ABSTRACT

STAM, CHRISTINA NICOLE. Development of Novel Biological Indicators to Evaluate the Efficacy of Microwave Processing. (Under the direction of Dr. Lee-Ann Jaykus).

Biological validation of traditional and alternative food processing technologies which result in commercially sterile products remains challenging. This is particularly true for multiphase food products, largely because of difficulties in proving that the fastest moving particles have been exposed to a sufficiently lethal treatment to inactivate the pathogen of concern, *Clostridium botulinum*. The purpose of this research was to investigate alternative approaches for application to biovalidation of commercial sterilization. In the first phase of the research, the spores of bacteria commonly used as surrogates in thermal inactivation studies, were immobilized in sodium alginate and the performance of the surrogates validated. The second study focused on developing a molecular-based method to rapidly quantify viable spores surviving thermal processing.

In the first study, spore crops for *Geobacillus stearothermophilus* and *Bacillus subtilis* were produced, suspended in a variety of media (water, alginate and salsa con queso), and their thermal inactivation kinetics determined. The D-values for *G. stearothermophilus* and *B. subtilis* at 121 °C were above the target D-value of 0.20 min for *Clostridium botulinum*; in most instances, suspension media did not affect D-values or resulting z_D values in a statistically significant manner. The spores were immobilized in a 3% sodium alginate suspension which was used to produce "beads" of approximately 30 µL in volume. These beads could be easily manipulated, colored with dyes, and had consistently high concentrations of spores whose thermal inactivation kinetics did not differ from spore stock suspension. Although efforts were made to track and recover the beads in a model aseptic continuous microwave process, mechanical difficulties complicated their timely recovery, making it difficult to conclusively

determine process lethality. However, the alginate-encapsulated spores maintained their physical integrity even after exposure to rigorous time and temperature combinations.

In the second study, a molecular method was developed for the detection and discrimination of viable and non-viable spores of *Clostridium sporogenes*. In the first phases of the research, a method to extract spore-associated DNA was developed, which included the combined steps of decoating and lysozyme digestion. The process resulted in recovery of high yields of quality DNA. A Sybr green-based quantitative real-time (qPCR) assay targeting the GerAB gene was also designed. When combined with the DNA extraction steps, the assay was log linear over a range of from 10^2-10^9 spores/mL, with a lower limit of detection of approximately 10^2 spores/mL. It was confirmed that exposure of spores to 121°C for as little as 2 min resulted in near complete degradation of the DNA and loss of PCR amplifiability, suggesting that under stringent heat treatment, the PCR-based method would be able to distinguish viable spores from those which had been killed. Under less stringent processing conditions, the DNA from non-viable spores remained detectable. In this case, the decoated spores were treated a 12.5 µg/ml concentration of propidium monoazide (PMA), a DNA intercalating agent, which selectively enters inactivated bacterial cells, binds to the DNA and makes it unavailable for amplification. Unfortunately, the PMA was not able to selectively inhibit the amplification of DNA derived from dead spores. This was evidenced by the fact that C_T values obtained for live and thermally treated spores were nearly identical, regardless of PMA treatment status. Further options for the selective detection of DNA derived from viable spores are under investigation.

Taken together, this research demonstrates the feasibility, as well as hurdles, involved in the design and use of novel methods to evaluate thermal process efficacy as applied to particulate

foods. Clearly, alginate-immobilization of spores is an effective method to produce large amounts of stable product for use in biovalidation. PCR-based detection methods to rapidly quantify process lethality have promise, especially when applied to stringent processes; application to less stringent processes requires further refinement. With additional research, it should be possible to move biovalidation forward to include emerging molecular-based technologies for rapid and reliable determination of process lethality.

Development of Novel Biological Indicators to Evaluate the Efficacy of Microwave Processing

by
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BIOGRAPHY

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CHAPTER 1
Literature Review

1. Introduction

Bacterial spores are known to be more resistant than their vegetative counterparts. Treatments such as heat, irradiation, chemicals and desiccation that inactivate vegetative cells will not completely inactivate spores (Sugiyama, 1951; Clouston and Wills, 1970; Yudkin, 1993; Stephens, 1998; Brown, 2000; Ricca and Cutting, 2003; Turnbough, 2003). Sporulation is the result of nutrient starvation creating metabolic inactivity, and providing stability for long periods of time (Stephens, 1998; Driks, 2002; Turnbough, 2003). When nutrients become limited, growth slows, and in the case of *Bacillus*, flagellar motility becomes activated as the organism seeks new food sources (Stephens, 1998). If no alternative food sources are found, the cell becomes metabolically inactive and spore formation can be completed (Stephens, 1998).

When nutrients are added to the environment the spore will usually germinate, converting back to the vegetative state (Driks, 2002). A germinant is required for this process. Germinants may include sugars, amino acids, nucleosides and can also be a mixture such as asparaginine, glucose, fructose and potassium (for *B. subtilis*) (Setlow, 2003). Spores can be triggered to germinate within several seconds of introducing the nutrients; germination can continue even if the nutrients are removed (Setlow, 2003).

From a food safety perspective, *Clostridium botulium* is the most serious sporeformer of concern (Brown, 2000). *C. botulinum* is a bacterium that causes foodborne intoxication. The neurotoxin is produced during growth of the bacteria, and if the *C. botulinum* strain is proteolytic, the neurotoxin becomes active during bacterial lysis. If it is a nonproteolytic *C. botulinum* strain, the toxin is activated by host proteases. Prior to the advent of modern

healthcare, botulism was almost universally fatal. Today, it is fatal in 5-10% of cases (CDC, 1998; Shapiro et al., 1998; Brown, 2000; Zubay, 2005). Patients need mechanical ventilation and antitoxin, making botulism a public health emergency that requires rapid recognition. The toxin is one of the most lethal known to man with an LD_{50} of 20-50 ng (Peck, 1997; Shapiro et al., 1998). In recent history, about 9 foodborne outbreaks occur annually with a median of 24 cases for all types of outbreaks (Arnon et al., 2001). Of these outbreaks, the mean size has been consistently 2.5 cases per outbreak (Angulo et al., 1998; Arnon et al., 2001). Although *C. botulinum* has caused outbreaks in the past (Table 1), it is more commonly a sporadic disease. In the United States, the Botulism Surveillance System was established because rapid diagnosis and treatment is the most important step in reducing death rates (Shapiro et al., 1998). For these reasons one case of *C. botulinum* is considered an outbreak and must be reported to the CDC (Shapiro et al., 1998).

There are seven different types of botulinum toxin, but types A, B and E are most often associated with human disease (Table 2) (Shapiro et al., 1998). The occurrence of different toxin types is in part regional, as in the US, type A is found west of the Mississippi River, type B east of the Mississippi River, and type E is found in Alaska and Arctic regions (Eisenberg and Bender, 1976; Horwitz et al., 1977; Shapiro et al., 1998). The majority of type E outbreaks occur in native Eskimo or Indian foods of Alaska which include raw, dried or fermented sea mammals and salmon (Eisenberg and Bender, 1976; Wainwright et al., 1988). More than half of all reported outbreaks have occurred in the western states of California, Washington, Oregon, Colorado and Alaska (Arnon et al., 2001). Of the botulism cases, there are 4 clinical presentations: foodborne, wound, infant and the extremely rare adult infectious botulism (Shapiro et al., 1998). From 1899-1990 there have been a total of 962 recorded outbreaks

involving 2320 cases and 1036 deaths (FDA-BAM, 2001). The yearly reports to CDC for botulism cases since 1973 include a median of 24 foodborne, 3 wound, and 71 infant botulism cases (Shapiro et al., 1998). Half of all foodborne cases are caused by toxin type A, with E and B equally accounting for the other 50% (Shapiro et al., 1998). Higher severity and fatality rates are associated with the type A toxin (Shapiro et al., 1998). In the United States the fatality rate from botulism was 25% in 1950-1959 and has decreased to 6% during 1990-1996 (Arnon et al., 2001).

The onset of botulism typically falls within 18-36 hours (Shapiro et al., 1998) of consumption of contaminated food. Initial symptoms include diarrhea, vomiting and nausea (Shapiro et al., 1998). Neurological symptoms follow and include dry mouth, blurred vision and diplopia (Shapiro et al., 1998). This is followed by dysphonia, dysarthria, dysphagia and peripheral muscle weakness that leads to decending paralysis (Shapiro et al., 1998). Muscular function returns after ventilatory support, which is usually needed for 2-8 weeks and sometimes up to 7 months (Shapiro et al., 1998). Recovery can take weeks to months following treatment (Shapiro et al., 1998).

Products that have been associated with botulism include meat, fish, vegetables, dairy, honey products, homemade salsa, baked potatoes, cheese sauce, sautéed onions with butter, garlic in oil, potato salad and home canned foods (Odlaug and Pflug, 1977; Shapiro et al., 1998; Brown, 2000). Of these food products, there is greatest concern for refrigerated and processed foods, especially the cook-chill and sous-vide foods (Evans et al., 1997). According to a review article by Brown (2000), most of the products implicated in *C. botulinum* outbreaks have a pH above 4.5, with few recorded outbreaks associated with products of pH lower than 4.5; the lowest was associated with a product with a pH of 3.5 and occurred in 1927 in California,

attributable to home-canned pears (Odlaug and Pflug, 1978). In this outbreak, *Lactobacillus*, yeast and *C. botulinum* spores (5 x 10^8 cfu/g) were found in the product (Odlaug and Pflug, 1978). In experiments with the organisms isolated from the outbreak, toxin production was present in test tubes inoculated with both yeast and *C. botulinum* at pH ranging from 3.33-4.22 (Odlaug and Pflug, 1978). Other studies have shown that *C. botulinum* can produce toxin in food products with a pH below 4.5 if spoilage organisms such as fungi are present. It appears that these fungi can create a microenvironment with non-homogeneous pH which can exceed 4.6 (Odlaug and Pflug, 1978; Raatjes and Smelt, 1979; Brown, 2000). In a study by Raatjes and Smelt (1979), the investigators reported that *C. botulinum* could grow and produce toxin at a pH of 4.0 in the presence of *Bacillus* spp. because the *Bacillus* removed residual oxygen and lowered the redox potential.

Mathematically, bacterial inactivation is characterized by D and Z values (Bellara et al., 1999). The D-value is defined as the time necessary at a given temperature to produce a one log or 90% reduction in the target bacterial population, and hence refers to the thermal resistance of the organism at a specific temperature (Bigelow and Esty, 1920; Montville and Matthews, 2005; Bellara et al., 1999; Singh and Heldman, 2001). The Z-value is derived from multiple D-values and refers to the influence of temperature on the D-value, defined as the temperature change needed to change the D-value by 90% (Bellara et al., 1999; Montville and Matthews, 2005). Commercial sterilization of thermally processed food is defined by the 21 CFR 113.3 as follows: *By the application of heat which renders the food free of microorganisms capable of reproducing in the food under normal nonrefrigerated conditions of storage and distribution; and viable microorganisms (including spores) of public health significance; or by the control of water activity and the application of heat, which renders the food free of microorganisms capable of*

reproducing in the food under normal nonrefrigerated conditions of storage and distribution. Commercial sterilization is based on a 12D approach to inactivate spores of *C. botulinum* (Collado et al., 2003). The 12D approach is used if the pH of the food product is above 4.5 (defined as low acid foods), which is based on the assumption that *C. botulinum* cannot grow below pH 4.5 (Hersom and Hulland, 1980). If other microorganisms in the product increase a normally lower pH to above 4.5 due to excessive growth, *C. botulinum* spores can subsequently germinate and grow (Kaplan and Williams, 1941). Thermal processing of high acid foods (foods with pH < 4.5) does not require the stringent heat treatments applied to low acid foods because *C. botulinum* growth is inhibited by the reduced pH and most spoilage organisms of acidic products (i.e., yeasts and molds) are inactivated readily at temperatures below 100ºC (Hersom and Hulland, 1980)

2. Biological Indicators

In food processing, there are three commonly used means by which to evaluate processing efficacy; these include the use of thermocouples, chemical indicators and biological indicators (Serp et al., 2002). Thermocouples are designed to measure the heat-penetration rates at a selected point in a product container (Hersom and Hulland, 1980; Holdsworth, 1997). Depending on the type of thermocouples that are used, they can be attached to a computer to give heat penetration parameters and F_o-values (Holdsworth, 1997). Chemical indicators use enzymes, sugar inversion, or color changes as a time-temperature indicator and provide information complementary to traditional thermocouple results (Holdsworth, 1997). An example of one such chemical indicator, which has been applied to microwave processing are the proteins M-1 [2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one] and M-2 [4-hydroxy-5-methyl-3(2H)-furanone] (Prakash et al., 1997). M-1 forms a Maillard reaction between glucose and

proteins, whereas M-2 is formed in meats with the reaction of ribose and proteins and has a detectable UV absorption maximum of 298 nm (Prakash et al., 1997). Anthocyanins have been used as an indicator of sterilization efficacy because their color is degraded at high temperatures (Holdsworth, 1997).

A biological indicator is a surrogate organism that has similar thermal inactivation properties to a pathogen of concern in the food product (Table 3) (FDA, 2000). For example, both *Geobacillus stearothermophilus* and *Clostridium sporogenes* have been used as surrogates for *Clostridium botulinum* (Quesnel, 1984; FDA, 2000). To date biological verification or the use of a biological indicator that is processed through the entire processing system, retrieved and then enumerated to determine lethality of the sterilization process has been the best approach to determine the efficacy of multiphase food product processing (commercial sterilization). The organisms can be incorporated into the product and treated in the same way as the natural product contaminants (Shintani and Akers, 2000; Serp et al., 2002; Marcy, 1997).

Selecting a biological indicator is dependent on the target microorganism and the epidemiological relevance of the organism to the product (NACMCF, 2004). From a public health perspective, in food processing we seek to inactivate the most resistant microorganism of public health significance. The identification of that (those) microorganism(s) is based on inherent microbial resistance to the process, initial load and the ability to grow in the food; that organism may not necessarily be the one present in the highest numbers (NACMCF, 2004). A surrogate organism is helpful for use during development and validation of a process and should contain several characteristics (although no biological surrogate contains them all): (i) non-pathogenic; (ii) thermal inactivation characteristics that can predict the target organism; (iii) behavioral similarities to the target organism, such as pH and oxygen tolerance; (iv) batch

stability; (v) thermal and growth stability; and (vi) maintenance at a constant and high population density (Quesnel, 1984; FDA, 2000). Lastly, for a biological surrogate to be reliably used, all other pathogenic microorganisms should be inactivated at a less stringent treatment when compared to the biological indicator (Quesnel, 1984).

Parameters for performance, and application of biological indicators need to be established due to lot-to-lot variations, differences in specifications and performance, and differences between biological indicators used and/or produced by different manufacturers (Smith et al., 1982; Shintani and Akers, 2000). Several parameters that should be monitored include D-value, the phase of growth in which organisms are harvested, the substrate in which the culture is grown, sample size and packaging conditions (NACMCF, 2004).

Clostridium sporogenes, which has similar thermal response characteristics and cultural, physiological and genetic similarity to *C. botulinum*, is a commonly used surrogate for validation of commercial sterilization (Chen and Hotchkiss, 1993; Welt et al., 1994). *C. sporogenes* has been frequently used in inoculated pack studies for low acid foods (Grischy et al., 1983; Brown, 2000), even though it can be as much as five times more resistant to thermal processing than *C. botulinum.* For example, it has been demonstrated that a 5-D reduction in *C. sporogenes* may be equivalent to a 12-D reduction for *C. botulinum* (Grischy et al., 1983). The z_D-values of *C. sporogenes* however are similar to those of *C. botulinum* (Grischy et al., 1983). The F-value, or the time it takes to completely inactivate an organism at 121.1ºC, ranges from 15 seconds to over 4.3 minutes for *C. sporogenes* as compared to 1.2 seconds to 18 seconds for *C. botulinum* (Russell, 1982; Holdsworth, 1997; Guan et al., 2003). Another reason that *C. sporogenes* is a good surrogate for *C. botulinum* is that it is a common spoilage organism found in sealed dairy products maintained under anaerobic conditions. Therefore, visual observation of gas expansion

in processed, packaged product can be used in place of enumerative assays for validation of thermal processing (Roberts and Zottola, 1993).

Geobacillus stearothermophilus has also been used as a surrogate for *C. botulinum.* Recently this organisms' genus was changed from *Bacillus* to *Geobacillus* because of phenotypic and phylogenetic similarity among group 5 isolates of thermophilic bacteria of the *Bacillus* genus (Nazina et al., 2001). This organism has D-values that are even higher than *C. sporogenes* (Gould, 2001). Of the spore-forming microorganisms examined to date, *G. stearothermophilus* is the most resistant, although that resistance occurs upon exposure to moist heat; the organism is considerably less resistant to dry heat (Bruch, 1964). Upon exposure to moist heat, survival of *G. stearothermophilus* would be considered a worst case scenario and, indeed, it would be impractical to process a product long enough to obtain even a one-log inactivation of *G. stearothermophilus*, as it has D-values ranging from 16-936 minutes at 121ºC (Allison, 1999; Brown, 2000). One limitation in working with this organism has been variability in growth, as changes in growth conditions and environment do not produce consistent sporulation (Allison, 1999).

3. Effect of Intrinsic and Extrinsic Factors on Sporulation, Germination and Thermal Resistance of Spores

A. Sporulation

Bacterial sporulation is caused by depletion of nutrients which alters the rod-shape of the bacteria to an oval dormant cell (Driks, 2002). As nutrients become limited, vegetative bacteria adapt. Specifically, they employ mechanisms such as chemotaxis to search for alternative food sources, production of antibiotics to kill competitive microflora, secretion of hydrolytic enzymes to break down proteins and polysaccharides, and integration of new genetic information

(Stephens, 1998). For those bacteria capable of forming spores, which includes only some Gram positive bacteria, sporulation is the last response to starvation (Hardwick and Foster, 1952; Foster, 1994; Stephens, 1998; Turnbough, 2003). Once sporulation occurs, the expression of a variety of genes is stimulated by the production of sigma factors stimulating RNA polymerase and DNA binding proteins (Doyle et al., 2001). However, the intracellular trigger to stimulate sporulation is currently unknown (Doyle et al., 2001).

The first step of sporulation is initiation, which occurs as a consequence of the depletion of nutrients, and/or induction of starvation (Yudkin, 1993; Serp et al., 2002). The second stage is chromosome segregation followed by sporulation-specific cell division, differential gene expression and specific signal transduction (Barak et al., 2005). Once sporulation starts, it takes approximately 8 hours to complete (Doyle et al., 2001; Driks, 2002). Upon completion of sporulation there will be no detectable metabolism (Setlow, 1992). Ultimately, the return pathway is germination followed by outgrowth (Barak et al., 2005).

The process of sporulation itself occurs in seven steps as follow (Figure 1):

- Vegetative growth of the bacterial cell
- Asymmetric septation, which is a visible line that can be seen microscopically on the mother cell, forming the forespore. In this case, both the forespore and the mother cell have identical chromosomes, although the forespore has a more condensed chromosome, the significance of which is currently unknown.
- The forespore becomes surrounded by the mother cell cytoplasm, enclosed in two membranes (inner and outer forespore membranes).
- Cell wall and cortex form between the two membranes. Synthesis of glucose dehydrogenase and acid-soluble spore protein (SASP) occurs, giving the

forespore UV resistance and chemical resistance. Then dehydration of the forespore begins and continues until spore maturation.

- The coat, comprised of protein, forms outside the cortex covering the forespore. The expansion and contraction of the flexible coat is necessary for dehydration and rehydration of the spore. Dipicholinic acid (DPA) synthesized in the mother cell is then accumulated in the core and the final process of dehydration is completed.
- Spore maturation, during which the spore coat becomes more dense and heat resistance develops.
- Lysis of the mother cell, releasing the spore (Yudkin, 1993; Foster, 1994; Doyle et al., 2001; Driks, 2002; Ricca and Cutting, 2003).

Spore populations are heterogeneous because lag phases of germination and outgrowth between individual spores are independent (Barker et al., 2005). Mature spores are 0.8-1.2 μm in length (Ricca and Cutting, 2003). Once sporulation has occurred it has been shown that spores can survive for years in the dessicated state (Ricca and Cutting, 2003; Barak et al., 2005). Several reasons exist for spore resistance, including genetic predisposition, sporulation conditions, spore coats, core permeability, core water content and mineral content (Setlow, 1992; Nicholson, 2000).

B. Germination

Spores survive well in harsh conditions until a stimulus, such as the addition of nutrients to the environment, cause activation, at which point the spores germinate and return to the vegetative state (Driks, 2002; Turnbough, 2003). Germination and growth is preceeded by the activation of dormant spores (Rodriguez et al., 1998). Activation occurs as a response to one of

four different conditions that can be categorized as follows: (1) nutritive, i.e., exposure to amino acids and/or sugars; (2) non-nutritive, such as exposure to metal ions and bicarbonate; (3) enzymatic initiation, usually with lysozyme; and (4) physical exposures such as heat and hydrostatic pressure (Russell, 1982; Johnson and Busta, 1984; Rodriguez et al., 1998; Collado et al., 2003). Of the different means by which to activate spores, heat is the most effective (Collado et al., 2003). For example, when suspended in water, spores can overcome dormancy through heat shock (Beaman et al., 1988). In the case of *G. stearothermophilus*, 10% of spores will germinate without heat shock and 50% form colonies after heat shock (Beaman et al., 1988).

Germination of spores is an irreversible process (Russell, 1982). During the process of germination, the spore structure changes, causing the cell to hydrate or swell; the cortex will then degrade and DPA is excreted (Russell, 1982). Outgrowth occurs when the swollen spores shed their coats, releasing the vegetative cell which subsequently divide (Russell, 1982).

C. Spore Structure

In the spore form, the bacterium is metabolically inactive and stable to extremes in environment including thermal processing and commercial disinfection (Sagripanti and Bonifacino, 1999; Driks, 2002). The spore structure consists of 5 layers, with the cortex and coats being most important in determination of heat resistance (Figure 2) (Russell, 1982; Driks, 2002). The core is the center of the spore and contains the DNA, RNA and DPA. Surrounding the core is the inner forespore membrane and germ cell wall. The germ cell wall is identical to the vegetative cell wall (Montville and Matthews, 2005). This is surrounded by the cortex which is comprised of peptidoglycan. Peptidoglycan is a peptide-polysaccharide layer that makes up the bacterial cell wall (Garrett and Grisham, 1999). A Gram-positive bacterial cell wall consists of several peptidoglycan layers that total approximately 25nm in thickness (Garrett and Grisham,

1999). The cortex peptidoglycan is somewhat different from a typical Gram-positive cell wall. Specifically, the spore peptidoglycan contains dipicholinic acid (DPA), and muramic acid residues are present as muramic acid lactam which are not in a typical cell wall (Doyle et al., 2001). The cortex is more or less a very thick cell wall but always contains DPA (Montville and Matthews, 2005; Russell, 1982). The cortex is responsible for spore resistance because it can dehydrate the core but the mechanisms involved in this process are currently unknown (Montville and Matthews, 2005). During germination and outgrowth the cortex is responsible for lysis (Russell, 1982). The spore coats are made of highly crosslinked peptides and comprise more than 50% of the spore (Driks, 2002). The coat is what protects the spore from chloroform or lytic enzymes, but also allows for the spore to respond to the presence of nutrients in the environment (Montville and Matthews, 2005; Driks, 2002). The coats are surrounded by the exosporium which is derived from the outer membrane layer and varies in size (Russell, 1982).

D. Environmental Effects on Spore Resistance

As previously stated, the critical pH level for commercial sterilization of foods is 4.5 because *C. botulinum* cannot grow and produce toxin below this pH. In many cases, the pH of a food may be adjusted to below 4.5 to inhibit *C. botulinum* outgrowth, allowing the processor to use reduced processing time, hence producing a more organoleptically acceptable product (Cameron et al., 1980). In general, for both *Clostridium* and *Bacillus* spp., the more acidic the pH, the less heat resistant are the spores (Cameron et al., 1980; Silla Santos et al., 1992). Even reduction of pH just slightly above 4.5 can reduce spore heat resistance (Cameron et al., 1980; Tejedor et al., 2001). Any drop in pH of the sporulation media can also cause a decrease in the thermal resistance of the propagated spores (Quesnel, 1984). In general heat resistance of spores has been shown to be correlated with the temperature at which the spore is produced (Warth,

1978). Specifically, production of spores at higher incubation temperature causes a decrease in the water content in the core, imparting a higher degree of heat resistance (Quesnel, 1984; Montville and Matthews, 2005; Nicholson, 2000).

G. stearothermophilus spore viability has been shown to decline over time, with spores having better survival when stored under frozen conditions compared to refrigerator storage (Reich et al., 1979). Evans and Curran (1960) found that *C. botulinum* 62A and *C. sporogenes* 3679 maintained viability after 3 years of storage at 30ºC when pH values ranged from 5-8. However, in the same study *G. stearothermophilus* had lost its ability to germinate at pH values less than 8.

E. Methods for Spore Propogation

Preparing vegetative bacteria for sporulation begins with the initiation phase, which involves propagating the bacterial culture to high density until nutrients are depleted. In the case of *Bacillus*, exhaustion of the carbon source will result in the initiation of sporulation (Nicholson, 2002). Little is known about the sporulation of bacteria in the natural environment and it is believed to occur very differently from the more artificial methods used for spore production in the laboratory (Nicholson, 2000).

Depending on the bacteria, either liquid or solid media can be used to induce sporulation. For example, both *C. sporogenes* and *G. stearothermophilus* can be sporulated in both liquid media or on petri dishes (Stewart et. al., 2000; Uehara et al., 1965; Serp et al., 2002). For *C. sporogenes*, sporulation can be increased with aeration every 24 hours over 5-6 days (Kaplan and Williams, 1941). Clifford and Anellis (1971) found that maximum sporogenesis for *Clostridium* spp. occurred in 60 hours at 37ºC.

To monitor the process of sporulation, phase-contrast microscopy is used (Evans et al. 1997; Raso et al., 1998; Serp et al., 2002). Under ideal conditions, sporulation efficiency, as evaluated microscopically, usually ranges from 80-100% (Clouston and Wills, 1969; Raso et al., 1998; Serp et al., 2002). Once optimal sporulation has been achieved, the spores are further purified to provide a high density spore crop that is stable to long-term storage (Uehara et al., 1965). Specifically, preparation of the spore crop requires harvesting of the spores in sterile distilled water or a specific buffer such as one containing potassium phosphate (Uehara et al., 1965; Futter and Richardson, 1970; Welt et al., 1994; Serp et al., 2002). Once in the buffer, the spores are washed 5-10 times by alternating steps of centrifugation and resuspension in fresh buffer, usually using sequentially high centrifugation speeds (Uehara et al., 1965; Welt et al., 1994; Serp et al., 2002). Sometimes the spores are heat shocked in a water bath before the wash steps, usually at 80° C for 15 minutes, to inactivate residual vegetative cells (Uehara et al., 1965; Serp et al., 2002). Additional steps may also be applied for purification. For example, Uehara et al., (1965) were able to remove non-spore material and cell clumps of *C. sporogenes* by preliminary filtration with Whatman no. 4 paper and a Millipore apparatus. The volume of the sterile water or buffer used to resuspend the purified spore crops can be adjusted during the washing steps; after the final wash is completed, volume is frequently adjusted to obtain the desired spore crop density (cfu/ml) (Uehara et al., 1965; Serp et al., 2002). Once purified, the spore suspensions are then stored at refrigeration or frozen temperatures until use (Welt et al., 1994; de Jong et al., 2002).

4. Using Spores in Thermal Inactivation Studies

Inoculated Pack Studies

For evaluation of the efficacy of heat to achieve commercial sterilization, the inoculated pack study design has been used for decades (Holdsworth, 1997). In the inoculated pack study, the food is inoculated with high levels of spores per container and subjected to the normal heat process (Hersom and Hulland, 1980). Spores used in the study must be appropriate in terms of heat resistance, capablilty to germinate and grow in the food (Hersom and Hulland, 1980). The inoculated pack is considered the simplest method, and the packs can be treated using several different process times and temperatures in an effort to identify proper processing conditions (Holdsworth, 1997). In the case of gas producing bacteria, sterility can easily be determined by swollen or blown cans and typically *C. sporogenes* 3679 is used in these types of studies (Hersom and Hulland, 1980; Holdsworth, 1997). For example, in a study by Guan et al. (2003), macaroni and cheese packs where inoculated with a concentration of 1.1×10^6 *C. sporogenes* 3679 spores/pack and then subjected to a microwave heat treatment. Control packs, which were not heat treated, displayed swelling associated with *C. sporogenes* growth and gas production within 2 weeks. Underprocessed packs all had gas production and those packs that were processed at or above target conditions showed no signs of gas production. This type of end-point approach to inoculated pack studies can be used along with enumerative cultural methods to provide complementary data.

Immobilization

Biological indicators such as *Bacillus* spores have been immobilized in or on solid matrices for use in inactivation studies. Specifically, they have been immobilized on paper strips and in gel matrices for use in thermal process validation (Serp et al., 2002). Paper strips

containing *G. stearothermophilus* spores are routinely used for autoclave sterilization validation and *B. subtilis* subsp. *niger* spores immobilized on paper have been used for validation of ethylene oxide sterilization (Smith et al., 1982). In a study by Reich et al (1979), investigators reported that commercially available *G. stearothermophilus* spore strips demonstrated decreased resistance with prolonged refrigerated storage, and more stable heat resistance profiles were obtained if the spores were stored in a frozen state. However, immobilizing microorganisms in a hermetic envelope, while convenient, does not allow for direct contact between the spores and the environment, and this may prevent the transfer of heat to the microorganisms, resulting in the bacterial survival (Serp et al., 2002).

Perhaps more relevant, spore crops can be immobilized in a sodium alginate solution to simulate food particles (Gaze, 2005). In this case, a 3-4% sodium alginate solution is made using sterile distilled water, to which the spore crops are added in approximately equal volume. The alginate-spore mixture can be dropped into a 100mM calcium chloride solution, instantaneously forming gels entrapping bacterial spores in the 3-dimensional lattice of cross-linked alginate (Smidsrod and Skjak-Braek, 1990). The alginate immobilized spores are viable for long periods of time and they have excellent mechanical and chemical stability (Smidsrod and Skjak-Braek, 1990). In a study by Marcy et al. (1997), the investigators demonstrated that alginate cubes could pass through mixing tanks, pumps, holding tubes and scraped surface heat exchangers at temperatures between 240-280ºF without damage. Furthermore, the alginate cubes were easily removed from the process. Marcy et al. (1997) also tested the storage stability of the alginate cubes for 6 months at 4ºC and found that the encapsulated spores were stable.

Determination of immobilized bacterial populations both before and after thermal (or other) processing requires dissolution of the alginate to release the bacteria before plating. The

alginate can be dissolved in a 50mM solution of sodium citrate or phosphate buffer at pH 7.0. Dissolving of the alginate can take from 30 minutes to several hours depending on the M-G ratio (β-D-mannuronic acid to α-L-guluronic acid ratio) of the sodium alginate used (Smidsrod and Skjak-Braek, 1990). Once the alginate is dissolved the bacteria is no longer immobilized and can be easily cultured. Neither immobilization nor dissolution of the alginate appear to impact spore viability (Smidsrod and Skjak-Braek, 1990)

Immobilization of microorganisms in alginate beads may be preferred to other immobilization approaches because of good product consistency, long term storage stability, and product resistance to impact and abrasion (Serp et al., 2002). In fact, data indicates that the thermoresistance of a microorganism does not change with such immobilization. For example, the D-values of *G. stearothermophilus* were the same for both the freely suspended and alginate immobilized spores (Serp et al., 2002). In addition, there is evidence that alginate beads maintain the same temperature on the inside as in the suspending environment (Serp et al., 2002). Leaching of materials has been found to be negligible, with model studies showing that <1% of yeast leaching due to abrasion, destabilization of alginate and/or the presence of nutrients (Serp et al., 2002).

5. Aseptic Processing/ Multiphase Foods

Aseptic processing is defined as sterilization of product and packaging material, whereby the product sterility is maintained during filling and sealing of the package (Hersom, 1985; Morris-Lee, 2004). Aseptic processing usually utilizes high temperature short time (HTST) treatment and results in higher quality products (Hersom, 1985) because the high temperature destroys target microorganisms while the short time allows for the retention of nutrients and product quality attributes (Lee and Singh, 1990; Zhang et al., 2001). Other advantages of aseptic

processing include reduced energy consumption and adaptability to automated control (Lee and Singh, 1990).

Products that have been suitable for aseptic processing are fluids of high viscosity, which are continuously pumped through a heat exchanger, held at target temperature for a designated time, and finally cooled to a temperature of 35ºC prior to aseptic fill (Hersom and Hulland, 1980). According to the Aseptic Packaging Council (2006), there have been over 30 different types of food products approved for aseptic processing in the United States and these include liquid eggs, tomato sauce, milk, wine, juices, tea/coffee beverages, tofu and syrups, to name few. Development of an aseptic process for multiphase foods or foods containing particles has been complicated, largely because of difficulties in proving that the fastest moving particle has been exposed to a sufficiently lethal treatment (Larkin, 1997; Morris-Lee, 2004). Validation of such a process for particulate foods remains challenging because it is not possible to measure the temperature of a food particle as it flows through the system (Digeronimo et al., 1997). Even though particle residence time has been used to determine process lethality, measuring the time and temperature of the fastest moving particle through a process is impossible using currently available technologies (Digeronimo et al., 1997). Consequently, there has been no reliable way to verify to the federal regulatory authorities such as the U.S. Food and Drug Administration (FDA) that there is a cost efficient way to assure the safety of multiphase aseptically processed products (Morris-Lee, 2004).

Requirements that need to be addressed in order to develop a multiphase aseptic process include the following: (i) identification and selection of an appropriate sterilization F_o value; (ii) development of a model to predict the sterilization of the slowest heating particle; (iii) microbial validation; and (iv) documentation of control of critical procedures and factors that impact the

process (Larkin, 1997; Marcy, 1997). The results from inoculated pack studies applied to multiphase food products are difficult to replicate because each particle of the food receives a different thermal process. It is not possible to identify the particle that receives the least thermal processing, therefore it would be advantageous to use a biological indicator that travels through the entire process, coming out intact and capable of microbiological analysis (Marcy, 1997). The indicator should simulate both the trajectory of the slowest heating particle and the fasting moving particle through the process (Gaze, 2005; Marcy, 1997). To validate the process in a statistically reliable manner, at least 299 particles should be recovered and enumerated, achieving a 95% confidence of collecting 1% of the fastest moving particles (Digeronimo, et al., 1997; Larkin, 1997).

NCSU has developed a particle flow monitoring system for foods with large particles so that process validation can be done. The system addresses four significant and critical design components: (i) use of simulated particles that are comparable to actual food particles with respect to both flow and thermal properties; (ii) measurement of particle behavior through the use of implants, sensors and data acquisition; (iii) measurement of time-temperature by thermosensitive and thermomagnetic implants; and (iv) bio-validation through inclusion of biological indicators and evaluation of spore viability both pre- and post-processing (Morris-Lee, 2004).

Microwave Processing

Microwave processing has been defined by the FDA as the use of electromagnetic waves of certain frequencies to generate heat in a material (FDA, 2000). Two frequencies have been approved for use in foods and these consist of (i) the Industrial and Scientific Medical (ISM) band (915 MHz), which is used industrially only in the US and the UK; and (ii) the 2450 MHz

treatment which is the frequency used in home microwaves but which may also be used industrially (Chipley, 1980; Welt et al., 1994; Ohlsson and Bengtsson, 2001; FDA, 2000).

Heating of foods by microwave energy arises as the product of both dielectric and ionic mechanisms (FDA, 2000). Dielectric heating is caused by the oscillation of water molecules that produce heat by rapidly changing direction to align themselves with the changing electric field that is associated with electromagnetic radiation (Ohlsson and Bengtsson, 2001; FDA, 2000). Because these oscillating molecules cannot keep up with the changing electric field, heat is generated (Sumnu, 2001). Ionic heating is caused by the migration of ions that will randomly transfer energy to water molecules by accelerating rapidly and colliding with other ions (Ohlsson and Bengtsson, 2001; FDA, 2000; Singh and Heldman, 2001). Adsorption of radiation generates heat, and water molecules are the most important component in creating this rise in temperature (Richards and Al-Shawa, 1981).

A microwave is made up of five major components which include (i) a power supply that converts an electric supply to high voltages; (ii) a magnetron that converts the power to microwave energy; (iii) a wave guide which transfers energy from the magnetron to the oven cavity; (iv) a stirrer or fan that scatters energy in the oven; and (v) the oven itself, which encloses the food within metallic walls (Singh and Heldman, 2001). When designing an effective microwave process, one needs to consider: (i) the type of food and its characteristics; (ii) the properties of the process; (iii) the equipment properties; (iv) the packaging material; (v) the need for achievement of lethal temperature in all parts of the product (i.e., absence of hot spots, or adequate consideration thereof); and (vi) the necessity of monitoring temperature reliability (NACMCF, 2004). Indeed, discontinuous dielectric properties between foods and air create uneven heating (Guan et al., 2003), as do corners or irregular shapes which cause hot and cold

spots (Prakash et al., 1997). Cold spots are the critical process factor because as food absorbs microwave energy, the cold spots change (NACMCF, 2004). Nonetheless, there are several advantages to microwave processing, including the fact that microwaves can be turned on or off instantaneously, that processing can be done in the package, and that microwaves are more energy efficient due to the speed of heating which is proportional to the power of the microwave (FDA, 2000; Singh and Heldman, 2001).

The efficiency of microwave heating is based on electromagnetic field strength and exposure time (Jeng et al., 1987). Heat generated by microwave occurs because of increasing molecular vibration which happens without breaking chemical bonds (Chipley, 1980; Wang et al., 2003). Therefore, microwave heating is a good alternative for processing of products that are not particularly heat stable, largely because of the shortened heating time (Sasaki et al., 1997). In fact, pasteurization of food has been done by microwave, resulting in shorter process times than used in conventional heating (Celandroni et al., 2004). For example, *S. enterica* serovar Typhimurium and *E. coli* were killed by microwave (915MHz) at lower temperatures and shorter times when compared to conventional heating (Chipley, 1980). However, for commercial sterilization applications, microwaves have only been successfully applied to liquid products (Wang et al., 2003).

Because microwaving is essentially a thermal process, microbial inactivation as a consequence of microwave treatment is thermally-based (Chipley, 1980; NACMCF, 2004). Numerous bacteria have been found to be inactivated by microwave; parasites have shown some resistance and at this time, virus susceptibility is unknown (NACMCF, 2004). Microwave heating has been found to be more effective in inactivating spores, including those of *G. stearothermophilus* and *C. sporogenes*, than conventional heating (Chipley, 1980). It is believed

that bacterial spores are killed by the heat generated from surrounding water molecules which absorb the microwave energy, since spores contain very little water themselves and do not readily absorb the microwave energy (Sasaki et al., 1997). In addition, dried spores and lyopohilized cultures seem to be unaffected by microwave processing, suggesting that the presence of water is critical to microwave effectiveness (Chipley, 1980).

Even though microwave processes may use somewhat lower temperatures and shorter times than conventional thermally-based processes, no known pathogenic or non-pathogenic microorganisms have been found to be particularly resistant to microwave heating. Consequently, the same surrogate microorganisms that are used in thermal process validation should be applicable for use as biological indicators in microwave processing (FDA, 2000). Indeed, Sasaki et al. (1997) found that *G. stearothermophilus* was the most resistant to microwave heating when compared to other spores of differing *Bacillus* species.

Table 1. Reported outbreaks of *Clostridium botulinum*

***C. botulinum* strain/ toxin**	**Food Product**	**No. of Persons**	**Country/State and City**	**Year**	**Reference**
B	Garlic Butter	30	Quebec/Montreal	1985	St. Louis et al, 1988
A	Potato and Eggplant Dip	30	Texas/El Paso	1994	Angulo et al, 1998
A	Chopped Garlic in Oil	3	New York/Kingston	1989	Bell and Kyriakides, 2000
A	Baked Potato	34	New Mexico/Clovis	1978	Bell and Kyriakides, 2000
A	Canned Cheese Sauce	8	Georgia	1993	Bell and Kyriakides, 2000
E	Beaver	3	Alaska	2001	Anonymous, 2001
E	Beached Beluga Whale (muktuk)	8	Alaska	2002	Anonymous, 2003
B	Home-canned pickled eggs (pH pickling juice 3.5)	1	Illinois	1997	Anonymous, 2000
A	Stew	1	Oklahoma	1994	Anonymous, 1995
B	Palani (surgeon fish)	3	Hawaii	1990	Anonymous, 1991

Table 2. *Clostridium botulinum* toxin types

Toxin Types	Source	Host	Reference
A	Can vegetable, fruits or meat	Human, Infant, Waterfowl	Odlaug and Pflug 1978; Bell and Kyriakides 2000
B	Can vegetable, fruits or meat	Human, infant, horses	Odlaug and Pflug 1978; Bell and Kyriakides 2000
C_1	Shrimp, crab, raw meat, seashores	Waterfowl, chickens	Montecucco 1995; Bell and Kyriakides 2000
C_2	Raw meat	Cattle, sheep, horses	Bell and Kyriakides 2000
D	Crab, soil	Cattle, horses	Bell and Kyriakides 2000
E	Fish/marine products	Human, fish, infants	Odlaug and Pflug 1978; Bell and Kyriakides 2000
F	Shrimp, liver paste	Human, infant	Montecucco 1995; Bell and Kyriakides 2000
G	Unknown, isolated from soil	Unknown, found in necropsy specimens with unexplained death in adults and infants	Montecucco 1995; Bell and Kyriakides 2000

Table 3. D-values of biological indicators for thermal heat inactivation

Strain	ATCC/Type	Temperature	Time	Reference
G. stearothermophilus	7953	120ºC	4.1 min	Quesnel, 1984
G. stearothermophilus	7953	121ºC	3.12 min	Russell, 1982
G. stearothermophilus	NCTC 10003	110ºC	50-92 min	Russell, 1982
C. sporogenes	NCTC 532	121ºC	0.15 min	Quesnel, 1984
C. sporogenes	PA 3679	121ºC	0.84 min	Russell, 1982
C. sporogenes	PA 3679	115ºC	2.77-3.63 min	Russell, 1982
C. sporogenes	NCTC 532	121ºC	0.15 min	Russell, 1982
C. sporogenes		100ºC	8-100 min	Montville and Matthews, 2005
C. botulinum	Type A	100ºC	50 min	Quesnel, 1984
C. botulinum	Type B	120ºC	0.1-0.2 min	Quesnel, 1984
C. botulinum	Type A & B	100ºC	7-30 min	Montville and Matthews, 2005
C. botulinum	62A	112.8ºC	1.23 min	Russell, 1982
C. thermosaccharolyticum		100ºC	400 min.	Montville and Matthews, 2005
B. cereus		100ºC	3-200 min.	Montville and Matthews, 2005

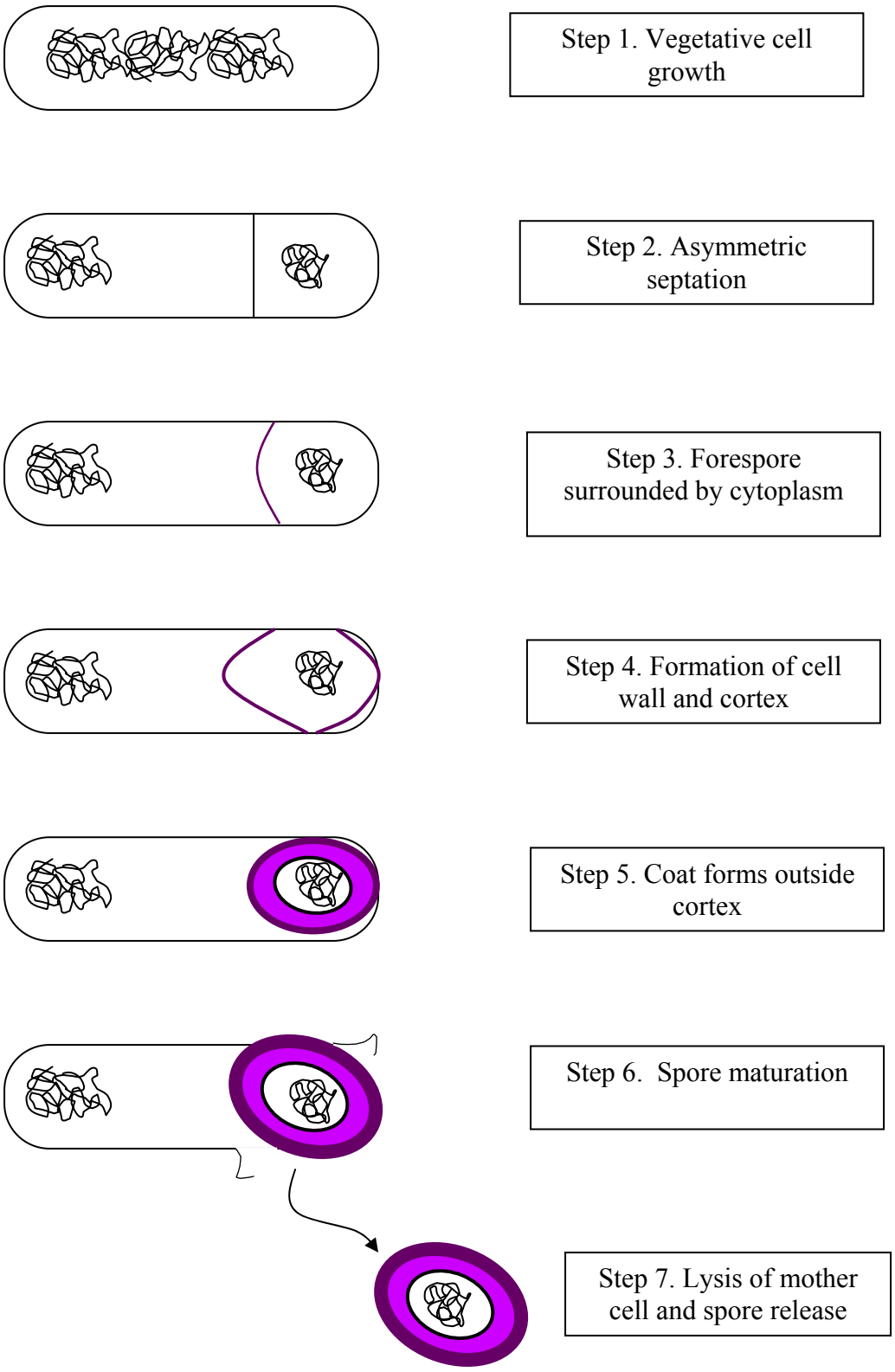

Figure 1. Sporulation Steps

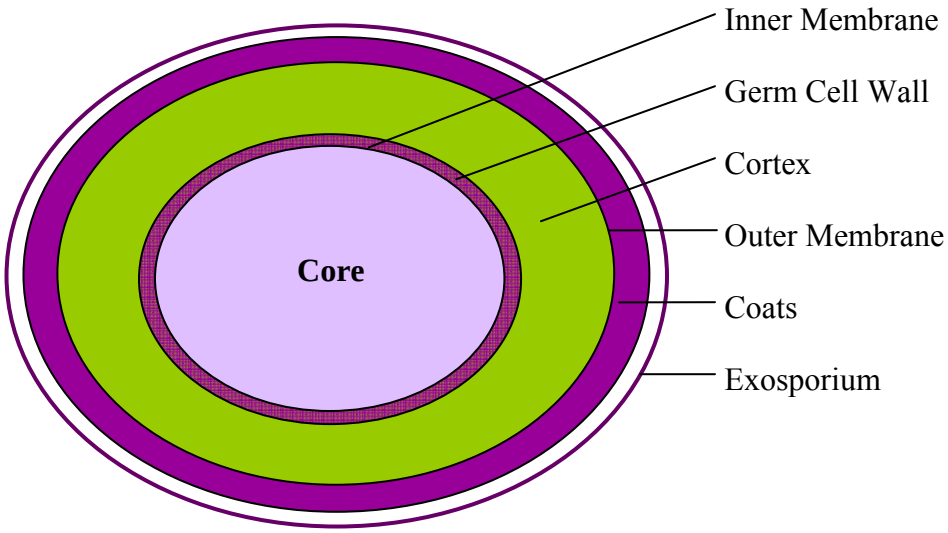

Figure 2. Spore Structure

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CHAPTER 2

Development of molecular methods to discriminate between viable and non-viable endospores of *Clostridium sporogenes*, a thermo-bacteriological surrogate

Abstract

DNA can persist in the environment long after cell death, making it difficult to accurately quantify bacterial cell populations using quantitative real-time PCR (qPCR). Recently ethidium monoazide (EMA) and propidium monoazide (PMA) have been used to selectively distinguish between viable and dead bacterial cells, but these agents have not yet been used to determine spore viability. EMA and PMA selectively permeate the membranes of dead bacterial cells and intercalate the DNA, making it unavailable for PCR amplification. In this project, we attempted to develop a PMA-based molecular method to quantify viable spores of *Clostridium sporogenes*, a commonly used *C. botulinum* surrogate, after thermal processing. Untreated and thermally inactivated spore suspensions were treated with a urea-based extraction buffer for spore coat removal. PMA was then added at a concentration of 12.5 μg/ml followed by crosslinking (to promote DNA intercalation) by exposure to a 500 watt light. This was followed by a lysozyme digestion and extraction of the total DNA. DNA and associated spore loads were quantified using a SYBR green-based quantitative real-time (qPCR) assay targeting the GerAB gene. The DNA extraction approach was efficient and the qPCR method was log linear over a range of from 10^2-10^9 spores/mL, with a lower limit of detection of approximately 10^2 spores/mL. Spore viability was not affected by removal of spore coats, as verified by direct plating and correlation with qPCR results. Despite these pre-treatments, PMA was not able to selectively inhibit the amplification of DNA derived from dead spores. This was evidenced by the fact that C_T values obtained for live and thermally treated spores were nearly identical, regardless of PMA treatment

status. Studies suggest that, even in the absence of the outer spore coat, the inner cortex layer still posed a considerable hurdle to complete incorporation of PMA to the interior of inactivated spores. Alternative approaches to facilitate molecular-based enumeration of surviving *C. sporogenes* spores are discussed and currently underway.

Introduction

Biological validation of commercial sterility obtained using traditional and alternative food processing technologies remains challenging. The traditional approach to determine lethality has been biological verification, or the use of a biological indicator that travels through the entire process stream is retrieved and then enumerated. In this case, the organism is incorporated into the product and treated in the same way as the natural product contaminant(s) (Shintani and Akers, 2000; Serp et al., 2002; Marcy, 1997). *Geobacillus stearothermophilus* and *Clostridium sporogenes* have been traditionally used as surrogates for thermal processing of low acid canned foods (FDA, 2000). In particular, *C. sporogenes*, which has similar thermal response characteristics and cultural, physiological and genetic similarity to *C. botulinum*, is a commonly used surrogate for validation of commercial sterilization (Chen and Hotchkiss, 1993; Welt et al., 1994), even though the organism can be as much as five times more resistant to high temperatures than *C. botulinum.* For *C. sporogenes* in particular, recovery of viable spores after exposure to thermal treatment is cumbersome, as the process requires substantial incubation time under anaerobic conditions. Many laboratories do not have the capabilities to perform such tests, and the extended time to results limits the ability to quickly evaluate the efficacy of existing and emerging food processing technologies.

Nucleic acid amplification approaches such as the polymerase chain reaction (PCR) have shown promise for improving time to detection for a wide range of applications in food microbiology. Recent real-time PCR methods (which employ simultaneous nucleic acid amplification and confirmation by hybridization in minutes to hours) are particularly promising, as these methods can be made at least semi-quantitative by correspondence to a standard curve. Nonetheless, PCR-based methods are rarely used for process validation, largely because the

DNA from dead bacterial cells (including inactivated spores) is highly stable and hence DNA-based amplification methods fail to discriminate between live and inactivated microorganisms (Nocker et al., 2007). Although the use of RNA targets in reverse-transcription (RT)-PCR has been purported as a means to circumvent this problem, bacterial spores have very low RNA content, making this a poor target for quantification of surviving spore populations.

DNA intercalating agents such as ethidium monoazide (EMA) have been used to selectively distinguish between viable and dead bacterial cells. Apparently, these dyes can only penetrate the membrane of dead cells (Rudi et al., 2005a; Nogva et al., 2003); once penetrated, they intercalate the DNA and upon photolysis using visible light, produce stable DNA monoadducts (Rudi et al., 2005b, Rueckert et al, 2005). Once the DNA is crosslinked, it becomes insoluble and is lost during genomic DNA preparation (Nocker et al., 2006). Theoretically, if EMA is used to treat bacterial cells prior to DNA extraction, only the DNA of viable cells should be available for PCR amplification (Nocker et al., 2007).

Nogva et al. (2003) provided proof-of-concept when they demonstrated that EMA-bound DNA cannot be amplified by PCR and therefore the compound can be used for the selective removal the DNA from mixtures of both live and dead bacterial cells. Since that time, numerous investigators have confirmed these findings, having applied EMA-PCR methodology for the selective detection, differentiation, and enumeration of living cells of *Salmonella*, *E. coli* O157:H7, *Listeria monocytogenes*, *Campylobacter jejuni*, and *Vibrio vulnificus* (Nogva et al., 2003; Nocker and Camper, 2006; Wang and Levin, 2006; Rudi et al., 2005a and b). These methods have also been used for the determination of total viable count (Rueckert et al., 2005). Most recently, Nocker et al. (2006b) described a related compound, propidium monoazide (PMA), which appears more selective than EMA and hence may have wider applicability.

Despite the promise of the methods described above, the use of DNA intercalating agents has not been applied for the discrimination of live and dead bacterial endospores, even though other types of viability assays (e.g., 5-cyano-2,3-diotolyl tetrazolium chloride (CTC), Live/Dead *BacLight*) have been used to in this regard (Laflamme et al., 2004).

The purpose of this research was to develop a rapid real-time PCR method which could be used to discriminate between surviving and inactivated spores of *C. sporogenes*. Because of the complexities of spore structure, including components responsible for substantial protection of nucleic acids, we anticipated methodological difficulties which are likely unique to spores. As such, the following specific objectives were undertaken: (i) to develop a reliable method to isolate DNA from both viable and inactivated spores of *C. sporogenes*; (ii) to develop a quantitative real-time *TaqMan*-based PCR assay for the detection and enumeration of the DNA obtained from *C. sporogenes* spores; and (iii) to demonstrate that DNA intercalating agents could be selectively taken up by dead bacterial spores but not viable spores.

Materials and Methods

Clostridium Strains and Spore Production

Clostridium sporogenes PA 3679 (ATCC 7955) was grown overnight in 10 mL of Reinforced Clostridial Medium (RCM) (BBL/Difco) and incubated at 35 ºC anaerobically. The overnight culture (0.5 mL) was inoculated into 150 mL of RCM broth and incubated anaerobically at 35 ºC for approximately 20 days or until greater than 75% sporulation was observed by phase contrast microscopy. Spores were harvested by centrifugation at 10,400 x g for 20 min and the supernatant was discarded. The pellet was suspended in 100 mL of cold sterile dH_2O. Washing of the spores was repeated five additional times, after which the spores were suspended in dH_2O and stored at 4ºC until use. After the washing and resuspension process, the final stocks consisted of >80% spores as determined by phase contrast microscopy. Spore concentrations were determined by surface plating serial dilutions onto agar-solidified RCM medium and incubating at 35 °C for 24 h under anaerobic conditions.

Spore-Coat Removal and Cortex Disintegration

C. sporogenes spores (1 mL containing10^8 spores) were pelleted (14,000 x g for 5 min) and suspended in 1 mL of spore-coat extraction buffer (8 M urea, 150 mM β-mercaptoethanol, 1% (w/v) SDS, 50 mM Tris, 1 mM EDTA, pH 8). Spores were incubated at 60 °C for 60 min, pelleted and suspended in 1 mL of 1x PBS, 25% (w/v) sucrose, pH 7.4. To promote degradation of the cortex, 50 µL of a 100 mg/ mL solution of lysozyme (Sigma; St. Louis, MO) was added followed by incubation at 37 °C for 60 min. After lysozyme treatment, spores were further pelleted and then subjected to DNA extraction.

PMA treatment of Spores

Treatment with the DNA intercalating agent propidium monoazide (PMA) was done after the spore coat removal step but before the lysozyme treatment, on spores which were were pelleted and resuspended in 500 µl sterile dH_2O. PMA was added to spores at a concentration of 12.5 µg/ml followed by incubation in the dark for 1 h. Thereafter, the spores were exposed to a 500-W halogen light placed 12 cm from the samples for 5 min. Spores were placed on ice during light exposure to prevent overheating. Following PMA treatment, spores were pelleted and resuspended in 1 mL of 1x PBS, 25% (w/v) sucrose, pH 7.4 and incubated for 60 min at 37 °C after the addition of 50 µL of a 100 mg/ mL solution of lysozyme (Sigma; St. Louis, MO). The spores were then again pelleted and subjected to DNA isolation.

DNA Extraction

DNA was isolated using the Mo Bio Ultra-Clean Microbial DNA Isolation kit following manufacturer's instructions (Mo Bio Laboratories Inc.; Carlsbad, CA). The efficiency of DNA isolation from both viable and heat inactivated spores was verified via 1% agarose gel electrophoresis and quantified by fluorescence measurement following binding of the DNA to the dye PicoGreen (Invitrogen; Carlsbad, CA).

Quantitative Real-Time PCR (qPCR)

All amplification reactions were performed in a final volume of 25 µL using a Cepheid Smart-Cycler real-time qPCR system (Cepheid; Sunnyvale, CA). The primers were designed using Beacon designer 6.0 (Premier Biosoft International; Palo Alto, CA) and targeted the GerAB gene (nucleotide accession id: AY046406) that encodes for the GerAB germination protein with an amplicon size of 120 bp. Typical reactions consisted of 1 µL of sample DNA, 12.5 µL SYBR Green Jumpstart Taq Readymix (Sigma; St. Louis, MO), 5 µL of forward (5'–

ACAGATGTAGCCGCAGGAATAAAC-3') and reverse primers (5'-GGTCCCTCCATAAACAGCATAAGC-3') (final concentration 200 nM) and nuclease-free H_2O to achieve the desired final volume. Amplification was done using the following cycle parameters: 95 °C for 2 min (1 cycle) followed by 95 °C for 15 sec, 57 °C for 30 sec, and 72 °C for 30 sec (40 cycles). To verify the absence of non-specific product amplification, melting curve analysis was performed over a range of 60-90 °C. The negative first derivative of the change in fluorescence was plotted as a function of temperature and amplification specificity was verified by the presence of a single peak.

The limit of detection of the qPCR assay was determined by constructing a standard curve using C_T values obtained from serially diluted DNA initially isolated from a suspension containing approximately 10^8 spores/ mL. The overall sensitivity of the assay was determined by constructing a standard curve using C_T values obtained from serially diluted spores (10^8-10^1/ml). Spores in these serial dilutions were pelleted at 14,000 x g, subjected to spore-coat removal and lysozyme treatment, and DNA was isolated as described above. All spore concentrations were verified by direct plating methods using solidified RCM media as described above. All experiments were done in triplicate. Statistics were performed using SigmaPlot software (Systat Software Inc.; San Jose, CA).

Flow Cytometry

To determine the efficiency of PMA penetration into spores and to correlate staining with viability, PMA-treated viable or heat inactivated spores were analyzed (individually and in combination) using a Becton-Dickinson FACSCalibur Flow Cytometer (North Carolina State University College of Veterinary Medicine Flow Cytometry and Cell Sorting Laboratory; Raleigh, NC). PMA-treated spores were excited using an argon laser (λ_{ex} = 488nm) and the

percentage of fluorescent spores were determined (λ_{em} = 525nm). A total of 200,000 spores were analyzed. Spores without PMA treatment were used as a control.

Results

Spore Coat Extraction

Nucleic acid extraction from bacterial spores is difficult because the DNA is tightly wound and confined to the core section of the spore; this is surrounded by an exosporium, a cortex, and several coat layers. The first attempt at DNA extraction provided low yields. It was determined that decoating, i.e., removal of the outer coats of the endospore, might facilitate DNA release, resulting in higher and more consistent yields. A solution containing urea, SDS and the β-mercaptoethanol, all of which are protein denaturants, was used for spore coat removal. Phase-contrast microscopy was used to evaluate the efficacy of the spore coat removal process on viable *C. sporogenes* spores. Extraction of the spore coats did not change the phase-bright appearance of the spores as shown in Figure 3-B. When comparing the coat-extracted spores to viable untreated spores, the spores still appeared phase bright and showed no other changes in morphology, suggesting that the structural integrity of the spores was still largely intact, probably due to the cortex structure. This would likely complicate DNA extraction. Therefore, lysozyme treatment of the spores immediately after spore coat extraction was done to promote cortex degradation. Lysozyme digestion following spore coat removal caused the spores to lose refractility or their phase bright appearance, turning the spores phase dark and causing them to clump together (Figure 3-C). Loss of refractility and clumping occurred within 25 min after the addition of lysozyme. When spores digested with lysozyme were compared to spores heated at 121°C for 90 min (Figure 3-D), both sets had the same loss of refractility. The change from a phase bright to a phase dark appearance after lysozyme digestion illustrates that the process of spore coat removal resulted in a spore that was susceptible to degradation by lytic enzymes.

qPCR

The detection limits for qPCR were obtained by constructing a standard curve after extracting the DNA from a high titer spore suspension, serially diluting, and performing qPCR in triplicate. The detection limit for the qPCR assay was approximately 10^2 spores/mL (Figure 4-A). In an effort to take into account any DNA lost in the extraction process, a standard curve was also constructed using serially diluted spores followed by DNA extraction, as shown in Figure 4-B. In this case, the overall limit of detection of the real-time qPCR assay, as determined by C_T values (Figure 4-B), was also in the range of 10^2 spores/ml. This was similar to the detection limit of the culture-based method. As expected, the assays performed using the diluted spore DNA (Figure 4-A) showed excellent linearity (r^2 of 0.99) within the range of 10^2-10^9 spores/mL. When spores were diluted and then extracted for DNA isolation (Figure 4-B), linear regression analysis resulted in an r^2 of 0.80. Clearly, the qPCR results obtained using this experimental protocol were more variable, most likely due to the multiple sample manipulations (i.e, spore coat extraction, lysozyme treatment, and DNA extraction) performed on each sample. Nonetheless, the assay was relatively log linear in the range of 10^2-10^9 spores/mL detection. The experimental conditions employed in these latter experiments are probably more reflective of conditions that would be encountered after thermal processing.

As stated above, destruction of microorganisms by heat may not necessarily degrade DNA, so the DNA associated with dead spores may be able to be amplified with the same efficiency as that derived from viable spores. During microwave thermal processing, extremely high temperatures (>121°C) for extended times are used, so it was necessary to establish the ease with which DNA is degraded/destroyed by such high temperatures. A comparison between viable and heat treated spores (121°C in 2 min intervals for up to 18 min) was done to determine

how heat treatment would impact degradation of the DNA when the spores are killed at excessively high temperatures. Indeed, heat treating the spores for only 2 minutes resulted in DNA destruction, as evidenced by the absence of a DNA smear using agarose gel electrophoresis, as compared to the smear seen for the DNA derived from viable spores (Figure 5). However, the DNA derived from the autoclaved spores was still marginally detectable by qPCR, having a C_T value of 35 compared to a C_T value of 12 for viable spores.

PMA Treatment

Fluorescence microscopy was used to determine the ability of PMA to penetrate the viable and heat-inactivated spores which had previously undergone the combined spore-coat extraction/lysozyme treatment. As shown in Figure 6, PMA could penetrate both live and dead spores, as evidenced by fluorescent staining of the spores. The penetration of PMA into the spores was further verified by flow cytometry, in which case both viable and heat inactivated spores demonstrated nearly identical levels of fluorescence following incubation with PMA. This suggested that PMA is capable of at least partial penetration of the spore and likely interacts significantly with the highly charged peptidoglycan layer of the spore cortex.

However, the ability of PMA to selectively intercalate the DNA of inactivated spores was not supported by the real-time PCR results. Specifically, when comparing qPCR results obtained from DNA derived from PMA-treated and control (untreated) live and heat inactivated spores, the characteristic shift in C_T values, which occurs because of amplification failure, did not occur (Figure 7). Clearly, it appeared that the PMA was unable to penetrate the inner membrane of heat-inactivated spores and hence could not intercalate the DNA.

Based on the hypothesis that PMA associated specifically with the spore cortex peptidoglycan which in turn prevented its entry into the DNA-containing core, we attempted to

precede PMA treatment with the combined steps of decoating and lysozyme digestion. As illustrated in Figure 8, when PMA was added after the lysozyme treatment, a negative shift in C_T value was observed. These results clearly suggest that lysozyme pre-treatment sensitized the spore to PMA entry, indicating that this approach is not feasible to selectively promote the entry of PMA into inactivated, but not healthy, *C. sporogenes* endospores.

Discussion

There is a continued need for more rapid approaches to the enumeration of bacterial endospores, especially as investigators seek to evaluate the efficacy of new food processing approaches. While standard plating methods remain the "gold standard," these may not be practical, especially when working with anaerobic sporeformers. Molecular amplification methods such as the polymerase chain reaction (PCR) have shown promise but are not ideal because (i) the DNA from dead bacterial cells (including inactivated spores) is highly stable and not indicative of viability; and (ii) the use of RNA targets is not an option because of the low RNA content of spores. The purpose of this project was to determine if it was feasible to produce a rapid molecular-based method for the post-process enumeration of endospores surviving thermal inactivation. Although we were not successful per se, the lessons learned here provide a basis upon which alternative molecular approaches may be designed.

Several challenges were encountered in this study. The first was developing a reliable method for extracting DNA from spores. The spore structure is complex and the same components which impart resistance to extreme environmental conditions are responsible for difficulties associated with DNA extraction. The center of the spore, or core, contains all components necessary to resume growth in the vegetative form, including the DNA (Montville and Matthews, 2005). This is surrounded by the cortex, which consists of multiple layers of peptidoglycan (Russell, 1982; Driks, 2002). This peptidoglycan is similar, but not identical to, the Gram positive cell wall, but is much thicker; it provides osmotic stability to the spore and is in large part responsible for heat resistance. The cortex is surrounded by a ketatin-like protein layer, with many disulfide linkages. This provides strength and also protects the spore from the action of chemicals and lytic enzymes (Riesenman and Nicholson, 2000). The cortex and coat

structures together are probably responsible for difficulties in releasing DNA from spores, leading to low and inconsistent yields during routine DNA extractions. Using a urea-based extraction buffer, we were able to remove the spore coats from the *C. sporogenes* spores prior to DNA extraction. The spore coat extraction buffer contains protein denaturants which degrade the outer spore coats. Nonetheless, even with the spore coats removed, DNA extraction remained inefficient. Because the spore coat was removed, however, the resulting spore was now susceptible to enzymatic degradation using lysozyme. Lysozyme activity was optimized and verified by loss of refractility under phase contrast microscopy. After lytic degradation, DNA extraction of both viable and dead spores was consistent in both yield and amplification efficiency.

The greatest obstacle in this study was facilitating the selective uptake of PMA by non-viable (heat inactivated) spores. In initial experiments, it was clear that control and heat inactivated spores both were penetrated by PMA, as determined by fluorescence microscopy and verified by flow cytometry. This was the case even after lysozyme treatment, which would theoretically remove all of the cortex peptidoglycan, and as confirmed by post-treatment loss of refractility. It is possible that the degradation of the cortex material and/or spore coat was incomplete, leaving residual organic material associated with the "naked" spore. If this occurred, the PMA might have been sequestered by this residual material before it could actually reach the DNA-containing core. In fact, similarities in structure between double stranded DNA and peptidoglycan have been reported by others (Schichnes et al, 2006). If this were the case, no PMA would have been available for binding to the DNA and qPCR signals would not been affected by PMA treatment. This was verified in the observation that C_T values obtained for live and thermally treated spores, regardless of PMA treatment status, were nearly identical. An

alternative explanation but less plausible explanation is that PMA may have bound to the DNA but failed to intercalate after light treatment. Regardless of the explanation, PMA was not able to selectively inhibit the amplification of DNA derived from dead spores, even after lysozyme pre-treatment.

Our data suggest that exposure to very rigorous heat treatment (>121 °C for 10 or more min) resulted in substantial degradation of spore-associated DNA, so much so that the remaining DNA was barely detectable by qPCR (Figure 5 and 7). This means that PMA pre-treatment of thermally inactivated *C. sporogenes* spores may not be necessary when using rigorous time/temperature combinations, such as have been proposed for microwave processing of particulate foods. In this case, the spore suspensions both before and after heating could simply be treated by the combined steps of decoating, lysozyme digestion, and DNA extraction followed by qPCR amplification and subsequent quantification based on qPCR standard curve. We are in the process of validating this approach.

However, it appears that at lower processing temperatures, the spore-associated DNA is not substantially degraded nor are qPCR signals significantly impacted (data not shown), meaning that PMA treatment, or an alternative, might be needed to facilitate molecular-based discrimination of live versus inactivated spores. An alternative approach to quantify surviving spore populations might be to use an RNA-based amplification method such as reverse transcription-PCR or nucleic acid-based sequence amplification (NASBA). Because spores contain very low levels of RNA, it would be necessary to precede RNA amplification with a rapid germination step. Unlike sporulation, germination is a very rapid process, usually occurring in the first 20-30 minutes following exposure to the germinant (Driks, 2002; Turnbough, 2003). Typical germinants include heat, sugars, enzymes and metal ions. During

this time, large amounts of mRNA (and rRNA) are produced as the spore prepares to enter into active vegetative growth. Candidate RNA sequences to be targeted might include those produced by the following genes: spore cortex-lytic (sleB) gene (gene accession id: 5395004), 16S Ribosomal RNA (gene accession id: 5399263) and the cell division protein (ftsZ) (gene accession id: 5399933). All of these genes are associated with the early phases of germination. For example, the protein produced by the sleB gene degrades the spore cortex; the 16S ribsosomal RNA is critical to initiation of protein synthesis; and the ftsZ gene product is a structural cell division protein that is expressed as the cell beings to replicate. Targeting germination-specific genes is a practical approach because only viable spores would be able to germinate and thus only viable spores would produce RNA corresponding to germination-specific genes (Nocker and Camper, 2006).

Currently no rapid molecular method exists to distinguish between live and dead bacterial endospores. The data presented here suggests that, while PMA was able to penetrate some structures of both viable and non-viable endospores of *C. sporogenes*, it did not impact DNA amplification efficiency, nor was the process selective based on viability. A number of different pre-treatments were attempted to facilitate viability-associated detection, and while such treatments resulted in improved DNA extraction efficiency, they did not resolve the PMA infiltration problem. We suspect that, even in the absence of the outer spore coat, the inner cortex layer still posed a considerable hurdle to complete incorporation of PMA into the interior of inactivated spores. The use of alternative RNA-based detection methods following a "flash germination" step may provide a means by which to overcome this problem, and investigation of this type of approach is currently underway at our location.

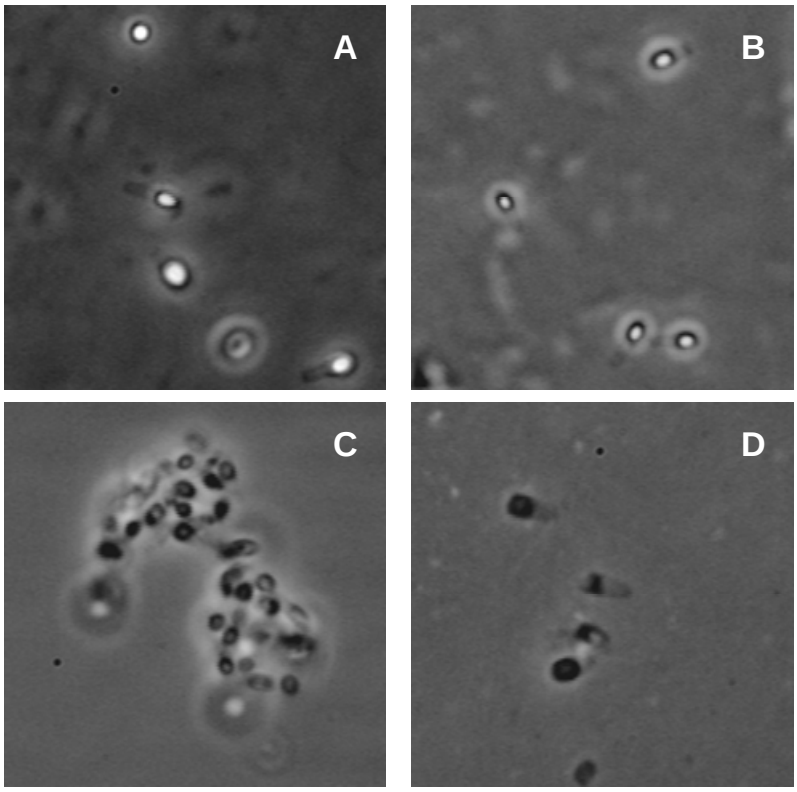
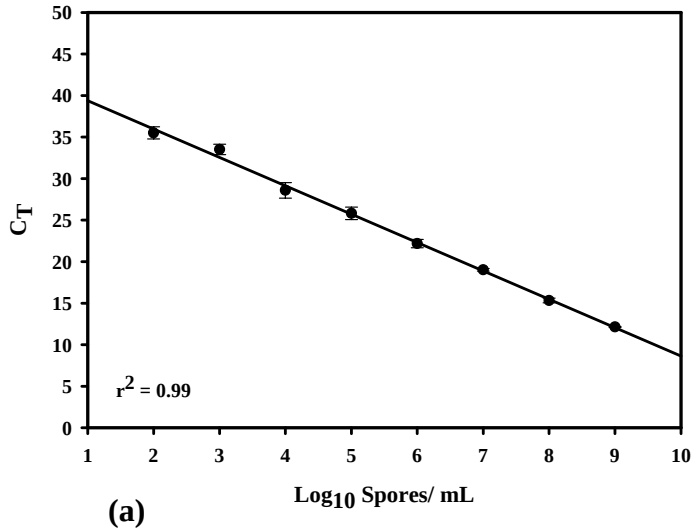
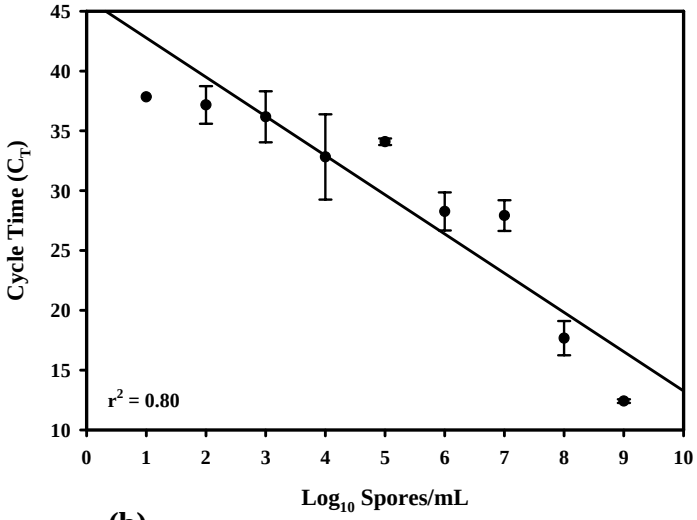


Figure 3: Lysozyme digestion of *C. sporogenes* spores previously treated with 8M urea, 1.0% (w/v) SDS, 140 mM β-mercaptoethanol in 50 mM Tris-HCl, 10 mM EDTA, pH 8.0 at 60 °C for 1h followed by incubation with 5 mg/mL Proteinase K at 37 °C for 1.5 h. Panel A. Phase-contrast microscopy image of non-treated viable spores. Panel B. Spores following a 1.5 h, 37 °C incubation spore coat extraction buffer. Panel C. Lysozyme digested, 25 min, urea-treated-spores. Panel D. Non-viable spores following heat treatment, 121 °C for 90 min.



(a)



(b)

Figure 4. *C. sporogenes* diluted spore DNA (a) and diluted spore suspension (b) standard curves. Isolation and detection, using real-time PCR, of *C. sporogenes* spores at levels from 10^9 to 10^2 spores/mL.

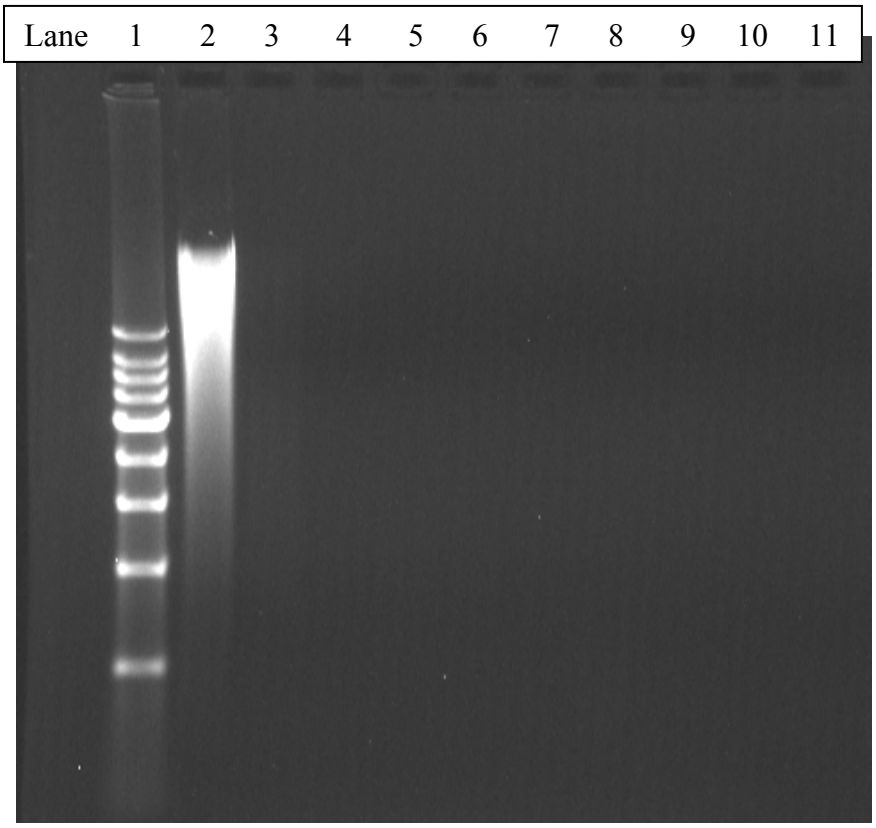


Figure 5. 1% agarose gel electrophoresis of genomic DNA isolated from thermally processed (121 °C) *C. sporogenes* DNA in 2 min intervals. Lane 1: 1kb ladder, Lane 2: Time 0 min, Lane 3: Time 2 min, Lane 4: Time 4 min, Lane 5: Time 6 min, Lane 6: Time 8 min, Lane 7: Time 10 min, Lane 8: Time 12 min, Lane 9: 14 min, Lane 10: Time 16 min, Lane 11: Time 18 min.

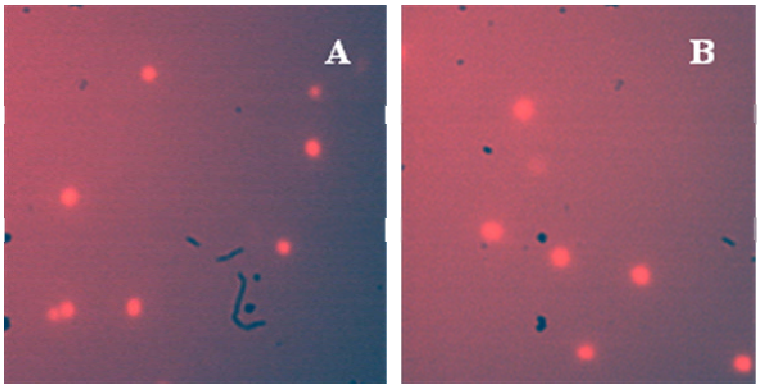


Figure 6. Fluorescence microscopy (385 nm excitation/ 565 nm emission) for propidium monoazide (PMA) stained live (Panel A) or heat inactivated (Panel B) *C. sporogenes* spores.

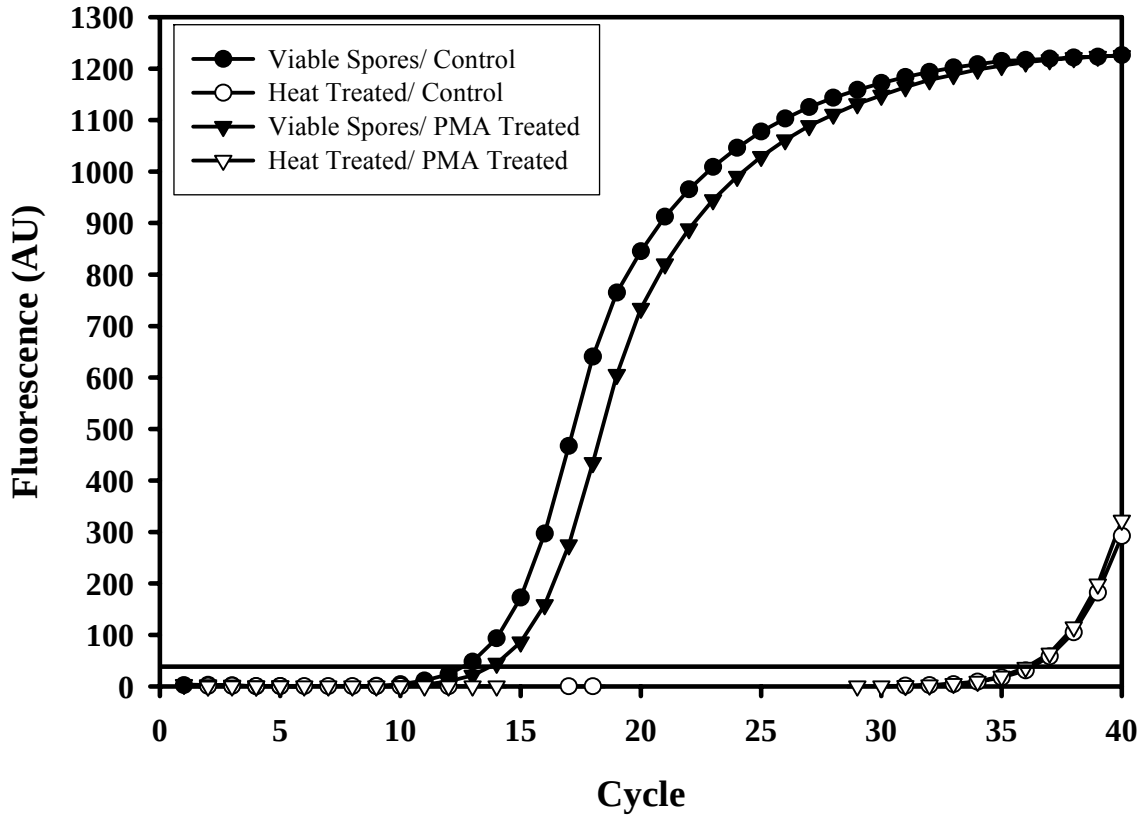


Figure 7. Viable and autoclaved (121 °C/20min) *C. sporogenes* spores with and without PMA treatment.

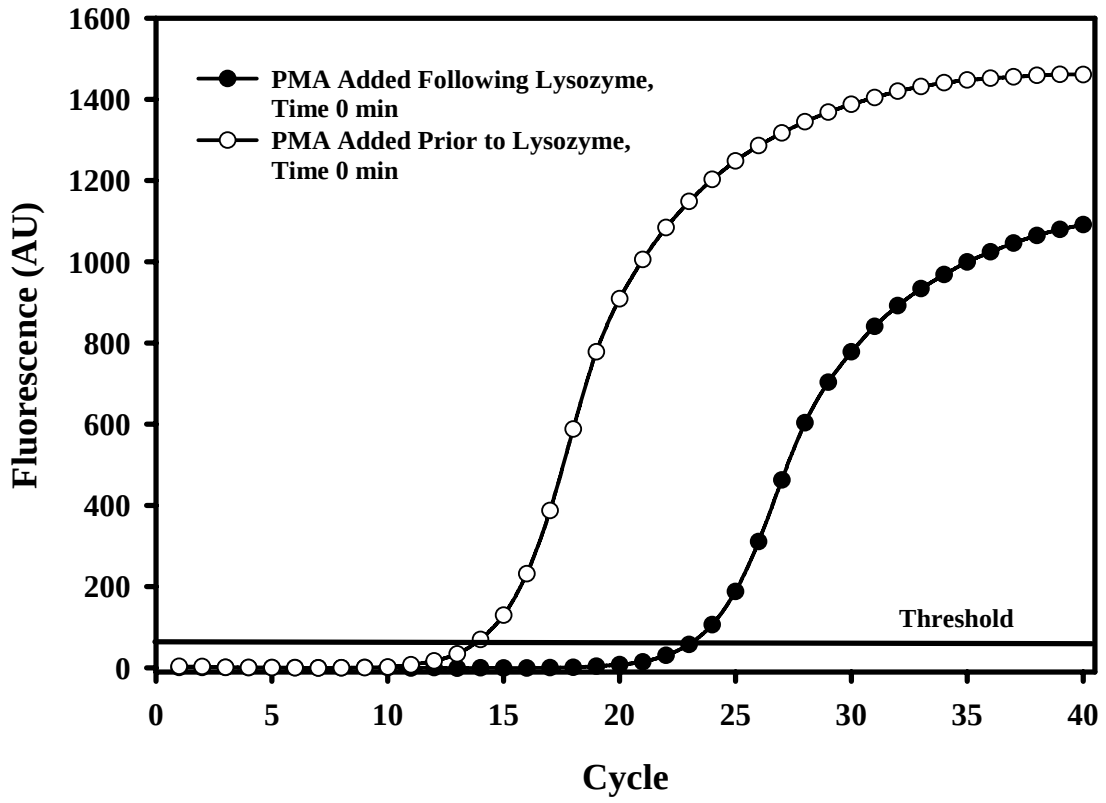


Figure 8. Viable *C. sporogenes* spores with PMA added before and after lysozyme treatment.

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CHAPTER 3

Immobilized *Bacillus* Spores for Use as Biological Indicators in Validating Aseptic Processing of a Multiphase Food Product

Abstract

Multiphase food products contain particles that move through a closed system at different rates of speed. The faster the particle moves through the system, the less stringent treatment it receives, leading to concern that the conventional 12D kill will not be obtained for all particles in the process. To date, the use of a biological indicator that travels through the entire process stream, retrieved and then enumerated to determine lethality of the process, has been the best approach to determine the efficacy of multiphase food product processing. The purpose of this research was to develop alginate-immobilized *Bacillus* spores to be used as biological indicators in simulated food particles to validate a continuous aseptic microwave process of a multiphase food. Spores crops for *Geobacillus stearothermophilus* and *Bacillus subtilis* were produced and their thermal inactivation kinetics, when suspended in water, alginate and salsa con queso, were determined. *G. stearothermophilus* and *B. subtilis* at 121 °C suspended in sodium alginate had D-values of 3.26 and 0.35 min, respectively. The D-values in the other suspension media at the same temperature were not statistically significantly different. The Z_D-values obtained for alginate-suspended spores were 7.14 and 9.2 °C for *G. stearothermophilus* and *B. subtilis*, respectively. The spores immobilized in 3% sodium alginate could be used to produce beads of approximately 30 μL in volume and when compared to non-immobilized spores, there was not statistically significant difference in viable counts. The beads were placed in simulated particles, inserted into an aseptic continuous microwave process, retrieved and enumerated. Mechanical difficulties complicated timely recovery of the particles; therefore, relevant microbiological data

were inconclusive. However, the alginate-encapsulated spores maintained their physical integrity even after exposure to rigorous time and temperature combinations. This study demonstrates the feasibility of using alginate-immobilized spores of *G. stearothermophilus* and *B. subtilis* as biological indicators in simulated food particles for validation of thermal processing.

Introduction

Aseptic processing is defined as sterilization of product and packaging material in batch, whereby the product sterility is maintained during filling and sealing of the package (Hersom, 1985; Morris-Lee, 2004). The process usually utilizes high temperature short time (HTST) treatment and has the advantage of resulting in higher quality products (Hersom, 1985) because the high temperature destroys target microorganisms while the short time allows for the retention of nutrients and product quality attributes (Lee and Singh, 1990; Zhang et al., 2001). From a food safety perspective, *Clostridium botulium* is the most serious sporeformer of concern in any sort of commercial sterilization process (Brown, 2000), which has lead to the 12D approach to inactivate spores of *C. botulinum* (Collado et al., 2003).

A multiphase food product is a food that contains solid food pieces, also referred to as particulates. Development of aseptic processes for multiphase foods has been complicated largely because of difficulties in proving that the fastest moving particle has been exposed to a sufficiently lethal treatment (Larkin, 1997; Morris-Lee, 2004). For example, validation of such processes is challenging because it is not possible to measure the temperature of a food particle as it flows through the system with currently available technologies (Digeronimo et al., 1997). Presently there are two methods to evaluate a thermal process efficacy, i.e., in-situ and physical-mathematical methods (Guiavarc'h et al., 2002; Van Loey et al., 1996). In situ methods measure safety or quality attributes such as microbial counts or other changes in organoleptic quality and is limited by frequent analytical or sampling problems. Mathematical-physical methods rely on recording of temperature data followed by generation of a mathematical model to predict lethality. Time-temperature integrators (TTI) can be enzymes (such as amylases or peroxidases) whose activity is time-temperature dependant; their enzymatic activity changes upon heating

which can be measured in a simple manner, usually by colorimetric means. To date there are no commercially available TTI's which has further complicated validation of process efficacy for multiphase aseptically processed food products.

In the absence of reliable TTIs, three methods are commonly used to evaluate process efficacy; including the use of thermocouples, chemical indicators and/or biological indicators (Serp et al., 2002). Similar to TTI's, chemical indicators are based on enzyme reactions, sugar inversion, or color changes which roughly correlate with time-temperature treatment and hence provide information complementary to traditional thermocouple results (Holdsworth, 1997). Thermocouples are designed to measure the heat-penetration rates at a selected point in a product container (Hersom and Hulland, 1980; Holdsworth, 1997). A biological indicator is a surrogate organism that has similar thermal inactivation properties to the target pathogen for the food product-process combination (FDA, 2000). Biological verification uses one or more non-pathogenic microorganisms with similar thermal inactivation profiles to the target pathogen(s). These are placed in the product pre-process, travel through the entire process stream, and are retrieved and enumerated to determine lethality. To date, this method has been the best approach to determine the process lethality for multiphase food products. This is because the surrogate is incorporated into the product and subjected to the same treatment as might be encountered by an naturally-occurring microbial contaminant (Shintani and Akers, 2000; Marcy, 1997).

Several alternative processing technologies exist that can be used in place of traditional thermal processing for food products. These alternative technologies include microwave and radio frequency, ohmic and inductive heating, high pressure, pulsed electric field, high voltage, pulsed light, oscillating magnetic fields, ultraviolet light, ultrasound and x-rays (FDA, 2000). For these alternative technologies to be used commercially, the most resistant pathogen(s) of

greatest public health significance must be determined (which is likely to be product and process-specific), and the mechanisms for microbial inactivation need to be identified. Of the above technologies, the two which are based on thermal inactivation are microwave and ohmic heating. The microorganisms of concern in microwave and ohmic heating are therefore similar to those used for evaluating traditional thermal processing efficacy (i.e., *C. botulinum* for commercial sterilization processes).

Of the various alternative technologies, the one of interest for this study was microwave. Numerous bacteria have been found to be inactivated by microwave energy; parasites have shown some resistance and at this time, and virus inactivation has not been studied (NACMCF, 2004). Some have reported that microwave heating is more effective in inactivating spores, including those of *Geobacillus stearothermophilus* and *Clostridium sporogenes*, than is conventional heating (Chipley, 1980). It is believed that bacterial spores are killed because of the heat generated from surrounding water molecules which absorb the microwave energy, since spores contain very little water themselves and do not readily absorb microwave energy (Sasaki et al., 1997). Even though microwave processes may use somewhat lower temperatures and shorter times than do conventional thermal processes, no known pathogenic or non-pathogenic microorganisms have been found to be particularly resistant to microwave heating. Consequently, the same surrogate microorganisms that are used in thermal process validation should be applicable for use as biological indicators in microwave processing (FDA, 2000).

Multiphase food products contain particles that move through a closed system at different rates of speed. The faster the particle moves through the system, the less thermal processing it receives, leading to concern that the conventional 12D kill will not be obtained for all particles in the process. A method needs to be developed to biologically validate that the fastest moving

particle through the system receives the required heat treatment to be considered commercially sterile. The purpose of this study was to develop highly heat resistant immobilized spores to be used in a particle flow monitoring system for biovalidation of multiphase food processing. Specifically, the first objective was to produce highly heat resistant spores to be used as *C. botulinum* surrogates and validate their heat resistance. A method to immobilize the spores in alginate beads was developed and the population consistency and thermal death kinetics in alginate were measured. Lastly, to evaluate concept feasibility, the immobilized spores were placed in simulated particles and used in microwave processing simulations.

Materials and Methods

Bacillus strains and spore production

Bacillus subtilis (ATCC 35021) (American Type Culture Collection; Manassas, VA) and *Geobacillus stearothermophilus* (ATCC 7953) were grown overnight in 10 ml of brain heart infusion (BHI) (BBL/Difco; Franklin Lakes, NJ) and incubated at 37ºC and 55ºC respectively. Overnight cultures (0.5 ml) were spread plated on 150 x 15mm Petri plates containing media consisting of 13g nutrient broth (BBL/Difco) supplemented with 0.51g $MgSO_4 \cdot 7H_2O$, 0.97g KCl, 0.2g $CaCl_2 \cdot 2H_2O$, 0.003g $MnSO_4 \cdot H_2O$, 0.0005g $FeSO_4 \cdot 7H_2O$ (Sigma Aldrich; Saint Louis, MO) and 1.5% agar per liter. Plates were incubated aerobically for 3-5 days at 37ºC for *B. subtilis* and 55ºC for *G. stearothermophilus*. Once greater than 95% spores were obtained as determined phase contrast microscopic smear, 10 ml of cold sterile distilled H_2O (dH_2O) were added to the plates and the surfaces scraped using a disposable hockey stick. Spores of *G. stearothermophilus* were sonicated (Branson Digital Sonifier 450; Danbury, CT) for 4 min at a 55 amplitude for a pulse of 10 sec on and 5 sec off; crude spore suspension of *B. subtilis* were not sonicated. Spores were washed repeatedly (5-10 times) with cold sterile dH_2O and centrifuged (16,270 x g) at 4°C for 20 minutes, until less than 5% vegetative cells remained, which was validated under phase contrast microscopy (Olympus BH-2; Center Valley, PA). Spores were resuspended in dH_2O and stored at 4ºC until use. The concentrations of the individual spore crops of *G. stearothermophilus* are approximately 10^8 spores/ml and *B. subtilis* are approximately 10^9 spores/ml.

Spore Enumeration

For enumeration of spore populations both before and after thermal exposures, 10-fold serial dilutions were prepared in buffered peptone water (BPW) and plated in duplicate on BHI

agar. Plates were incubated at 35ºC (*B. subtilis*) or 55ºC (*G. stearothermophilus*) for 24-48 hours. All plating was done in duplicate or triplicate.

Alginate immobilization of spores

A 3% sodium alginate (Fluka; Switzerland) solution was prepared by suspending the alginate in distilled water followed by sterilization by autoclaving for 20 min at 121ºC. The sterilized alginate solution was mixed with an equal volume of undiluted spore crop, resulting in a colorless suspension. This colorless suspension was used for production of the *G. stearothermophilus* alginate particles; the *B. subtilis* spore suspension was colored using FD&C Blue No. 1 Aluminum Lake (Hilton-Davis; Cincinnati, OH). In this case, a 1.2% solution of the blue color was made by suspending the dye in sterile water and the resulting solution was added to the alginate-spore mixture bringing the color concentration to 2.5%. The alginate-spore suspension were pipetted using a finnpipette repeater set (Thermo Fisher Scientific; Waltham, MA) in approximately 30µl volumes into a 100mM $CaCl_2$ (Sigma Aldrich; Saint Louis, MO) solution. Alginate-spore beads formed instantaneously. The beads were removed from the calcium chloride solution using sterile tweezers and placed in a sterile screw cap conical tube containing sterile dH_2O. They were stored at 4ºC and plated monthly to assure population consistency.

The spore-alginate complex was disrupted, releasing the spores, by placing the beads in a 1:10 dilution of 50mM sodium citrate for 30 minutes with continuous vortexing at room temperature. Validation experiments were done to assure that the alginate suspension and resulting particle formation did not inactivate the spores, and that the spore populations were consistent between individual beads. These experiments were done by comparing the bead populations using the statistical software InStat (GraphPad Software; San Diego, CA).

Capillary tube method for determination of D-values

Sterile glass capillary tubes (Rupe Zellner; Tonawanda, NY) with a 3 mm outer diameter were prepared by adding 50 µl of spore crop (undiluted spore suspensions at a concentration of approximately 10^8 - 10^9 spores/ml) and heat sealed with a Bunsen burner. Capillary tubes were placed in an oil bath (Haake L/D8 Fisions; Waltham, MA) at 110, 115, 118, 121ºC for *B. subtilis* and at 115, 121, 124 and 126ºC for *G. stearothermophilus* at various time intervals. The time intervals for *B. subtilis* were 3 min, 1 min, 30 sec, and 15 sec at 110, 115, 118 and 121ºC, respectively. Those for *G. stearothermophilus* were 8 min, 2 min, 1 min and 30 sec at 115, 121, 124 and 126ºC, respectively. Capillary tubes were removed from the oil bath and placed immediately in an ice bath for 5 min, and then sterilized by immersion in a 3% hypochlorite solution for 30 sec. The ends of the capillary tube were removed using a glass cutter and 5 ml of peptone was flushed through the tube to remove the spore sample followed by 10-fold serial dilution and plating for enumeration.

Thermal inactivation kinetics were also determined using the same method as applied to spores suspended in 3% sodium alginate (1:1 v/v), and for spores supended in Tostitos® salsa con queso (pH 5.99) (Frito Lay; Dallas, TX). In the latter instance, the spore suspension was mixed 1:1 (v/v) with the salsa con queso. All other experimental considerations, including the capillary tube method and time-temperature combinations, were the same as those described above.

D-values were calculated by graphing the time (min) versus $\log_{10}$ CFU/ml in Microsoft Excel™ (Microsoft; Redmond, WA) and expressed as the negative reciprocal of the slope. Z_D-values were determined by averaging the three D-value replicates for each organism-time combination and plotting the temperature (^oC) versus the $\log_{10}$ of the average D-value. The Z_D,

defined as the temperature change necessary to change the D-value by one $\log_{10}$, was calculated as the negative reciprocal of the slope of this line. Comparison of D-values was done by analyzing the differences in the means (t-test) using the Mixed Procedure of SAS (Cary, NC). A t-test on the Z_D values was done using the statistical software InStat (GraphPad Software; San Diego, CA).

Simulated particle biovalidation

Simulated "particles" were developed and manufactured at North Carolina State University. The particles were made of polymethylpentene (PMP) in the shape of a cube with 1.3 mm outer dimensions and a cylindrical inner cavity able to hold a maximum volume of 1 ml. A magnet that can track residence time was placed in the cap of the particle and covered with a gasket. Two spore-alginate beads of either *G. stearothermophilus* (1.1×10^7 spores/bead) or *B. subtilis* (5.9×10^8 spores/bead) were added to the inner cavity of the particle along with 150 µl of sterile dH_2O and the particles were sealed by pressing the lids together. Two beads each of *G. stearothermophilus* and *B. subtilis* were also added to the inner cavity of the same particles so that one particle would contain both types of *Bacillus* spores. In this case, the beads were suspended in 100 µl of sterile dH_2O and sealed. Each particle was weighed to make sure they fell within the range of 1.88 – 2.03 g. Particles containing spore beads were stored at 4ºC until ready to use and were held no longer than 4 months.

60 kW Continuous Flow Microwave Heating Unit

A pilot scale 60 kW continuous flow microwave heating unit (Industrial Microwave Systems; Research Triangle Park, NC) operating at 915 MHz was used in this study. The temperatures were measured with thermocouples positioned at the inlet of the system, the inlet and exit of each applicator and the holding tube, as shown in Figure 2. The temperatures were

recorded using a datalogging system (HP 3497A, Agilent Technologies; Palo Alto, CA). The power supplied to the microwave system was adjusted to control the experimental temperatures of 126 ˚C, 132 ˚C, and 138 ˚C. The system was preheated by pumping hot water at 130 ˚C and recirculating it for approximately 30 minutes. The matrix was then loaded into the system and the biological indicators were injected either through the hopper or the injection tubes A/B (Figure 9). The matrix was then heated to each processing temperature, with a residence time of 25 s in the holding tube, followed by rapid cooling to 70 – 80 ˚C in a tubular heat exchanger. Biological indicators were collected at the end of the cooling tubes, placed in an ice slurry, and immediately taken to the laboratory for microbiological analysis.

Results

Production of spore crops

Sporulation of *B. subtilis* was achieved using a modified nutrient media which produced a consistently high yield of spores. For *G. stearothermophilus*, the same media did not work consistently, with initial attempts providing spore yields of less than 50%. It soon became clear that harvested *G. stearothermophilus* had to be stored for at least a month at 4°C after sonication before sporulation would improve to over 95%. Storing the mixture of spores and vegetative cells at refrigeration temperatures helped to create a more stressful environment for the vegetative cells that would lead to increases in sporulation. Even then, there were lot-to-lot differences in sporulation for *G. stearothermophilus*.

Producing a highly heat resistant spore crop within a specified D-value range was also challenging. Several attempts were made to increase heat resistance by pre-treating spores at high temperatures (100 – 121°C), plating, and choosing the survivors to be used as the inoculum for more resistant spore crops. This method was both time consuming and relatively ineffective. We then obtained highly resistant spore inoculum (both *B. subtilis* and *G. stearothermophilus*) from an outside source (SGM Biotech Inc, Bozeman, MT), and used these to propagate the heat resistant spore crops used in subsequent studies. These crops functioned as anticipated within the thermal inactivation ranges of interest and produced minimal variability in survivor curve analysis.

Thermal Inactivation of *G. stearothermophilus* and *B. subtilis* spore crops

The individual D-values for each replicate at each temperature and in each suspending medium are shown in Appendix C. For ease of comparison, these data are summarized, along with the corresponding statistical analysis, in Figure 10 and Table 4. The *B. subtilis* spores were

inactivated at temperatures ranging from 110°C to 121°C and at temperatures of 115°C to 126°C for *G. stearothermophilus*. With the exception of the lowest temperature tested, there were no statistically significant differences in D-values when comparing type of suspending medium; this relationship held true for both organisms. At the lowest temperature tested for *B. subtilis*, (110°C), the D-value corresponding to spores suspended in queso was 7 min longer than the D-value for the other two matrices. On the other hand, the D-value for *G. stearothermophilus* suspended in queso and treated at the low temperature of 115°C was actually 15 min shorter than that for the other matrices. In both of these instances, the D-values corresponding to the queso suspensions were statistically significantly different from the other matrices. On the other hand, for both organisms, the alginate and water suspensions gave D-values that were not statistically significantly different from one another at the lowest temperatures tested.

The temperatures and log D-values were plotted to obtain the decimal reduction time curves and calculate the z_D-values for each suspension medium as shown in Figure 11. The *B. subtilis*-alginate suspension showed a higher z_D-value (9.2°C) compared to those for *B. subtilis* suspended in water or queso (both around 7°C). The z_D values for *G. stearothermophilus* were higher in the queso (8.3°C) compared to both the alginate and water suspensions (7.14°C). None of these differences, however, were statistically significant.

Validation of spore population consistency in sodium alginate beads

Alginate-immobilized spores of *B. subtilis* and *G. stearothermophilus* were evaluated by microbiological plating to assure that no spores were inactivated in the process, and for consistency of population density. Results reported in Table 5. The *B. subtilis* particles were evaluated with and without the addition of Aluminum Lake Blue #1 dye to verify that the addition of color did not negatively impact the spore population. No statistically significant

decreases in spore population occurred as a consequence of alginate immobilization or addition of Aluminum Lake Blue. Initial population of beads was determined by plating a one bead equivalent of the spores mixed with alginate without immobilizing the suspension in calcium chloride. All beads of both *G. stearothermophilus* and *B. subtilis* had a greater than 91% recovery when compared to the one bead equivalent. The standard deviation between spore immobilized beads populations was 0.04 for *G. stearothermophilus* and 0.08 for blue *B. subtilis* beads, showing a high degree of consistency which was verified by statistical analysis that revealed no significant differences in populations when comparing individual beads.

Microwave with Simulated Particles

In the first microwave run, simulated particles carrying alginate immobilized spores of *B subtilis* (approximately 10^9 spores/bead) and *G. stearothermophilus* (approximately 10^6 spores/bead) were inserted into a salsa con queso product in the microwave system and the time that each particle entered the system was recorded. Both the temperature at the time the particles were inserted, and the amount of time in the system, were erratic due to mechanical difficulties. The product temperature was well below the set temperature (118 °C), even though the hold time was for several hours; however, a good portion of this hold time was in the holding tube, as the cubes became lodged at this location in the system. Although 10 particles containing *B. subtilis* were retrieved from the system, spore populations were over 10^8 CFU/alginate bead, suggesting minimal thermal inactivation (data not shown).

In the second microwave run, carboxymethylcellulose (CMC) solution (TIC Gums; Belcamp, MD) was substituted for the salsa con queso due to similar viscosity and was used as the heating menstrum. Although the set temperature was 118°C, the actual processing temperature is unknown. Again, mechanical problems impacted the experiment, and particles

were retrieved from the system within several hours of insertion. In this case, there were no detectable surviving spores of either *B. subtilis* or the *G. stearothermophilus*. Based on the initial input and limit of detection, the approximate $\log_{10}$ reduction for *B. subtilis* was estimated at 7.0 and the log reduction for *G. stearothermophilus* was approximately 4.0 (data not shown).

In the final microwave attempt, again using CMC, the set temperature was 115°C with the true measured temperature being 125°C. All 30 particles were retrieved from the system within several hours of insertion. Two of the particles containing *G. stearothermophilus* had counts of approximately 10^5 spores/per particle. One of the particles with a positive plate count also contained *B. subtilis* beads that were inactivated. All other particles containing alginate beads had no detectable level of spores surviving (detection limit of <100 spores/ml) the microwave process (Table 6).

Discussion

Spore crops of *B. subtilis* and *G. stearothermophilus* were produced and used in a variety of ways in an effort to develop a means by which to validate thermal inactivation targeting sporeforming pathogens in particulate food products. For a biological indicator to be a relevant surrogate, it must have similar thermal inactivation properties to the pathogen of concern (FDA, 2000). The thermal resistance properties of the *B. subtilis* and *G. stearothermophilus* spore crops developed in this study were similar to values reported previously by others (Russell, 1982; Montville and Matthews, 2005; Hernandez et al., 1994). For example, the thermal death time that we observed for *B. subtilis* spores suspended in deionized water was 0.23 min at 121°C, which is right at the targeted reference D-value for *C. botulinum* of 0.20 min at 121.1°C (Quesnel, 1984). As expected, the D-value for *G. stearothermophilus* suspended in deionized water exceeded the *C. botulinum* target. The *G. stearothermophilus* D-value of 3.26 min at 121.1°C, would equate to approximately 16.3 $\log_{10}$ kill of *C. botulinum*, or a 16D process. *G. stearothermophilus* is the most heat resistant organism of the *Bacillus* genus and Sasaki et al (1998) found it to be the most resistant to conventional and microwave heating when comparing the thermal resistance profiles of the spores of 12 different *Bacillus* species.

A 1:1 (v/v) mixture of the spores and 3% sodium alginate, without subsequent particle formation, was used to provide an estimation of the thermal resistance of immobilized spores. Although there was a slight increase in thermal resistance for alginate suspensions of both *B. subtilis* and *G. stearothermophilus*, this was not significantly different from values achieved for spores suspended in water, suggesting that the sodium alginate would not adversely affect the thermal inactivation kinetics. For immobilization, the spore-alginate mixture was dropped into a 100 mM solution of calcium chloride. Although calcium chloride has been shown to increase

overall heat resistance when used as a suspension medium, others have shown that when alginate particles are suspended in water during thermal inactivation (as they were done in this study), the small amount of calcium chloride used in making spore beads provided no additional increase in heat resistance (Serp et al., 2002).

Thermal inactivation kinetics were somewhat different, but not statistically significant, when both spore crops were suspended in a 1:1 solution of a model food product, in this case, salsa con queso. For *B. subtilis* there was an increase in heat resistance at the lowest temperature (110°C) tested, which may be attributed to a protective effect associated with the high lipid content of the salsa con queso. In a study by Molin and Snygg (1967), *B. subtilis* had an increase in heat resistance when suspended in various oils relative to a phosphate buffer-based spore suspension. In the same study, the authors did not observe the same effect for *G. stearothermophilus*, where oil versus buffer suspension did not significantly impact D-values. In our study, the D-values of *G. stearothermophilus* in salsa con queso actually decreased relative to water and alginate suspensions at the lowest treatment temperature of 115°C. This is similar to the findings of Fernandez et al. (1994), who reported that the D-values for *G. stearothermophilus* heated in a mushroom substrate were 10 min lower at 115°C when compared to a water suspension. In another study of *G. stearothermophilus* heated in a mushroom substrate, the organism was again less heat resistant in comparison to the reference medium (Periago et al., 1998). The observed increases or decreases in heat resistance for the two different types of organisms could be associated with synergistic or inhibitory interactions between the suspending matrix and the organisms. For example, salsa con queso contains 0.8 % sodium and could also contain other additives including, monosodium glutamate, sodium citrate, sodium phosphate and sodium hexametaphosphate that could increase heat resistance or decrease heat

resistance, a relationship which is likely organism-dependent. Previously reported Z_D values for *B. subtilis* and *G. stearothermophilus* have ranged from 5.7 – 9.5°C (Feeherry et al., 1987; Bender and Marquis, 1985; Nakayama et al., 1996; Periago et al., 1998; Haas et al., 1996; Serp et al., 2002). These are consistent with the Z_D values observed for all suspension media used in our study. Serp et al. (2002) found that the z-values of *B. subtilis* and *G. stearothermophilus* were consistent when suspended in water or in immobilized form. The standard Z_D value for *C. botulinum* is 10°C. Ideally, it would be best to have a Z-value that was higher than *C. botulinum* so any change in temperature would reflect overkill in *C. botulinum*. In this case both organisms are lower than the standard, and the lethality rate at the processing temperature would have to be calculated against the standard to account for the discrepancy in Z_D values. Developing a surrogate organism to have a D-value at or higher than the target organism is difficult, which makes creating a surrogate with a set Z_D value virtually impossible.

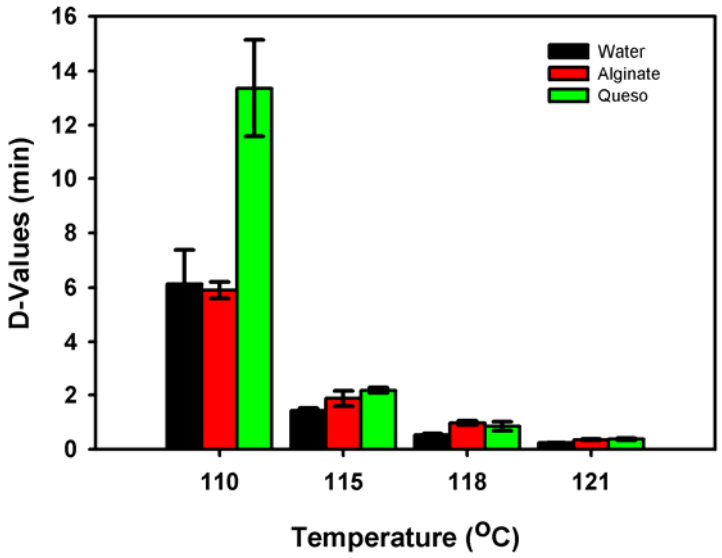
When individual beads containing alginate-immobilized spores were compared to one another, spore populations were relatively consistent. Furthermore, predicted spore population was consistent with what was recovered by plating, demonstrating that immobilizing spores in alginate would be an effective means by which to use them in thermal processing experiments. When comparing spore populations using the one-bead equivalent value, there was a slight increase in the population of the *B. subtilis* in the immobilized bead form, but no statistical significance was found. One explanation for this could be that upon gelling, water is lost due to gel shrinkage, causing the spore population to become more concentrated (Smidsrod and Skjak-Braek, 1990). One of the concerns in using the alginate beads was storage stability and leaching. In a study on the effect of storage on *C. sporogenes* and *G. stearothermophilus* spores immobilized in alginate, Dallyn et al. (1977) found that after a year at 4°C, there were no

changes in the estimated and actual populations nor was there a significant change in heat resistance of the spores. In our study, the *B. subtilis* and *G. stearothermophilus* alginate particles that were created with and without FD&C Blue #1 stored at 4°C showed no changes in population during the 4 months of storage from when the beads were made until they were used (data not shown).

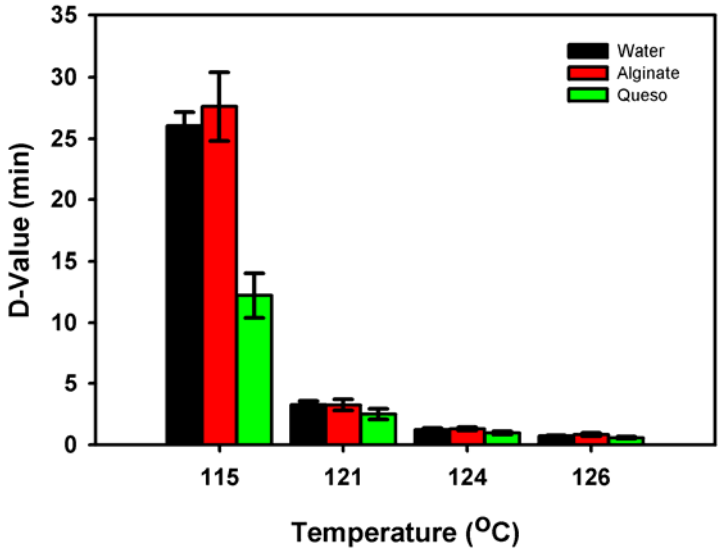
The results obtained from the simulated particles that were inserted into the microwave process are inconclusive. Several mechanical issues complicated recovery of the particles in a timely manner; further, there were consistent difficulties in with the particles lodging in the hold tube, as well as significant temperature flux. Nonetheless, the alginate-encapsulated spores that were encased within the particles maintained their physical integrity throughout such extensive processing time and temperature combinations. For the two particles that entered and exited the system properly in the last microwave processing attempt, it shows that there is promise for the future experiments to demonstrate the safety of aseptically processing multiphase food products using simulated food particles. In one of the particles that contained both *B. subtilis* and *G. stearothermophilus*, the *B. subtilis* was completely inactivated and the *G. stearothermophilus* had a 2-log decrease in population. Combining multiple types of spores with various D-values could further add to processing validation showing the target organism was inactivated and the over target organism could be used to determine process lethality, which could cut down the total number of samples to be used. This shows promise for the near future that it is possible to determine the lethality of the microwave process at lower processing temperatures.

This study demonstrates the feasibility of using alginate-immobilized *B. subtilis* and *G. stearothermophilus* as biological indicators in thermal process validation. The alginate-immobilized spores were consistent in size and spore population, and remained stable for long

periods of time when stored at 4°C. The alginate beads facilitated the use of a standardized inoculum population in thermal inactivation studies. When placed in the simulated particles, the alginate beads were protected from the product, preventing product contamination; the beads could also be easily removed from the particles after they were retrieved from the process stream. Because the particles have a cylindrical inner cavity capable of holding a 1 ml volume, and the alginate beads are of low volume, there is plenty of additional space available for placement of a magnet to track residence time, a small thermometer, and/or a miniaturized TTI device. To our knowledge, this is the first attempt to produce a model system to monitor thermal process efficacy in a size and shape consistent with that of a food particle. Future research will focus on using the immobilized spores in conjunction with chemical (enzymatic) indicators and time/temperature monitors in an effort to develop a simulated particle that is able to monitor all major process parameters while simultaneously providing microbiological data on process lethality.



(a)



(b)

Figure 9. ***B.subtilis*** **(a) and** ***G. stearothermophilus*** **(b) spore crop D-values suspended in water, sodium alginate and salsa con queso. Based on mean and standard deviation of 3 replicates.**

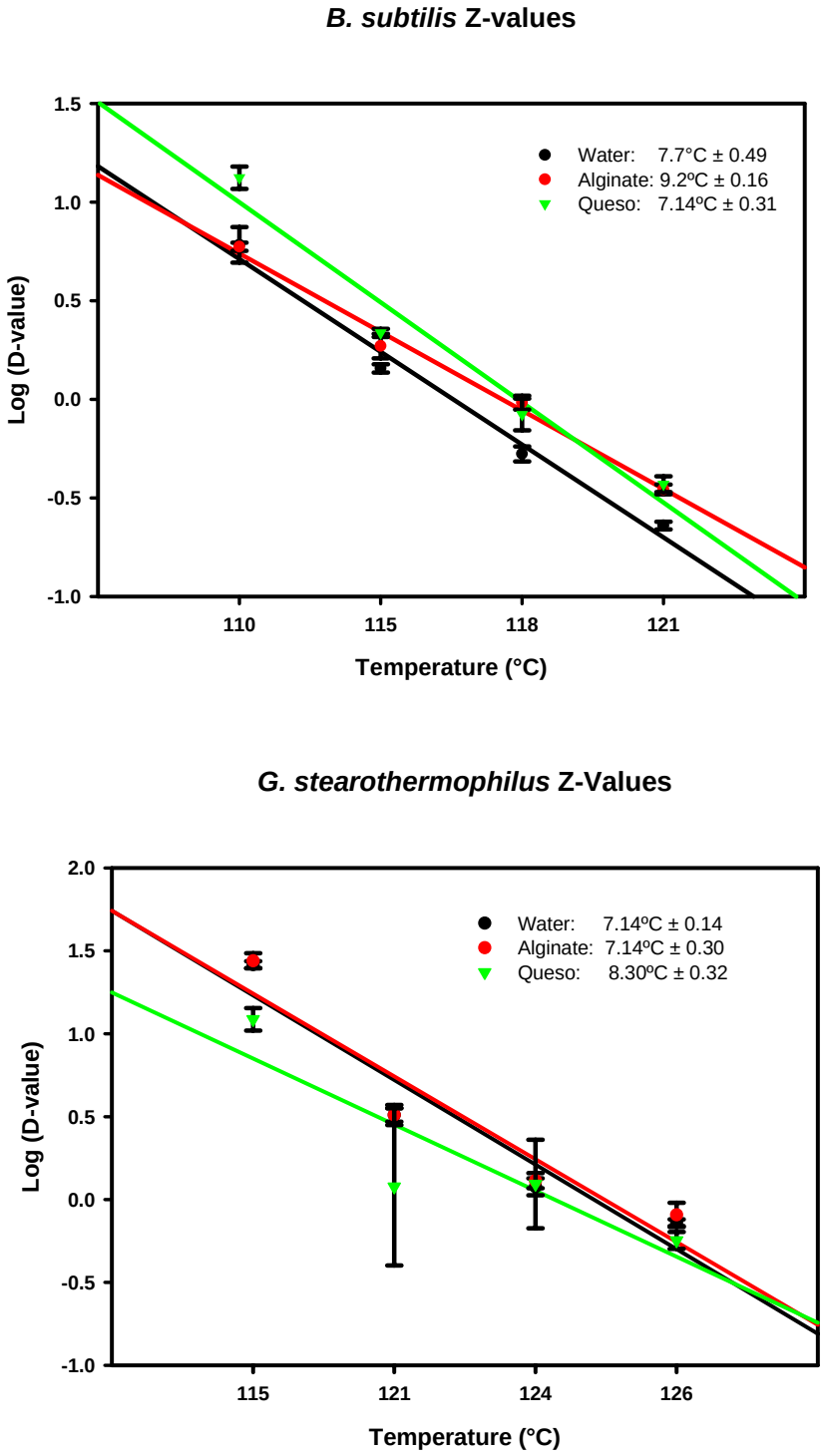


Figure 10. Z_D-values of *B. subtilis* and *G. stearothermophilus* spores suspended in water, sodium alginate and salsa con queso. Based on mean and standard deviation of 3 replicates for survivor curve.

Table 4. Means of D-values of *G.stearothermophilus* and *B. subtilis* in the differing types of media and temperatures.

G. stearothermophilus				
Media	**115°C**	**121°C**	**124°C**	**126°C**
Water	26.1^{a}	3.26^{b}	1.2^{b}	0.72^{b}
Alginate	27.61^{a}	3.24^{b}	1.3^{b}	0.82^{b}
Queso	12.22^{c}	2.48^{b}	0.95^{b}	0.57^{b}

B. subtilis				
Media	**110°C**	**115°C**	**118°C**	**121°C**
Water	6.14^{a}	1.43^{b}	0.53^{b}	0.23^{b}
Alginate	5.91^{a}	1.88^{b}	0.97^{b}	0.35^{b}
Queso	13.36^{c}	2.18^{b}	0.84^{b}	0.37^{b}

a, b, c: means that share the same superscript numbers are not significantly different from each other; means with different superscript numbers are significantly different ($p < 0.05$)

Table 5. Consistency (log spores/mL) of alginate-immobilized beads of *G. stearothermophilus* and *B. subtilis*. Mean and standard deviation of 5 repetitions. No statistical differences between means ($p > 0.05$).

Microorganism	Crop	Alginate Mix	Bead Equivalent	Alginate Beads	% Recovery
G. stearothermophilus	8.36 ± 0.05	8.11 ± 0.06	8.81 ± 0.02	8.08 ± 0.04	91.7
B. subtilis	9.28 ± 0.03	8.86 ± 0.06	8.95 ± 0.03	9.66 ± 0.04	107.9
B. subtilis Blue	9.21 ± 0.03	8.84 ± 0.04	8.76 ± 0.08	9.41 ± 0.08	106.8

Table 6. Encapsulated *Bacillus* spores in simulated particles, processed through the continuous microwave and enumerated.

B. subtilis	Particle Weight (g)	Time Entered System	Time Exited System	Plate Count
C51	1.92	Control	Control	4.3×10^9
C52	1.88	Control	Control	9.95×10^8
C46	1.94	14:51:18		<100 spores/ml
C70	1.93	14:59:18		<100 spores/ml
C33	1.98	14:53:26		<100 spores/ml
C29	1.9	14:55:32		<100 spores/ml
C69	1.9	15:07:32		<100 spores/ml
C66	1.94	15:04:38		<100 spores/ml
C30	1.92	15:10:30		<100 spores/ml
C39	1.97	15:06:04		<100 spores/ml
C58	1.89	15:02:22		<100 spores/ml
C68	1.93	14:57:48		<100 spores/ml

G. stearothemophilus	Particle Weight (g)	Time Entered System	Time Exited System	Plate Count
F116	1.94	Control	Control	2.7×10^6
F126	1.95	Control	Control	2.9×10^6
F119	1.93	14:54:52		9.0×10^5
F135	2.03	15:01:32		<100 spores/ml
F136	1.98	15:09:45		<100 spores/ml
F107	1.97	15:03:53		<100 spores/ml
F124	1.93	15:11:13		<100 spores/ml
F93	1.92	15:09:00		<100 spores/ml
F99	1.98	15:08:15		<100 spores/ml
F133	1.93	14:57:02		<100 spores/ml
F98	1.99	15:00:47		<100 spores/ml
F95	1.95	14:52:41		<100 spores/ml

Table 6. (cont)

Combination Particle Code	Particle Weight (g)	Time Entered System	Time Exited System	Plate Count G. *stearothermophilus*	Plate Count *B. subtilis*
215	1.90	Control	Control	4.85×10^6	1.6×10^9
230	1.89	Control	Control	5.5×10^6	2.7×10^9
239	1.95	15:03:07		<100 spores/ml	<100 spores/ml
227	1.94	15:06:47		<100 spores/ml	<100 spores/ml
204	2	14:54:07		<100 spores/ml	<100 spores/ml
203	1.95	14:56:16		<100 spores/ml	<100 spores/ml
249	1.9	14:58:33		<100 spores/ml	<100 spores/ml
242	1.95	15:12:01		<100 spores/ml	<100 spores/ml
214	1.94	14:51:56		1.45×10^5	<100 spores/ml
213	1.96	15:00:03		<100 spores/ml	<100 spores/ml
201	1.93	15:05:19		<100 spores/ml	<100 spores/ml
244	1.93	15:12:57		<100 spores/ml	<100 spores/ml

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APPENDIX A

BACILLUS SPORULATION PROTOCOL

Bacillus Heat Resistant Media

- 13 g Nutrient Broth
- 0.51 g $MgSO_4 \cdot 7H_2O$
- 0.97 g KCl
- 0.2 g $CaCl_2 \cdot 2H_2O$
- 0.003 g $MnSO_4 \cdot 7H_2O$
- 0.00055 g $FeSO_4 \cdot 7H_2O$
- 15 g Agar
- 1000 mL dH_2O

Sporulation Protocol for *B. subtilis*

- Grow stock culture overnight in 10 mL BHI
- Streak culture on BHI and incubate at 35 °C for overnight
- Take a single colony and transfer to 10 mL BHI
- Spread plate overnight culture on *Bacillus* heat resistant media
- Incubate at 35 °C for 3 – 5 days
- Harvest spores using sterile dH_2O and scraping the surface of the plate with a sterile hockey stick
- Follow with cleaning proctol

Sporulation Protocol for *G. stearothermophilus*

- Grow stock culture overnight in 10 mL BHI
- Streak culture on BHI and incubate at 35 °C for overnight
- Take a single colony and transfer to 10 mL BHI
- Spread plate overnight culture on *Bacillus* heat resistant media
- Incubate at 55 °C for 3 – 5 days
- Incubate plates at 4 °C for 2 days
- Harvest spores using sterile dH_2O and scraping the surface of the plate with a sterile hockey stick
- Follow with cleaning protocol

BIPHASIC MEDIA FOR SPORULATION OF *CLOSTRIDIUM*
(Zuylen, Andre-Van, personal communication, April 11, 2007)

Biphasic medium consists of a liquid phase and a solid phase.

Liquid phase (TPGS)

tryptone (Difco)	5 %
bactopeptone (Difco)	0.5 %
glucose	0.2 %
soluble starch	0.2 %
cysteine	0.05%

pH 6.8
sterilise 15 min at 120 °C

Solid phase

meat extract (Liebig)	1.67 %
bactopeptone (Difco)	1 %
tryptone (Difco)	1 %
gelatin (Gelatine Delft)	1 %
agar	2 %

pH 7.0
sterilize 15 min at 120 °C.

Approximately 0.1 ml of a stock cooked meat (difco) culture is inoculated into 10 ml of liquid TPGS medium. After incubation for 24 h at 30 °C. the entire culture is inoculated into 1000 ml TPGS. This TPGS is than poured over the agar phase (at least fifteen plates (diameter of 14 cm)). The biphasic culture is incubated anaerobically for 5-7 days at 30 °C. When sufficient spores are present (10 - 70 %, as observed by microscopic examination), spores are harvested by collecting the liquid phase.

The spore suspension is washed 3 times with distilled water by repeated centrifugation at about 5 °C at 12000 g (= 10000 rpm for rotor SS34) for 10 min each time. It is recommended to treat the spore suspension in an ultrasonic bath (e.g. D-50 model sonogen, Branson Instruments Comp, USA) after washing to ensure loose spores. The treated suspension is heated for 10 min at 80 °C to ensure elimination of botulinum toxin and/or vegetative cells and enumerated at TSA according to procedure 004 (Enumeration of *Clostridium botulinum*).

The suspensions are dispensed in vials of 2 -3 ml, deep-frozen at -20 °C until use.

HARVESTING AND CLEANING SPORES

Add cold sterile dH_2O (10 ml/application) to agar surface
Scrape spores from agar with a sterile hockey stick
Place scraped surface into sterile centrifuge tube
Repeat above with another addition of 10 ml cold sterile dH_2O and scrape surface
Once all plates are harvested and collected in centrifuge tubes:
Centrifuge at 4 °C for 20 min
Discard supernatant
Resuspend in cold dH_2O
Repeat wash step 5 more times or until no vegetative cells are present
Store at 4 °C in cold sterile dH_2O until use

PREPARATION OF ALGINATION FOR CELL IMMOBILIZATION
(Smidsrod and Skjak-Braek. 1990. Trends Biotech. 8: 71-78)

Preparation of alginate solution:
1. 2-4% w/v suspended in distilled water
2. Suspension stirred for 6 hours by magnetic stirrer at room temperature
3. Filter sterilize through a 0.22μm filter

Mixing with Cells:
1. Sterilized alginate solution is mixed in an equal volume with the suspended cells

Formation of Beads:
1. Alginate-cell suspension is added to a $CaCl_2$ solution with 20-100mM solution Ca^{2+} ions.
2. Beads left to harden in $CaCl_2$ for 5-30 minutes.

Dissolving of beads:
1. Immerse beads in a solution with phosphate or citrate (50mM Na^+ citrate or phosphate buffer at pH 7.0, this will sequester calcium ions).
*High-G alginate takes several hours, compared to 10-30 minutes for a low-G alginate

BACILLUS ENDOSPORE ENCAPSULATION PROTOCOL

100 mM $CaCl_2$ Solution

- 14.7 g $CaCl_2$
- 1000 mL dH_2O

50 mM Sodium Citrate

- 14.7 g sodium citrate
- 1000 mL dH_2O

Spore Encapsulation

1. Dilute and plate spore crop 5 times on BHI
2. Mix spore crop 50:50 with alginate dilute and plate 5 times on BHI
3. Take 50:50 spore/alginate equivalent to amounts of beads being used and dilute with sodium citrate and plate in duplicate on BHI
4. Make beads in 100 mM $CaCl_2$ solution
5. Dissolve 5 beads in a 1:10 solution of 50 mM sodium citrate
6. Dilute beads with 50mM sodium citrate and plate on BHI in duplicate (5 reps w/beads)

D-VALUES PROTOCOL

1. Add 50 µl spore crop to capillary tube (Fisher Cat # 11-365A)
2. Heat seal ends of capillary tube with flame
3. Wrap wire around capillary tube (makes inserting and removing tube from bath easier)
4. Place capillary tube in oil bath (Haake L/D8 Fisons) at set temperature and time
5. Remove capillary tube and place in ice bath for 5 minutes
6. Place cooled capillary tube in hypochlorite for 30 seconds
7. Break ends off capillary tube using glass cutter
8. Place capillary tube in 15 or 50 ml conical tube and add 5 ml peptone water to tube to flush out sample
9. Prepare dilutions for plating in duplicate
10. Pour plate using desired media
11. Incubate at set temperature for 24 – 48 hours

Dilution scheme

Add 5 ml peptone water to the 50 µl sample (10^{-2})
Take 1 ml add to 9.0 ml test tube (10^{-3}), continue to appropriate dilution
*can also prepare sample with 25 µl, just add 3 ml peptone; prepare dilutions using 0.5 ml into 4.5 ml peptone

SPORE COAT EXTRACTION PROTOCOL

Spore Coat Extraction Buffer

In a 15 mL Conical Tube:

- 2.4 g Urea (Sigma)
- Add 2.5 mL Tris-EDTA (Sigma)
- Mix by vortexing – takes some time
- Add 150 µL β-mercaptoethanol (Sigma)
- Add 500 µL 10% SDS
- Mix gently by inverting
- Add Tris-EDTA until it reaches 5 mL on conical tube

Lysozyme (100 mg/mL)

- 0.1 g lysozyme (Sigma)
- 1 mL sterile H_2O
- Mix gently
- Aliquot 50 µL into eppendorf tubes and freeze at -20 °C

Spore Coat Extraction

- Place 1.8 mL spores in eppendorf tube and centrifuge
- Resuspend pellet in 1 mL spore coat extraction buffer
- Incubate at 60 °C for 1 hr in dry water bath
- Centrifuge spores
- Resuspend pellet in 950 µL PBS with 25% sucrose
- Add 50 µL lysozyme, gently invert tube
- Incubate at 37 °C for 1 hr
- Centrifuge spores
- DNA extraction – MoBio UltraClean DNA isolation kit (Cat. # 12224-50), resuspend in bead solution

REAL-TIME PCR FOR *C. sporogenes*

Primers designed to GerAB gene with nucleotide accession number: AY046406
CspGerF1: 5' ACAGATGTAGCCGCAGGAATAAAC
CspGerR1: 5' GGTCCCTCCATAAACAGCATAAGC
Product is 120 bp, region 1835 – 1954 of GerAB gene

Real-time PCR using Jumpstart SYBR green master mix (Sigma S4438):

- 12.5 µL 2X Jumpstart master mix
- 0.25 µL ROX reference dye
- 0.5 µL CspGerF1 (200 nm final concentration)
- 0.5 µL CspGerR1 (200 nm final concentration)
- 10.25 µL water
- 1 µL DNA
- Total = 25 µL

PCR Conditions

- 95 °C 120 seconds x 1 cycle
- 95 °C 15 seconds
- 57 °C 30 seconds
- 72 °C 30 seconds

} 40 Cycles

Program in Cephid Smart Cycler: Spore SYBR Green

SWEET POTATO MICROWAVE PROTOCOL

9 runs per organism
40 pouches per run (10 for plating, 20 for color indicator)
B. subtilis 0.1ml pouches
G. stearothermophilus 0.1ml pouches

Procedures

1. Collect 40 pouches from microwave run
2. Place 20 pouches in sterile labeled test tubes in the incubator (35C for *B. subtilis*, and 55C for *G. stearothermophilus*)
3. Place 10 pouches in sterile labeled tubes in the refrigerator – these are the extras
4. Use other 10 pouches for plating with BHI spread plates

BHI Spread Plates undiluted:

1. Cut the corner of 3 *B. subtilis*/*G. stearothermophilus* units with sterile scissors and place in 15ml conical tube
2. Allow contents to drip into the tube.
3. Cut the remaining part of the unit into small sections letting it fall into the tube
4. Vortex the tube for several minutes
5. Spread Plate 0.1ml on BHI, in duplicate
6. Place in incubator for 48 hours

BHI Spread Plates using Dilutions:

1. Cut the corner of the 2 *B. subtilis*/*G. stearothermophilus* units with sterile scissors
2. Allow contents to drip into a sterile 15ml conical tube.
3. Cut the remaining part of the unit into small sections letting it fall into the tube
4. Add 1.8ml of USP-fluid D into the tube with the 2 cut up units (10^-1 dilution)
5. Vortex the tube for several minutes
6. Make serial dilutions in eppendorf tubes using 0.9ml buffer with 0.1ml suspension
7. Spread Plate 0.1ml of each dilution on BHI, in duplicate
8. Place in incubator for 48 hours

APPENDIX B

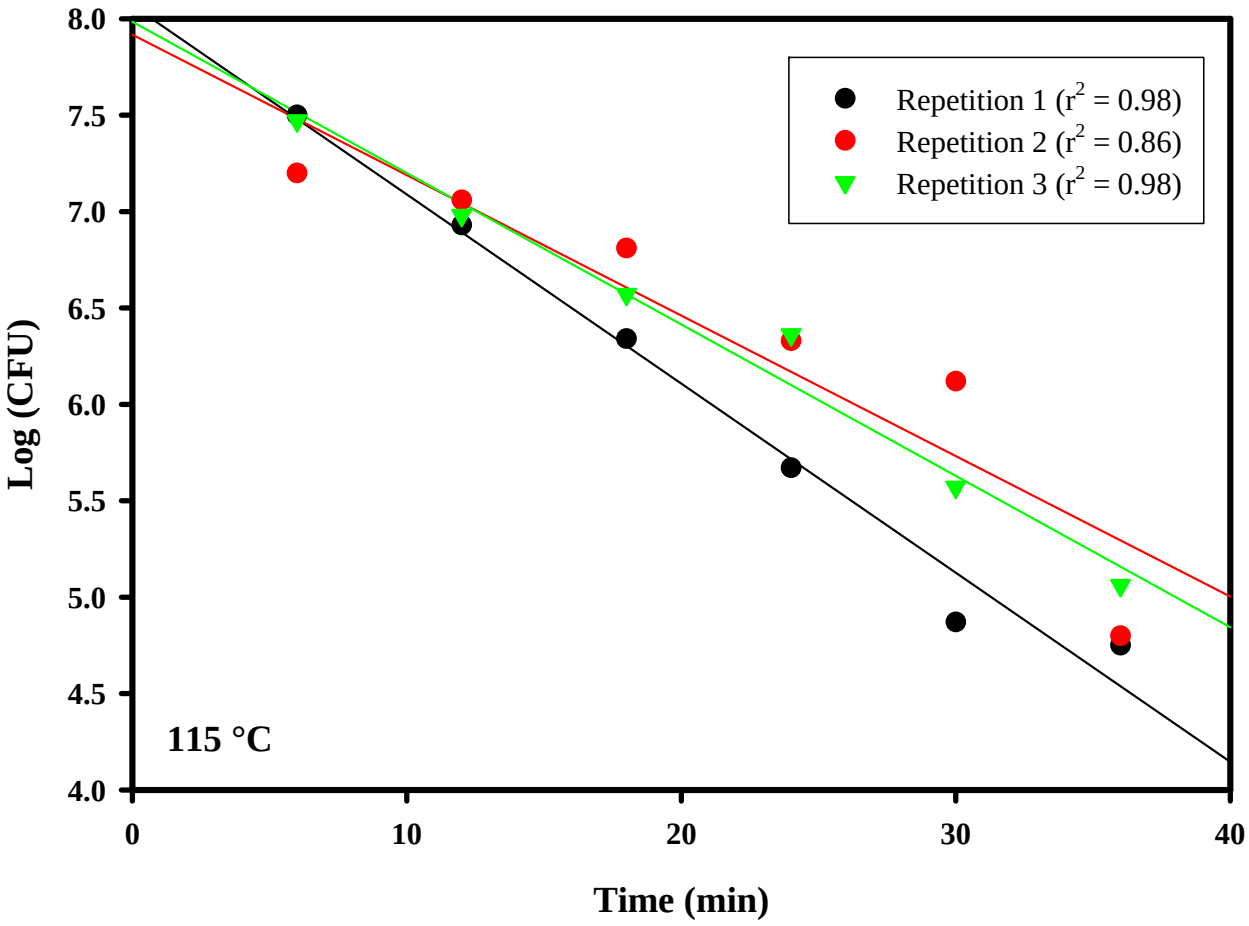

Figure 11. ***G. stearothermophilus*** **D-values in salsa con queso**

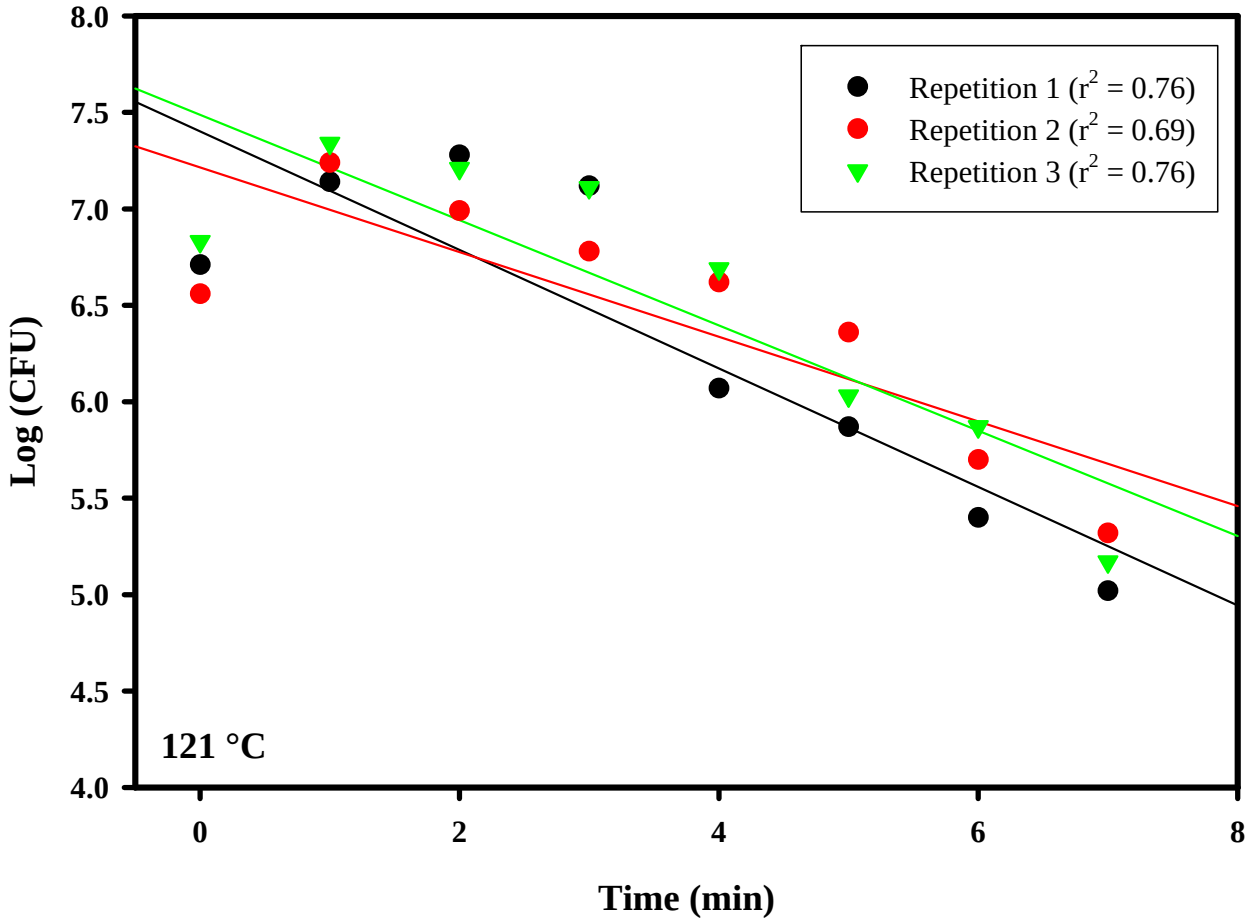

Figure 12. ***G. stearothermophilus*** **D-values in salsa con queso**

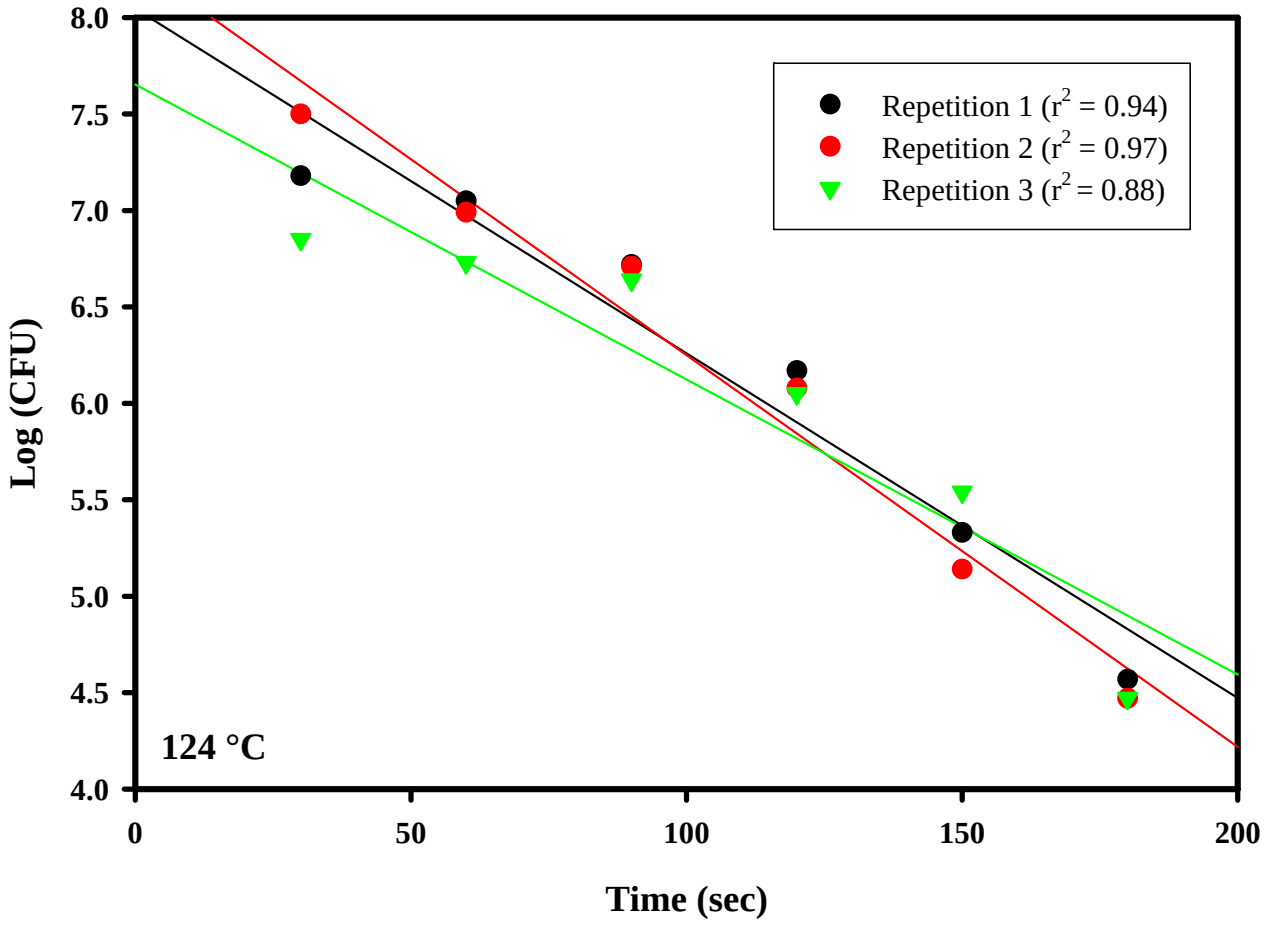

Figure 13. ***G. stearothermophilus*** **D-values in salsa con queso**

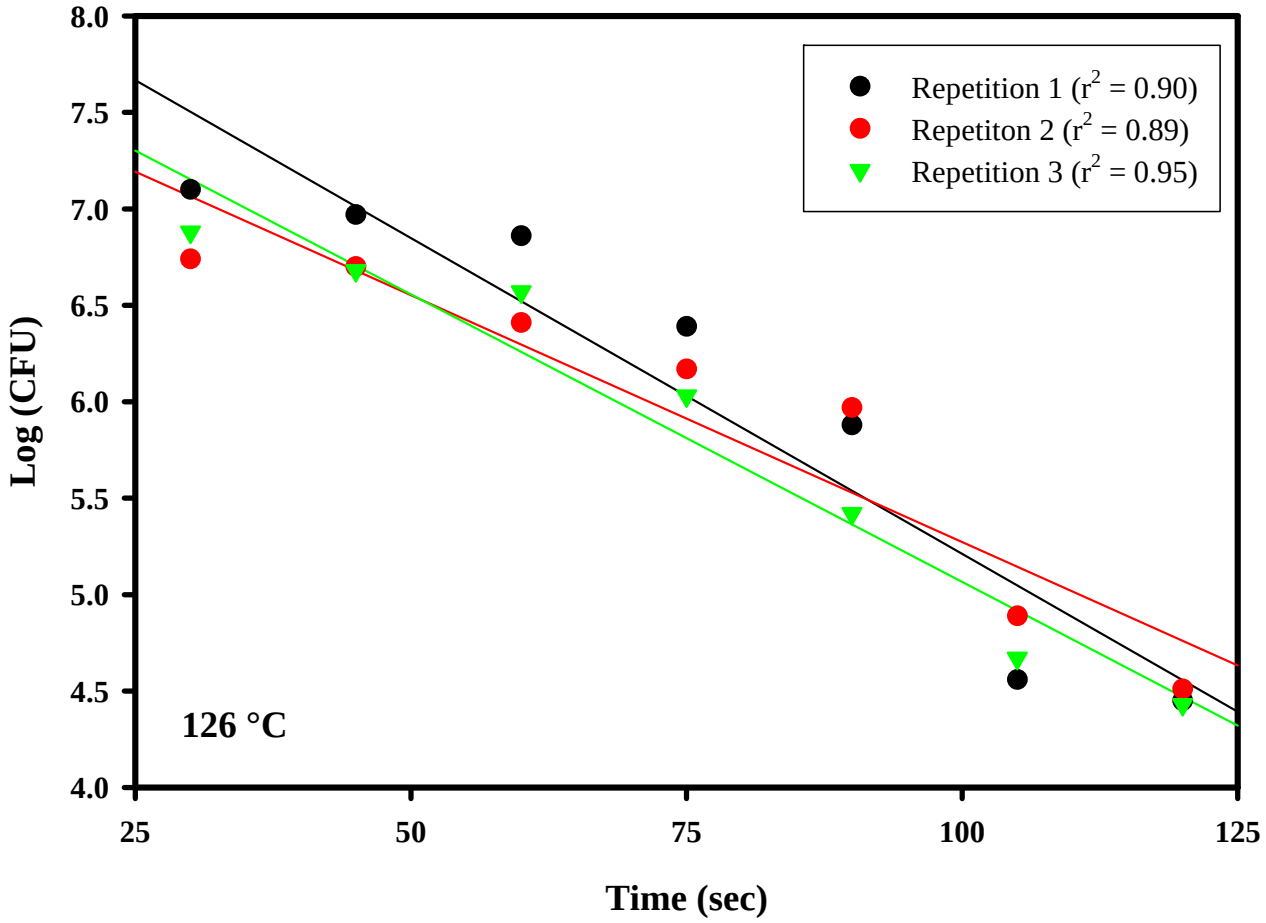

Figure 14. ***G. stearothermophilus*** **D-values in salsa con queso**

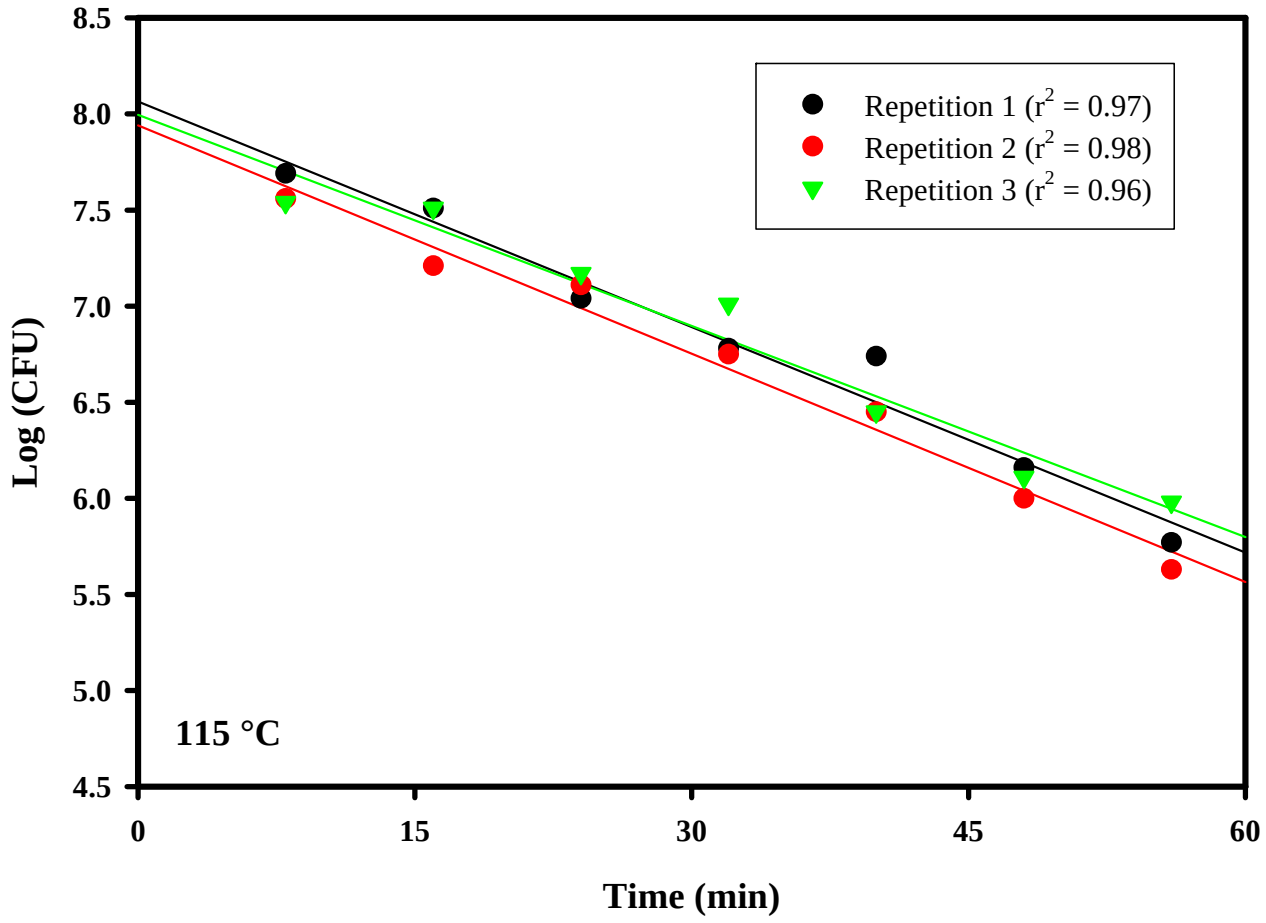

Figure 15. ***G. stearothermophilus*** **D-values in water**

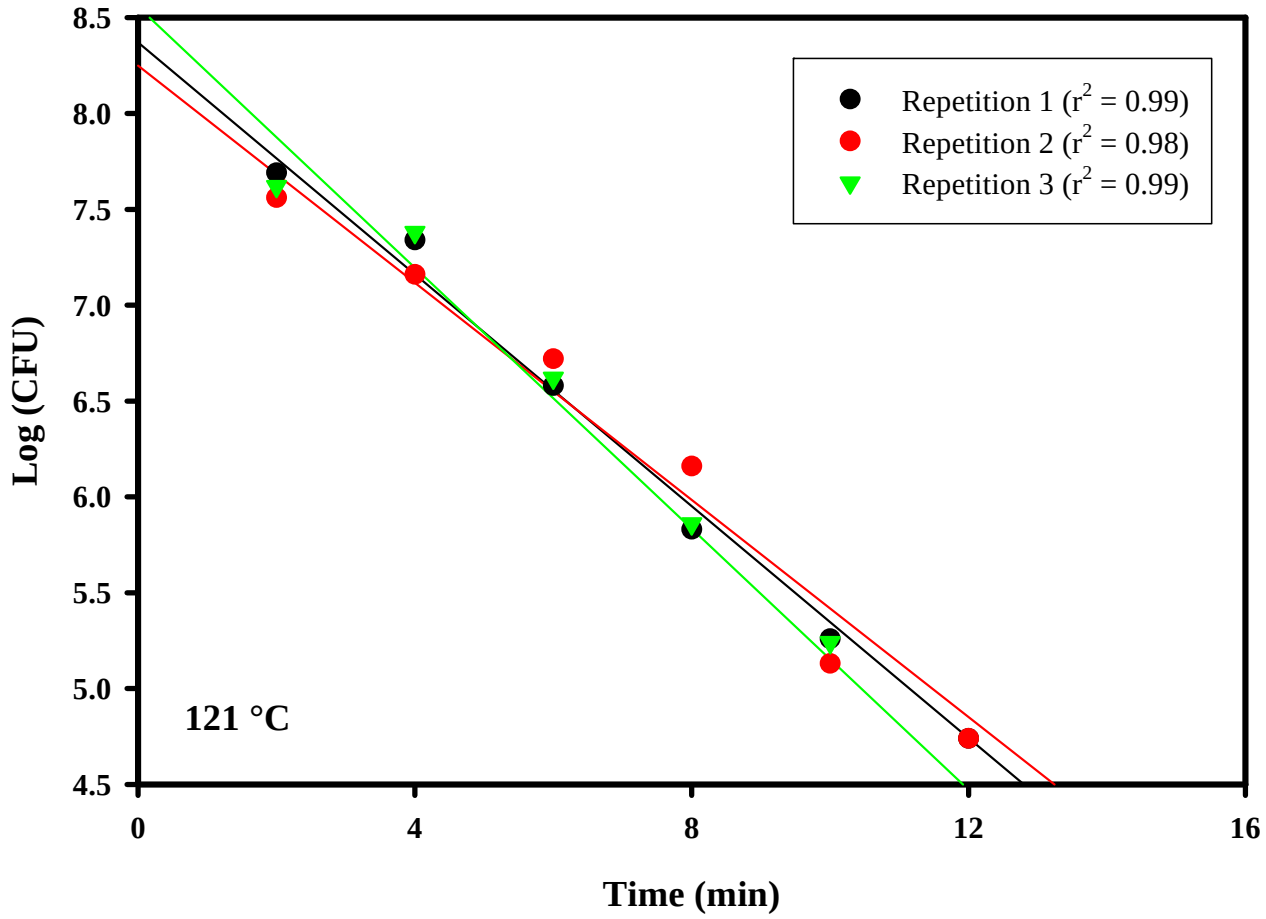

Figure 16. *G. stearothermophilus* D-values in water

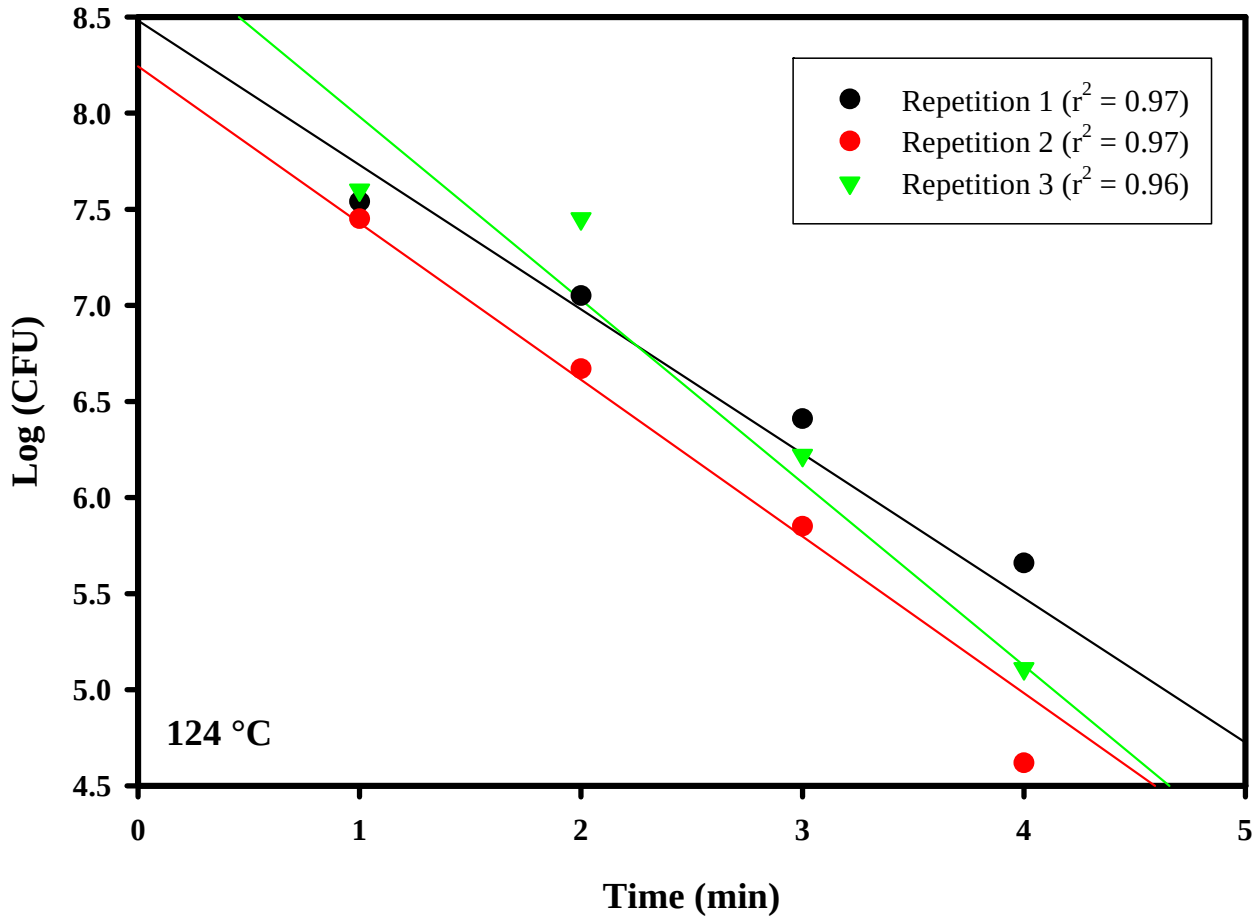

Figure 17. ***G. stearothermophilus*** **D-values in water**

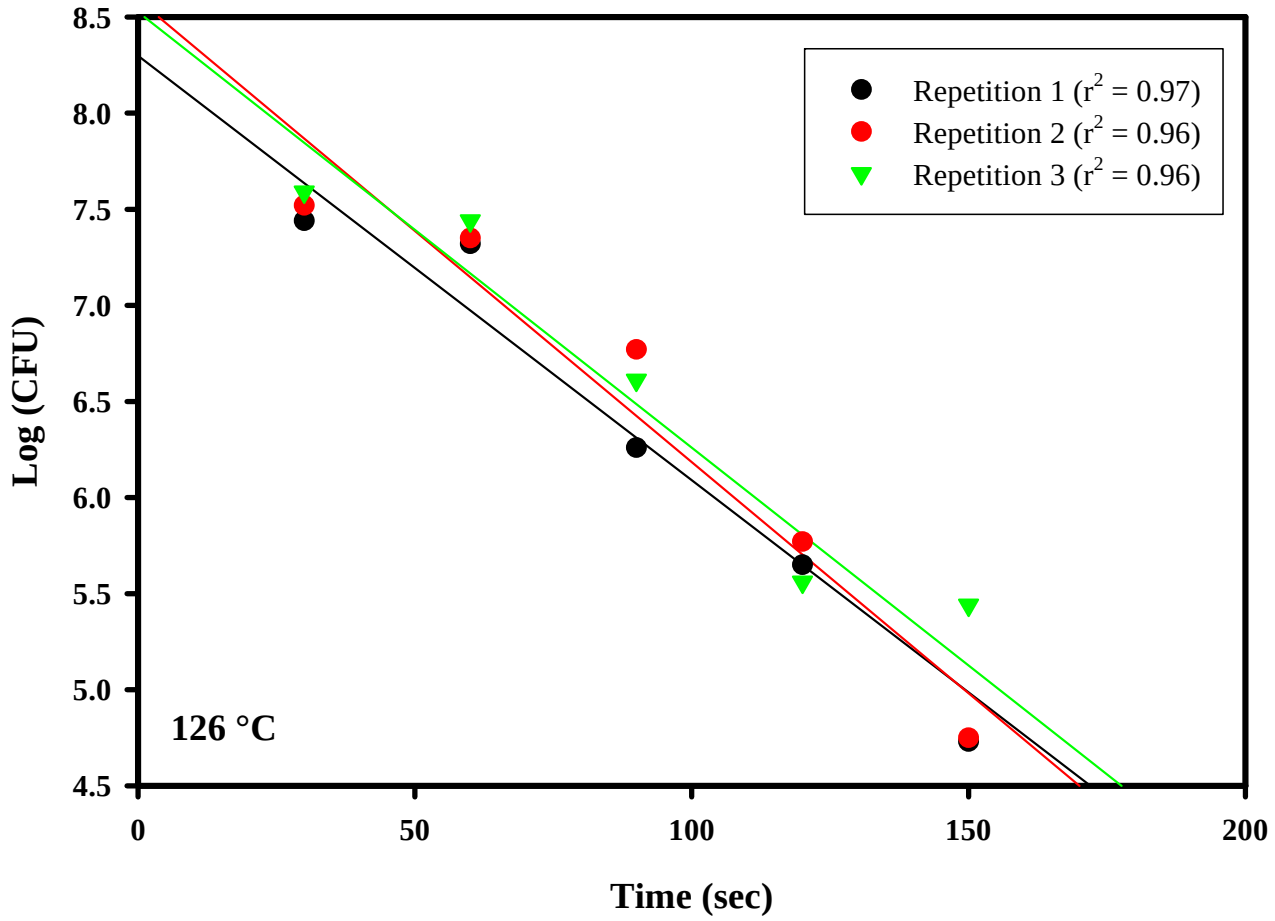

Figure 18. ***G. stearothermophilus*** **D-values in water**

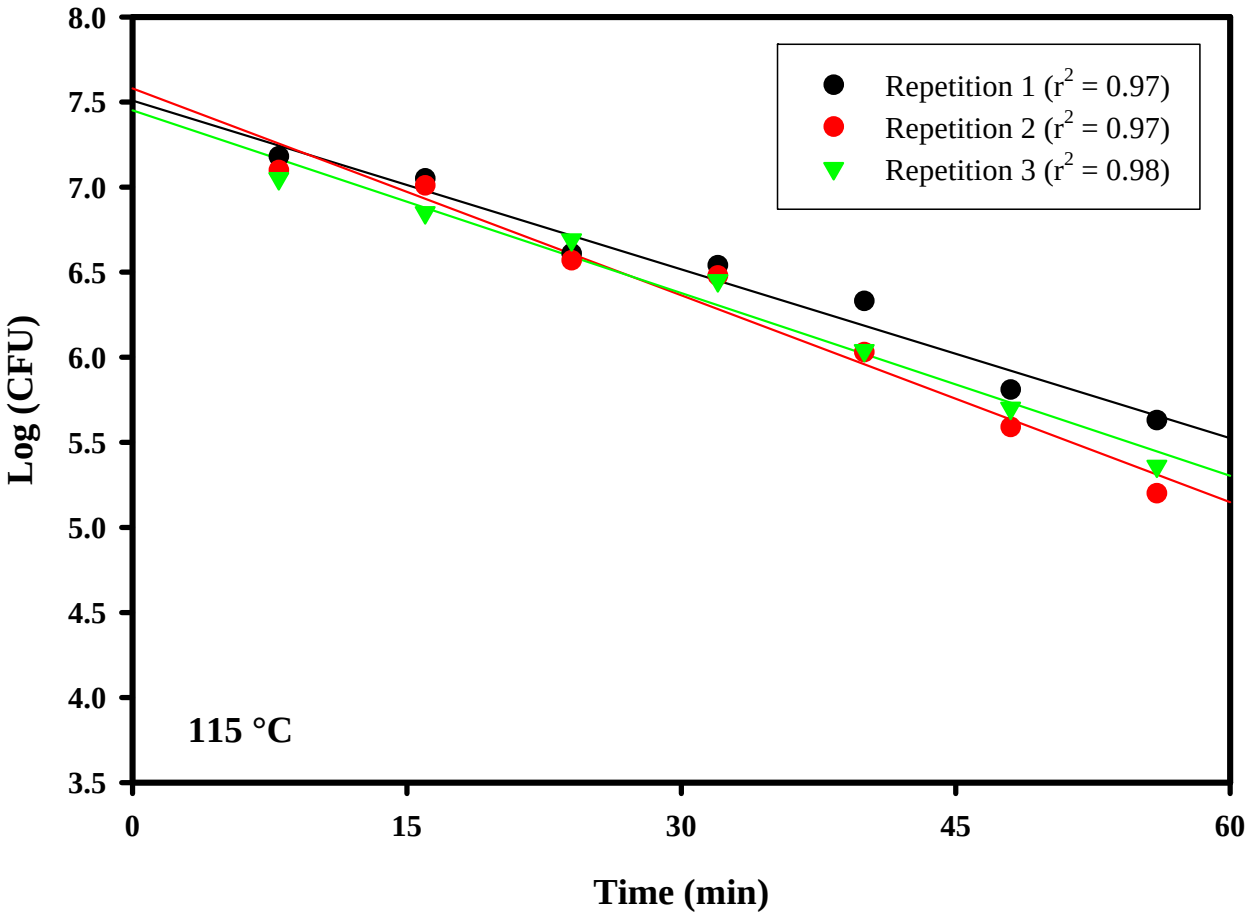

Figure 19. ***G. stearothermophilus*** **D-values in Alginate**

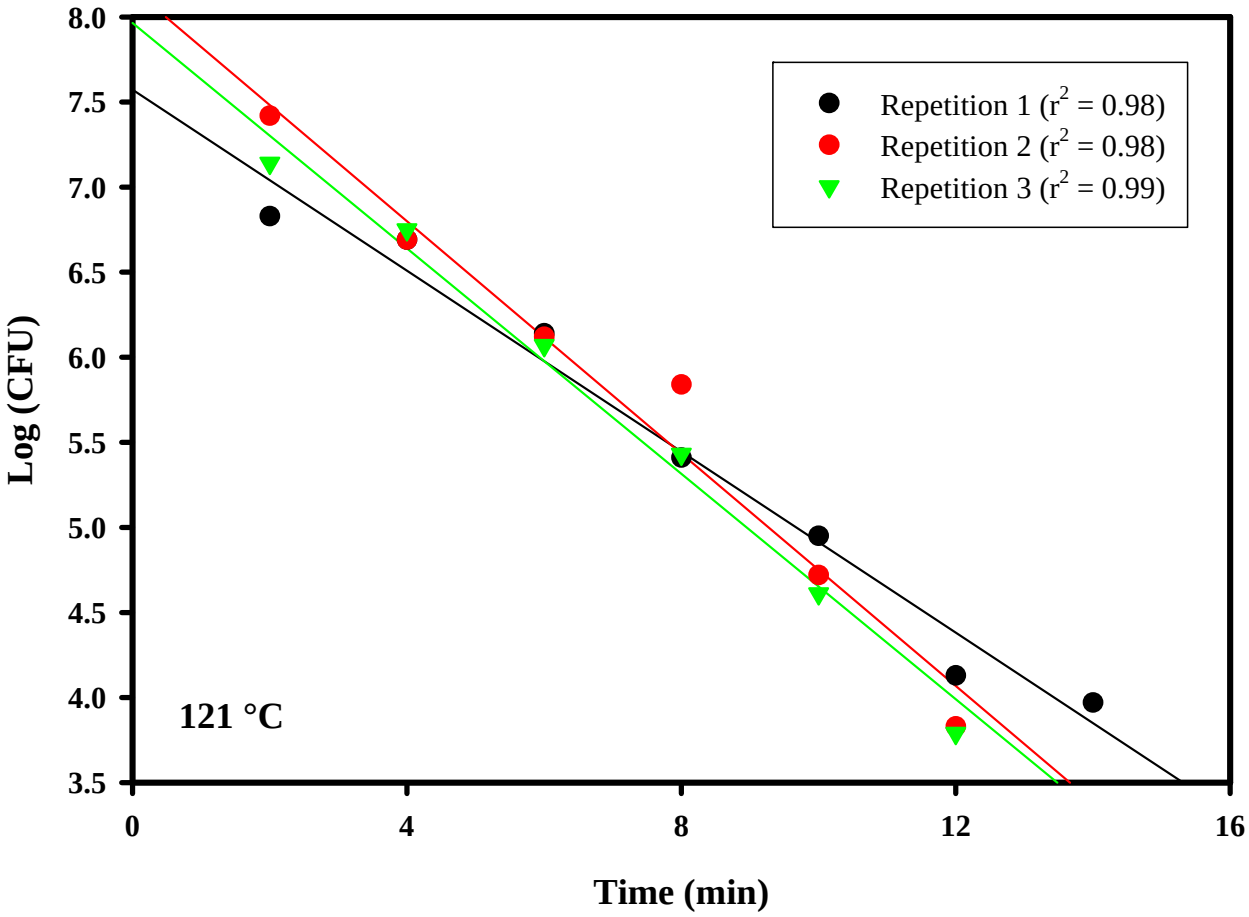

Figure 20. ***G. stearothermophilus*** **D-values in Alginate**

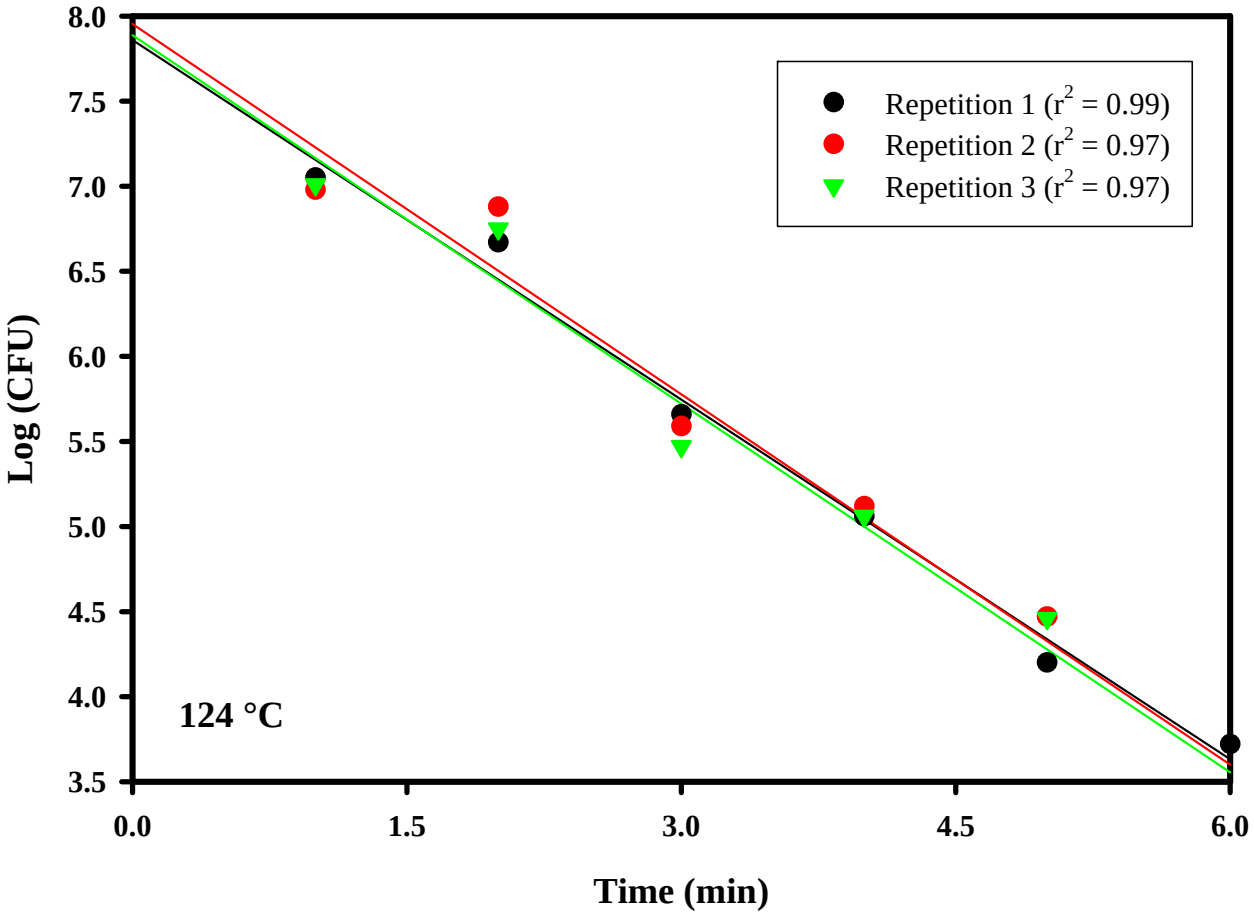

Figure 21. ***G. stearothermophilus*** **D-values in Alginate**

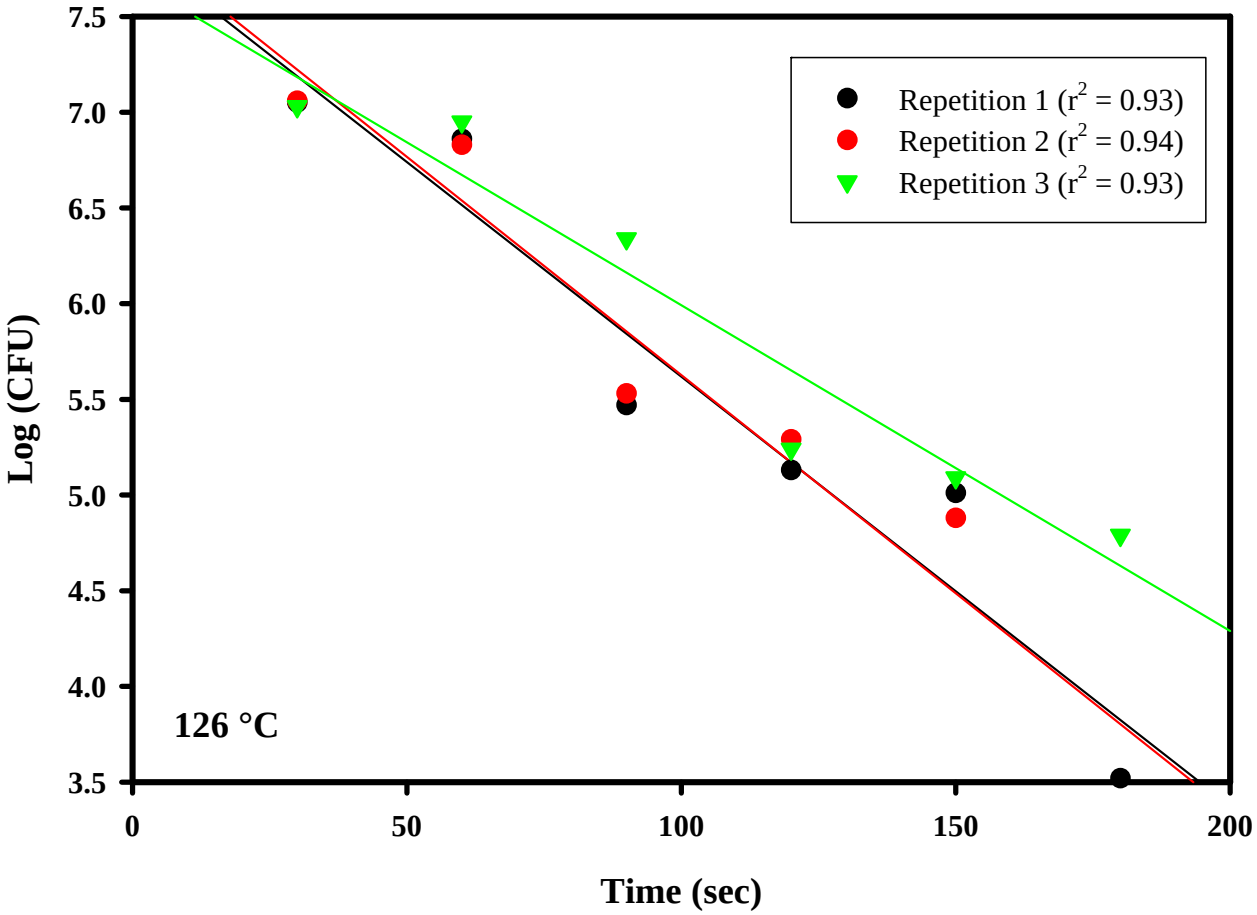

Figure 22. ***G. stearothermophilus*** **D-values in Alginate**

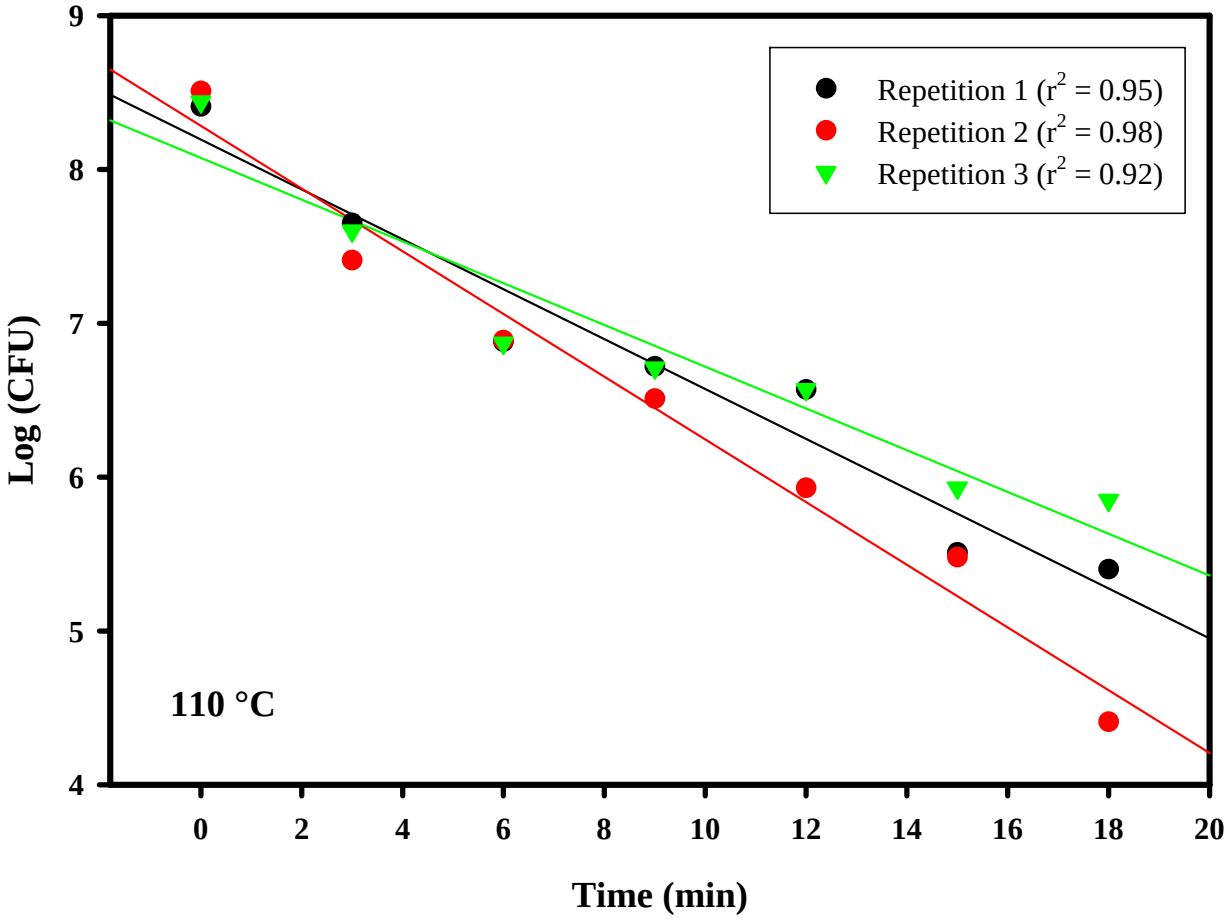

Figure 23. *B. subtilis* D-values in Water

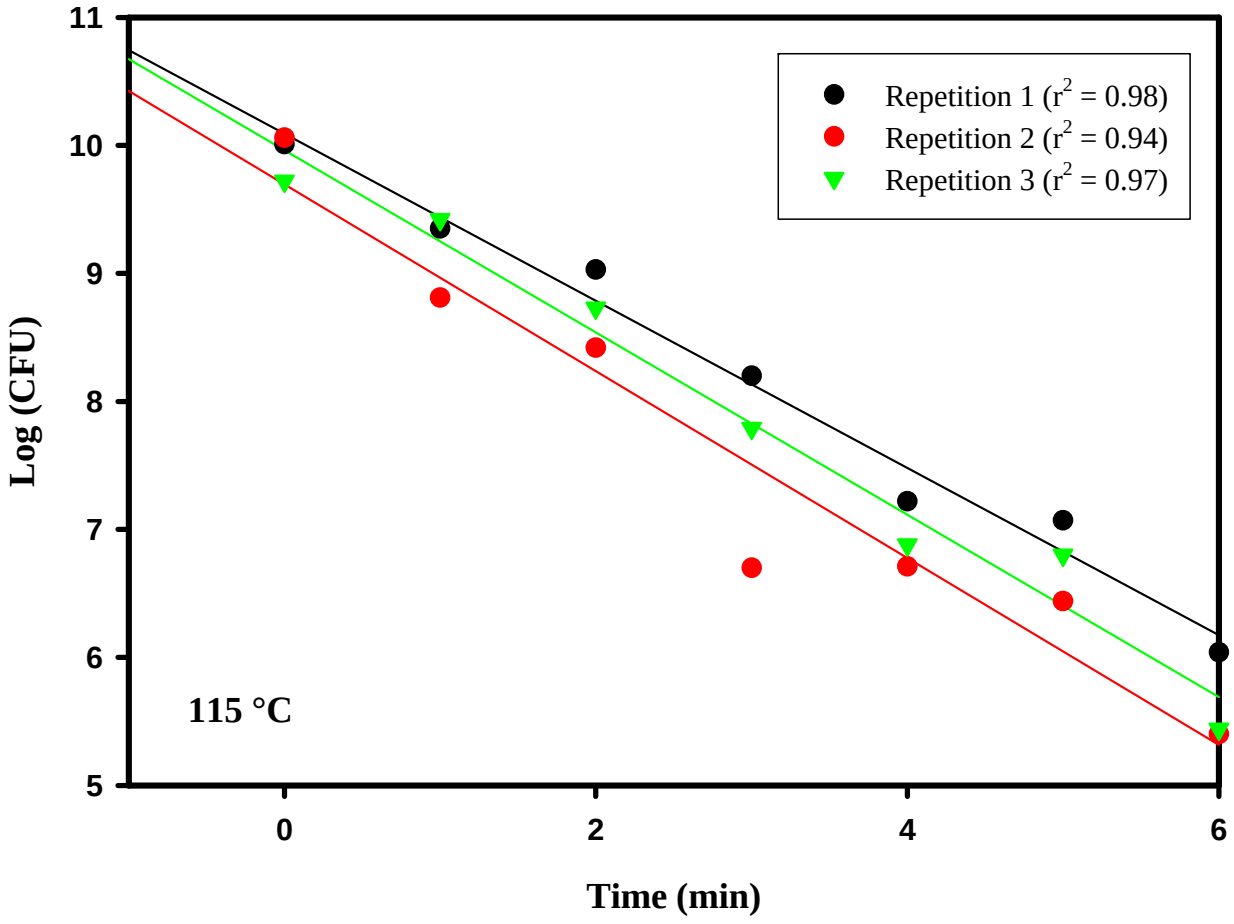

Figure 24. *B. subtilis* D-values in Water

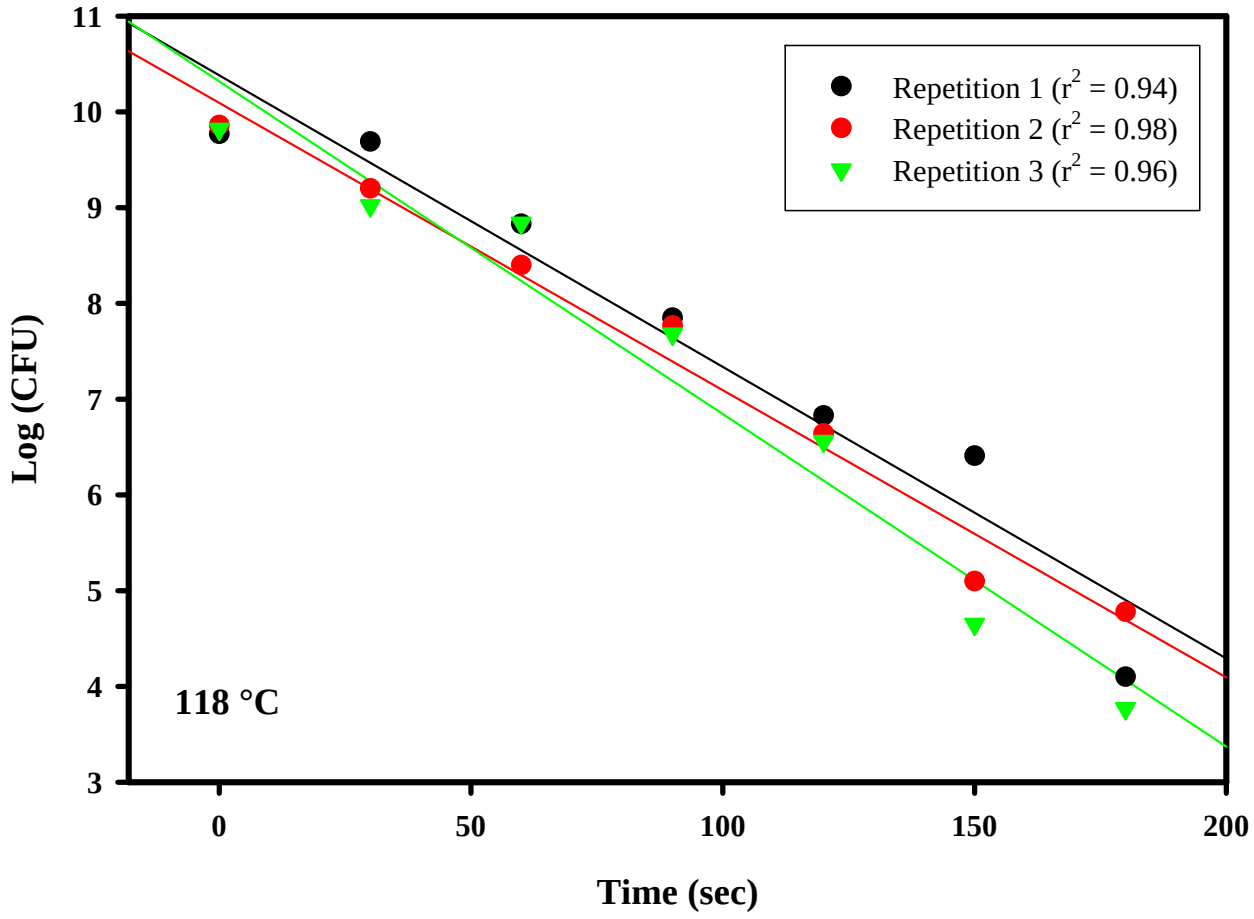

Figure 25. ***B. subtilis*** **D-values in Water**

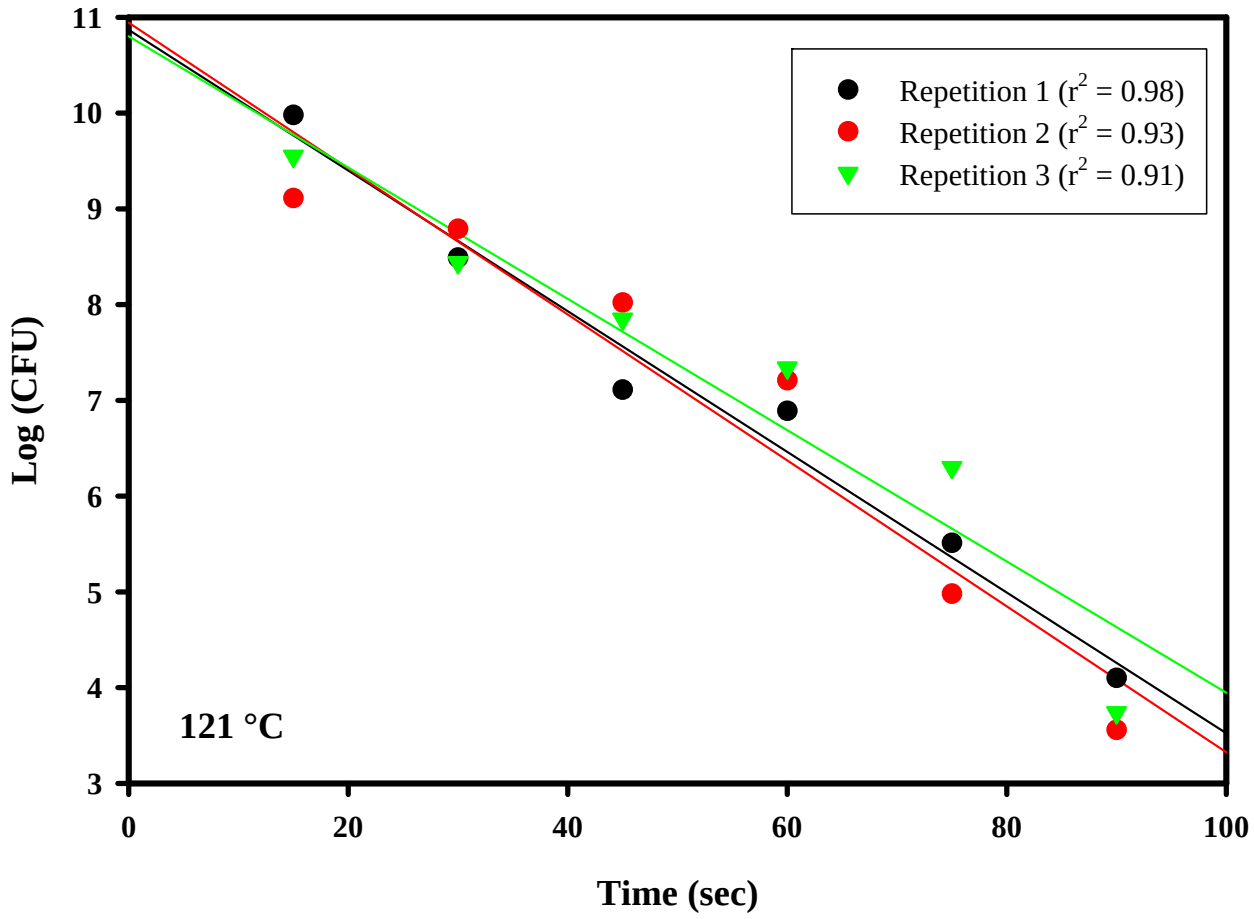

Figure 26. *B. subtilis* D-values in Water

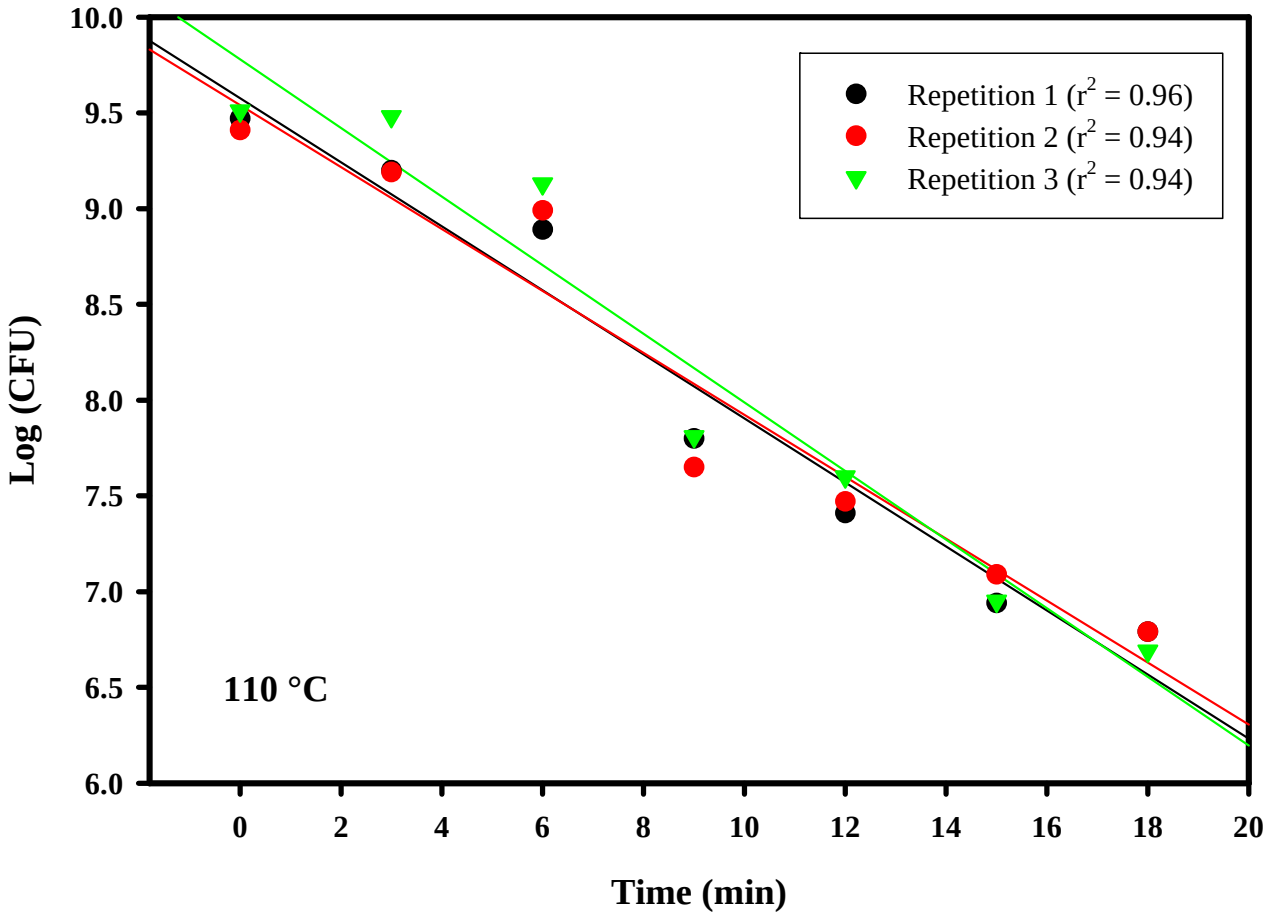

Figure 27. *B. subtilis* D-values in Alginate

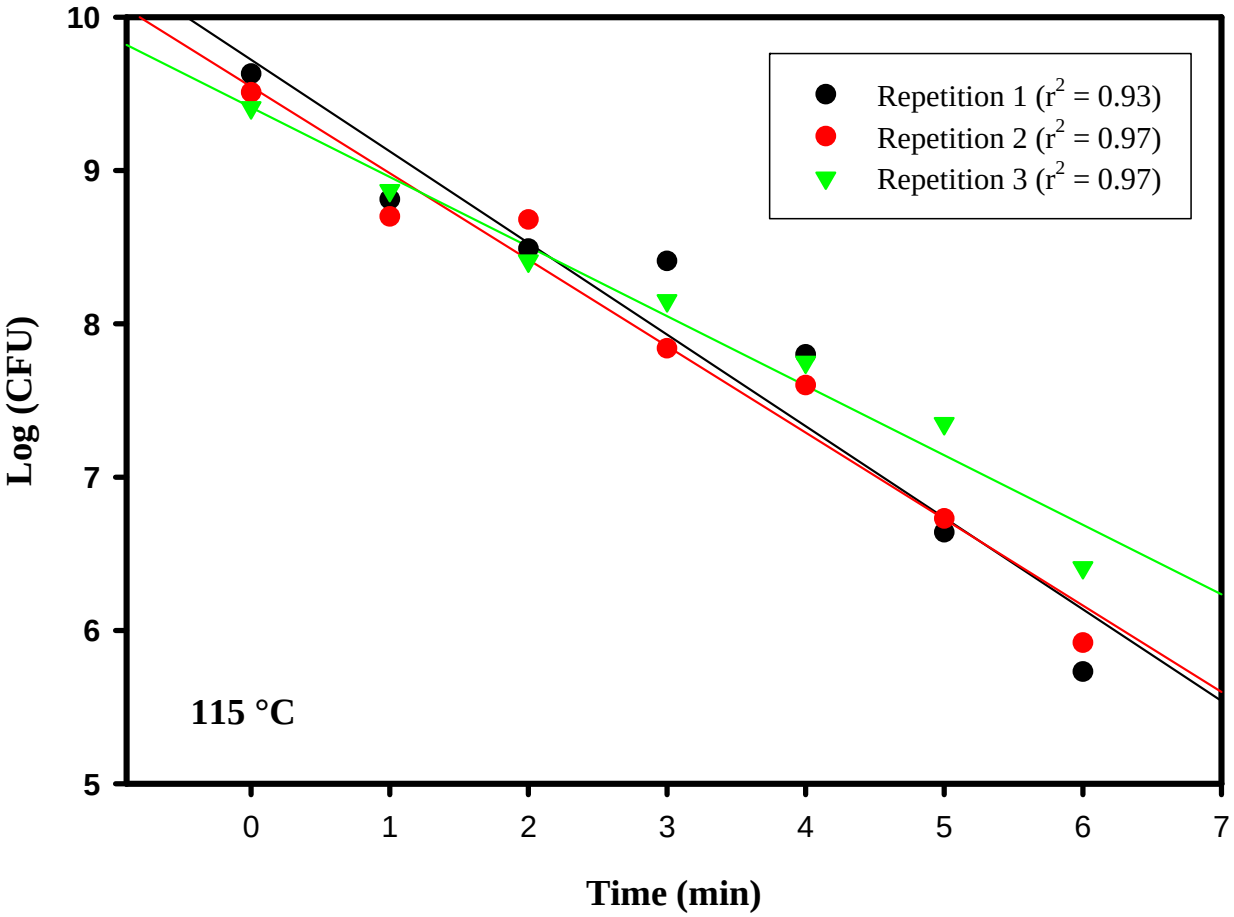

Figure 28. ***B. subtilis*** **D-values in Alginate**

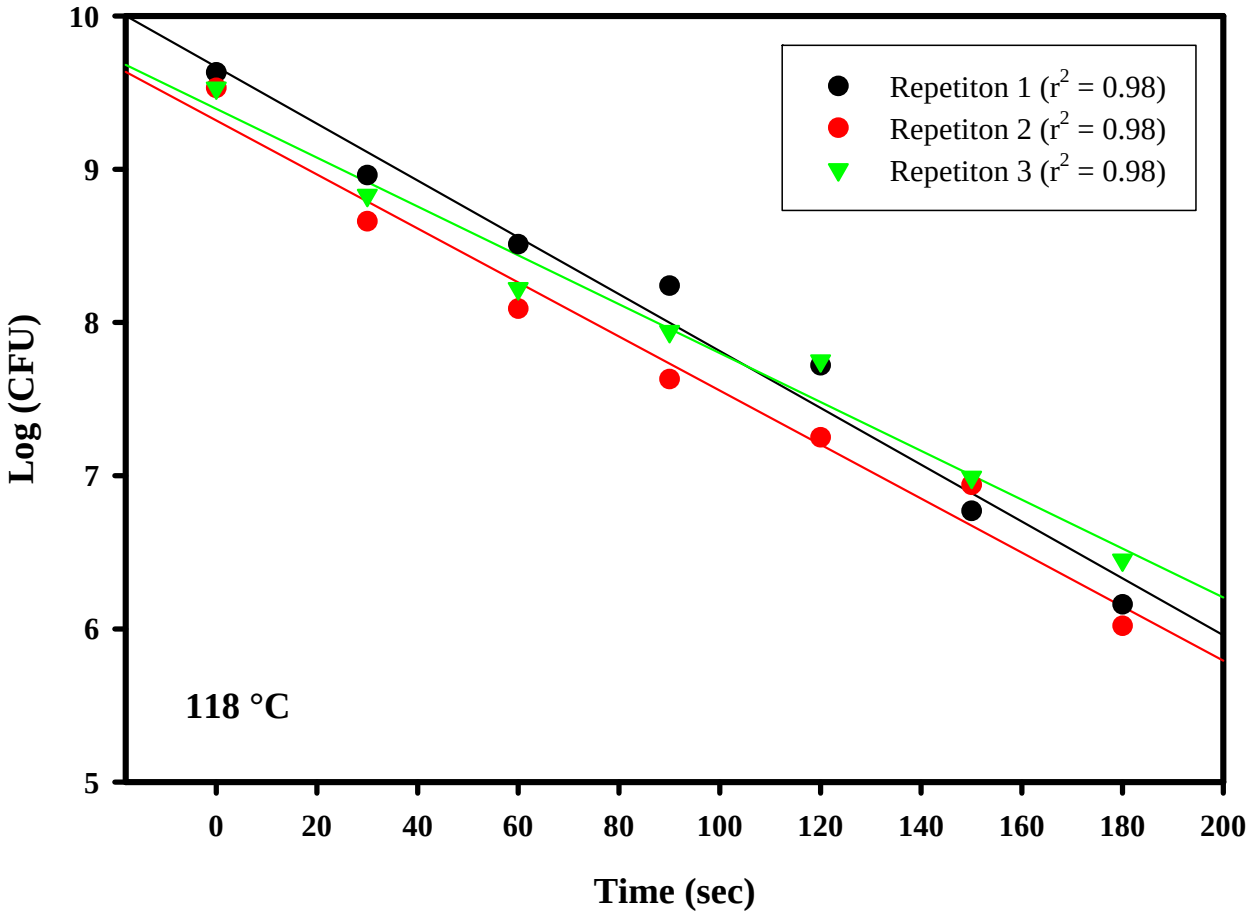

Figure 29. ***B. subtilis*** **D-values in Alginate**

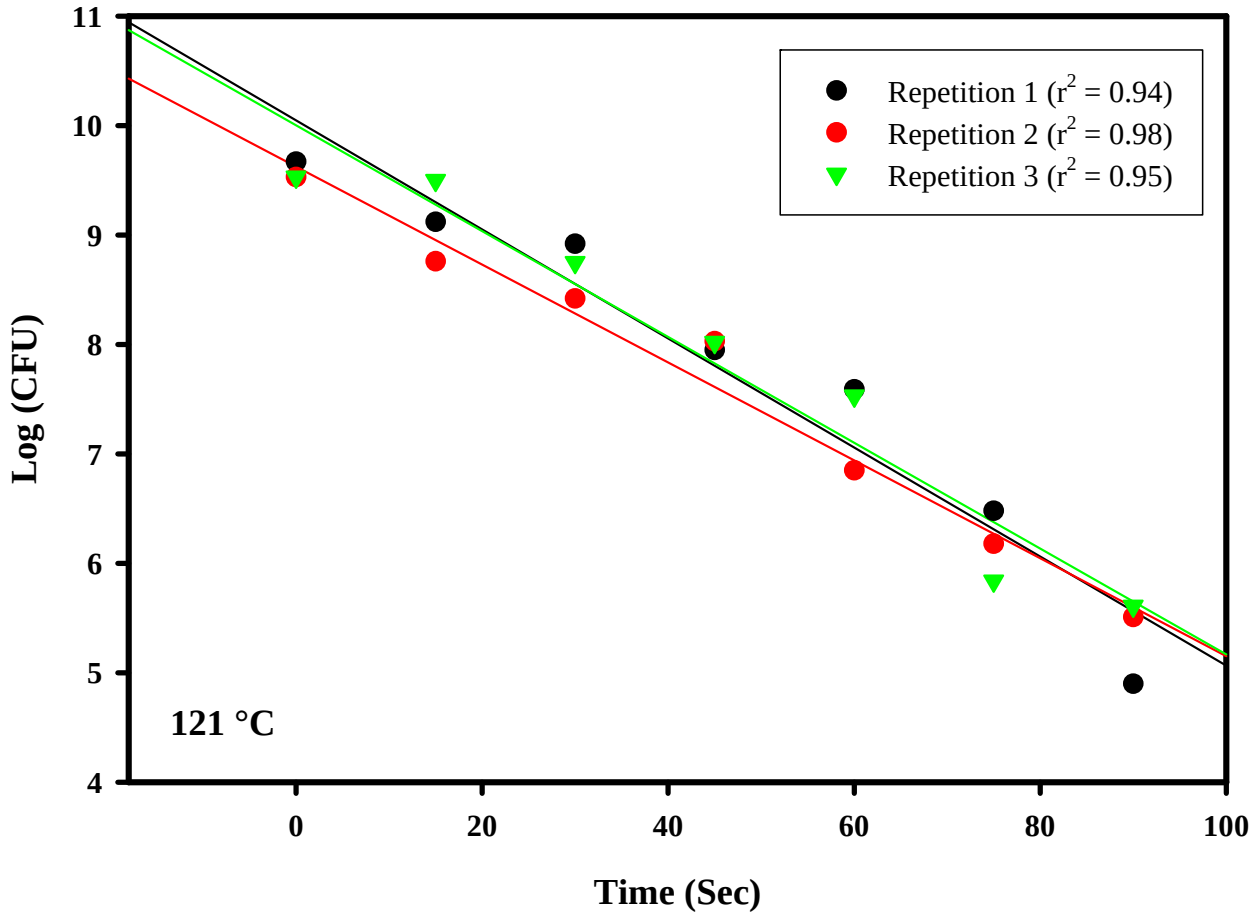

Figure 30. *B. subtilis* D-values in Alginate

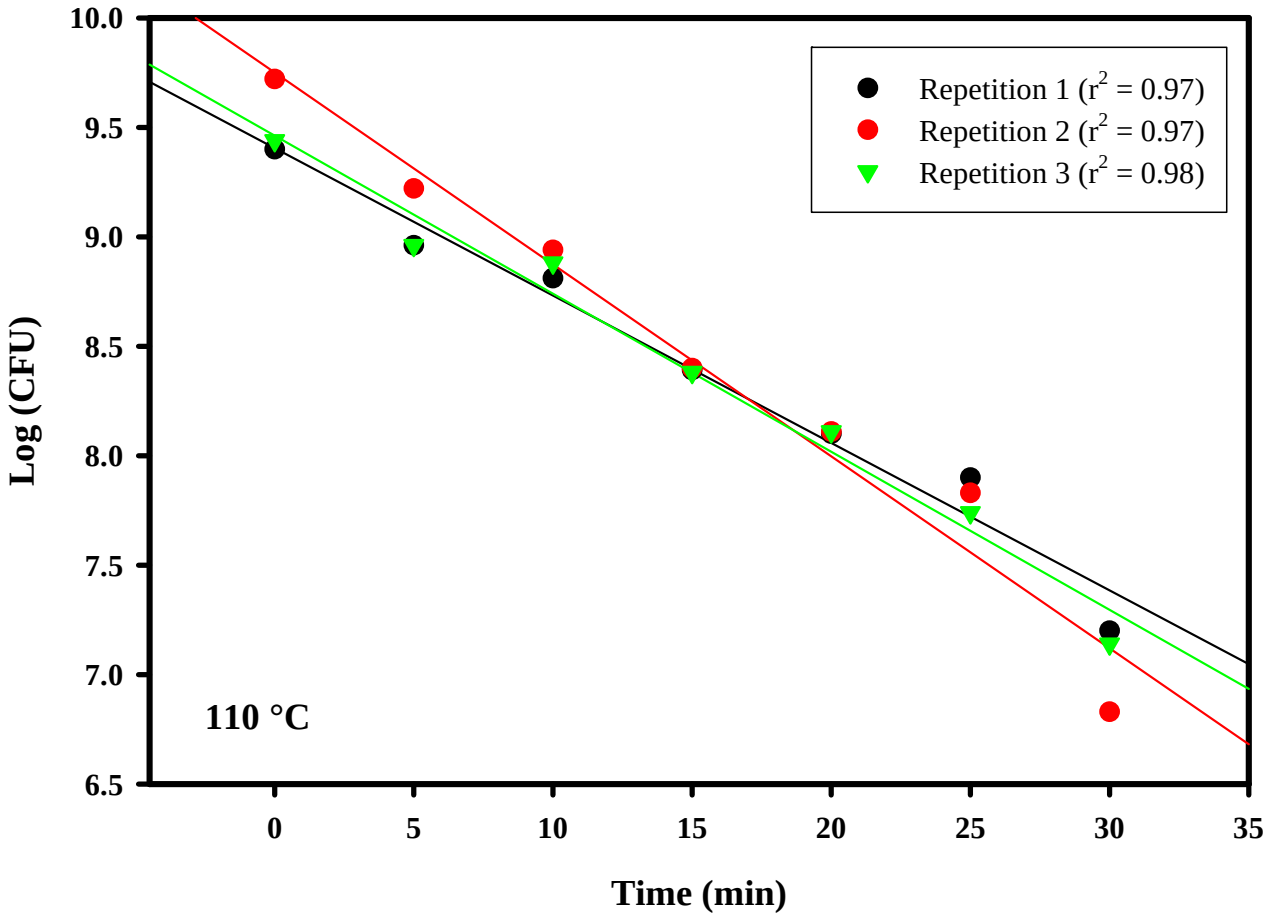

Figure 31. *B. subtilis* D-values in salsa con queso

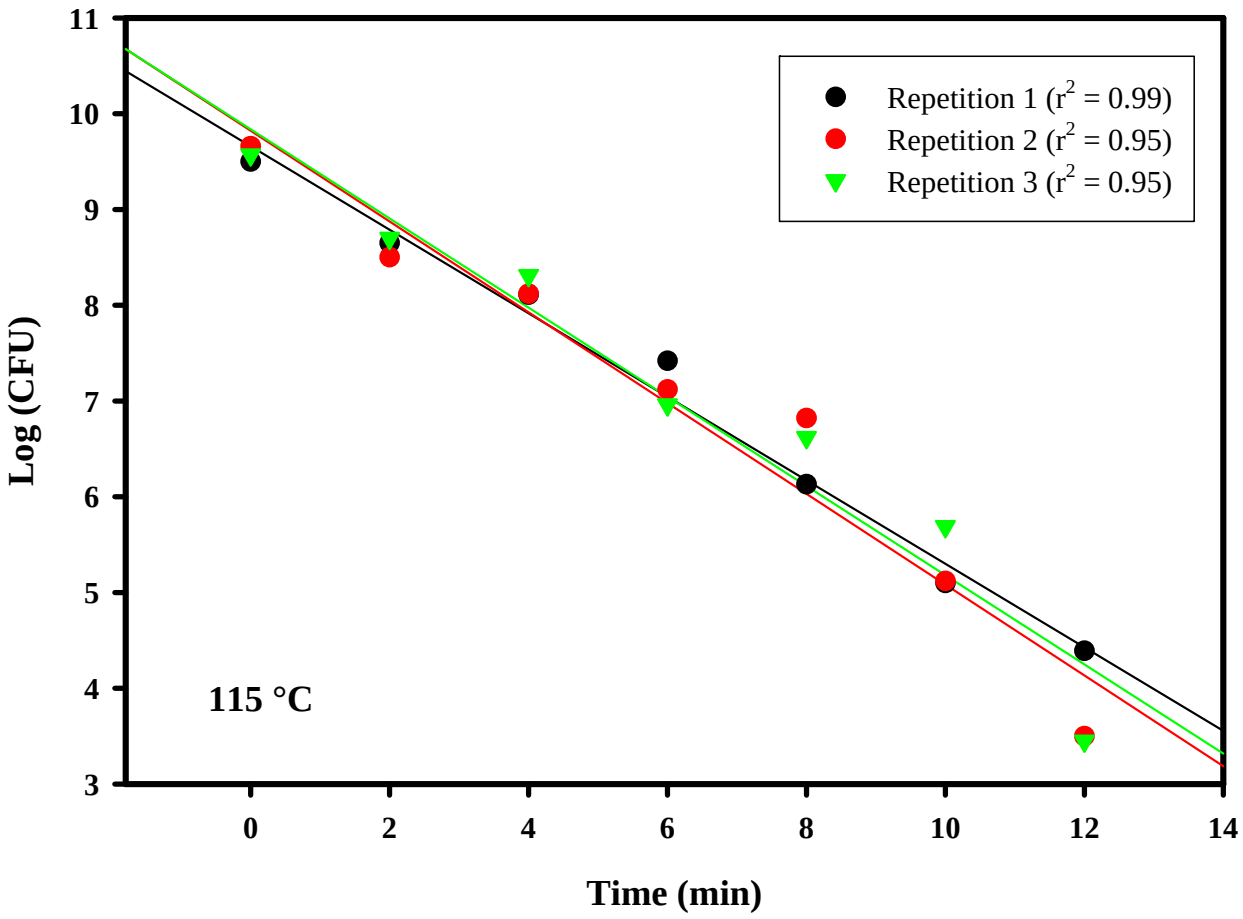

Figure 32. ***B. subtilis*** **D-values in salsa con queso**

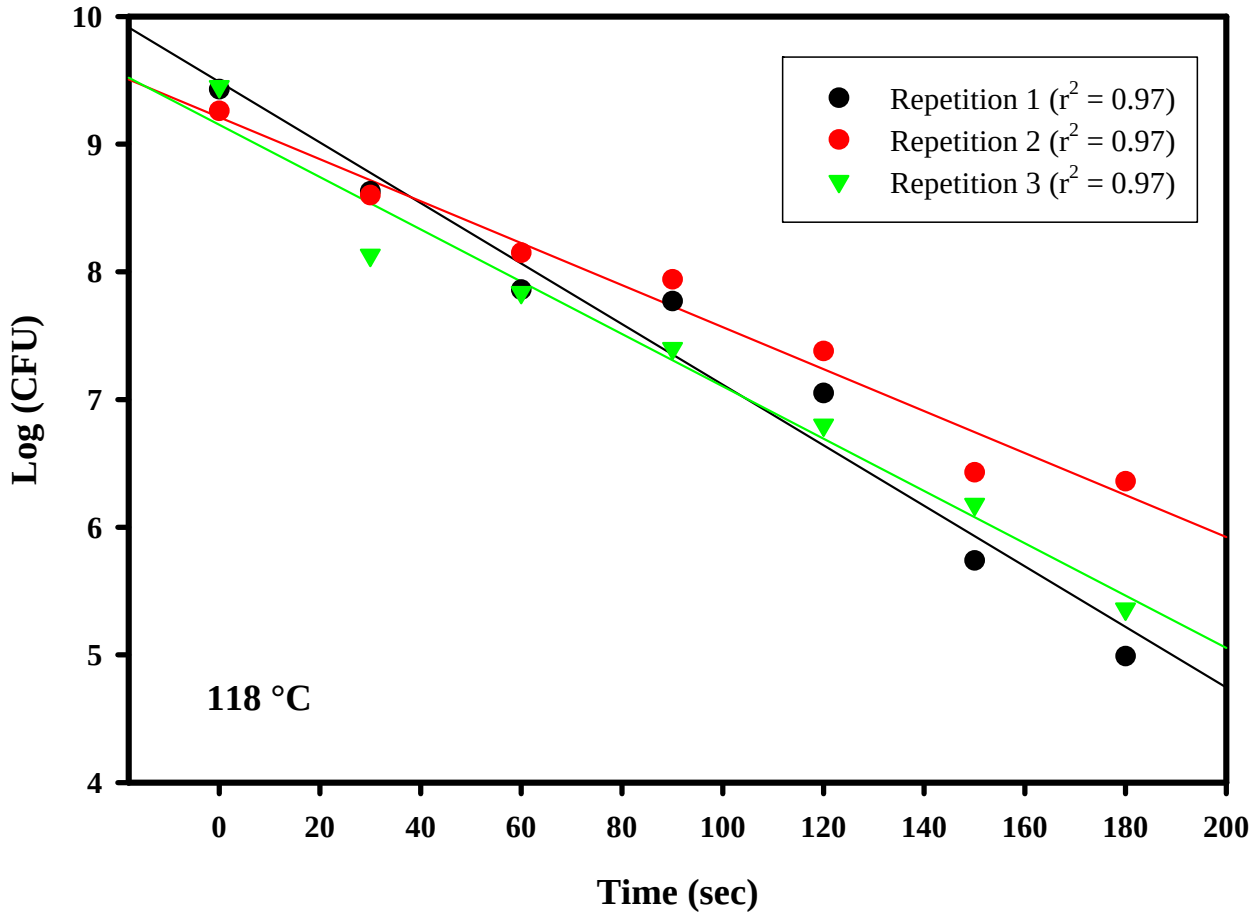

Figure 33. ***B. subtilis*** **D-values in salsa con queso**

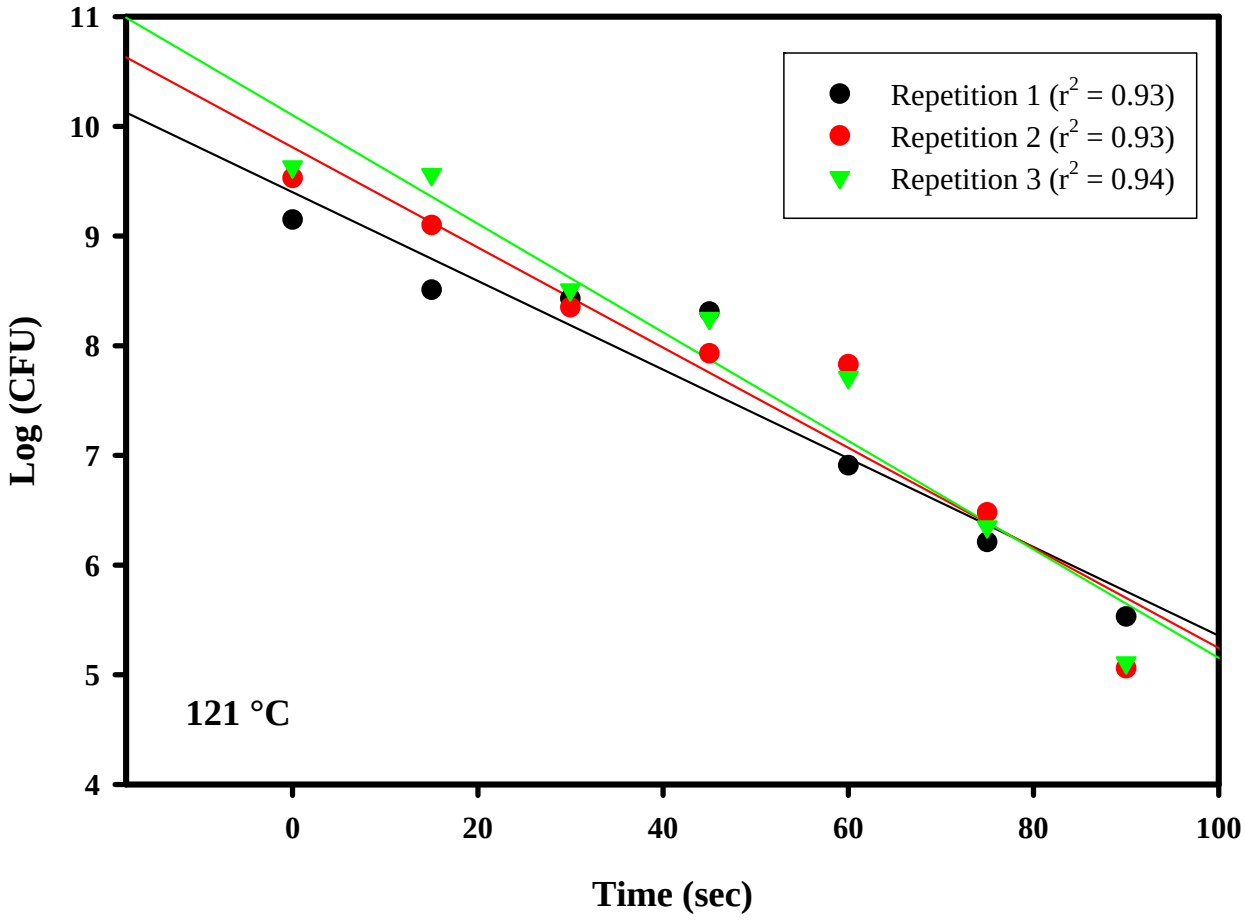

Figure 34. ***B. subtilis*** **D-values in salsa con queso**

APPENDIX C

Feasibility of Utilizing Bio-indicators for Testing Microbial Inactivation in Sweetpotato Purees Processed with a Continuous Flow Microwave System

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Running Head: Bio-indicators in continuous microwave system

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ABSTRACT

Continuous flow microwave heating has potential in aseptic processing of various food products, including the purees from sweetpotatoes and other vegetables. Establishing the feasibility of a new processing technology for achieving commercial sterility requires evaluating microbial inactivation. This study aimed to assess the feasibility of using commercially available plastic pouches of biological indicators containing spores of *Geobacillius stearothermophilus* ATCC 7953 and *Bacillus subtilis* ATCC 35021 for evaluating the degree of microbial inactivation achieved in vegetable purees processed in a continuous flow microwave heating unit. Sweetpotato puree seeded with the bio-indicators was subjected to three levels of processing based on the fastest particles: under-target process (F_0~0.65), target process (F_0~2.8), and over-target process (F_0~10.10). After initial experiments, we found it was necessary to engineer a set up with two removable tubes connected to the continuous flow microwave system to facilitate the injection of indicators into the unit without interrupting the puree flow. Using this approach, 60% of the indicators injected into the system could be recovered post-process. Spore survival after processing, as evaluated by use of growth indicator dyes and standard plating methods verified inactivation of the spores in sweetpotato puree. The log reduction results for *B. subtilis* were equivalent to the pre-designed degrees of sterilization (F_0). This study presents the first report suggesting that bio-indicators such as the flexible, food grade plastic pouches can be used for microbial validation of commercial sterilization in aseptic processing of foods using a continuous-flow microwave system.

Keywords: microwave, aseptic, continuous flow, purees, bio-indicators

INTRODUCTION

Continuous flow microwave processing has potential in thermal processing of foods. A process for rapid sterilization and aseptic packaging of viscous products using a continuous flow microwave system operating at 915 MHz has been successfully developed in the Department of Food Science at North Carolina State University (Raleigh, NC). It has been demonstrated previously that this technology has the ability to produce a high-quality, shelf-stable sweetpotato puree with no detectable microbial growth based on 90 days of storage at ambient temperature (Coronel and others 2005). However, further research is needed to validate the process for use in commercial production with respect to microbial inactivation. For validation of commercial sterilization, it is necessary to quantitatively demonstrate adequate inactivation of *Clostridium botulinum* or a relevant surrogate spore former.

Studies have shown that microwave heating inactivates vegetative bacterial cells and spores in a manner indistinguishable from that of conventional heating (Welt and Tong 1994). Thus, the sporicidal activities of microwave energy are simply a function of thermal heat converted from the microwave energy (Jong and others 1987). Indeed, an equivalent degree of inactivation of the spores of *B. subtilis* and other *Bacillus* species has been demonstrated using microwave as compared to conventional thermal processing (Celandroni and others 2004, Wang and others 2003).

Nonetheless, there are important considerations which are unique to microwave processing. For example, dielectric properties determine the extent of heating of a material subjected to electromagnetic waves. Dielectric properties consist of dielectric constant (ε') and dielectric loss factor (ε"). Dielectric constant is a measure of the ability of a material to store electromagnetic energy whereas dielectric loss factor is a measure of the ability of a material to

convert electromagnetic energy to heat (Metaxas and Meredith 1983). Loss tangent ($\tan\delta$), a parameter used to describe how well a product absorbs microwave energy, is the ratio of dielectric loss factor (ε") to the dielectric constant (ε'). A product with a higher loss tangent is heated faster under microwave field as compared to a product with a lower loss tangent (Nelson and Datta 2001). Assuming no magnetic losses in the materials, the microwave power absorbed per unit volume (Q) in a material is given by the following equation (Metaxas and Meredith 1983).

$$Q = 2\pi f \varepsilon_0 \varepsilon'' E_{rms}^2 \qquad (1)$$

where f is the frequency of the microwave in Hz, ε_0 is the permittivity of free space ($8.86H10^{-12}$ F/m), ε" is dielectric loss factor, and E_{rms} is the root mean square value of the electric field.

The exposure to electromagnetic energy to achieve a desired temperature is determined by the dielectric properties of the food, which significantly changes as the temperature of the food material changes (IFT 2000). For microwave processing, the lethal kill for sterilization efficiency is defined as the time and temperature history at the coldest location of the product being processed. Within a microwave system, critical process factors affecting lethality include the chemical and physical properties of the food material (ionic content, moisture, density, and specific heat), temperature, microwave frequency, and the microwave applicator design.

Microbial validation has been widely used for various thermal processes including retorting, high-temperature short-time (HTST) processes, and microwave heating (Pflug and others 1980, Smith and Kopelman 1982, Marcy 1997, Guan and others 2003). There are in fact many different types of biological indicators and methods which can be used in this regard, the appropriateness of which depends on the process and the target pathogen. For example, Smith and Kopelman (1982) used the inoculated pack method with *B. subtilis, B. stearothermophilus,*

and *Clostridium sporogenes* spores as indicators to validate commercial sterility of sweetpotato puree processed by steam flash sterilization and aseptic filling. The inoculated pack technique was also used by Guan and others (2003) for validation of a pilot scale 915 MHz microwave-circulated water combination heating system. However, the inoculated pack technique would not be suitable for the large volume of the material required in continuous flow processes. Pflug and others (1980) have developed biological indicators made of plastic to validate sterilization processes for canned products. Several others have produced encapsulated spores for process validation of canned products (Pflug and Smith 1977, Jones and others 1980). A recent and popular trend has involved the immobilization of viable bacterial spores (usually *B. subtilis* or *B. stearothermophilus*) in calcium alginate gel beads which are then seeded into the product pre-process and recovered post-process. This approach has been used to evaluate ultra high temperature (UHT) processing (Dallyn and others 1977) and microwave heating (Serp and others 2002). In the latter case, the immobilized spores were placed throughout the sterilization chamber for reliable multipoint mapping of the thermal treatment.

Recently, commercial biological indicators (SGM Biotech, Inc., Bozeman, MT), which contain high densities of purified bacterial spores encased in a thermoplastic polymer polypropylene pouch, have been released. The packaging material allows the indicators to tolerate high temperature processes. The bio-indicators contain growth media (pH = 6.85) with a pH indicator which changes color to signal microbial growth, providing a single endpoint detection. Alternatively, the liquid spore suspensions can be recovered from the pouches and plated for enumeration using standard cultural procedures. Successful utilization of the biological indicators to monitor an array of thermal processes used in the medical and industrial sectors has been reported (Gillis and McCauley 2006). Although they have the potential for use in thermal

process validation for foods, such studies have yet to be done. Accordingly, the objective of this study was to assess the feasibility of using the plastic self-contained biological indicators to evaluate spore inactivation using a continuous flow microwave system as applied to processing of sweetpotato puree.

MATERIALS AND METHODS

Sweetpotato Puree

Sweetpotato puree was purchased from the Bright Harvest Sweet Potato Co. (Clarksville, AK). The puree was made from the orange-fleshed Beauregard cultivar and had approximately 82% moisture content on a wet weight basis. The puree was packed in 20 lb bag-in-box containers, shipped frozen and stored at -20 ˚C until use.

Biological Indicators

The flexible, food grade plastic pouches of biological indicators were manufactured by SGM Biotech, Inc. (Bozeman, MT) and contained 0.1 ml of spore suspension of *G. stearothermophilus* ATCC 7953 or *B. subtilis* ATCC 35021 in sealed polypropylene (PP) tubing. These totally self-contained indicator pouches were 15 mm in length and 2 mm in thickness (Figure 1). The spore suspension had a pH of 6.85 and contained spores at populations sufficiently high enough to determine their log reduction for under target (126 ˚C), target (132 ˚C) and over target (138 ˚C) processing temperatures. Specifically, the *B. subtilis* indicator contained approximately 4.85×10^6 viable spores and the *G. stearothermophilus* indicator contained approximately 1.8×10^6 viable spores per pouch. The indicator pouches were stored under refrigeration (4 ˚C) until used to prevent germination and outgrowth of the spores.

D and z value Determination

The determination of D and z values was done by SGM Biotech, Inc. and reported upon receipt of the indicators. The fraction negative (FN) and most-probable-number (MPN) analyses were used to calculate the D-values based on the Stumbo-Murphy-Cochran method (Pflug 2003). Briefly, 10 replicates of biological indicator units were subjected to lethal stress sufficient to inactive all spores in some but not all replicate units. In the FN analysis, the units were subjected to different stress levels (heating times), all other factors held constant. Determining the fraction of replicate biological units that were negative assisted in estimating critical parameters of the surviving microbial population. Each stress level used 10 replicate pouches and the various heating times differed from one another by a constant interval of time. The exposures were done in a steam biological indicator evaluator resistometer (BIER) vessel (Joslyn Corporation, Macedon, NY) at the specific temperatures. After completion of each exposure, test units were incubated for 7 days at 55 ˚C for *G. stearothermophilus* and 35 ˚C for *B. subtilis*. After incubation, spore units were evaluated for colorimetric endpoint (color change = positive for growth, no color change = negative for growth) and the fraction of replicate biological units negative and positive for growth was used to calculate the critical parameters of the surviving microbial population. During the incubation period the spore test units were monitored for growth. After incubation, the suspensions were recovered from the pouches and evaluated to determine the MPN of survivors. Based on MPN data, the Stumbo-Murphy-Cochran (SMC) procedure was used to calculate the D-values at 115.6 ˚C, 118 ˚C, 121.1 ˚C, 124 ˚C and 126.7 ˚C for *G. stearothermophilus* and at 110, 115.6 ˚C and 121.1 ˚C for *B. subtilis*. The data collected during the determination of the D-values were used to calculate the Z-values for both spores using log linear regression (Association for the Advancement of Medical Instrumentation 2006). For *B. subtilis* ATCC 7953, the D-values at 121.1 ˚C, 126 ˚C, 132 ˚C, and 138 ˚C were 0.4, 0.12,

0.029 and 0.006 min, and the calculated Z-value was 9.5 ˚C. The D-values for *G. stearothermophilus* ATCC 35021 at these temperatures were 2.0, 0.51, 0.097 and 0.018 min, respectively, and the Z-value was 8.3 ˚C.

Measurement of Dielectric Properties

An open-ended coaxial probe (HP 85070B, Agilent Technologies, Palo Alto, CA) equipped with an automated network analyzer (HP 8753C, Agilent Technologies, Palo Alto, CA) was used to determine the dielectric properties of the spore suspension and sweetpotato puree. The calibration of the system was performed using a short block, air, and water. The spore suspension was heated in an oil bath (Model RTE111, Neslab Instruments Inc, Newington, N.H., U.S.A.) at a range of temperatures (20 ˚C, 75 ˚C, 90 ˚C, 100 ˚C, 110 ˚C, 120 ˚C, 125 ˚C and 130 ˚C) with 915 MHz frequency. The sweetpotato puree was heated in an oil bath (Model RTE111, Neslab Instruments Inc, Newington, NH) from 15 ˚C to 145 ˚C in 5 ˚C intervals with 915 MHz frequency. The dielectric constant and dielectric loss factor were calculated using the software provided with the probe based on the phase shift and magnitude of the reflected signal. Three repetitive measurements were performed for each duplicate sample.

Measurement of Viscosity

Viscosity of the puree was determined using a controlled stress rheometer (Stress Tech Rheological Instruments AB, Lund, Sweden) equipped with a pressurized sealed cell and a covette bob and cup. Compressed air was applied to the sealed cell at 29 psi to prevent boiling and excessive moisture loss of the sweetpotato puree. All measurements were performed in duplicate. Samples were pre-sheared for 30 s at 50 s^{-1} and allowed to equilibrate for 25 s before testing began. Shear rate sweeps were performed on the samples at 70 ˚C and 130 ˚C with shear rate ramped up and down from 1 to 250 s^{-1}.

60 kW Continuous Flow Microwave Heating Unit

A pilot scale 60 kW continuous flow microwave heating unit (Industrial Microwave Systems, Research Triangle Park, NC) operating at 915 MHz was used in this study. The temperatures were measured with thermocouples positioned at the inlet of the system, the inlet and exit of each applicator and the holding tube, as shown in Figure 2. The temperatures were recorded using a Datalogging system (HP 3497A, Agilent Technologies, Palo Alto, CA). The power supplied to the microwave system was adjusted to control the experimental temperatures of 126 ˚C, 132 ˚C, and 138 ˚C at the center point of the holding tube exit. The system was preheated by pumping hot water at 130 ˚C and recirculating it for approximately 30 min. The sweetpotato puree was then loaded into the system and the biological indicators were injected when the temperature at the center of the holding tube exit reached the experimental temperature. The biological indicators were mixed with the puree, filled in a tube, and released into the puree stream through the hopper or the injection tubes A/B as illustrated in Figure 2. The puree was heated to each processing temperature, with a residence time of 25 s in the holding tube, followed by rapid cooling to 70 – 80 ˚C in a tubular heat exchanger. Biological indicators were collected at the end of the cooling tubes, placed in ice slurry, and immediately taken to the laboratory for microbiological analysis.

Microbiological Tests

Biological indicators (3-10 units) were placed in sterile whirl pak bags and immediately incubated for 48 hours at 55 ˚C for *G. stearothermophilus* and 35 ˚C for *B. subtilis* after which they were evaluated colorimetrically as described above. After incubation of the indicators, an unchanged purple color indicated that all spores were inactivated. For biological indicators

containing surviving spores, acid production associated with bacterial growth caused a change in the pH, resulting in a color change from purple to yellow.

A second set of indicators were used for enumeration. Specifically, biological indicators obtained post-process were pooled (2 sets of 5 each, i.e., duplicate evaluations) for each processing run. Indicators were placed in a 10% hypochlorite solution for 1 minute to decontaminate the pouch surface, and then removed and air dried for 30 s. Dissecting scissors sterilized with 70% ethanol and flamed were used to cut open the indicator pouches. A P200 micropipette was used to remove 75 µl of the indicator spore suspension from each of the five pouches, and these aliquots were pooled in a 1.5 ml Eppendorf microcentrifuge tube to achieve a 375 µl volume of spore suspension for a pooled sample set. The suspensions were spread plated on brain heart infusion (BHI) agar (Becton Dickinson Co., Franklin Lakes, NJ) undiluted and after 10-fold serial dilution up to 10^{-5} using fluid D (Millipore, Billerica, MA) as the diluent. Plates were incubated for 48 hours at 55 ˚C for *G. stearothermophilus* and 35 ˚C for *B. subtilis* prior to manual counting. Untreated indicators were processed for colorimetric endpoint detection of growth and by plating to establish the baseline microbial population prior to thermal treatment.

Thermal Death Time and D-Value Calculations

The thermal death time or F-value was determined to evaluate the efficiency of the processing method. Using equation 2, the recorded temperature profile was utilized to calculate the F_0 to establish the sterilization value, where T is temperature (˚C) and t is the processing time (minutes). The sterilization value is used to ensure safety for low-acid (pH ≥ 4.6) foods processed to commercial sterility. Sweetpotato puree has a pH of 5.8 to 6.0. The reference

temperature used was 121.1 °C with a Z-value of 10°C for *C. botulinum* (Eszes and Rajko´ 2000, Heldman and Hartel 1997).

$$F_0 = \int_0^t 10^{\left(\frac{T(t)-T_0}{z}\right)} dt \tag{2}$$

Using a reference temperature (T_2) and the thermal resistance of the specific microorganism (z), the D-value at another processing temperature (T_1) can be estimated using Equation 3.

$$D_1 = D_2 * 10^{\frac{T_2-T_1}{z}} \tag{3}$$

RESULTS AND DISCUSSION

Dielectric Properties and Apparent Viscosity of the Processing Materials

The dielectric properties of the spore suspension and sweetpotato puree were measured at 915 MHz in a temperature range from 20 ˚C to 130 ˚C (Figure 3). Dielectric constant and dielectric loss factor were similar to the values reported by Fasina and others (2003) and Coronel and others (2005) for sweetpotato purees. Similar trends were reported by Guan and others (2004) for mashed potatoes, and for other food materials by Nelson and Datta (2001). The dielectric constant values for the spore suspension and the puree had similar values. However, dielectric loss factor values for sweetpotato puree were higher than those of the spore suspension, which is desirable because we did not want the indicators to receive a more extensive heat treatment than that applied to the carrier fluid during microwave processing. The apparent viscosity was measured at 70 ˚C and 130 ˚C (Figure 4). As expected, the puree

viscosity decreased as temperature increased, and the results are in agreement with those of Grabowski and others (2006). These changes in viscosity would be among the factors affecting the dielectric properties of the sweetpotato puree during microwave heating.

Injection of the Biological Indicators into the Microwave System

In an initial experiment, 40 biological indicator units were added to the puree (60 - 70 ˚C) and loaded into a tube (1.7 cm in diameter, 110 cm in length). The tube was then dipped to the base of the hopper to release the bio-indicators into the sweetpotato puree stream which was being pumped at a rate of 3.8 l/min. The intent was to collect the indicators at the end of a cooling tube with a side-entry strainer (W25E15SCC420B, Waukesha Cherry Burrell, Delavan, WI). Unfortunately, very few of the initial indicators injected into the process (20% from 3 replicated runs) were collected at the end of the runs. Several problems were noted: (i) indicator pouches tended to become trapped at the bottom of the hopper and were never processed; (ii) pouches tended to cluster, causing blockage of the system; and (iii) pouches adhered to the side of the pipe walls and blades of the static mixers.

An improved method was designed for injecting the biological indicators into the system. This included the use of two removable tubes connected to the continuous flow microwave system, which allowed injection of the units into the process stream without interrupting the puree flow (Figures 2). Each tube was connected to the system with inlet and outlet valves which were tightened using sanitary clamps. The injection tube was 91 cm in length with an internal diameter of 2.3 cm. The larger diameter facilitated the loading of the puree-bio-indicator mixture. In an effort to avoid cold slug, the tube was held in a warm bath to maintain the temperature of the puree at between 60 and 70 ˚C. In addition, the puree flow rate was increased from 3.8 l/min to 5.7 l/min in order to push the indicators more effectively through the static

mixers. The increased flow rate also assisted in overcoming the increased viscosity of sweetpotato puree in the cooling tube which occurred when the puree temperature was dropped from 130 ˚C to 70 ˚C (Figure 4); this also reduced the number of bio-indicators trapped during the cooling phase of the process. It was also necessary to decrease the total number of indicators used per replicate run from 40 to 30 which provided additional distance between individual units and reduced their tendency to associate with one another and clump. This also reduced warping of the indicators which occurred as processing temperatures approached the 160 ˚C melting point of polypropylene (Kissel and others 2003). Taken together, these improvements allowed us to retrieve about 60% of the input indicator units (Table 1 and 2). Most of the remaining bio-indicators were distributed along the static mixers during the tests, and they did not appear to hinder the movement of the pouches through the system.

The temperature of the sweetpotato puree was measured with thermocouples positioned at the inlet of the system, the inlet and exit of each applicator, and the inlet and exit holding tube (Figure 2). Figures 5a and 5b show time-temperature profiles recorded during the microwave processing of sweetpotato puree containing biological indicators for the target temperature of 132 °C. The inlet temperature of the puree fluctuated between 60 and 70 ˚C (Figure 5a). The temperature was approximately 100 ˚C at the exit of the first applicator, and was the target processing temperature after the exit of the second applicator. The outlet temperature of the puree after the cooling tube was cooled to between 70 and 80 ˚C is detailed in Figure 5b. For each injection, 2.3 liters of sweetpotato puree carrying bio-indicators passed through the two microwave applicators and entered a 2.4 m long holding tube with an internal diameter of 22.9 mm and a residence time estimated at 25 s. The radial temperature distributions for processing temperatures of 132 ˚C and 138 ˚C were narrow, which are in accordance to the temperature

profile reported by Coronel and others (2005). The average hold time within the holding tube for the volumetric flow rate was 25 s, and that of the fastest particles (center of the tube) was 12.5 s. The F_0-value was calculated based on the hold time required to achieve the specific log reduction in the population at the set of target processing temperatures used in this study. The fastest fluid elements (center, about 2 times of the mass mean residence time) at the under target (126 ˚C), target (132 ˚C) and over target (138 ˚C) processing temperatures received a thermal treatment equivalent to F_o = 0.65 min, 2.80 min, and 10.10 min, respectively (Table 1 and 2).

As described in the Materials and Methods section, the D-values for *B. subtilis* (0.4 min) and *G. stearothermophilus* (2.0 min) were much higher than those of *C. botulinum*, which generally has been reported to be 0.25 min at 121.1 ˚C (Holdsworth 1997). Thermal process guidelines stipulate the need to achieve a 12 D drop in *C. botulinum* for commercially sterile low acids foods, a process usually equivalent to a full exposure of 121.1 ˚C for approximately 3 min (Pflug and others 1990, Holdsworth 1997). In this study, at the target process temperature of 132 ˚C, a 8.55 D inactivation of *B. subtilis* and 2.50 D inactivation of *G. stearothermophilus* were equivalent to a 12 D inactivation of *C. botulinum.*

Microbiological Data

Microbiological results are detailed in Tables 1 and 2. The *B. subtilis* indicators receiving under target, target, and over target processes demonstrated log reductions exceeding 4.69 as evaluated using enumerative assays (Table 1). Furthermore, the *B. subtilis* units subjected to target and over target process temperatures showed no indication of spore survival in endpoint detection by colorimetric assays, confirming a log reduction >4.69 and in agreement with the predicted F_0 value. However, the *B. subtilis* bio-indicators subjected to the under target process demonstrated color changes in 10/53 pouches, suggesting that viable spores survived the 126 ˚C

thermal treatment. These results were anticipated because the calculated average degree of sterilization for a 126 ˚C process was 10.5 D reduction for the fluid element and a 5.32 log reduction for the fastest particle using *C. botulinum* as the target.

For the under target process, the *G. stearothermophilus* indicators demonstrated log reductions ranging from 1.34 to >4.26 (Table 2), with positive color changes in 29/37 processed pouches (Table 2). Consistent with predictions, *G. steraothermophilus* spores, which are quite heat resistant, should have displayed approximately 1.3 to 2.5 log reductions when exposed to under process conditions. The >4.26 $\log_{10}$ reduction of *G. stearothermophilus* in some pouches exposed to the under-target process was not expected. It was possible that several indicator pouches adhered to the surface of the static mixers and the tubes throughout the system, and in so doing, received longer heat treatment. For target and over target processes, log reductions exceeding 4.26 were seen for the *G. stearothermophilus* indicators (Table 2), which agrees with the expected log reductions for these processes. The indicators processed at target displayed color changes in 50% (15/30) of the pouches (Table 2), suggesting that the color-based assessment of growth is more sensitive than the enumerative assay. This is logical because plating detection limits were 100 CFU/ml while with the colorimetric endpoint, we could detect as few as 1 CFU per 0.1 ml of spore suspension per pouch. No color change was noted for the over target processed indicators, confirming the destruction of all viable spores and consistent with the predicted sterilization (F_0) value.

CONCLUSIONS

This study demonstrates a potential of using polypropylene packaged bio-indicators to evaluate thermal inactivation efficacy as applied to a continuous flow microwave process intended for

viscous food materials. Engineering the system was achieved, and we were able to recover approximately 60% of the input indicators injected into the system. Viability of the spores in the pouches could be evaluated by an endpoint colorimetric method or by cultural enumeration, and the results obtained were consistent with predicted thermal inactivation kinetics. In fact, the log reduction results for the *B. subtilis* indicators were equivalent to the pre-designed degrees of sterilization (F_0). Further refinements to of the system are necessary to improve the post-process recovery of the indicators and to facilitate monitoring their residence time within the holding tube. Overall, the use of bio-indicators for verification of spore inactivation in continuous-flow microwave processing of sweetpotato puree is feasible. This approach may be useful in evaluating microbial inactivation in aseptically processed viscous food products.

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Table 1. Growth Indicators and Count Reduction of Biological Indicators containing *Bacillus subtilis*

Initial Spore Count	Process Level	Degree of Sterilization F_0[a]	Degree of Sterilization F_0 [b]	Reps	Log Reduction value[c]	Indicators Positive	Indicators Negative
$4.85x10^6$	Under target process	1.29	0.65	1a	>4.69	4	14
				1b	>4.69		
$4.85x10^6$		1.29	0.65	2a	>4.69	2	13
				2b	>4.69		
$4.85x10^6$		1.29	0.65	3a	>4.69	4	16
				3b	>4.69		
$4.85x10^6$	Target process	5.13	2.80	4a	>4.69	0	12
				4b	>4.69		
$4.85x10^6$		5.13	2.80	5a	>4.69	0	16
				5b	>4.69		
$4.85x10^6$		5.13	2.80	6a	>4.69	0	13
				6b	>4.69		
$4.85x10^6$	Over target process	20.41	10.10	7a	>4.69	0	9
				7b	>4.69		
$4.85x10^6$		20.41	10.10	8a	>4.69	0	10
				8b	>4.69		
$4.85x10^6$		20.41	10.10	9a	>4.69	0	10
				9b	>4.69		

[a]F_o, based on volumetric holding time (Unit: minutes)
[b]F_o, based on fastest particle/element (Unit: minutes)
[c]Log_{10} reduction for *Bacillus subtilis* based on enumerative assay; inactivation was calculated based on the difference between the log of the initial counts (4.85 x 10^6) and the log of the final counts. The limit of detection of the assay was 100 CFU/ml, so the absence of colonies after processing constituted a >4.69 log inactivation (i.e., 6.69-2.0=>4.69).

Table 2. Growth Indicators and Count Reduction of Biological Indicators containing *Geobacillus stearothermophilus*

Initial Spore Count	Process Level	Degree of Sterilization F_0[a]	Degree of Sterilization F_0 [b]	Reps	Log Reduction value[c]	Indicators Positive	Indicators Negative
1.8 x 10^6	Under target process	1.29	0.65	1a 1b	1.34 >4.26	14	0
1.8 x 10^6		1.29	0.65	2a 2b	>4.26 1.57	11	3
1.8 x 10^6		1.29	0.65	3a 3b	1.68 2.99	4	5
1.8 x 10^6	Target process	5.13	2.80	4a 4b	>4.26 >4.26	7	3
1.8 x 10^6		5.13	2.80	5a 5b	>4.26 >4.26	5	8
1.8 x 10^6		5.13	2.80	6a 6b	>4.26 >4.26	3	4
1.8 x 10^6	Over target process	20.41	10.10	7a 7b	>4.26 >4.26	0	9
1.8 x 10^6		20.41	10.10	8a 8b	>4.26 >4.26	0	1*
1.8 x 10^6		20.41	10.10	9a 9b	>4.26 >4.26	0	10

[a]F_o, based on volumetric holding time (min)
[b]F_o, based on fastest particle/element (min)
[c]Log_{10} reduction for *Geobacillus stearothermophilus* based on enumerative assay; inactivation was calculated based on the difference between the log of the initial counts (1.8 x 10^6) and the log of the final counts. The limit of detection of the assay was 100 CFU/ml, so the absence of colonies after processing constituted a >4.26 log inactivation (i.e., 6.26-2.0=>4.26).

*1 bio-indicator was removed from the run due to the problem on surface adherence.

Figure Legends

Figure 1. Bio-indicators Containing Spores of the Tested Microorganisms

(A = Unprocessed, B = Processed with Negative Result, C = Processed with Positive Result)

Figure 2. Schematic Diagram of the Processing System (Adapted from Cornel and others, 2005, with modifications)

Figure 3. Dielectric Properties of the Biological Indicators Spore Suspension and Sweetpotato Puree at 915 MHz

Figure 4. Apparent Viscosity of Beauregard Sweetpotato Puree

Figure 5a. Typical Temperature-Time History at the inlet of the Microwave Heating Section and Exit of Second Microwave Heating Applicator

Figure 5b. Typical Temperature-Time History at the Exit of Holding and Cooling Section

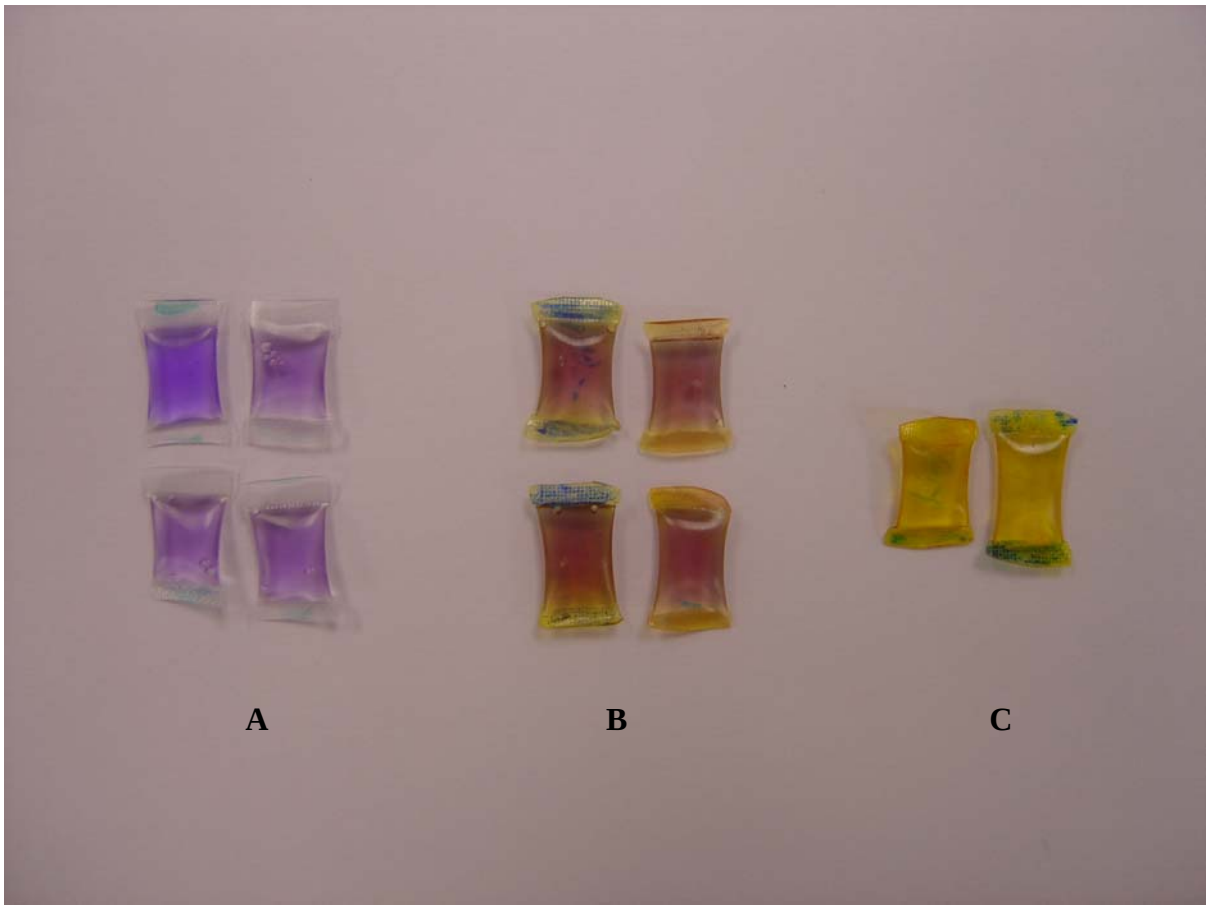

Figure 1. Bio-indicators Containing Spores of the Tested Microorganisms
(A = Unprocessed, B = Processed with Negative Result, C = Processed with Positive Result)

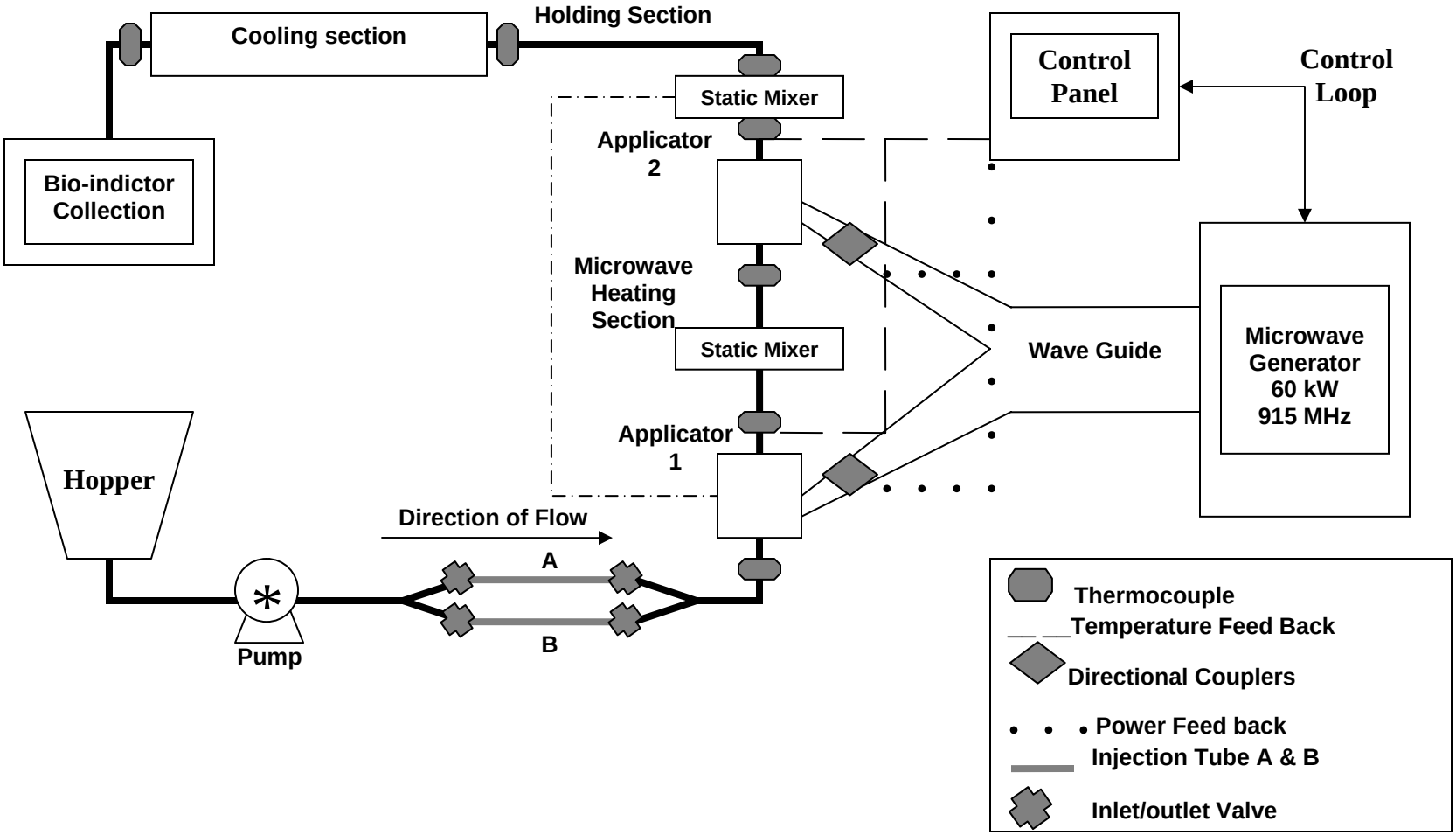

Figure 2. Schematic Diagram of the Processing System (Adapted from Cornel and others, 2005, with modifications)

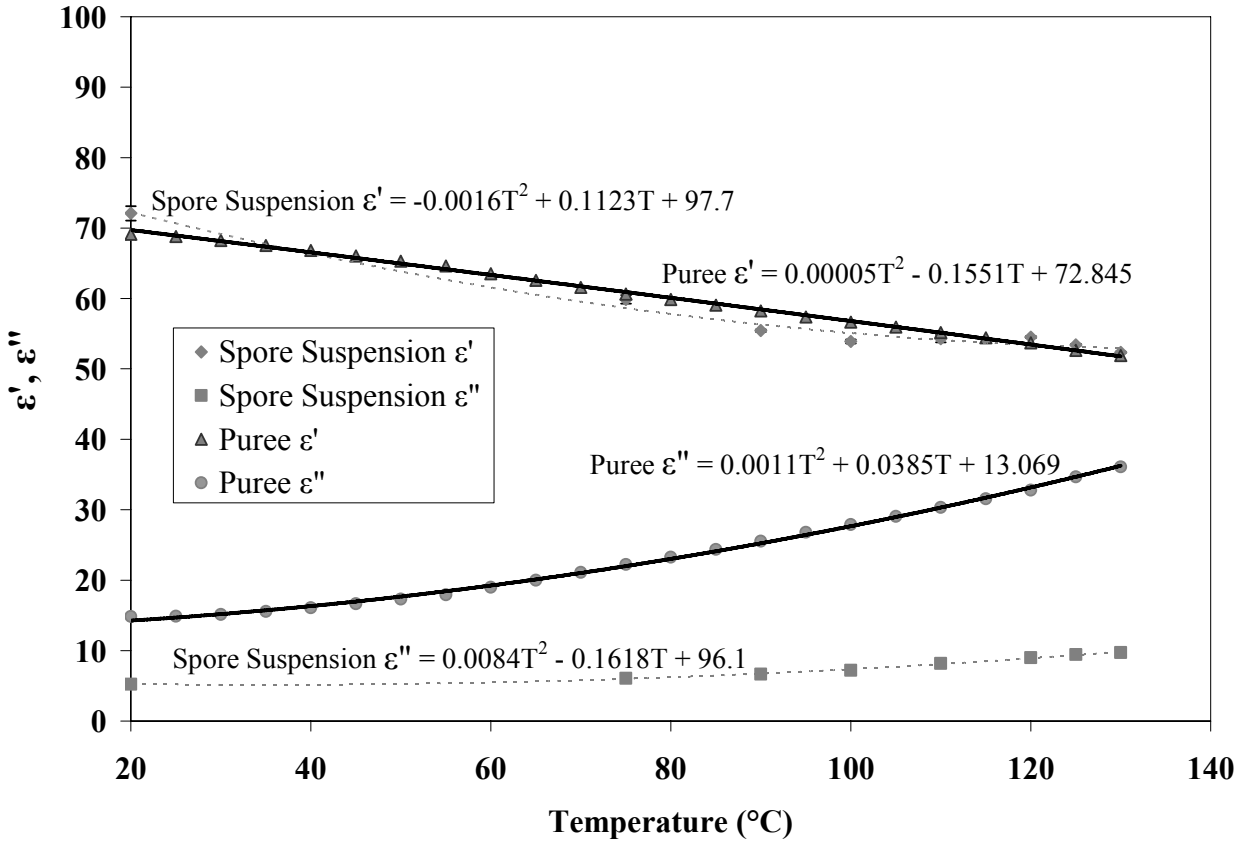

Figure 3. Dielectric Properties of the Biological Indicators Spore Suspension and Sweetpotato Puree at 915 MHz

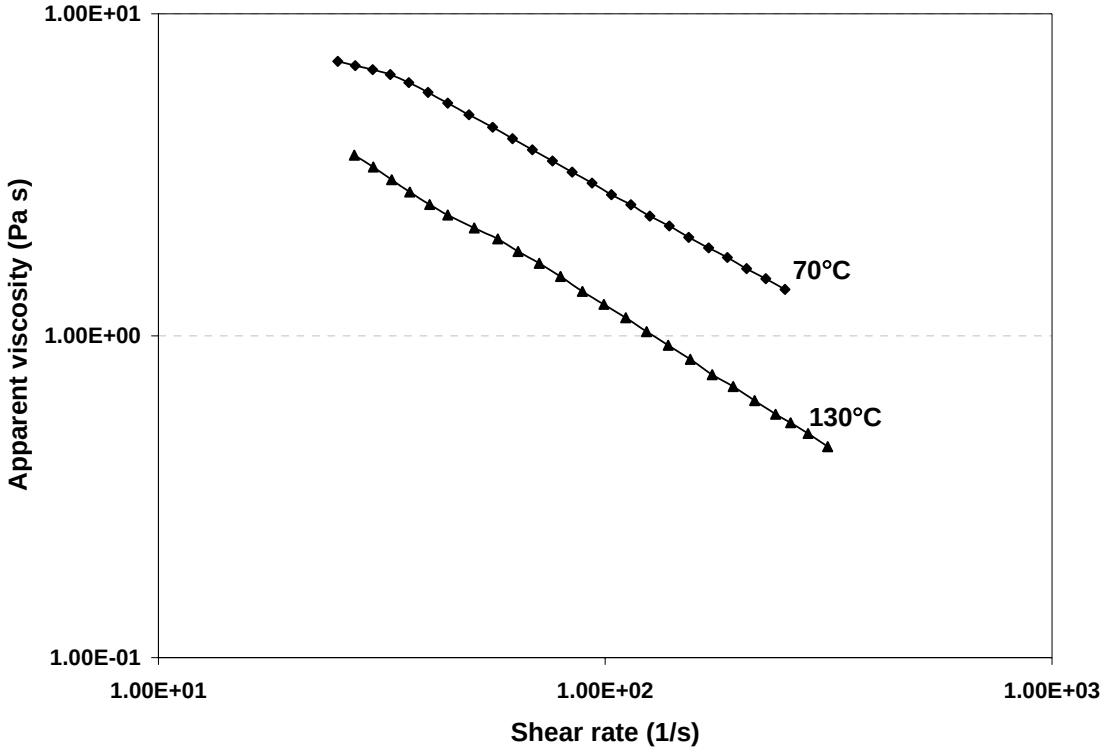

Figure 4. Apparent Viscosity of Beauregard Sweetpotato Puree

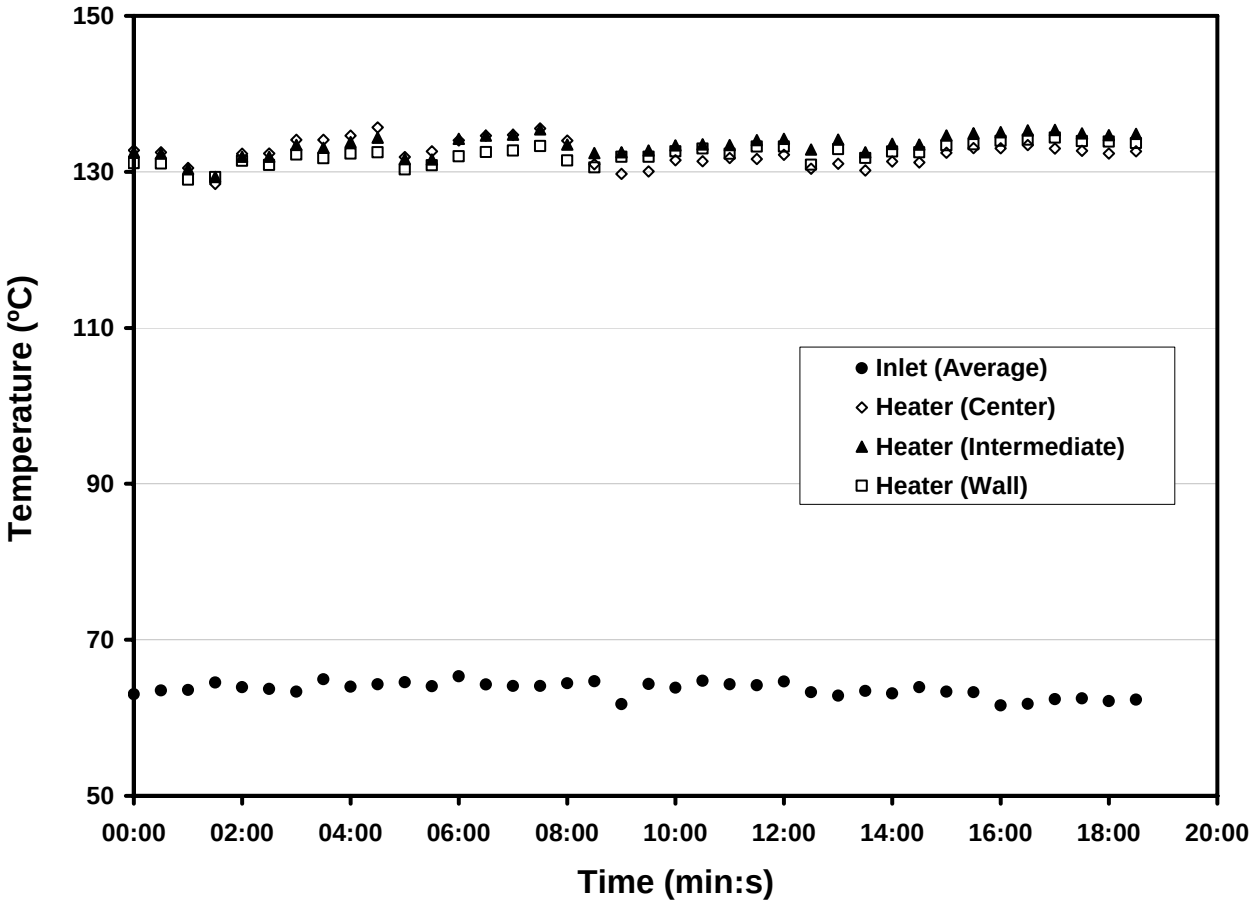

Figure 5a. Typical Temperature-Time History at the Inlet of the Microwave Heating Section and Exit of Second Microwave Heating Applicator

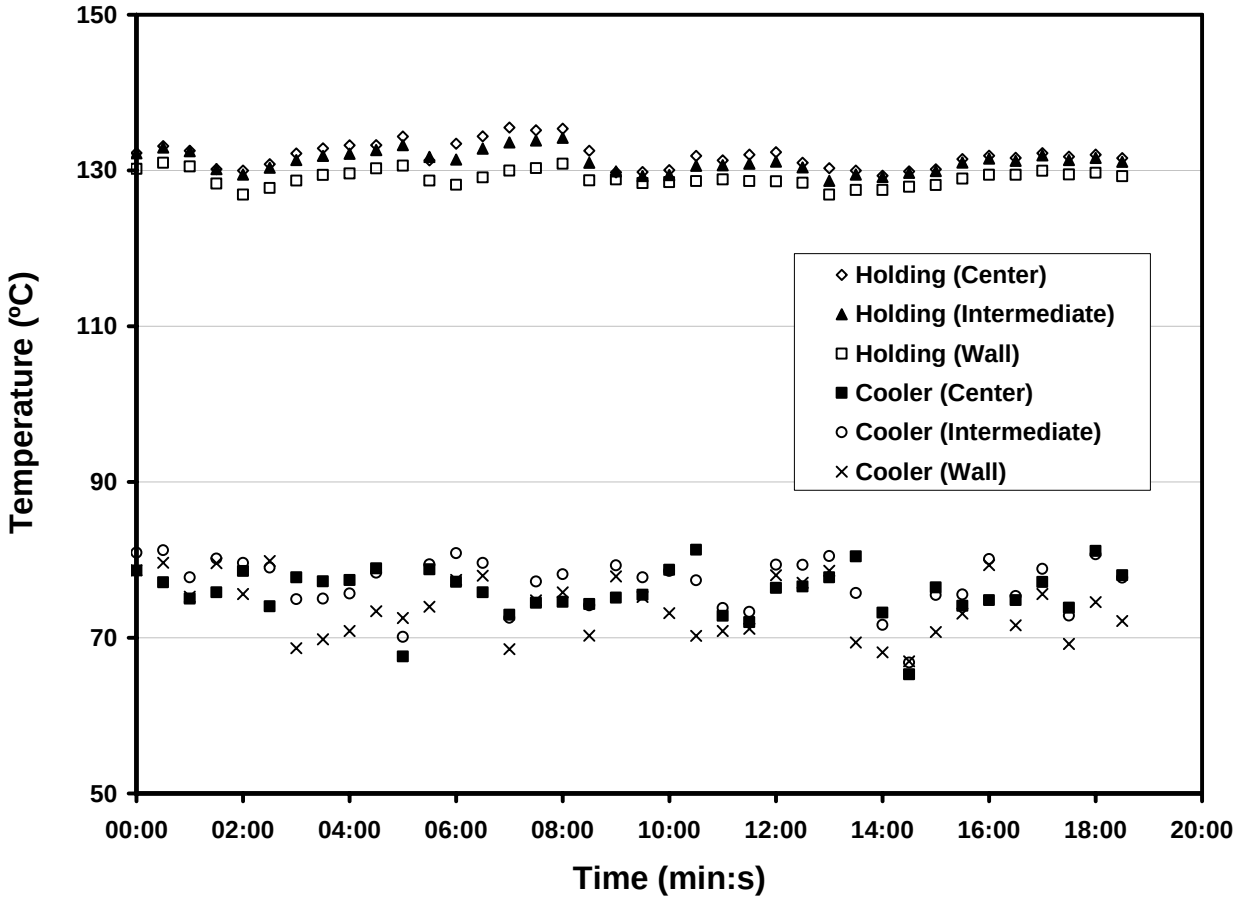

Figure 5b. Typical Temperature-Time History at the Exit of Holding and Cooling Section

NOMENCLATURE

D	Thermal Death Time (min)
F_0	Sterilization Value (min)
T	Temperature (˚C)
t	Time (s)
V	Volume (m^3)
z	Thermal Resistance of Microorganisms (˚C)
ΔT	Change in Temperature (˚C)
ε'	Dielectric Constant
ε"	Dielectric Loss Factor
Q	Microwave Power Absorbed Per Unit Volume
ε_0	Permittivity of Free Space
f	Frequency (Hz)
E_{rms}	Root Mean Square Value of the Electric Field.