaminate by the spores during food processing.

There is an increased consumer demand for fresher foods which are minimally processed with low heat treatment. The consumption of refrigerated foods of extended durability (REPFED) is increasing worldwide; these foods are processed with maximum temperature reaching 65-95 °C. This temperature can eliminate vegetative cells but does not destroy the bacterial spores like Clostridia (Hyytiä-Trees et al., 2000; Eija Hyytiä, Hielm, Mokkila, Kinnunen, & Korkeala, 1999).

Heat treatment for REPFED foods are followed by rapid cooling and chilled at 1-8 °C, thus microbial safety mainly relies on refrigerated storage temperature. Many REPFED foods are packaged with modified atmosphere (MA) to ensure anaerobic conditions which also favor the growth of psychrotrophic spore forming bacteria (Del Torre, Della Corte, & Stecchini, 2001).

Many factors increase the risk of nonproteolytic (group II) *Clostridium* in REPFED food which includes (a) raw material that can contain spores; (b) insufficient heat treatment time to inactivate spores; (c) Non-proteolytic Clostridia spores can grow at chilled temperature (3 °C) (Graham et al., 1997; Stringer et al., 2013) and (d) limited use of salt and other preservatives in REPFED foods.

Contamination of *C. botulinum* in fish was studied worldwide and has been documented regular positive samples of the proteolytic type A, B and F and nonproteolytic type B and E (Fach et al., 2002; E. Hyytiä, Hielm, & Korkeala, 1998). These studies reported that the type E was found in the most botulism cases which were related to consumption of fishing product.
A survey is given in Table-2.3 indicates the presence of different *C. botulinum* types in North American fish.

Raw fruit and vegetables are very vulnerable to spore-forming pathogens (*C. perfringens, C. botulinum* and *B. cereus*) (Nguyen-The, 2012) and can cause a hazard in a healthy kitchen. Contamination can be due to the processing steps of fertilizing and cultivation (Hauschild & Dodds, 1993). Botulinum type A toxin was identified in south-east and north-eastern France during early September 2011 from incriminated olive paste (Pingeon et al., 2011). The reason behind that was incorrect sterilization during processing in the industry. In Finland, two persons were intoxicated in 2011 by *C. botulinum* type B after having eaten olive and almonds (Jalava et al., 2011).

Table-2.3 Incidence of *C. botulinum* spores in prepared fish in North America (Reviewed by Hauschild & Dodds, 1993)

Product	Origin	% Positive samples	Types identified
(Whitefish chubs)	Great Lakes		
Eviscerated		13	E, C
In brine tank		20	B, C, E
Brined, Washed		14	E
Fresh smoked		1	B, E
Haddock fillets	Atlantic	24	E
Frozen flounder Vacuum-packed	Atlantic	10	E
Frozen packed fish	Canada	<1	A, B, E
Filleted Cod	New York City	<1	E
Salmon	Washington	8	E
Salmon	Alaska	100	A

2.4 *C. sporogenes* as surrogate for *C. botulinum*

C. sporogenes belongs to the genus *Clostridia* and has been discussed as a good surrogate for nontoxigenic version of proteolytic *C. botulinum* (Collins & East, 1998). According to Bradbury et al. (2012), *C. sporogenes* strain PA 3679 (CCUG 7489, ATCC 24143, ATCC 9755) was first isolated from spoiled canned corn in 1927 and later designated as putrefactive anaerobe. It is a good thermal processing surrogate for *C. botulinum* (Janelle L. Brown, Nai Tran-Dinh, & Belinda Chapman, 2012; McClung, 1937) and has same morphology. *C. sporogenes* was used as a test organism in this thesis because of its nontoxigenicity and its spores can exceed the heat resistance of proteolytic *C. botulinum* (Townsend, Somers, Lamb, & Olson, 1956). Moreover, working with *C. botulinum*, health hazard is strongly concerned and high biosecurity is required (Janelle L Brown, Nai Tran-Dinh, & Belinda Chapman, 2012).

Phylogenic analysis showed that *C. sporogenes* and group I *C. botulinum* are very close to each other (Figure 2.2). Further studies reported that 16S rRNA indicates 99-100% nucleotide similarity between *C. sporogenes* strains and a number of proteolytic *C. botulinum* (Bradbury et

al., 2012). The optimal growth temperature (35-40 °C), pH and growth environment are all same as for *C. botulinum*.

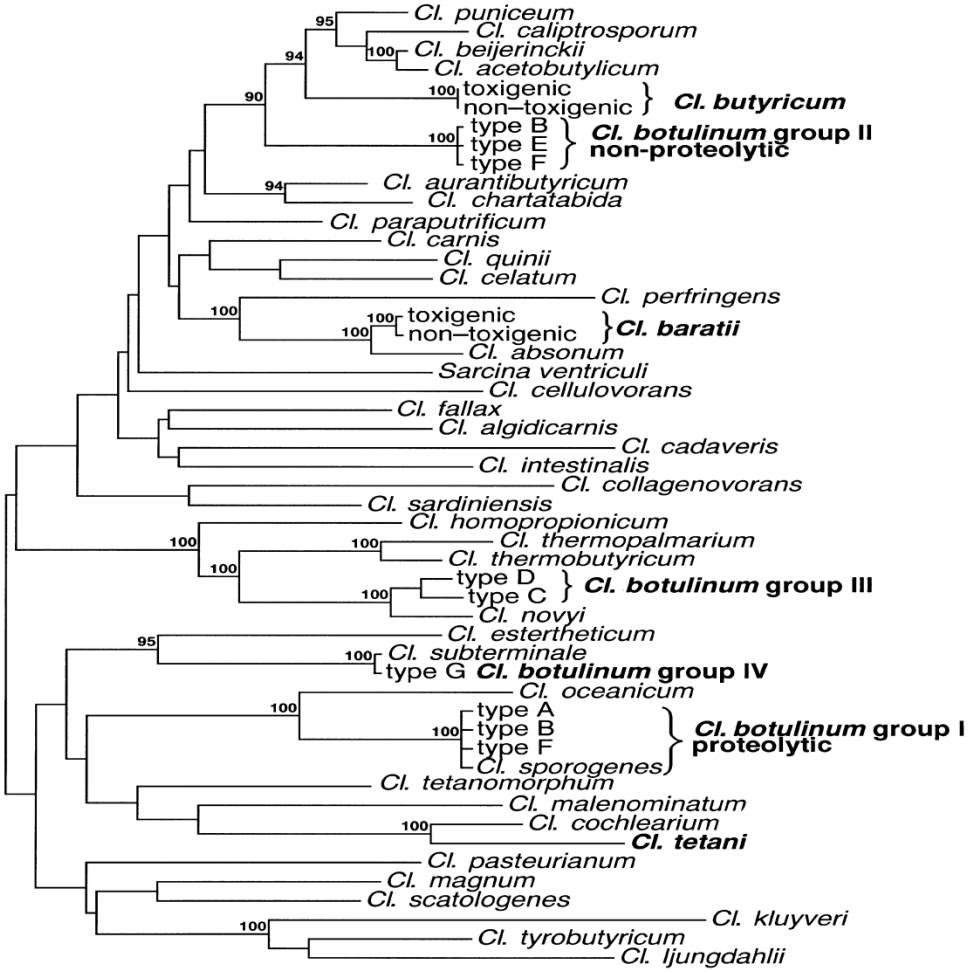

Figure 2.2 Interrelationship among the *Clostridium* spp. (Collins & East, 1998)

2.5 Clostridia inactivation

2.5.1 Factors affecting heat resistance of Clostridia

Several intrinsic and extrinsic factors have an effect on the inactivation kinetics of Clostridia. Temperature and incubation time in a growth media can influence the number of bacteria cells and their physiological status. As *C. sporogenes* grows at anaerobic conditions, it should be

controlled. Even at optimal growth temperature the physical condition of bacterial cells can be hampered by the longer incubation time.

As the spores are more heat resistant than the mother cells of *C. sporogenes*, the sporulation media is also an important factor. The physiological condition of spores in the sporulation media can also vary according to different pH, temperature, atmospheric condition and most importantly age of the spore. Incubation at suboptimal growth temperature for a longer time may have an effect on the spore's physical condition.

2.5.2 Effect of temperature in different phases and time

Optimal growth temperature for *C. sporogenes* and *Clostridia* in general is between 35 °C and 40 °C (Cato, George et al. 1986). The number of vegetative cells that have sporulated will increase with enlarged incubation time as there will be a lack of nutrition.

C. sporogenes cannot grow at 4 °C (Cato et al., 1986). However, the temperature and time period can have an effect on both viable counts. Studies found that suspensions stored at -20 °C and 4 °C has approximately 1-log10 reduction of viable vegetative cells compared to cells stored at 25 °C after 1 week (J. H. Mah, Kang, & Tang, 2009). The same study has reported that spore concentration have a statistically significant difference and about 1.5-$\log_{10}$ higher within 4 weeks if stored at 4 °C. The same study also suggests that heat resistance of spores stored at 4 °C for more than 4 weeks is significantly higher than storage at 25 °C.

2.5.3 Media

Medium plays an important role for growth, sporulation and regeneration of Clostridia. Sometimes one particular medium can be used for two or more cases depending on experimental design. All type of media may have influence on the growth of microorganism and different heat treatment medium may have an effect on their heat resistance. For example, growth medium and sporulation medium has a difference in the nutrition content, however, in this research RCM was used in both purpose.

Growth medium is used for growth and enrichment for microorganism. Growth media can be a liquid (broth) or solid depending on the experimental condition with added selective nutrition and atmospheric condition. In this work, growth medium has been reinforced clostridial medium

(RCM) broth at anaerobic conditions. Researchers found that *C. sporogenes* cells were deficient in glycine reductase activity when grown in rich medium containing pyruvate and proline (Venugopalan, 1980). The needs of essential nutrition in growth medium are also varying between different groups of *Clostridia*. High concentration of arginine and phenylalanine are required for group-I whereas for non-proteolytic group-II required glucose and other carbohydrate energy sources rather than arginine and phenylalanine (Whitmer & Johnson, 1988).

Sporulation medium is a medium where bacteria cells are able to start sporulation. Sporulation normally occurs in sporulation medium at suboptimal temperature (30 °C). Most of the sporulation media are composed of different nutrition levels which may have an impact on the heat resistance of the spores. For example, calcium is most important for developing of full heat resistant spore whereas manganese has higher contribution in HHP (heat and high pressure) resistant spore production (Lenz & Vogel, 2014). The lack of these elements in the sporulation medium may not give spores with higher heat resistant.

The medium that is used during heat inactivation of bacterial cells or spores are called heat treatment medium. The Medium used in the laboratory was distilled water and salt water (NaCl). The medium that is used for heat treatment has a major role on the inactivation kinetics. Different studies have shown different heat resistance at the same pH and temperature with different medium. Thus, for example D-value (min) was found to be 15.9 and 25.3 min in phosphate buffer and buffered pea puree respectively during heat treatment of *C. sporogenes* (PA 3679) at 110 °C with pH 7.0 (Cameron et al., 1980).

Depending on the microorganism, there is a wide range of selective and nonselective media that can be used in the laboratory for regeneration of heat treated spores. Selective media are especially designed for recovery and growth of damaged spores after heat treatment. For example, MCP (Oxoid CM0992) medium designed to identify *C. perfringens* that has a lack of β-D glucosidase activity. In MCP medium, *C. perfringens* does not cleave the chromogen, indoxyl β-D glucoside. Furthermore, it also ferments the sucrose in the medium, reducing the pH and finally bromocresol purple changes from purple to yellow. Other *Clostridium* spp. will appear either as purple colonies or blue/green colonies. Purple colonies indicate that the organism is not able to ferment glucose, whereas blue/green colonies indicate fermentative. On the other hand for a non-selective medium, RCM is a good example which was used in this thesis

experiments as a non-selective enrichment medium. RCM is favorable for growing various anaerobic and facultative microorganisms when incubated in proper condition (Mac Faddin, 1985). Yeast extract acts as a major stimulator for growth of *clostridium* cells in RCM. Pflug, Scheyer, Smith, and Kopelman (1979), has reported that Yeast extract agar with additives during plating gives the highest recovery for heated *C. sporogenes* spores.

2.5.4 Effect of pH during inactivation

It is now well known that organisms are not inherently dependent on genetic factors; moreover it depends on the environment during spore formation and actual real time heat treatment conditions. Several studies have shown that lowering the pH values can increase the inactivation of spores e.g., a significant reduction of about 3-$\log_{10}$ of spore germination was notable when pH was lowered from 7.0 to 4.0 for the same temperature/pressure combination (Stewart, Dunne, Sikes, & Hoover, 2000). Other investigators have found higher pH effect at lower rather than higher temperature (Cameron et al., 1980). At pH 4.0, 25 °C and 404 megapascals pressure, *C. sporogenes* were reduced by 2.5-$\log_{10.}$ However, it was reduced only <0.5-$\log_{10}$ spore at the same treatment with pH 7.0 (Stewart et al., 2000).

2.5.5 Kinetic parameters

Bacterial growth can be described by binary fission in which two identical cells are produced from one mother cell and regenerates cell components. To discuss microorganism growth by binary fission, it can be plotted as the logarithm of viable cells number versus time of incubation. Curves derived from the graph has four phases which can be named as (A) lag phase, (B) exponential phase, (C) stationary phase, (D) death phase (Figure 2.3). The kinetic parameters are the mathematical illustration of these distinct phases.

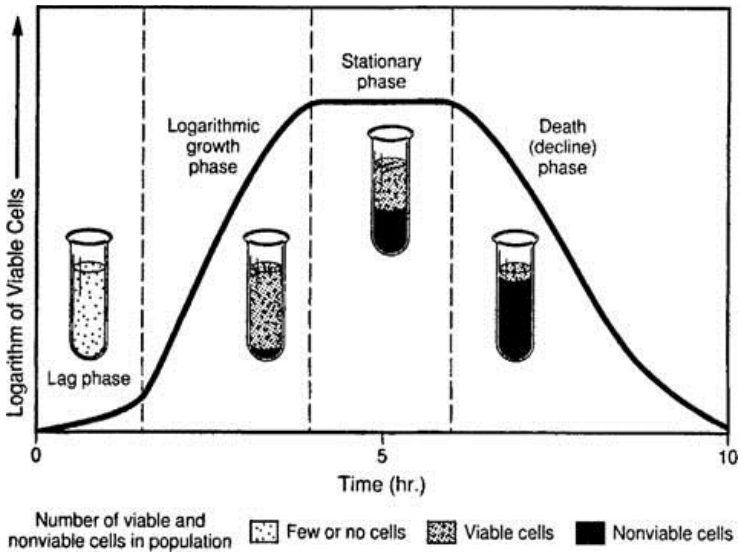

Figure 2.3 Microbial growth showing as log of viable cells against time in hr.

(A) Lag phase: Bacteria get a good condition in lag phase to adapt and before grow up they start to synthesize essential enzymes and RNA. This phase acts as a maturation phase for the bacteria life cycle where actually bacteria cells get bigger before dividing into two new cells. It is the phase of high biosynthesis where nutrients are necessary for a rapid growth of microorganism. Studies have shown that two elements can be determined the lag time: (i) total time required for the cells to adapt into a new environment and (ii) the rates at which those repairs are made (Robinson, Ocio, Kaloti, & Mackey, 1998).

B) Exponential phase: Exponential phase begins when a mature cell starts doubling into two. At this time, bacteria start to produce wastes or byproducts and the nutrition level of the growth medium falls rapidly. This is an interesting phase for biochemistry since many byproducts can be produced which could be for the next generation's fuel source. For example, H_2 is produced during exponential growth of *Clostridia* in a typical anaerobic process (Minton & Clarke, 1989). This reaction shifts from an H_2/acid production phase to a solvent production phase when the pH drops to pH 4.5 or below (Byung & Zeikus, 1985). Studies have shown that by decreasing pH and other biosynthesis during the exponential phase, hydrogen production is slowed whereas if the pH can be controlled in optimal level (pH 6-7), a high volume of hydrogen can be produced in the industrial scale (Khanal, Chen, Li, & Sung, 2004).

(C) Stationary phase: During stationary phase, the growth curve becomes horizontal which indicates the growth of bacteria has stopped. It occurs because the nutrition level declines and

production of other toxic products increases. In stationary phase it is possible for bacteria to grow up to 10^9 CFU/mL in a population. Moreover, bacteria (vegetative) of stationary phase are more heat resistant (Doyle, Mazzotta, Wang, Wiseman, & Scott, 2001).

(D) Death phase: When the number of viable cells decline due to a lack of proper nutrients and the accumulation of toxic components in the environment, the death phase begins. The death of microbial organism is not logarithmic like exponential phase. After this phase, microorganism will either survive or be inactivated or turn into dormant cells. After long time incubation, even at the same temperature, spore forming bacteria can start sporulation. In this experiment, death phase was selected to start the sporulation process.

Thermal inactivation

Inactivation kinetics normally follows log-linear kinetics which can be characterized by D and z-values. D-values (decimal reduction time) can be defined by the time required when 90% of the bacteria is killed at a certain temperature (T) and can be called 1-D. However, D-values are the primary method for modeling traditional first order kinetics of thermal inactivation. Studies found difficulties to discuss when survivor curves of *Clostridium* spp. are deviated from log-linearity (Anderson, McClure, Baird-Parker, & Cole, 1996).

In order to calculate D-values, inactivation rate is common to use first order kinetics based on the theory that all bacteria are equally heat resistant and sensitive to a specific heat and time. Equation (1) describes the killing inactivation of bacteria;

$$N = N_o e^{-kt} \qquad (1)$$

'N' represents the change in bacterial or spore concentration for each heat treatment, 'N_o' is the initial concentration of cells or spores, the rate constant for specific temperature is 'k' and 't' represents time.

In logarithm expression of equation (1) we get a simplified equation (2)

$$\log (N) = \log (N_o) - t/D \qquad (2)$$

We can plot the logarithm of surviving bacteria (CFU/mL) at time (min) and get a semi-logarithmic linear curve (Figure 2.4).

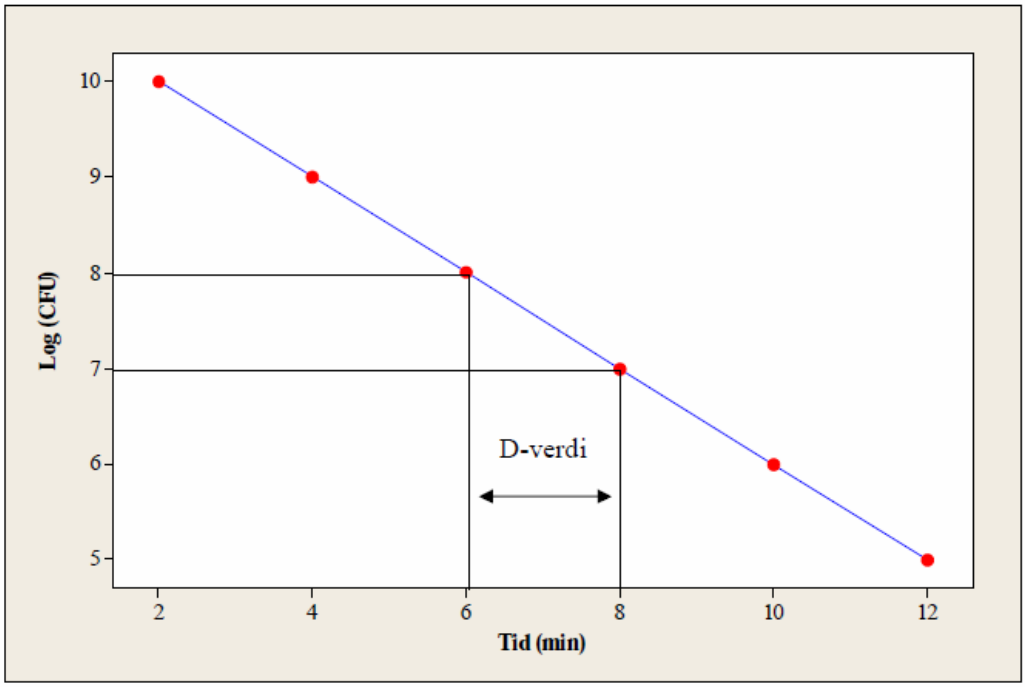

Figure 2.4 A basic inactivation kinetic model presenting D- value.

By using this curve slope, D- values can be calculated with the Equation (3).

$$D = -1/a \qquad (3)$$

Here 'a' indicates the slope of the line and 'D' for D-values in min or sec.

To obtain z-value from the slope, several D-values are needed to calculate for the corresponding temperature. Equation (4) is used for calculating z-value.

$$Z = (T_1-T_2) / (\log D_1 - \log D_2) \qquad (4)$$

Here, T = temperature and D = D-values. The temperature coefficient (z-value) can be defined as temperature (°C) needed to change the D-value according to logarithmic unit (Figure 2.5).

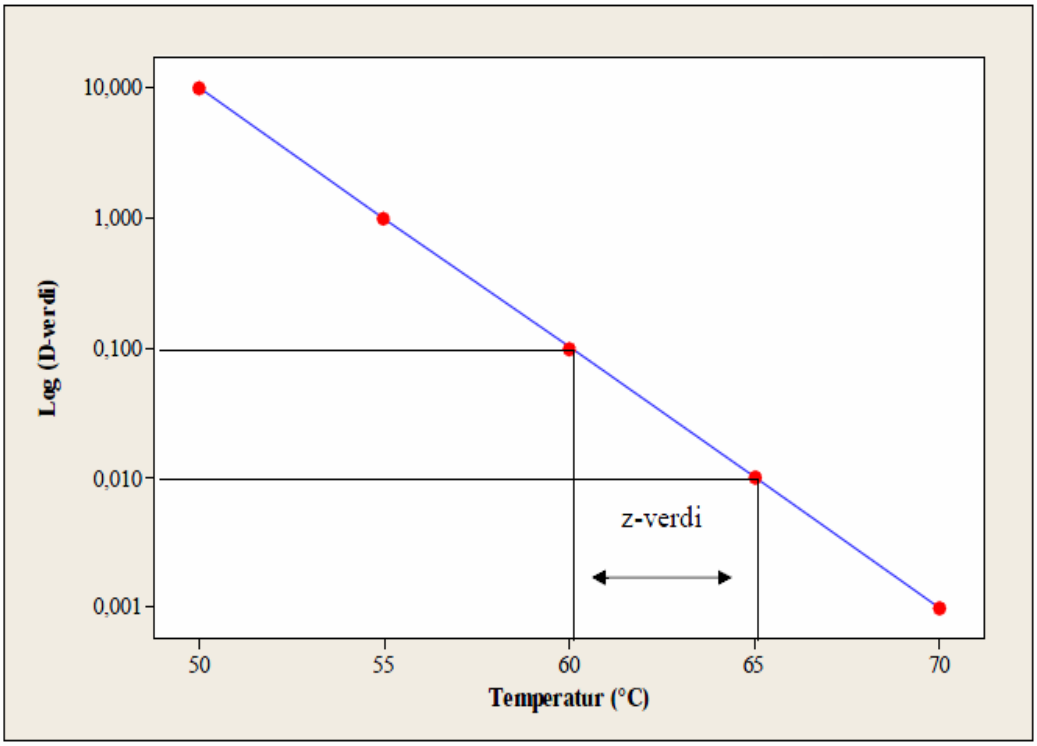

Figure 2.5 A basic inactivation kinetic model representing z-value.

Although mathematical expression of thermal inactivation kinetics and development of better software dates from the early 20th centuries, heat processing was established and patented by Appert in early 19th century (J. Smelt & Brul, 2014).

Microorganisms are more heat resistant in dry heat than wet heat. In food microbiology, wet heat resistance has been more emphasized because protein denaturation (Setlow & Setlow, 1998) and membrane damage (Coleman, Chen, Li, Cowan, & Setlow, 2007) plays an important role in this process.

Studies have shown different D-values using different medium for heat inactivation of *C. sporogenes* 3679 (Table 2.4) indicates that thermal inactivation varies in different food materials.

Table 2.4 $D_{121\,°C}$ and z-values for *C. sporogenes* strain PA 3679 suspended in phosphate buffer and selected foodstuffs

Media and foodstuffs	$D_{121\,°C}$ (min)	z-values (°C)	pH	references
Phosphate buffer	0.62	12	7	Luechapattanaporn et al. (2004)
Phosphate buffer	2.6	14	7	Cameron et al. (1980)
Phosphate buffer	1.2	10.5	7	Gaze (1990)
Phosphate buffer	1.1	11	5	Cameron et al. (1980)
Asparagus (puree)	1.48	9.28	5.88	Silla, Nuñez, Casado, and Rodrigo (1992)
Mushroom (extract)	1.5	9.59	6.65	Rodrigo, Martinez, Sanchez, Peris, and Safon (1993)
Potatoes (mashed)	0.61	10	6.30	Luechapattanaporn et al. (2004)
Water (distilled)	0.80	9.78	7	Stumbo, Murphy, and Cochran (1950)

Thermal inactivation of group II *C. botulinum* with different temperature has been examined in a range of media (Table 2.5). It has been found that variation in the inactivation media, fat content, protein, pH and nutrition concentration have a significant effect on heat resistance of group II *C. botulinum* (Lindström et al., 2006).

Table 2.5 Heat resistance of group II *C. botulinum* spores at low temperature in various media

Heating medium	Group II *C. botulinum* type	Temperature (°C)	D-value (min)	Reference
Phosphate buffer	B	90.0	0.4-8.3	J. P. P. M. Smelt (1980)
Phosphate buffer	E	90.0	12.6	M. Peck, Fairbairn, and Lund (1993)
Distilled water	E	90.6	5.0	Alderton, Chen, and Ito (1974)
Crab meat	B	90.6	8.2	Peterson et al. (1997)
Craw fish	E	90.0	2.5-3.1	de Pantoja (1986)

3. Materials and methods

3.1 Test organism

Three different strains of *C. sporogenes* strain PA 3679, CCUG 24143 and CCUG 7489 were purchased from Cultural Collection; University of Gothenburg (CCUG). The strains arrived as freeze dried powder in vacuumed glass vessels and stored at room temperature before use.

3.1.1 Storage procedure

Microbank® (Prolab Diagnostic) is a freezing procedure for storage of microorganisms. It is a vial that contains 25 sterile colored beads with cryopreservative (glycerol) (Figure-3.1).

Figure 3.1 Microbank® (Prolab Diagnostic) with inoculated strain.

C. sporogenes strain PA 3679, CCUG 24143 and CCUG 7489 was grown from freeze dried cultures in the laboratory of Nofima. The vials containing PA 3679, CCUG 24143 and CCUG 7489 were cut aseptically and a solution was prepared with adding 1 mL reinforced clostridial medium (RCM) and then inoculate into a 100 mL glass bottle containing 75 mL RCM and TSAYE Petri dishes. The incubation was performed under strict anaerobic conditions with 100% N_2/CO_2 headspace for glass bottles and anaerobic jar for Petri dishes. After storage at 37 °C for two days bacteria colonies were collected from the TSAYE Petri dishes with a sterile plastic loop and inoculated to the Microbank® tubes. Microbank® was shaken 4-6 times for proper penetration of the bacteria into the pores. All the tubes were stored at -70 °C. For further experiment four Microbank® tubes were prepared for each bacterial strain.

3.1.2 Sporulation

In the laboratory experiments, two different kinds of sporulation techniques were applied. For PA 3679 and CCUG 24143, a sporulation medium (SM) was prepared containing 3% trypticase, 1% peptone and 1% $(NH_4)_2SO_4$ (W.-W. Yang & Ponce, 2009). A 10% inoculum from RCM was transferred to 75 mL of sporulation medium (SM) containing 100% N_2/CO_2 headspace. The SM was then heat shocked at 80 °C for 30 min and incubated at suboptimal temperature at 30 °C while shaking at 155 rotations per min (rpm). Only PA 3679 was heat shocked before incubated at suboptimal (30 °C) temperature. Incubated samples were monitored by phase contrast microscopy (LEICA 1000) at 400 magnifications for spore production (phase bright spores) rate (W.-W. Yang & Ponce, 2009). Phase bright spores were seen bright at phase 2 contrast while vegetative cells were black (Figure-3.2). After 7-8 days incubation period at suboptimal

temperature (30 °C), about 90% spores (phase bright) were found. According to the methods described for *C. sporogenes* endospore suspension was then centrifuged at 12,850 g for 10 min at 4 °C (Yang & Ponce, 2009). After discarding the supernatants the endospores were washed with autoclaved MilliQ water and once re-suspended again with autoclaved MilliQ water (20 mL) and centrifuged again. Phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, pH 7.34) was used for re-suspending the pellets. PBS contains 500 µg/mL lysozyme (L7851-5G) was filtered with 0.2 µm filter before adding to the PBS. To release the spores from vegetative cells, ultra-sonicator (COLE PARMER) with 50/60 Hz was used for 8 min and incubated at 37 °C for two hr. This incubation helps to digest the vegetative cells. Free spores were washed with 20 mL of autoclaved MilliQ water and centrifuged at 2050 g for 20 min. To obtain a pure spore suspension without mother cells/cells debris, the suspension was washed and spores observed with phase contrast microscopy until less than 1% vegetative cells remained.

No special sporulation medium was used for CCUG 7489 (Figure-3.3). The strain CCUG 7489 was inoculated on RCM plates and incubated for 10 days at 37 °C followed by 7 days incubation at 30 °C. Colonies were then collected by sterile plastic loops and suspended into 15 mL falcon tubes containing 10 mL sterile water. All other procedures (washing, centrifugation etc.) remained the same for PA 3679 and CCUG 24143.

The concentration of the spores was determined by making a serial dilution of the original stock suspension and plated on RCM agar medium followed by incubation in anaerobic condition at 37 °C for 3 days. The colonies were counted and the spore concentration was confirmed for PA 3679 to about 10^7 spores/mL, CCUG 24143 was about 10^9 spores/mL whereas CCUG 7489 showed 10^8 spores/mL in suspension.

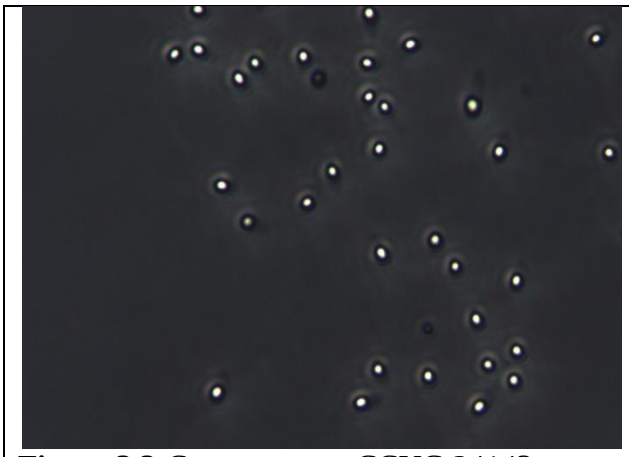

Figure 3.2 *C. sporogenes* CCUG 24143 spores under 400 magnification phase contrast microscope.

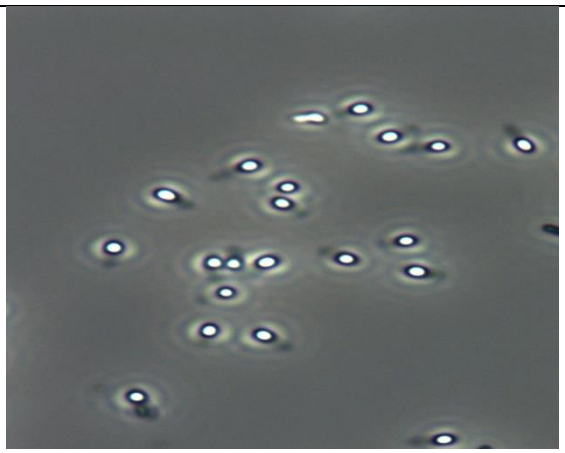

Figure 3.3 *C. sporogenes* CCUG 7489 spores under 400 magnification phase contrast microscope.

3.2 Isothermal heat treatment of *C. sporogenes* in capillary tubes

3.2.1 Capillary tube method with water

From the stock suspension (10^9spore/mL), 10^7 spores/mL concentrations were prepared and 100 µL was transferred using a pipette into a 200 µL volume of sterile capillary tube (Figure 3.4). The tubes were then sealed by a gas burner. To confirm that the tubes were airtight, they were placed vertically (no movement of liquid).

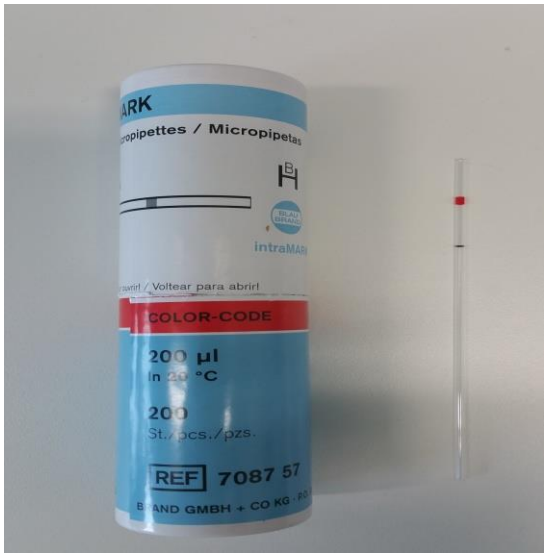

Figure 3.4 Capillary tube, color-code, ref708757

Specially prepared magnetic rails (Figure-3.5) were used on which capillary racks can be attached and the capillary tubes are easily removable during heat treatment. Each rack can hold three tubes at a time and can be tightened with adjustable wire if necessary. Heat treatment was done in the water bath at different temperatures for specific time and also at specific temperature for different time periods for both strains.

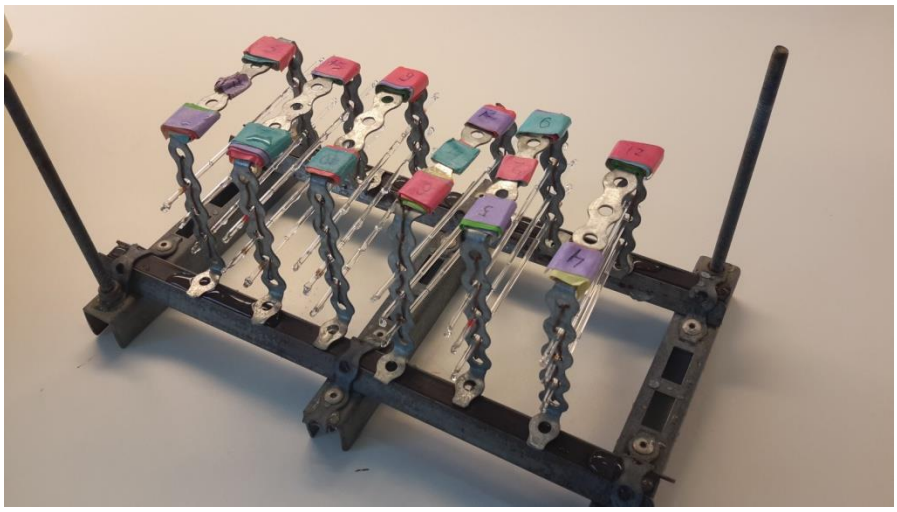

Figure 3.5 Capillary tubes inside the tube racks on the magnetic rail.

The capillary tubes were placed in the tube racks and transfered into the hot water very immediately after being placed on ice. After heat treatment for 2-12 min with 2 min interval of each parallel, the tubes were put on the ice again to cool down rapidly. The tubes were washed with 70% ethanol; air dried and cut at one end. The inoculated samples were then taken (Figure 3.6) by a sterile syringe to transfer into a 1.5 mL Eppendorf tube.

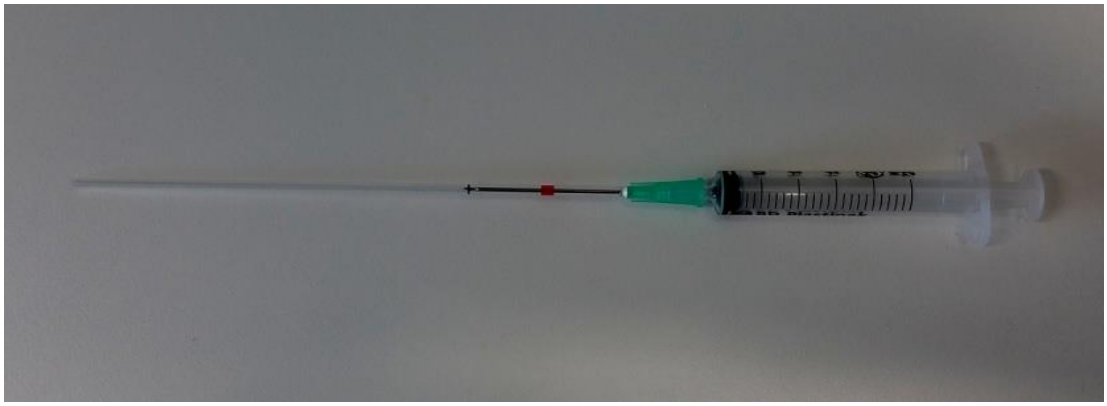

Figure 3.6 Collecting samples from capillary tube by sterile syringe.

Water bath (Lauda) was used for heat treatment experiment with capillary tubes. *C. sporogenes* strains PA 3679, CCUG 24143 and CCUG 7489 were heat treated with water medium at

different temperatures for different time periods. All the three strains were heat treated at 85 °C and 100 °C for 4 min. PA 3679 was first examined for the maximum lethal temperature for 4 min at 60 °C, 80 °C and 100 °C. Finally CCUG 7489 was selected for higher heat treatment (90 °C) with water and two different concentrations (3% and 6% w/v) of salt (NaCl) medium.

3.2.2 Heat treatment of *C. sporogenes* with different salt water concentration

C. sporogenes was heat treated with 3% and 6% w/v NaCl at 90 °C for different time periods. In this case CCUG 7489 was selected as it was found more heat resistant. 200 µL of stock solution was prepared with 3% and 6% w/v NaCl which had a concentration 10^8 spores/mL. A series of 10 fold dilution was prepared and inoculated 100 µL samples into capillary tubes. After heat treatment in water bath, they were placed into crushed ice water and then cut in one end prior to collect the samples by sterile syringe. The samples were then plated by EDDY JET spiral plater.

3.2.3 Heat treatment equipments for capillary tube

A water bath (Lauda) was used for capillary tube heat treatment (Figure 3.7). The temperature was adjusted to targeted heating level. The capillary tube racks were then put into hot water when the temperature stabled at the targeted label.

Figure 3.7 Water bath for capillary tube heat treatment

In addition to the digital displayed temperature on the water bath, an external thermometer was also used to monitor the temperature of the water (Figure 3.8). After finishing the heat treatment

the tubes were transferred immediately into ice water to cool down. Ice water stop further heating of the spores and takes short time to decrease the temperature.

Figure 3.8 Temperature detector

3.3. Isothermal heat treatment of *C. sporogenes* in meat product (minced farce) by using shaka autoclave.

3.3.1 Meat product sample preparation and inoculation

A minced meat 'model-product' of 1% and 2.5% salt concentration was transferred from a freezer (-20 °C) to refrigerator (0-4 °C) and thawed overnight. Both of these products were prepared for single and double heat treatment by an autoclave, Steriflow, France (Figure 3.6).

A Portion of minced meat (750 g) with 1% and 2.5% salt were placed into two different buckets. Before adding the bacterial sample, 180 g of each model product (1% and 2.5% salt) was transferred from the main product package into four cans for temperature control. There was a total of four cans, two (1% and 2.5%) of them for single and two (1% and 2.5%) for double heat treatment. Four more cans were filled with 180 g model product for examination of background flora from each salt concentration for a single and double heat treatment. For inoculation with spores, 4.5 mL of 10^8 CFU/mL stock solutions (CCUG 7489) were added into each bucket

containing 1% and 2.5% salted model product and mixed well to achieve homogeneity. The concentration of *C. sporogenes* was $6.0x10^5$spores/g. A total of four cans were filled with each salt concentration product with two of them being single and two double heat treatments. In total, there were eight cans with inoculated spores. Gelatin (0.83 g) was added into each can before sealing.

3.3.2 Autoclave heating

The autoclave heating program was designed according to the D-values obtained from capillary tube methods for the strain CCUG 7489 at 90 °C temperature. The temperature in an autoclave has a long come-up time compared to the capillary tubes, resulting in a non-isothermal heating. A shaka autoclave was used for this experiment while it was static during the experiment (Figure 3.9). To monitor and measure the temperature; a software, ValSuite Pro version 3.1.3.3 (Ellab AS, Denmark) was used. An experimental design was prepared to follow the autoclave procedure which explains the temperature range, heating time, cooling time and preheating time etc. The heating time at a specific temperature e.g., 90 °C was first measured when the temperature reached 90 °C. This experimental design for single and double heat treatment is given in Table 3.1 and 3.2.

Table 3.1 Schedule program for single heat treatment by autoclave

Period	°C	Bar	Min
Heat	70	0.5	1
Heat	90	0.8	2
Heat	90	0.8	30
Cooling	30	0.5	10
Cooling	20	0.2	15
End			

Table 3.2 Scheduled program for double heat treatment by autoclave

Heating process 1	**°C**	**Bar**	**Min**
Heat	80	0.5	2
Heat	80	0.8	60
Cooling	20	0.5	2
Cooling	20	0.2	8
Cooling	20	0.2	10
Holding time			
Heat	25	0.2	10
Cooling	25	0.2	65
Heating process 2			
Heat	70	0.5	1
Heat	90	0.8	2
Heat	90	0.8	30
Cooling	30	0.5	10
Cooling	20	0.2	15
End			

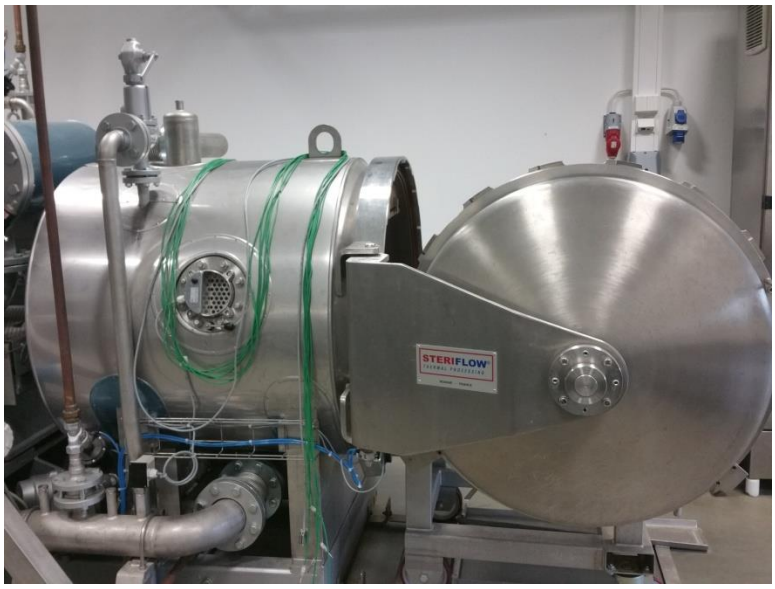

Figure 3.9 Steriflow autoclave.

For this experiment four EVal Flex thermocouples (type SSA-TS) were used to measure the temperature in the center of the cans and two thermocouples were measuring temperature in the

autoclave water (Figure 3.10a). The thermocouples were inserted into the filled cans which were selected for temperature monitoring (Figure 3.10b).

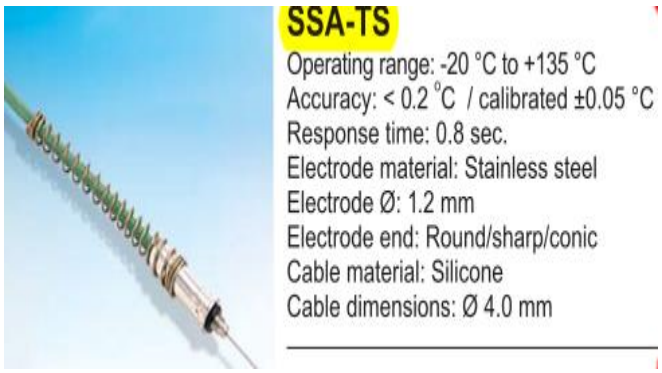

Figure 3.10a EVal Flex thermocouples (type SSA-TS)

Figure 3.10b Thermocouple with cans

The meat product was filled into aluminum cans which were 99/93.6x31.5 mm in dimension and 0.23 mm of thickness (Figure 3.11). It was confirmed that the internal food contact lacquer of the cans and lids were formulated in accordance with the requirements from the FDA in the USA (Food and Drug Administration, 21 CFR 175.300) and the European Regulation No. 1935/2004/EC. It was the latest generation of lacquers for the internal protection of aluminum cans which ensure the integrity and safety of the can and full compliance with the latest food contact legislation. No Bisphenol A (BPA) is used in the formulation of raw materials for the internal food contact lacquer.

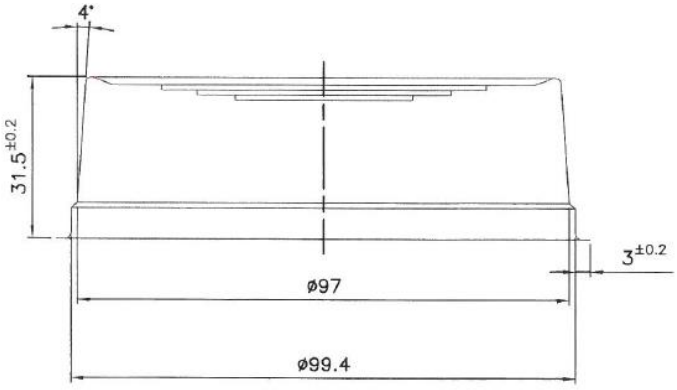

Figure 3.11 Physical parameters of the can. The maximum diameter is 99 mm on top and minimum 93.6 mm in bottom which gives a 4° angle.

All the cans were filled with 180 g of meat product and sealed (Figure 3.12a-b).

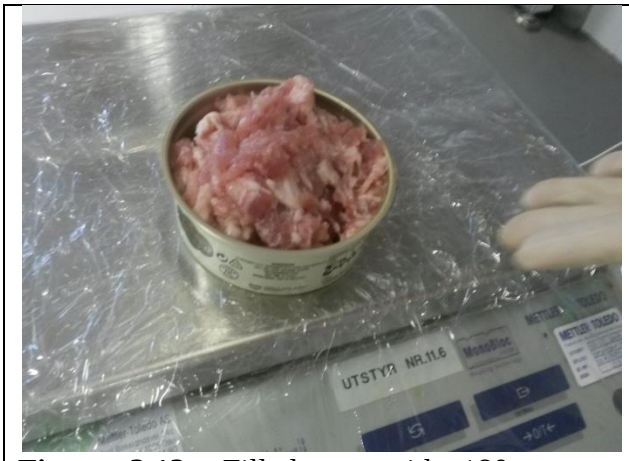

Figure 3.12a Filled can with 180 g meat product

Figure 3.12b Can was sealed with sealing machine

3.4 Calculation of results

After heat treatment in capillary tube and the autoclave, both RCM and MCP were used as recovery medium. Plate count agar (PCA) media was used to count the background flora.

3.4.1 Dilutions

After heat treatment in capillary tubes, autoclaved distilled water and salt water (when heat treatment media was salt water) were used for dilution in Eppendorf tubes. For dilution of meat product, buffered peptone water was used.

Dilution of *C. sporogenes* suspension for heat treatment in capillary tubes

A series of 10-fold dilution was prepared. After collecting the heat treated samples (100 µL) from the capillary tubes by sterile syringe, 50 µL samples were taken into an Eppendorf tube and 450 µL of sterile distilled water was added to make a 10-fold dilution. Water was used when heat treated media was water and salt water for salt media.

Dilution of meat product with and without *C. sporogenes* before and after heat treatment

To prepare a dilution, 25 g of farce (meat product) was taken into a stomacher bag from samples with and without spores. Both meat product samples are of 1% and 2.5% salt which are also before and after autoclaved. Distilled buffered peptone water was added until the total weight is 250 g. After stomacher for 4 min the filtrates were transferred into a 15 mL falcon tube. A series

of 10 fold dilution were prepared by taking 100 µL suspensions into Eppendorf tube prior to add 900 µL of buffered peptone water.

3.4.2 Colony counting

Spiral plating is a well-known method which is convenient to reduce the number of plates and is easily applicable to measure the concentration of the microorganisms. The objective is to spread the sample in a decreasing rate followed by Archimedes spiral. The spiral starts from the center of the plates followed by gradually decreasing to the edge. The volume of the sample is given by equation (5);

$$Vs = (DF)(A) \quad (5)$$

Here, DF indicates the number of microliter samples plated per square mm and the distance of near spiral is expressed by A.

For spiral inoculation, EDDY JET machine was used for counting the bacterial colonies. Bacterial cultures were plated for regenerating in MCP, RCM and PCA. Separate needles were used for different dilutions. Inoculation volume for each plate was 49.2 µL (D mode). Manual plating procedure was applied for strain CCUG 7489. For manual plating, 500 µL samples were inoculated by a sterile plastic loop. According to the experimental procedure no colonies were detected at 100 °C below 4.3 log CFU/mL which was selected as a detection limit.

3.4.3 Statistical method

The data are analyzed by linear regression using the built-in “parallel lines” macro in SigmaPlot 13. This macro uses Analysis of covariance (ANCOVA) to determine whether regression lines are parallel and subsequently if they have the same intercept. The assumption made by ANCOVA is called the homogeneity of regression slopes.

Alpha error levels for rejection of the null hypothesis were set at 0.05

4. Results

4.1 Inactivation of *C. sporogenes* by capillary tube methods

C. sporogenes strain PA 3679 was heat treated at different temperatures (60 °C, 80 °C and 100 °C) for 4 min. No surviving spores were found at 100 °C (below detection limit of log 4.3), a 1.3 log reduction was observed at 80 °C and no log reduction was found between 60 °C and 80 °C (Figure 4.1). An inactivation temperature of 85 °C was then selected.

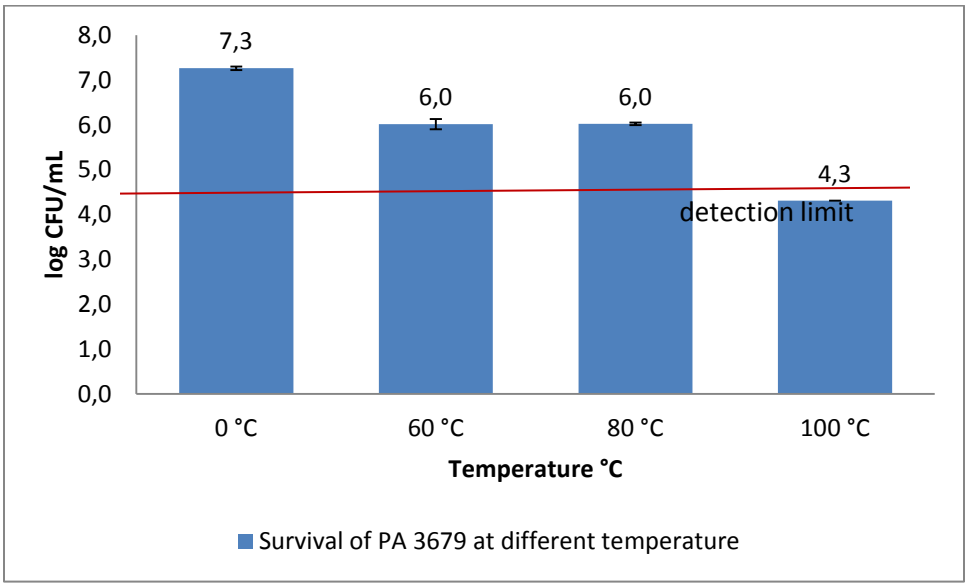

Figure 4.1 PA 3679 survival after heat treatment for 4 min at different temperature (°C). Red line indicates the detention limit.

C. sporogenes strains (PA 3679, CCUG 24143 and CCUG 7489) were examined for the maximum heat tolerance at 85 °C and 100 °C for 4 min to select a suitable strain for further experiments. CCUG 7489 was found to be the most resistant (Figure 4.2). After four min heat treatment at 85 °C, 1.43 log CFU/mL reduction was observed for CCUG 7489, whereas 1.94 and 3.02 log reduction was found for PA 3679 and CCUG 24143, respectively (Figure 4.2).

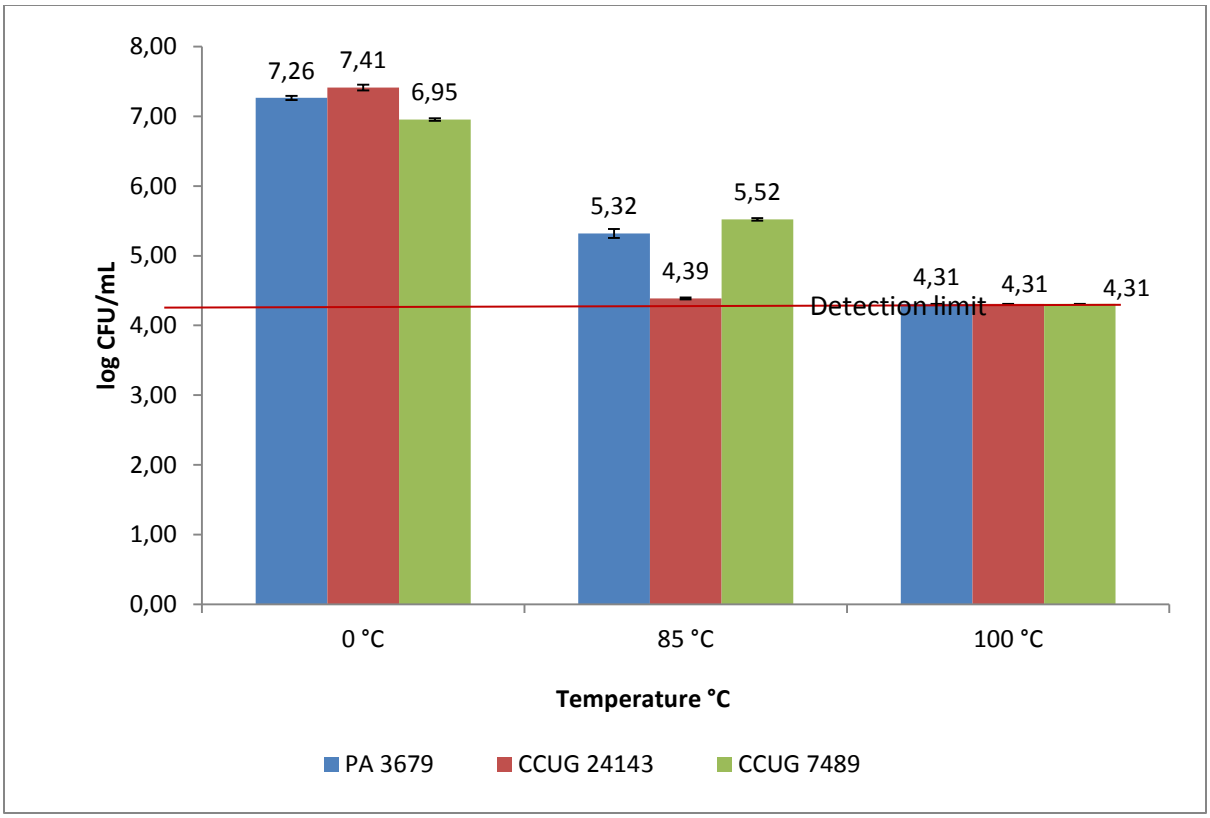

Figure 4.2 log CFU/mL of *C. sporogenes* at different temperature for 4 min heat treatment. Red line indicates the detection limit.

Four different experiments were done with CCUG 7489 at 90 °C when deionized water was the heat treatment medium. To examine the *C. sporogenes* lethality in salt medium, three different experiments were carried out for CCUG 7489 with 0% w/v, 3% w/v NaCl and one with 6% w/v NaCl. All the experiments had three parallels for each temperature and time which are represented as single dots in the graph (Figure 4.3, 4.4 and 4.5).

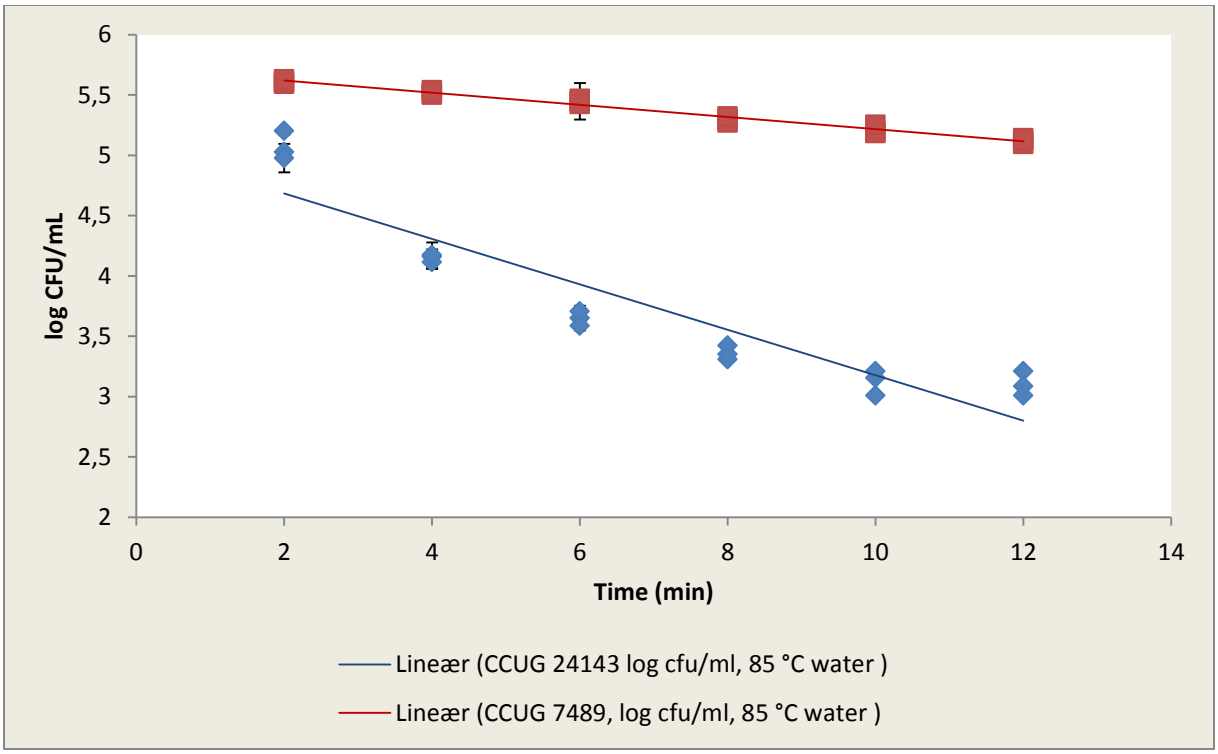

Figure 4.3 Survivors of *C. sporogenes* (CCUG 24143 and CCUG 7489) at specific heat treatment temperature (85 °C) as a function of time.

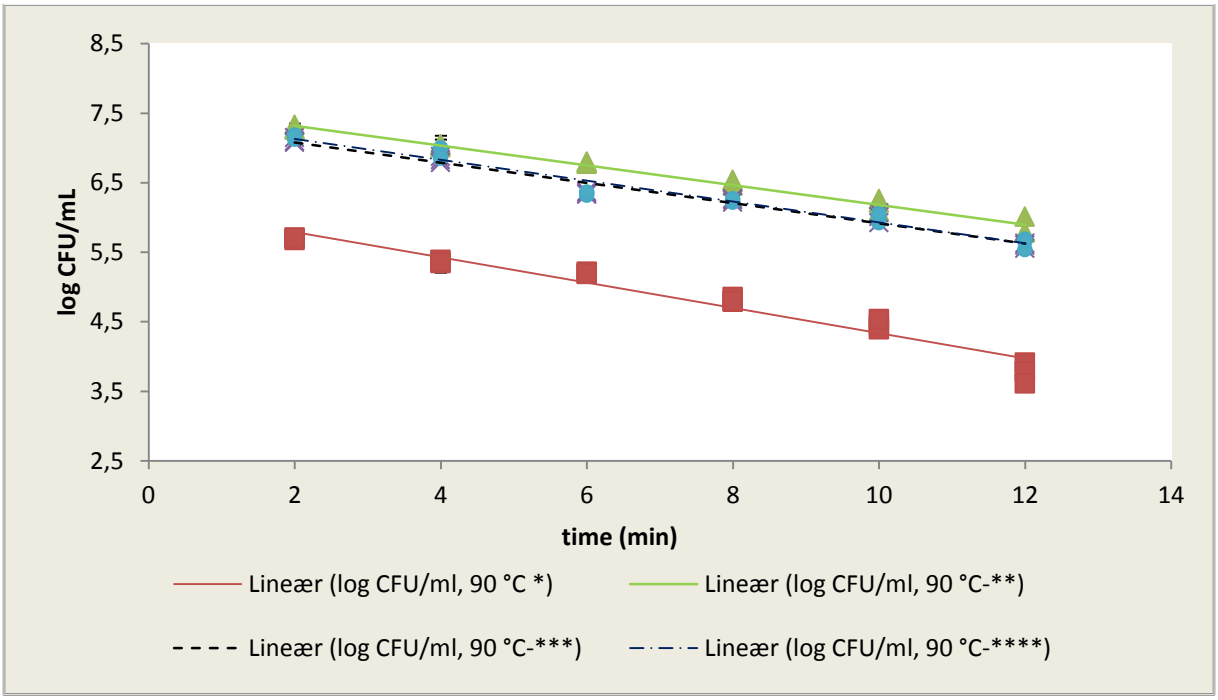

Figure 4.4 Survivors of *C. sporogenes* (CCUG 7489) at specific heat treatment in deionized distilled water as a function of time. * = Three replicates for each strain

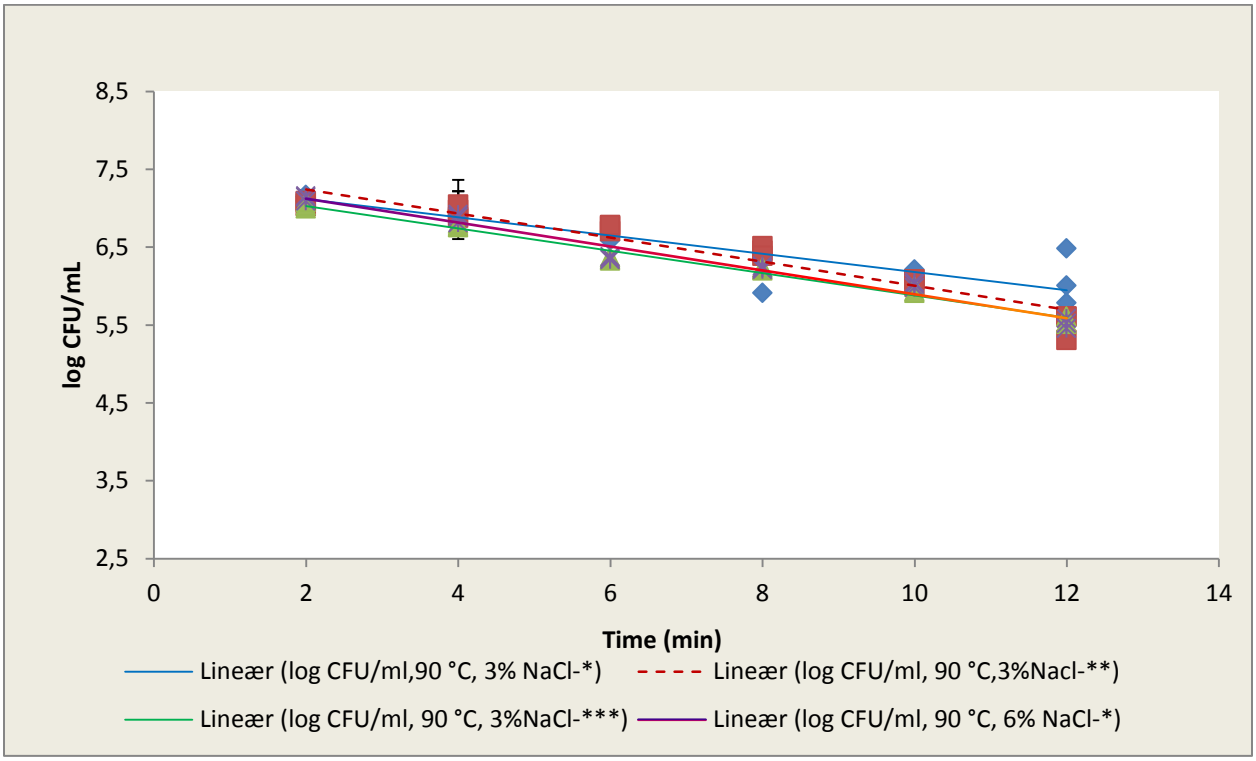

Figure 4.5 Surviving spores of *C. sporogenes* (CCUG 7489) after heat treatment in salt media as a function of time. *= represents different replicates

Table 4.1 shows the D-values (min) and regression coefficient (R^2) for CCUG 24143 and CCUG 7489 in water media after heat treatment. The D-values were calculated by using the equation (3) which was described in the theory section.

Table 4.1 D-values (min) in water medium and regression coefficient (R^2) of *C. sporogenes* strains in different temperature; S1 and S2 = experiment at the same date with water and salt

***C. sporogenes* strain**	**Temperature °C (water medium)**	**D-values (min)**		**R^2**
CCUG 24143	85	5.319		0.857
CCUG 7489	85	20		0.963
CCUG 7489	90	5.524	average	0.949
CCUG 7489	90	7.042 (S2)	6.532	0.983
CCUG 7489	90	6.896 (S1)		0.972
CCUG 7489	90	6.666		0.963

Heat treatments with salt media of different concentrations were done for *C. sporogenes* CCUG 7489. D-values (min) and regression coefficient (R^2) are given in table 4.2. D_{90}-values were calculated by the equation (3) which was described in the theory section.

Table 4.2 D_{90} values and regression coefficient (R^2) for CCUG 7489 in salt media; * = represents the number of replicates for each media; S1 and S2 = experiment at the same date with water and salt.

Salt media (NaCl) concentration % w/v	D_{90} value (min)	R^2
3*	8.547	0.815
3**	6.66 (S2)	0.92
3***	6.99 (S1)	0.98
6*	6.53	0.96

Heat treatment of *C. sporogenes* (CCUG 7489) with distilled water, 3% and 6% w/v NaCl was done at the same date to examine the survival. The objective of this experiment with the same age of the spores was to see the effect of salt media during regeneration after heat treatment. Figure 4.6 showing the survival in water and 3% w/v NaCl medium and the slopes show no significant difference (p = 0.83, NB: for statistical analysis the regression lines are not based on the means) between the medium for survival.

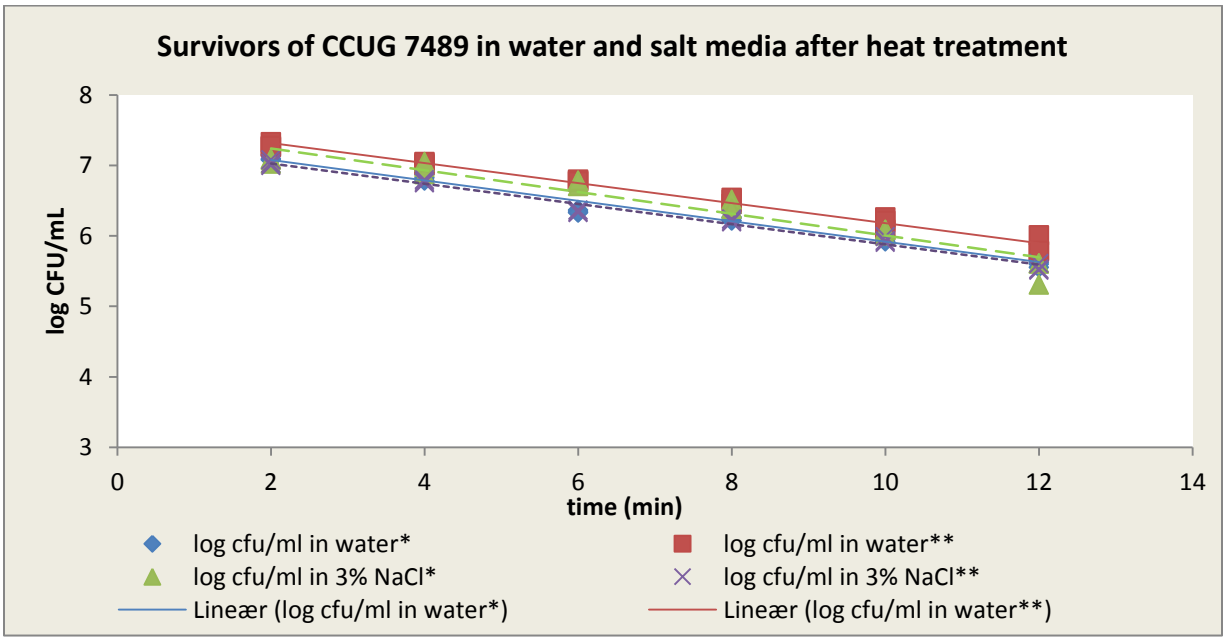

Figure 4.6 Survivors of *C. sporogenes* (CCUG 7489) after heat treatment in water and 3% w/v NaCl media as a function of time. * = represents the replicates.

The slopes for water and 6% w/v salt (NaCl) medium are not significantly different (p = 0.98). Figure 4.7 illustrates the survivals in water and 6% w/v NaCl media.

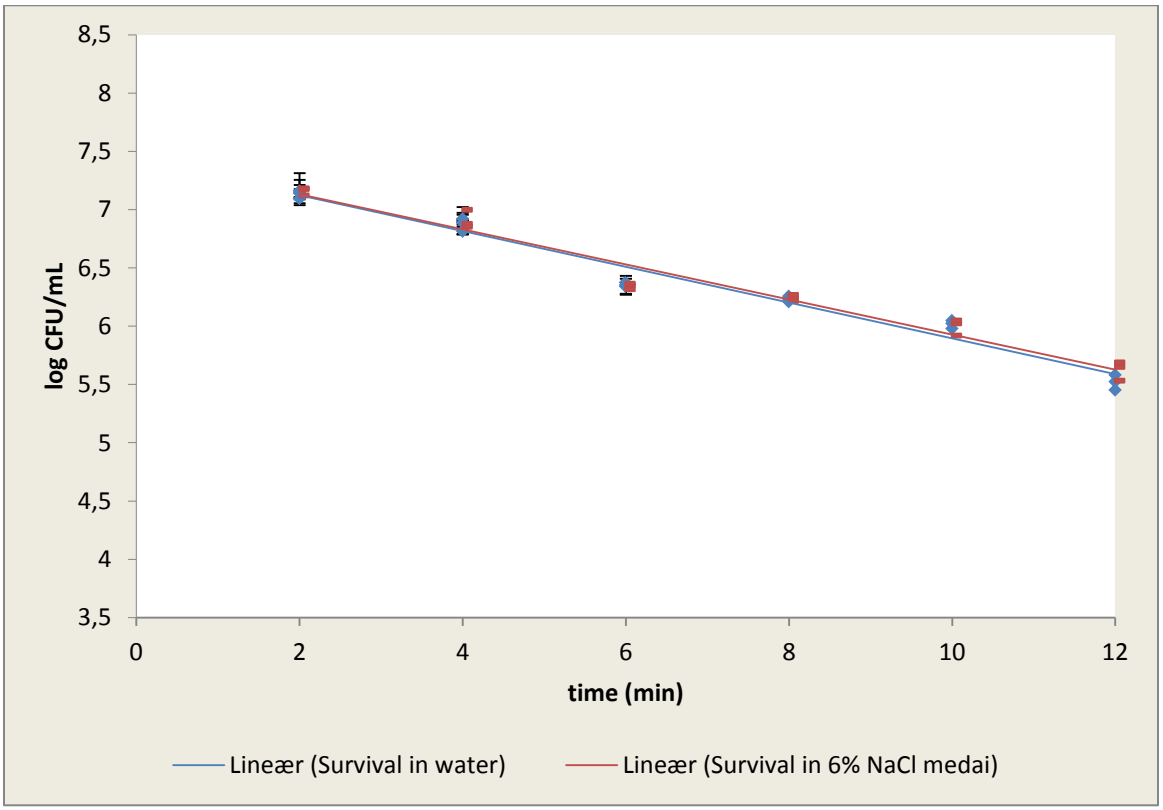

Figure 4.7 Survivors of *C. sporogenes* (CCUG 7489) after heat treatment in water and 6% w/v NaCl media as a function of time

4.2 Inactivation of *C. sporogenes* in meat product by autoclave

C. sporogenes (CCUG 7489) was used for wet heat pasteurization in meat product to examine the survivors after single and double heat treatment. The actual experimental program by autoclave for single heat treatment is given in Table 4.3.

Table 4.3 Actual program run for single heat treatment in the autoclave

Period	°C	bar	Min: sec
Heat	70	0.5	1
Heat	90	0.8	02:08
Heat	90	0.8	30
Cooling	30	0.5	10:01
Cooling	20	0.2	15
End			

The temperature curves were plotted in Microsoft Excel by measuring the temperature every 5 sec given by the autoclave. Figure 4.8 shows four different probes which were used to measure the core temperature of the cans as a function of time.

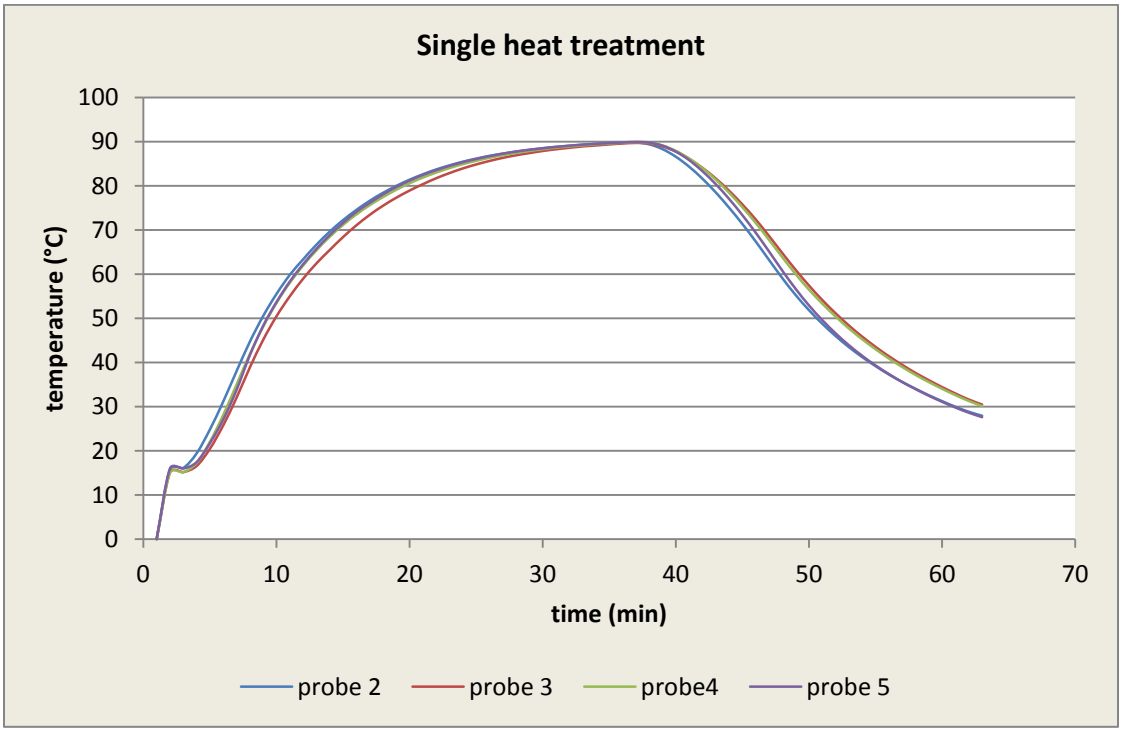

Figure 4.8 Temperature data in different time during single heat treatment

C. sporogenes (CCUG 7489) was also used for double heat treatment to examine the pasteurization effect on meat product. The first part of the double heat treatment was heating to 80 °C for 60 min, then the products were chilled to 25 °C for 75 min and heated a second time at 90 °C for 30 min. Table 4.4 illustrates the actual programs of autoclave.

Table 4.4 Actual double heat treatment program run by autoclave

Program run1	**°C**	**bar**	**Min: sec**
Heat	80	0.5	02:16
Heat	80	0.8	60:00
Cooling	20	0.5	06:46
Cooling	20	0.2	08:00
Cooling	20	0.2	10:00
Holding time	**°C**	**bar**	**Min: sec**
Heat	25	0.2	10
Cooling	25	0.2	65
Program run 2	**°C**	**bar**	**Min: sec**
Heat	70	0.5	01:30
Heat	90	0.8	02:04
Heat	90	0.8	30
Cooling	30	0.5	10:03
Cooling	20	0.2	15
End			

The temperature curves were plotted by taking the temperature data at every 5 sec. The graph shows different probes for the core temperature of the cans (Figure 4.9).

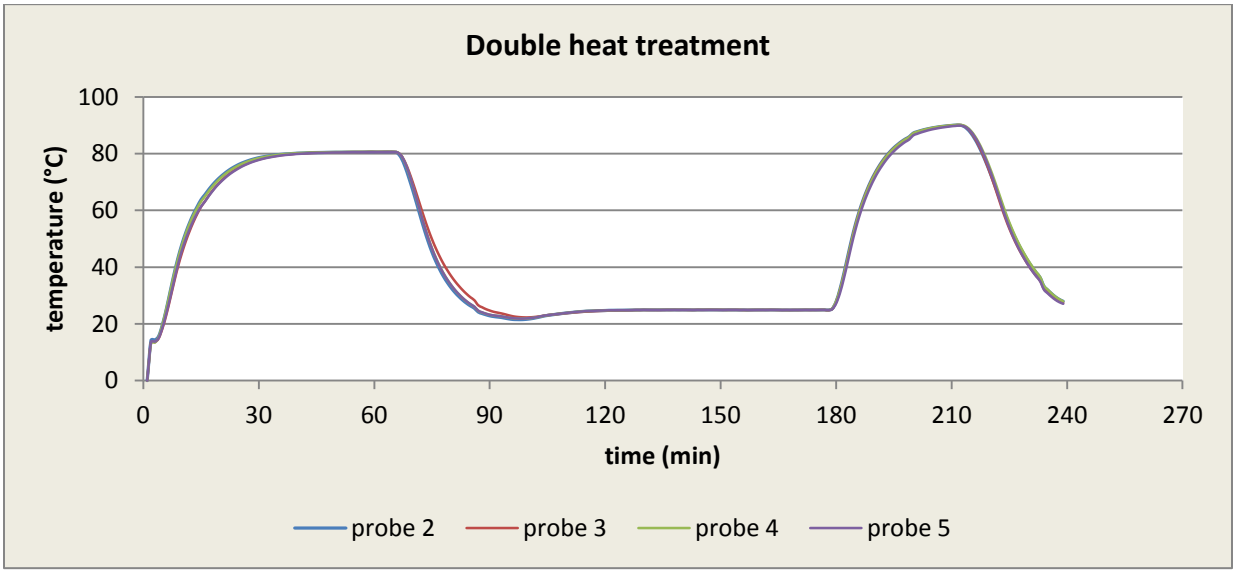

Figure 4.9 Temperature data in different time during double heat treatment

After single and double heat treatment, the samples were diluted and plated by using EDDY JET at D mode (49.2 µL). For each sample and dilution two parallels were used and average values were calculated. All the plates were incubated at 37 °C for 3 days. The colonies were counted and the difference between single and double heat treatment were plotted (Figure 4.10). This graph also represents both MCP and RCM media for regeneration. Results showing in graph that

there are no significant difference (p=0.03) in single and double heat treatment. However, the numbers were higher in MCP (6.2) than RCM (5.5) before heat treatment.

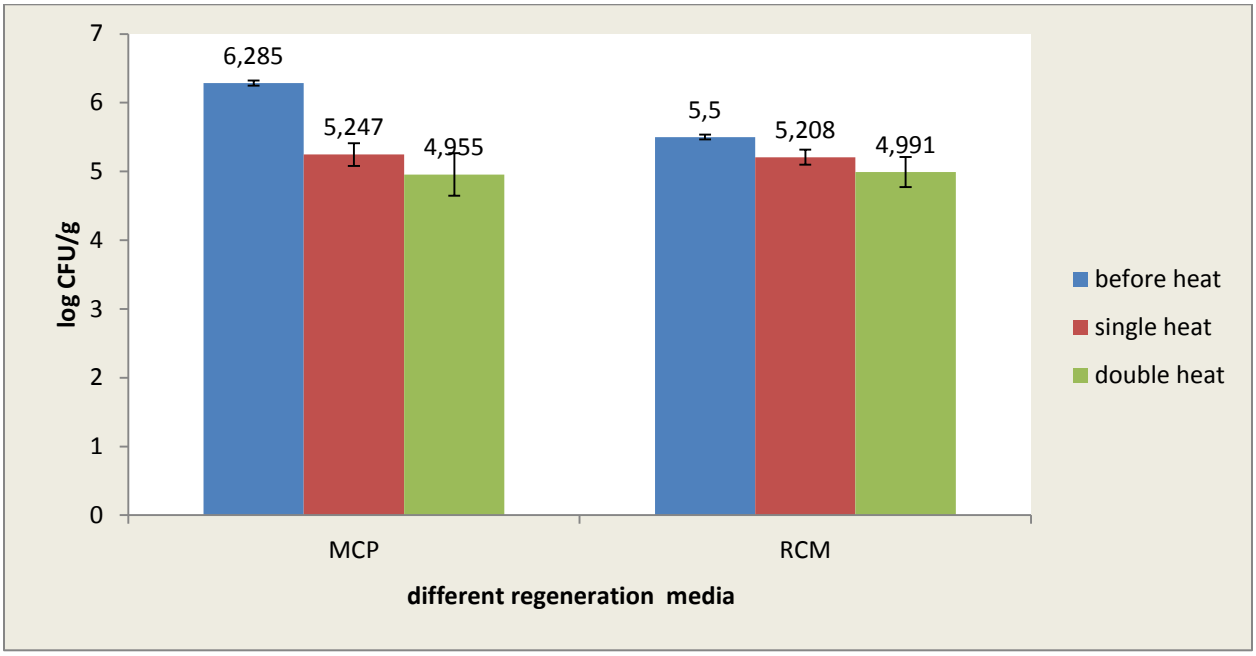

Figure 4.10 Regeneration of CCUG 7489 after different heat treatment by autoclave

5. Discussion

5.1 Thermal inactivation by capillary tube method

Special care was taken to confirm the capillary tubes being fully emerged during heating and they were quickly transferred from hot water to ice cold water. The data were analyzed by the log-linear approach. Care was taken to avoid the spore clumping, uneven heating and post contamination. Survival curve was found difficult to fit the linear log line when primary concentration of spores without heat treatment was used, and it also decreased the regression coefficient (R^2). The same problem was faced by Anderson et al. (1996), where survivor curves were deviated from log-linearity at low temperature (<121 °C) and found difficulties to describe by using traditional first order kinetics. The curves were better understood by using vitalistic approach and log-logistic transformation.

The most heat resistant spores were found from strain CCUG 7489 at 90 °C whereas PA 3679 was the least heat tolerant. In this experiment, no colonies were detected below 4.3 log CFU/mL

which was set as a detection limit. No spores of PA 3679 and CCUG 24143 were able to regenerate after heating at 100 °C in water for 4 min. However PA 3679 was heat activated at 80 °C for 15 min (at original chosen method was 30 min) before starting the sporulation process at suboptimal temperature (30 °C), whereas the other strains (CCUG 24143 and CCUG 7489) did not pass through this process and it did not have any negative impact on sporulation. However, heat shock in bacterial cells can increase the heat resistance ability of the spores (Setlow & Setlow, 1998), it was not the objective to strengthen the spores by other means. Heat shock is selective for spores which are highly heat resistant. Weaker spores which are more heat labile may lose its protective proteins and some enzymes that are important to regenerate (Setlow & Setlow, 1998). Another reason for PA 3679 and CCUG 24143 do not tolerate the heat above 90 °C for 4 min which can be discussed by the theory that these strains were not come into any contact of external nutrition (amino acids, vitamins) for growth, during sporulation and regeneration except RCM. None of the strains experienced any modified growth media, sporulation media or regeneration media. As *C. sporogenes* is a spore forming bacteria, it can trigger sporulation in any minimum environmental stress condition. However, the composition of regeneration media can lead to major difference in their physiology. Studies reported that PA 3679 has high heat tolerance at 121 °C, 126 °C and 130 °C and these were cultivated in ox-heart broth and inoculated in MPA 3679 agar after heat treatment (Santos & Zarzo, 1998).

It has been found that CCUG 7489 has higher heat resistant value than other strains (PA 3679 and CCUG 24143) at 85 °C for 4 min (Figure 4.2). However CCUG 7489 and CCUG 24143 were very close (Figure 4.2) at 85 °C, CCUG 7489 showed less log reduction (D_{85}= 20 min) than CCUG 24143 (D_{85}= 5.31 min) (Figure 4.3). Other study reported that D_{121}= 0.6 min for PA 3679 in deionized water which is much higher than this result (Janelle L. Brown et al., 2012). The average D_{90} values for CCUG 7489 found in deionized water by this experiment was 6.53 min which is also different to other studies (Table 4.1). This can be described in theory that methods and experimental designs can have significant effect on heat resistance. For example, W. W. Yang, Crow-Willard, and Ponce (2009), used digital dry bath for inactivation of *C. sporogenes* at 100 °C while they removed the samples in every 5 min directly from the tubes for plating in RCM to avoid temperature decrease and have found a D-value of 15 min.

Survival of *C. sporogenes* in different salt concentration (during simulated processing) was the major focus in this study. It has been noticed that 75% of daily salt intake comes from processed foods, whereas, meat (18%) contributes a major part (Mhurchu et al., 2011). It is now a worldwide concern for salt intake because it can lead to high blood pressure, cardiovascular disease and strokes (Elliott et al., 1996). Thermal inactivation in capillary tubes with deionized water and salt water was done in the same day to ensure the age of the spores were same for both experiment. CCUG 7489 was heat treated in 3% and 6% w/v NaCl medium at 90 °C for 12 min and 2 logs reduction was observed between 2 to 12 min. it was not found a significant difference found between 3% and 6% NaCl for the survival of *C. sporogenes* (Figure 4.5). At the same time the regression lines of the survivals for water (D_{90}= 6.97 min in average) and 3 % salt medium (D_{90}= 6.83 min in average) were parallel (Figure 4.6) which also proved there is no statistically difference (p= 0.83) between salt (3% w/v NaCl) and water for the survival of the spores. The slopes for water and 6% w/v salt (NaCl) medium were parallel and was not significantly different (p = 0.98). From this, it can be deduced that there is no significant difference in D-values (kinetics) between water and salt medium.

The number of dead and injured spores increase during the heating time and most of the injured bacteria will regenerate and start growing during favorable and, optimal recovery conditions, thus the D-values appear higher. If recovery condition is unfavorable, injured cells are stressed by the environmental conditions, low pH and $a_{w,}$ thus the D-values appear low. The growth rate may also depends on the type of degree of injury during heat treatment.

5.2 Thermal inactivation by autoclave

The main goal of thermal treatment is the elimination of vegetative pathogenic microorganisms which may be present in raw food. A single and double heat treatment was applied by using an autoclave to observe the difference in survival of *C. sporogenes* before and after heat treatment. During the autoclaving, the heat rise gradually from 0 °C to 90 °C for 40 min (single heat) the heat treatment is delayed in the model product (minced meat). It is a completely different heat treatment compared to capillary tube methods where heat rise instantly when sample tubes were submersed into the hot water from ice cold water. Figure 4.8 show that the autoclave takes about 30-40 min to come-up time to reach the temperature at 90 °C and 20 min to cool down to room temperature. During this time approximately 1 log reduction occurred (Figure 4.10) for *C. sporogenes* (CCUG 7489). This reduction value cannot be compared with capillary tube results because the heating media, process of heating and time is different from each other. In the double heat treatment, model product was heated at 80 °C for 60 min. A regeneration time was applied for 75 min at 25 °C to germinate the spores followed by second heat treatment at 90 °C for about 30 min. After counting, a 1.3 log reduction was found (Figure 4.10) which has no significant difference ($p=0.03$) from single heat treatment. Pasteurization inactivates most viable microorganisms but not heat resistant spores. During double heat treatment some spores were able to germinate in germination temperature (25 °C) and then died after second time heating and yet the result found 0.3 logs less than single heat treatment. A sharp decrease of thermal death times at higher temperatures indicates the holding time at the lower temperatures has a little contribution to the sterilization. So the holding time at highest temperature can virtually determine the pasteurization value of the whole process. A complete destruction of proteolytic spores is not an option as a measure to prevent botulism because a 'botulinum cook' (F=3) of meat causes undesired appearance and flavor. So for a shelf-stable meat products can be cooked to F_o values between 0.1 and 1.5 which could reduce the number of proteolytic *C. botulinum* by about 1 to 7 $\log_{10}$ cycle.

It is known that free fatty acids are non-toxic compound which have antimicrobial activity. There are low concentrations of free fatty acids in foods. However foods containing fats and natural oils have higher concentration of free fatty acids which are sufficient enough to affect the microbial growth. Lekogo, Coroller, Mathot, Mafart, and Leguerinel (2010), has reported

significantly reduced D-values down by 30% for *C. sporogenes* while studied with different levels of free fatty acids. Higher fat content can give a negative impact on thermal inactivation of microorganisms. Higher D-values were found for *proteolytic C. botulinum* when heat treated with fatty media like beef, pork and milk (Janelle L Brown et al., 2012). The reason for the lower log reduction by the autoclave can be assumed that the inactivation was greatly influenced by the intrinsic factors (fat, pH, a_w) of the meat products.

6. Conclusion

The experimental designs for capillary tube method was followed by W. W. Yang et al. (2009), except preheat treatment in order to trigger sporulation. It was finally decided not to activate because it did not have significant effect on sporulation by external preheat treatment. This procedure is more close to real life where the spores do not experience any artificial heat to such degree (80 °C for 30 min) (Løvdal, Granum, Rosnes, & Løvdal, 2013).

The biggest challenge faced during spore cultivation was the unsynchronized way of growth which consists of all possible cell forms e.g., young vegetative cells, spores of different ages and germinated spores. The production of spore was achieved by 10^7 spores/mL for PA 3679 which was heat shocked at 80 °C for 30 min. For CCUG 24143 and CCUG 7489, cultivation of spores achieved by 10^9 spores/mL and 10^8 spores/mL in sporulation medium (broth) and RCM plating methods, respectively. None of these two strains were heat shocked. By the heat shock, more heat resistant spores can be achieved.

The D-values found for all three strains (PA 3679, CCUG 24143 and CCUG 7489) were very low in water in comparing to the other published studies (Nygaard & Lie, 2011; J. Smelt & Brul, 2014; W. W. Yang et al., 2009). A sharp log-reduction was observed during the first 2 min of heat treatment for all the strains which could be explained by that the weakest spores died during this time and they were large in number (> 50%). The overall D-values are also showing that the

resulting spores from the process of spore cultivation were more heat labile; however, more studies are required to prove this statement. The D_{90} value found for CCUG 7489 was 6.53 min.

What difference was observed during heat inactivation by using 0%, 3% and 6% w/v NaCl solution?

C. sporogenes CCUG 7489 was studied in presence of different salt concentrations (0%, 3% and 6% w/v NaCl) for better understanding about the inactivation in compare to deionized distilled water. The inactivation experiment was done in both salt and water medium at the same time to confirm the age of the spores was same. No significant difference (p = 0.84 and 0.98 for 3% and 6% with water, respectively) was found between salt and water as a medium for inactivation of *C. sporogenes*. This result shows that the applied salt concentration is not sufficient to inactivate the *C. sporogenes* spores. It can be concluded that the capillary tube results (D-values) cannot easily be extrapolated to industrial process.

Autoclave was used to see the difference between single and double heat treatment in spore inactivation process. During single heat application, concentration of cells declined from 6.29 log CFU/g to 5.24 log CFU/g. Double heat treatment was modeled to have a holding period for spores to germinate in-between two heating schedules. During double heat application, the concentration of cells declined from 6.29 log CFU/g to 4.59 log CFU/g.

Future work

Different salt types e.g., KCl should be used to study the effect on the inactivation kinetics of *C. sporogenes*.

A modified log-logistic approach should be done to compare the recent results.

More data is needed to confirm the amount of fat and other factors in meat product which can lead an effect on the thermal killing of bacterial spores.

7. References

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