INACTIVATION OF WILD-TYPE BACILLUS SPORES IN A SOY MEAT ANALOG MODEL BY EXTRUSION COOKING

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by

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The undersigned, appointed by the Dean of the Graduate School,

have examined the thesis entitled

INACTIVATION OF WILD TYPE BACILLUS SPORES IN A SOY MEAT ANALOG MODEL BY EXTRUSION COOKING

presented by Rosemary Mwangi

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and hereby certify that in their opinion it is worthy of acceptance.

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INACTIVATION OF WILD-TYPE BACILLUS SPORES IN A SOY MEAT ANALOG MODEL BY EXTRUSION COOKING

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ABSTRACT

The heat resistance of spores continues to be a challenge in food processing applications. Suitable methods that effectively inactivate spores without producing adverse effects on quality and nutrition of food products are constantly being sought. In this study, the efficacy of extrusion cooking (180°C, 125 rpm, 1 MPa pressure) in inactivating wild-type spores in a soy meat analog was determined. The effect of several media in influencing heat resistance was also tested. The recovery media used were Nutrient Agar (NA), Nutrient Agar with 1 ppm Calcium plus starch (NACaS) and fortified concentrated Tryptone glucose extract (TGE) agar with Calcium and dipicolinate (Ca-DPA TGE).

The wild-type spores were isolated from soy flour, soy protein concentrate and wheat gluten. Several sporulation media were tested to determine the media that would yield the most heat resistant spores. Sporulation Agar I was determined to result in the most heat resistant spores. Next, the D-values of *B. cereus*, 4II 1, *B. stearothermophilus* and 3III 1C spores suspended in peptone water were obtained. Both *B. cereus* and 4II 1 spores that were less heat tolerant had a comparable D-value$_{100°C}$, while *B. stearothermophilus* and 3III 1C spores that were more heat resistant also had a comparable D-value$_{150°C}$. Extrusion processing of the soy meat analog inoculated with *B.*

stearothermophilus, 4II 1 and 3III 1C spores also revealed that *B. stearothermophilus* spores were the most heat resistant. However, cooking significantly reduced the numbers of *B. stearothermophilus*, 4II 1 and 3III 1C spores inoculated into the soy meat analog.

CHAPTER 1

INTRODUCTION

The protein nutritional value from experimental and commercial extruded texturized vegetable products (TVP) has been assessed in adolescents and adults. In adults, protein digestibility and nitrogen balance (g N/ day) was 66.1% and 1.16 in TVP from defatted soy flour and 63.4% and 1.31 g N/ day in TVP from soy protein concentrate TVP, respectively. In comparison, beef possessed 73.2% protein digestibility and 0.42 g N/day (Cheftel 1986). Thus, TVP, such as soy meat analogs, provide a good source of protein that is readily digestible. Soybeans are also a good source of calcium and linoleic acid. Alpha-linoleic, an essential fatty acid, is an omega-3 fatty acid present in soybeans. A consistent consumption of soy foods also confers certain health benefits. Thus, soy foods that provide a good source of nutrition are especially vital for vegetarians, and provide a good substitute for meat products (Wardlaw and Kessel 2002).

In the production of TVP by extrusion cooking, the unfolding and aggregation of protein that occurs when they are heat processed is utilized in generating the resultant texture. After dissociation of the protein structure by high extrusion temperatures and shear, the protein forms dry, insoluble, expanded and fibrous structures upon aggregation that readily rehydrate into elastic and chewy meat analogs (Cheftel 1986).

The *Bacillus cereus* group consists of six genetically related species: *Bacillus anthracis*, *Bacillus cereus*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus pseudomycoides*, *Bacillus thuringiensis* and *Bacillus weihenstephanensis* (Ankolekar and others 2008). *B. cereus* is a gram-positive, facultative anaerobe, spore-forming, motile microorganism. *B. cereus* is considered a human opportunistic pathogen. *B. cereus*

causes diarrhea and emetic types of food poisoning (Zhou and others 2008). The toxin cereulide that is produced in the food, is responsible for emetic food poisoning, while diarrheal food poisoning results from the production of enterotoxins. It is thought that the enterotoxins are produced in the intestine after ingestion of *B. cereus*-like organisms (Rosenquist and others 2005). *B. cereus* produces three enterotoxins, the most widely characterized enterotoxins are haemolysin BL (HBL) and non-haemolytic enterotoxin (NHE) that are composed of three different protein complexes. The third enterotoxin, cytotoxin K (CytK), is composed of a single protein (Rosenquist and others 2005; Ankolekar and others 2008, Zhou and others 2008). *B. cereus* has been isolated from a wide variety of foods, such as fresh vegetables (Valero and others 2002), pasteurized milk (Zhou and others 2008), rice, desert mixes, infant foods, spices, ready to serve foods, seafood and meat products (Ankolekar and others 2008). As a result, *B. cereus* presents a potential risk in food safety.

Extrusion processing also permits the ultra-high temperature processing of products for very short period of time (30-120 s), thereby limiting the adverse effects of heat treatment, such as loss of nutrients, Maillard reactions, and so forth. The treatment conditions used in extrusion cooking have also proven to be effective in significantly reducing spores that are very resistant to heat treatment. Consequently, extrusion cooking was used in this study to determine its efficacy in inactivating wild-type spores inoculated into a soy meat analog. The objectives of the study were:

1) To isolate wild-type bacilli from soy flour, soy protein isolate and wheat gluten.
2) To determine the D-values of the wild-type bacilli spores and compare them to those of *B. cereus* and *B. stearothermophilus* spores.

3) To determine the possible use of the isolated wild-type bacilli as indicators of pathogenic *B. cereus* in heat inactivation studies and, in particular, in the effect of extrusion processing in inactivating the wild type spores.
4) To compare the effect of extrusion cooking in inactivating the wild-type spores to the inactivation of *B. stearothermophilus* spores in a soy meat analog.

A review of the studies conducted has proven that *B. stearothermophilus* spores are more heat resistant than *B. cereus* spores, and are also among the most heat resistant aerobic spores. Accordingly, inferences on the heat resistance of the wild-type spores in comparison to the heat resistance of *B. cereus* spores and other bacilli spores could be deduced.

CHAPTER 2

LITERATURE REVIEW

2.1 History of Bacilli

Bacilli were first studied by Cohn in 1876 and a little later in the same year by Koch. Cohn wondered why a 100°C heat treatment was not sufficient to kill the bacteria present in hay infusions, probably *Bacillus subtilis* spores, while most living organisms died at much lower heat treatment temperatures. Cohn detected numerous straight motile rods in the hay infusion broth and also observed refractile bodies in these rods. He also determined that these refractile bodies did not germinate in the liquid they were formed but they would germinate in new hay infusion broth. Koch, on the other hand, sought to find cures for diseases, specifically anthrax, by better understanding this new science of bacteriology. About 12 years later, in 1888, Koch observed the sporulation of *Bacillus anthracis* and other spore-formers. Moreover, Koch described the first complete cycle of sporeformers, from sporulation to germination and then multiplication and back to re-sporulation, of spore-forming microorganisms (Gould 2006).

2.2 Bacilli

Bacilli are rod-shaped cells that are more or less straight. These rods occur singly or in chains. They are mostly Gram-positive. However, while some are Gram-positive during the early stages of growth, they become Gram-negative as they age. Bacilli are aerobic or facultatively aerobic. They have endospores that are formed during adverse conditions that confer high resistance to these unfavorable conditions. Each cell contains only one endospore. The formation of these spores is enhanced under aerobic conditions.

Bacilli possess variable colony morphology, as they vary in size as well as form pigments in some media. They can withstand a wide range of environmental conditions, some are psychrophilic, others mesophilic and still others are thermophilic. Some are acidic-loving while others prefer alkaline environments. Lastly, some are salt tolerant while others have specific salt requirements. Catalase is formed by most species. Finally, some are oxidase positive while others are oxidase negative (Sneath 1984).

2.3 Sporulation

2.3.1 Stages of the sporulation sequence

After Koch observed the entire life-cycle of sporeformers, Knaysi (1938) described sporulation in detail as observed under a light microscope. The onset of electron microscopy allowed researchers like Fitz-James, in 1960, to describe the processes that led to the transformation of a vegetative cell to a spore. In 1966, Ryter and a group of other researchers finally subdivided the sporulation sequences into the seven stages of sporulation that are used today (Gould 2006).

Sporulation usually follows after the end of the logarithmic growth and division in sporulating microorganisms. On the other hand, it possible to circumvent this period of exponential growth and proceed directly from a spore to spore "microcycle". In each case, the duration of time between germination and sporulation is the same in similar species. Under both normal and microcycle sporulation, sporulation proceeds at the same rate and the same morphological changes that accompany each sporulation stage are observed (Fitz-James and Young 1969).

Stage I Formation of the axial filament

At the end of exponential growth that precedes sporulation, chromatin bodies appear compact and discrete as seen under the electron microscope. In addition, each cell has two chromatin bodies that are suspended to the peripheral membrane via associated mesosomes. As the logarithmic growth phase comes to an end, DNA synthesis ceases. The chromatin bodies, on the other hand, continue to change shape, flowing into one another until, cytologically, they form one rope or axial thread in the cell. Now the cell has one chromatin body that is still attached to the mesosomes at each pole of the cell. While the significance of the axial thread of the chromatin is not clear, it has been determined in several varieties of *Bacillus cereus*, *B. subtilis*, *B. anthracis*, *Clostridium pectinovorum* and *Clostridium histolyticum*. However, this deformation of chromatin is reversible in standard culturing conditions when the sporulation media is replaced with fresh media (Fitz-James and Young 1969).

Stage II Formation of spore septum and Stage III Engulfment of the forespore

"The formation of the forespore septum is the first definite sign that a cell is entering sporulation" (Fitz-James and Young 1969). The relationship between the mesosomes anchoring the axial filament to the mesosomes at the forespore fold is not clearly understood. Morphological evidence as observed under the electron microscope indicates that one pair of DNA attached mesosomes a short distance away from the pole of the cell are the site of septum that proliferates into the forespore membrane.

The cell membrane forms a pocket within the cell and chromatin material and its mesosome for the future spore are deposited into this pocket (forespore). The membrane induced pocketing arises from a pair of DNA attached mesosomes that form a septum

that asymmetrically divides the mother cell. Then, "two mesosomes that are not attached to DNA develop outside the forespore (compartmentalized DNA)", and these continue to develop as the future spore membrane with "continued proliferation around the cell end", completely surrounding the forespore. The mesosomes and the membranes that surround the forespore are newly synthesized (formed de novo) and not "shifted into folds" from membranes formed at other sections of the sporangium (Fitz-James and Young 1969).

Stage IV Formation of the cortex

The cortex is a "critical" structure in the maintenance of the dormant spore. In sections of spores observed under an electron microscope, the cortex is observed as a dense narrow band around the forespore profile. The cortex is formed by the deposition of peptidogylcan between the inner forespore membrane and the outer forespore membrane in a two stage process. In the first step the vegetative cell-type polymer is laid down, while in the second step the spore specific peptidoglycan of the cortex is laid down. Thus, during early development the cortex appears as an undifferentiated band. When the cortex is fully formed as in a mature spore an inner band appears that is slightly more dense than the cortex. This inner band lies on the surface of the plasma membrane or inner forespore membrane. As the inner band is located above the plasma membrane and is present even in the mature spore, it plays a role as the primordial cell wall (cortical membrane or germ cell wall) (Fitz-James and Young 1969; Russell 1982).

Various methods are used to extract the spore cortex for analysis, including acid treatment to induce lysis. An analysis of the cortex has revealed four major bands that make up the cortex. The inner layer is reminiscent of a primordial cell wall and is folded and less elastic as it fails to adjust with the increased cortical swelling. The cortex is

striated and more elastic, while the coat and exosporium were not changed much by the acid treatment. The striated and plastic nature of the cortex has also been observed from negatively stained "coat-cortex" preparations that have revealed a rigid spore coat. However, spores treated with Cleland's reagent to remove the spore coat have revealed a spongy fibrous matrix making up the cortex. The cortex has also been observed as the main covering of spores treated with chloramphenicol to dissolve the spore coat (Fitz-James and Young 1969).

The cortex possesses elasticity that is conferred by the introduction of Ca^{2+} or Ca-DPA during sporulation, which decreases the repulsion of carboxyl groups present in the peptidoglycan making up the cortex. During sporulation, the contraction of this layer may dehydrate the protoplast (spore core) that is thought to be one of the mechanisms by which heat resistance of the spore is enhanced (Vinter 1969).

With the complete formation of the forespore and formation of the cortex, the sporulating cell is now committed to completing the sporulation cycle before it can germinate and enter the vegetative cycle (Fitz-James and Young 1969).

Stage V Formation of the spore coat

Although, formation of the spore coat takes place in this stage, deposition of the part of the layers of the spore coat also takes place during the early stages of spore formation. The deposition of proteins in forming a visible coat is comparatively late and is cystine-dependent. The deposition of the spore coat is also first observed after the formation of the cortex. The synthesis of the proteins that are deposited to form the spore coat can be traced back to the forespore stage. In *C. pectonovorum* the spore coat first develops as discontinuous masses of dense coat material deposited in various regions on

the cortex that then join to form a continuous spore coat. On the other hand, in *Bacillus* spores the spore coat develops as a continuous spreading coat layer. The above differences in coat formation between *C. pectonovorum* and *Bacillus* microorganisms has been revealed by studies with the electron microscope that have not revealed the sites of protein synthesis (Fitz-James and Young 1969).

While perisporal mesosomes appear to play a role in the deposition of spore coats, this is yet to be proven as spore coats have been formed in *Bacillus megaterium* that does not possess mesosomes or when present are far from the spore surface.

Stage VI Spore maturation and Stage VII Lysis of the sporangium and liberation of the mature spore

In this stage, the density of the spore coat increases, spore refractility and heat resistance develops. The spore also becomes dehydrated and resistance to UV radiation develops. Refractility of the spore begins to develop during late coat formation. Refractility as well as heat resistance are both associated with the synthesis of DPA and the uptake of a significant amount of calcium by the mature spore (Fitz-James and Young 1969; Russell 1982). Once the spore reaches maturation, the mother cell lyses releasing the spore.

2.3.2 Components of the mature spore

Calcium is incorporated in *B. cereus* during the development of the forespore. However, the calcium incorporated is unstable and can be removed by washing the spores. Thus, it is not clear whether the calcium was incorporated into the spore or bound to the spore surface. Binding of calcium in bacilli precedes DPA synthesis. "The same

phenomenon has been observed in *Clostridium roseum*". However, calcium induced DPA synthesis is dependent upon the time of incorporation of the calcium into the spore. The addition of calcium after cortex formation in *B. cereus* T spores had commenced did not stimulate increased DPA synthesis. In *B. cereus* var *alesti* grown in complex but calcium deficient medium, the addition of calcium after the beginning of "whitening" of forespores greatly increased DPA synthesis (Vinter 1969).

DPA is synthesized in the mother cell and is taken up into the forespores. DPA synthesis has been observed to be concomitant with the development of refractility in the spores, accumulation of calcium, iron and changes in distribution in hexosamine-containing material. Hence, synthesis and uptake of DPA from the mother cell is paralleled by the above prevailing conditions. DPA appears in bacilli during cortex formation and maturation of the spores. DPA is located only in the spore core of the mature spore (Vinter 1969; Setlow 2005).

A very important group of proteins, small acid soluble spore proteins (SASP) occurring in alpha and beta conformity, are found in the spore core. These proteins have short peptide chains of 60-75 amino acids. These proteins are produced only in sporulating species. They are synthesized in the developing forespore during late sporulation, just before the incorporation of DPA into the forespore. The α/β SASP confer significant heat resistance to the spore in addition to resistance against many chemicals. The amino acid sequences of these proteins are very highly conserved both within and across species (Setlow 2006).

Another integral component of the mature spore, cystine rich proteins, are formed relatively early during sporogenesis as there is a higher requirement of sulfur amino acids during sporogenesis. These cystine-rich proteins are located in the spore coat.

Other integral proteins found in the new spore coat include diaminopimelic (DAP) acid and trichloroacetic acid (TCA). "Increased incorporation of exogenous radioactive diaminopimelic acid into the hot TCA-insoluble fraction was observed in *B. cereus* during sporogenesis" (Vinter 1969). DAP that is found in the cortex, is first incorporated at septation and then during the DPA synthesis and calcium accumulation. Both TCA and DAP in spores are formed fresh (de novo) and pre-existing DAP in the vegetative cell is not incorporated into the de novo formed DAP in the spore. Only exogenous C-DAP (cytoplasmic DAP) can be incorporated into the DAP in the newly formed spore coat (Vinter 1969).

2.4 Activation of bacterial spores

Ending the dormant nature of bacterial endospores and transforming them into vegetative cells involve three sequential processes, namely, activation, germination and outgrowth (Keynan and Evenchik 1969). The first process, activation, is a reversible process. Activation ends dormancy of spores and prepares the spore for germination. However, the spore still retains its properties of refractility, non-stainability, heat resistance and dipicolinic acid content (DPA). Activation does not occur in all spores and is dependent upon the sporulation medium, the chemical composition of the spore, the history of the spore and the nature of the germinants used to germinate the spore, (Keynan and Evenchik 1969; Berg and Sandine 1970). Activation does not involve metabolism, but is brought about by changes in the configuration of macromolecules,

particularly the tertiary structure of macromolecules. The kinetics of activation resemble the melting curves of macromolecules (Berg and Sandine 1970).

2.4.1 Mechanism of activation

Spores differ from their vegetative cells in that they contain five times more sulfur. This additional sulfur is present in the form of cystine, which is contained in the spore coats. It is postulated that the spore coat proteins, which are rich in cystine, are responsible for maintaining spore dormancy. These spore coat proteins are stabilized in a specific arrangement by disulfide (S-S) linkages. Thus, it is thought that activation is brought on by the reduction of S-S bonds resulting in the unblocking of an enzyme system or by a change in the permeability of a structure controlling the dormant state of the spore (Berg and Sandine 1970). Activation has been brought on by several mechanisms including, heat, calcium-DPA, low pH and reducing agents, chemical treatment, ionizing radiation and aging.

2.4.1.1 Heat activation of bacterial spores

The activation of spores by heat is also dependent on other variables, including the particular genus of bacteria as well as species of *Bacillus* being activated. Some strains, such as *Bacillus megaterium* are activated at 60°C, while other species, such as *Bacillus stearothermophilus* require 115°C. Moisture is also needed in heat activation. Additionally, heat activation is affected by pH, but this is also strain dependent. Keynan and Evenchik (1969) showed that the optimal pH for the heat activation of *B. cereus* T spores was between 2 and 3 when heated at 60°C. Activation at the same temperature at pH lower than 2 took 10 min. It has also been shown that *B. cereus* T spores are not

activated by high pH above 8.5. On the other hand, *Clostridium bifermentans* is heat activated at high pH but not at low pH (Keynan and Evenchik 1969; Berg and Sandine 1970).

Heat activation of *Bacillus* spores is also impeded by the presence of certain cations. The composition of the suspending medium as well as the composition of the sporulating medium also influences heat activation (Keynan and Evenchik 1969; Berg and Sandine 1970).

In an experiment carried out exposing *B. subtilis* to a temperature range of 5-94°C, the activation energy (ΔH), involved in bringing about activation at each temperature, was the same. Furthermore, the change in entropy (ΔS), resulting from the formation of the activated complex was very small 6.1 Kcal/°C. Thus, it was concluded that heat activation was brought about by the breakage of a few strong bonds and not many weak bonds (Berg and Sandine1970).

2.4.1.2 Activation by calcium-dipicolinic acid (DPA)

The addition of equal moles of calcium and DPA to *B. subtilis* 168, *B. cereus T* spores and *B. megaterium* has brought about their activation even in the absence of heat. However, *B. subtilis* 168 and *B. megaterium* do not germinate in the calcium-DPA solution. But, *B. megaterium* spores germinate spontaneously in distilled water (Keynan and Evenchik 1969; Berg and Sandine 1970).

It is thought that the Ca^{2+} and DPA ions in the saturated solution attract and bond with the Ca^{2+} and DPA ions in the spore, thereby uncovering receptors that bind with

germinants, such as L-alanine. Calcium-DPA treatment may also increase permeability of the spore (Berg and Sandine 1970).

2.4.1.3 Activation by low pH and reducing agents

Low pH reduces the time needed to activate *B. cereus* T and *B. subtilis* spores. While it takes about 30 min-3h to activate both bacilli in distilled water, it took only 12 min to activate both bacilli at a pH of 1.0 (Berg and Sandine 1970).

Reducing agents, such as mercaptoethanol and thioglycollate, have also been shown to induce reversible activation of *B. cereus* T spores. Activation by these reducing agents takes longer than activation by low pH, as in the case of mercaptoethanol and thioglycollate, where it took at least 12 h before activation occurred. With *C. bifermentans*, however, no activation was observed. Furthermore, activation by reducing agents appears to be influenced by pH and correlates to spontaneous activation at low pH. Activation by reducing agents is greatly enhanced at pH 4.5-7.2 (Keynan and Evenchik 1969; Berg and Sandine 1970).

Activation at pH extremes, low pH and high pH, occurs in the range at which proteins are denatured. Reducing agents are also known to cause denaturation of proteins.

2.4.1.4 Activation by chemical agents

Bacillus pantothenticus spores are activated by strong polar solvents such as dimethylformamide and dimethylsulfoxide and concentrated solutions of urea, at room temperature. *B. megaterium* spores have been activated by aqueous ethyl alcohol coupled with heat treatment at 30°C, as well as by treatment with water vapor at room

temperature and germinated by glucose. The effectiveness of activation by water vapor was influenced by exposure time, relative humidity and temperature of treatment. Spores activated by ethyl alcohol and water vapor treatments could also be deactivated.

Very polar solvents rupture hydrogen bonds, causing changes in tertiary structures of proteins. Solvents that cause protein denaturation by breaking the hydrogen bonds have been shown to readily induce activation of bacteria spores, even at low temperatures.

As water is needed for activation, it seems that activation site, whose location is unknown needs to be hydrated before activation commences. Thus, some activation mechanisms act by opening the spore structure, thereby, permitting this activation site to be hydrated. Treatment of *B. megaterium* spores with aqueous ethyl alcohol and water vapor as stated above activated spores, while aqueous *B. megaterium* spore suspensions were not activated until heated. Thus heating ethyl alcohol may alter the water molecules as they enter the vapor state, such that they are able to reach and hydrate the critical activation site. This takes place as water in its vapor state has weaker hydrogen bonds. Ethyl alcohol may also act by decreasing the surface tension of water or changing the structure of water enabling the water to reach critical spore sites (Berg and Sandine1970).

2.4.1.5 Activation by ionization radiation

Irradiated spores of *B. megaterium* have been shown to germinate even when the germinated spores could not divide. Irradiation activated spores can also be de-activated (Keynan and Evenchik 1969; Berg and Sandine 1970). Ionizing radiation induces changes in the tertiary structure of macromolecules directly and indirectly. Indirect

changes occur from the secondary effects of generated free active radicals from water, which result in the rupture of hydrogen bonds, peptide bonds and covalent linkages (Berg and Sandine 1970).

2.4.1.6 Activation by aging

Storage of *B. subtilis* spores at 5°C for 525 days causes activation. In addition, in an experiment where *B. subtilis* was exposed to a temperature range of 5-94°C, heat activation at lower temperatures was obtained over a longer time period, and corresponded to heat activation obtained at higher temperature but in a shorter time period. Therefore, aging and heat activation both appear to work via the same mechanism. However, while both activation and aging bring about the end of spore dormancy, the loss of dormancy by activation is a reversible process but aging leads to an irreversible loss of dormancy (Keynan and Evenchik 1969; Berg and Sandine 1970).

With respect to aging, where spores are stored in an aqueous suspension, it is thought that as time passes, some of the water molecules in the suspension are vaporized. The vaporized water molecules are then able to enter the spore, and reach and hydrate the critical site for activation.

In conclusion, it is postulated that the unknown spore activation site may be an enzyme or an enzyme system that is dehydrated and inactive in dormant spores, whose activity is required for germination (Berg and Sandine 1970).

2.5 Inactivation of spores

The ubiquitous nature of spores in the environment as well as their resistance to acute stress increases the potential of these organisms as agents of food spoilage and

thereby food-borne diseases. Thus, there is a growing need to find effective methods in killing them or reducing their numbers in food ingredients to an inconsequential level.

2.5.1 Inactivation by heat

2.5.1.1 Inactivation by wet heat

The core water content is the main factor influencing the heat resistance of spores to moist heat. Most *Bacillus* spores have a core water content between 27-55%. The lower the core water content, the higher the resistance to moist heat. Some of the factors regulating the core water content in bacillus spores include the uptake of DPA into the core, core mineralization, cortical contraction and high growth temperatures (Russell 1971; Setlow 2006).

The higher the spore core mineralization, the higher the heat resistance. Spores with high Ca^{2+} levels are the most heat resistant, followed by those with high Mg^{2+} or Mn^{2+} cations that are moderately heat resistant, and lastly, those in which K^{+} or Na^{+} ions have been substituted for Ca^{2+} ions are the least heat resistant. Also, it is not clearly understood how core mineralization confers heat resistance beyond reducing the core water. It is thought that mineralization may confer resistance by interaction with some of the spore macromolecules including proteins via a DPA-metal ion lattice that saturates the spore core (Russell 1971; Setlow 2006).

Saturation of the spore core DNA with the α/β-type SASP protects spore DNA from damage by moist heat treatment, preventing DNA damage, such as depurination that occurs in α^{-}/β^{-} spores subjected to moist heat treatment. Alpha/ beta-type SASP also protect the spore from cytosine deamination (Setlow 2006).

The mechanism by which low core hydration protects the spore from inactivation by moist heat is unclear. However, it is thought that as the spore is kept in a dehydrated state, the proteins in the spore are also in a relatively dehydrated state. Thus, they are more difficult to denature than hydrolyzed proteins. It has been hypothesized that dehydrated proteins are more heat resistant than hydrated proteins due the reduced molecular motion of dehydrated proteins.

Although the mechanisms by which wet heat inactivation proceeds remain unclear, moist heat inactivation results in the inactivation of core enzymes and rapture of the spore's inner membrane permeability barrier. The inner membrane is a strong permeability barrier that protects the spore from chemicals and other agents that could damage the spore DNA. Nonetheless, inactivation of spores by wet heat does not happen as a result of DNA damage. It is hypothesized that the inactivation of enzymes may play a role in moist heat inactivation of spores (Roberts and Hitchins 1969; Setlow 2006).

2.5.1.2 Inactivation by dry heat

The α/β-type SASP also protect spores from dry heat inactivation in the same manner they protect spores from wet heat inactivation. Inactivation by dry heat is caused by DNA damage, thus DNA repair in recovering dry heat injured spores is important. *B. subtilis* spores, transformed to possess the recA gene (gene coding RecA restriction endonuclease), are more sensitive to dry heat inactivation than their corresponding wild-type spores. Thus, it appears that RecA DNA enzymes are involved in repairing the DNA damage in the spore that results from dry heat inactivation (Russell 1971; Setlow 2006). As inactivation by dry heat in vegetative microorganisms occurs as a result of oxidation processes, it may be inferred that some of the DNA damage resulting from dry

heat inactivation could be as a result of oxidative stress. Minerals present in the core, coupled with the elevated temperature of dry heat inactivation and lipids (1-13% in *Bacillus* and up to 38% in *Clostridium* lipids by dry weight) (Murrell 1969) present optimal conditions for the autoxidation of lipids in the spore that generate free radicals. When the free radicals are not quenched they cause DNA damage that leads to mutations. Mutations in dry heat treated spores have been reported by Zamenhof (1960).

2.5.2 Inactivation by radiation

Radiation treatment of spores has been carried out with gamma (γ) rays and ultra violet (UV) radiation. Low water content in the core enhances spore resistance against inactivation by radiation. Low core water is thought to induce resistance by reducing the potency of γ-radiation in producing hydroxyl radicals. DPA also induces resistance against UV radiation inactivation of spores. UV absorbing pigments located in the spore's outer layers also protect spore components including spore DNA, from UV radiation. However, this UV absorbing pigments are not present in all *Bacillus* species (Setlow 2006).

UV radiation at 254nm is the most efficient wavelength for spore inactivation. On the other hand, spores still exhibit resistance against UV radiation by the formation of novel DNA photoproducts at this wavelength. These photoproducts include cylobutane dimers (CPDs) and (6-4) photoproducts (64PPs) formed between adjacent pyrimidines in the same DNA strand and thymidyl-thymidine adduct (spore photoproduct). While the first two are predominant in vegetative cells treated with UV radiation, very minute contents are found in their spore counter parts. The last photoproduct, spore photoproduct (SP), is the principal photoproduct generated in UV treated spores.

Spore photoproduct as the name implies is formed between adjacent thymidine residues on the same DNA strand. In spores, SP is not as lethal a lesion as CPDs and 64PPs, as SP is repaired immediately after spore outgrowth commences by a minimum of three repair mechanisms. SP is also repaired by recombination and excision. Recombination and excision is dependent on RecA endonuclease. Therefore, efficient and rapid repair of SP is a critical factor in spore resistance to UV radiation (Setlow 2006). As such it can be drawn that inactivation of spores by UV radiation that is most efficient at 254nm takes place when the formation of SP lesions overrides their repair leading to DNA damage and mutations leading to loss of viability.

The spore DNA photochemistry also plays an important role in the formation of SP. The α/ β-type SASP that saturate DNA in the spore core promote SP formation in the spore while suppressing CPD and 64PP formation. The low core water in the spore also enhances SP formation in the spore. DPA also increases DNA photosensitivity. Thus, while these are mechanisms that confer resistance against UV radiation by promoting production of the less lethal SP products, nonetheless, when repair does not occur efficiently, DNA mutations that lead to cell death occur (Russell 1971; Setlow 2006).

While the mechanism by which γ radiation kills spores is not clear as mentioned before, γ radiation generates hydroxyl radicals probably from unsaturated lipids in the spore. Once generated, hydroxyl radicals continue to react, forming new free radicals, thereby, causing oxidative stress that leads to mutations in the DNA and consequently death.

2.5.3 Inactivation by chemicals

The spore coat is the main spore integument conferring resistance against chemical inactivation. The spore coat is responsible for spore resistance against most oxidizing agents such as chlorine dioxide, hypochlorite and ozone (Setlow 2006). The spore coat detoxifies these chemicals before they penetrate, thus, preventing the damage of sensitive spore components within the spore. While the respective proteins responsible for the detoxification property have not been determined, superoxide dismutase (reacts with reactive oxygen species and peroxidases to neutralize them in oxidative reactions) has been found in the exosporium and spore coat of some *Bacillus* species. The spore inner membrane is extremely impermeable, restricting the diffusion of small hydrophilic and hydrophobic molecules (Setlow 2006).

Saturation of the spore DNA by α/ β SASP also prevents damage of spore DNA by hydrogen peroxide resulting from treatment with chemicals such as formaldehyde and nitrous acid.

Chemicals induce DNA damage in spores, causing mutations that accumulate. If these mutatitions include recA mutation then DNA repair mechanisms are also impaired leading to cell death. However, such instances are very few and only very few chemicals are genotoxic (Setlow 2006).

Spore inactivation by oxidizing agents is usually as a result of damage caused to the spore's external layers, particularly the inner membrane. The inner membrane of the oxidized spore ruptures upon germination of the spore, killing the spore. Mild treatment of spores with oxidizing agents increases the permeability of the inner membrane, thus, enhancing sensitivity to future treatments, such as wet heat treatment. While the specific

mode of damage of the inner membrane is not known, it is not attributed to the oxidation of unsaturated fatty acids (Setlow 2006).

Strong acids result in the rupturing of the inner membrane that acts as a barrier against hydrophilic and hydrophobic molecules. Thus, molecules in the acid diffuse into the spore causing the spore to lyse. Inactivation of spores with organic solvents at high temperatures may also proceed via the same mechanism (Setlow 2006).

2.5.4 Inactivation by high hydrostatic pressure (HPP)

Bacterial spores exhibit resistance to high pressures above 400 MPa (Moerman and others 2001). This range of pressure is known to induce protein denaturation by ionization. Ionization occurs as charged groups in the peptide chain form ionic bonds, which alters protein solubility. DPA protects spore proteins against excessive ionization and solvation. Hence, spores are more resistant than vegetative cells to high pressure treatments. Thus, increased pressure treatment does not lead to increased lethality, and infact, protects spore DNA against thermal inactivation. *B. subtilis* DNA melted at 86-92°C, treatment with 274 MPa of pressure increased the melting point of *B. subtilis* DNA (Russell 1982).

HPP inactivates spores via a two-step mechanism. In the first step, low pressure, less than 100 Mpa, combined with temperatures from 40°C, are used to germinate the spores. In the second step, high pressure about 300-400 Mpa, is used to inactivate the spores. Temperatures of 25-75°C have been combined with this pressure treatment in various research (Sale and others 1970; Russell 1982). Spores that have been subjected to high pressure treatment have undergone some biochemical changes, including, loss of DPA, calcium and hexosamine containing material. HPP-treated spores also become

phase bright. These are changes associated with germinated spores. Hence, it can be concluded that HPP induces germination, then inactivates the germinated spores (Sale and others 1970; Russell 1982; Moerman and others 2001; Yuste and others 2001).

HPP is influenced by ionic solutes. NaCl and CaCl, at low $A_{w,}$ greatly reduced spore inactivation by HPP, whereas glycerol and sucrose, at low A_w, had no effect on HPP efficacy. Thus, ionic solutes greatly increase spore resistance against HPP treatment. Inactivation is also greater at neutral pH and resistance is increased at extreme pH, both acidic pH and alkaline pH (Sale and others 1970; Russell 1982).

2.6 Factors affecting heat resistance of spores

There are various factors affecting the heat resistance of spores. These factors can be divided into four main categories:

1) Inherent resistance of the spores.
2) Environmental factors during growth and sporulation, including the sporulating media.
3) Environmental factors active during heat treatment.
4) Environmental factors active after treatment, including the recovery medium.

2.6.1 Inherent resistance of the spores

Spores of both *Clostridium* and *Bacillus* exhibit a wide range of heat resistance. In addition, sensitivity also varies within the genus and among the different strains of the same species (Russell 1971). *B. megaterium* ($D_{100°C}$ = 1 min) and *Clostridium* type E spores ($D_{77°C}$=0.77-1.95min) are more sensitive to heat, while *B. stearothermophilus* ($D_{115°C}$ = 22.6 min) and *Clostridium thermosaccharolyticum* ($D_{132.2°C}$ = 4.4 min) are heat

resistant (Roberts and Hitchins 1969). Ohye and Scott (1957) showed that *Clostridium* type A and B ($D_{112.8°C}$ = 0.15-1.32 min) spores are 1000 times more resistant than 10 strains of *Clostridium* type E spores.

While the number of spores present influences the amount of time taken to inactivate the spores, it does not influence the kinetics of heat inactivation. The survivor curve shape is not affected by the initial spore population. In an experiment conducted by Bigelow and Esty in 1920, the time necessary to kill *B. subtilis* increased as the numbers of spores in the suspension were increased. However, as the temperature of heat inactivation was increased, the time necessary to kill the *B. subtilis* spores was decreased (Russell 1971). Additionally, viable cells of other species conferred some protective effect against heat inactivation.

2.6.2 Conditions during sporulation

2.6.2.1 Temperature

Spores of several bacilli have been shown to exhibit increased heat resistance with increased sporulation temperature, including *B. subtilis* and *Bacillus coagulans* (Lechowich and Ordal 1962), *B. cereus* 4332 and *B. cereus* 9818 (Gonzalez and others 1999) and *B. stearothermophilus* (Gilbert 1966). As the sporulation temperature was increased, the cation to DPA ratio in the spores increased, which is thought to be responsible in enhancing heat resistance of the spores. In addition, the higher the incubation temperature, the lower the core water of the resultant spores that further enhances heat resistance of the spores (Russell 1971; Setlow 2006).

2.6.2.2 Ionic content

The addition of cations to growth and sporulation media has led to increased heat resistance of the generated spores. *B. megaterium* grown in media supplemented with calcium chloride was observed to exhibit increased heat resistance. The higher Ca:DPA molar ration was thought to confer the increased heat resistance. In an experiment with *B. megaterium* sporulated with media containing Mg^{2+} and Fe^{2+} as the sole cations, replacing Fe^{2+} with Ca^{2+} and Mn^{2+} resulted in the most heat resistant spores, while the addition of Mn^{2+} or (Ca^{2+} and $Mn^{2+)}$ resulted in spores with intermediate heat resistance. The addition of Ca^{2+} had no effect on heat resistance, while spores produced on the original media containing Mg^{2+} and Fe^{2+} resulted in the least heat resistant spores (Russell 1971). *B. subtilis* spores with increased heat resistance have been produced by adding 10-50ppm Ca, 1-10ppm Mn and 1-5ppm Fe to the sporulation medium. Magnesium has been determined to significantly increase spore heat resistance. High levels of phosphate in dipotassium hydrogen phosphate (K_2HPO_4) drastically decreased the heat resistance of *B. coagulans* var *thermoacidurans* spores. However, 0.05% (K_2HPO_4) increased the heat resistance of the same spores compared to those produced in media lacking phosphate (Roberts and Hitchins 1969; Russell 1971).

2.6.2.3 Organic compounds

While organic compounds such as p-Aminobenzoic acid increased the sporulation of *B. cereus*, the resultant spores did not possess increased heat resistance. *B. cereus* spores formed in media supplemented with cysteine or thioproline had reduced heat resistance and their spores had a reduced amount of DPA. L-glutamate and L-proline also lowered the heat resistance of *B. cereus* and *B. megaterium* spores. *B cereus*

sporulated in media containing DL or L-phenylalanine had increased heat resistance. The reduction of the amount of yeast in *B. cereus* var *terminalis* sporulating media decreased the DPA content from 74 to less than 0.2µg/mg spores and consequently decreased its heat resistance (Roberts and Hitchins 1969; Russell 1971).

2.6.2.4 Lipids and other factors

The heat resistance of *Clostridium botulinum* was moderately increased when acetate, propionate, butyrate and caprylate were added to growth media but greatly increased when palmitate, stearate and oleate were added to the growth media. The longer the carbon chain, the greater was the increased heat resistance (Roberts and Hitchins 1969; Russell 1971).

While studies have been conducted on the effects of pH, water activity and oxygen on sporulation, there have been no studies on the effect of these phenomena with respect to heat resistance of the resultant spores.

2.6.3 Conditions during heat treatment

2.6.3.1 pH and buffer components, sodium chloride, ionic environment and water activity

The heat sensitivity of spores increases at extreme pH values with maximum resistance at neutral pH values for most spores. A variation between pH 5 and 9 has little effect on heat resistance of the spores. However, optimal heat resistance can also be exhibited at slightly alkaline pH, or slightly acid pH. For example, *B. anthracis* possess maximum heat resistance at pH 8, while *Clostridium welchii* spores possess maximum heat resistance at pH 5. Buffer constituents also affect the heat resistance of spores.

Phosphate ions reduced the heat resistance of *B. coagulans* and *B. stearothermophilus* spores but increased that of *B. megaterium* spores (Roberts and Hitchins 1969). But even among *B. megaterium* strains, variation in heat resistance occurs when they are heated in phosphate buffer. However, a drastic reduction in pH, 7 to 4 greatly reduced the heat resistance of *C. botulinum* (strain 62A spores) by a factor of 5 at 110°C (Roberts and Hitchins 1969), and of *B. stearothermophilus* strains 7953, 12980 and 15951 by a factor of 7-10 at 115°C (Lopez and others 1996). The decreased heat resistance was dependent on temperature and was greatest at the given temperatures above than at higher temperatures.

Studies conducted on the effect of the sodium chloride (NaCl) concentration in the heating menstrua have resulted in varied results with respect to elevating or diminishing heat resistance of the suspended spores. In studies with *B. coagulans* var *thermoacidurans* sporulated in tomato juice, increasing the sodium chloride concentration in the tomato juice increased the death rate of the resultant spores (Roberts and Hitchins 1969). On the other hand, Esty and Meyer in 1922 found no changes in heat resistance when they heated the spores certain aerobic spore-formers in 6% NaCl, but increased heat resistance with 0.5-1.0% NaCl and decreased heat resistance in 8, 10 and 20% NaCl solutions. NaCl solutions of 2% and 3%, respectively, conveyed the greatest heat resistance to *Clostridium tetani* and *Clostridium perfringens* spores respectively. Thus, different concentrations of NaCl have been determined to have varying effects on the heat resistance of spores of various aerobic and anaerobic sporeformers (Russell 1971).

Low concentrations of magnesium and calcium reduce spore heat resistance in phosphate buffer. Tris buffer (M/200) also causes a reduction in heat resistance.

Chelating agents such as M/100 ethylenediamine tetracetic acid (EDTA) or M/100 glycine also reduce spore heat resistance (Roberts and Hitchins 1969).

Reducing the water activity greatly increased the heat resistance of the spores (Roberts and Hitchins 1969).

2.6.3.2 Organic environment

While high carbohydrate concentrations confer increased heat resistance to spores, equimolar concentrations of different sugars did not result in similar protective effects. Carbohydrate concentrations increase heat resistance of spores by inducing plasmolysis leading to partial dehydration (Roberts and Hitchins 1969; Pflug and others 2001). While the heat resistance of aerobic sporeformers was increased by 70% sucrose, this sugar concentration did not result in any changes in heat resistance for the spores of certain *Clostridium* species. Sucrose, glucose, and glycerol, at concentrations of 10-50% in addition to 2.5% pectin, gum arabic, glycogen and dextrin did not cause any change in the heat resistance of *Clostridium sporogenes* spores (Roberts and Hitchins 1969).

Serum albumin, ovalbumin, and yeast nucleic acid conferred protective effects dependent on concentration to *Clostridium* species (Roberts and Hitchins 1969).

Chilies and mustard oil decreased the heat resistance of *B. subtilis* spores, while garlic oil and allylisothiocyanate (organosulphur compound, derived from the essential oil of mustard) decreased the heat resistance of *C. sporogenes* spores. Organic sulfur compounds were also very effective in reducing the heat resistance of spores of both aerobic and anaerobic sporeformers (Roberts and Hitchins 1969).

2.6.3.3 Lipids

Although lipids have been shown to increase heat resistance of spores, studies under clearly defined conditions need to be conducted that can clearly demonstrate increased resistance conferred by lipids during heat treatment. An experiment conducted with spores of *B. cereus*, *B. megaterium* and *B. subtilis* that had been previously freeze dried showed that soybean oil conferred more heat resistance than olive oil, triolein, or liquid paraffin. The different fatty acids also conferred greater heat resistance than phosphate buffer. The degree of heat resistance conferred varied among the three species. The D-values recorded for the experiment with soybean oil vs. phosphate buffer are as follows; with phosphate buffer, $D_{95°C}$ of 13, 8, and 4.5min resulted, while with soybean oil, $D_{121°C}$ of 30, 108 and 6min resulted respectively with spores of the above Bacilli. It is thought that lipids increase the heat resistance of spores by localizing the spore in an environment of low water activity. However, it has been argued that if this were the only mechanism by which lipids conferred increased heat resistance, then spores equilibrated at a particular A_w before being re-suspended in lipids would portray D-values associated with the particular A_w in an aqueous system. Therefore, it is postulated that the peroxide content of the lipid might also bring about resistance, whose degree is associated with the range of unsaturation of the given lipid (Roberts and Hitchins 1969).

2.6.3.4 Gaseous environment and other factors

Heating spores in inert gases is slightly more effective in inactivating them than heating in air or oxygen. Heating spores in nitrogen has similar efficacy to heating spores in air (Roberts and Hitchins 1969). *B. subtilis* spores occluded in calcium carbonate

crystals heated at 121°C in moist and dry heat conditions were 909 times more heat resistant than their un-occluded counterparts (Roberts and Hitchins 1969).

2.6.4 Recovery conditions

2.6.4.1 Growth inhibitors

Injured spores that survive heat inactivation are sometimes more exacting in their growth requirements than unheated spores. Starch increased the colonies formed from heated spores of *C. botulinum*, *B. coagulans* and *B. stearothermophilus* by adsorbing growth inhibitors such as unsaturated fatty acids. This was concluded as the addition of glucose and maltose (the monosaccharides that make up starch) did not result in the same effect (Roberts and Hitchins 1969; Russell 1971; Foegeding and Busta 1981). The addition of charcoal and serum albumin in the recovery medium also increased the number of heated spores' recovered (Roberts and Hitchins 1969; Russell 1971; Foegeding and Busta 1981).

Glutamic acid is important in ensuring maximum recovery of *B. subtilis* spores. Furfural (1ppm) greatly increased the number of colonies formed from spores of heated thermophiles. Glutamic acid mediates amino acid deficiency brought on by heat inactivation of the alanine or glucose systems (Adams 1978).

Heated spores of *Bacillus* and *Clostridium* are also more sensitive to curing salts. Severely heat treated *C. sporogenes* (PA 3679) spores were more sensitive to low concentrations of NaCl than mildly heated or unheated spores of the same strain. *Bacillus pumilus*, *B. stearothermophilus* and *B. subtilis* var *niger* were also sensitive to NaCl that inhibited their growth (Roberts and Hitchins 1969; Foegeding and Busta 1981). Increased sensitivity was observed only after more than 99% of the population had been

inactivated, thus it was concluded that this was a natural property of the heat resistant fraction of the spore population. The surviving heat treated spores of *B. subtilis, C. perfringens*, and PA 3679 spores were also very sensitive to nitrite. The inhibition by nitrite on both heated and unheated spores was greater at pH6 than at pH 7 (Adams 1978).

Heat injured spores of *C. perfringens* are also sensitive to certain antibiotics such as polymyxin, kanamycin, streptomycin and neomycin, while their unheated counterparts are not. This increased sensitivity is caused by inactivation of the lytic system and spore membrane. On the other hand, antibiotics such as chloramphenicol and tetracycline mitigate the increased sensitivity of heat injured spores to sodium lauryl sulfate, sodium deoxycholate and quaternary ammonium compounds. The antibiotics act as surface active agents, forming a thin layer that coats the spores (Adams 1978; Foegeding and Busta 1981).

2.6.4.2 pH value

Heated spores may be more sensitive to pH that unheated spores. In certain media, unheated *B. stearothermophilus* spores had reasonable colony counts at pH 6.5 and pH7. However, D-values of surviving *B. stearothermophilus* spores on the same media rose by 28-54% when enumeration occurred at pH 7 than at pH 6.5 (Adams 1978).

2.6.4.3 Temperature

Spores that survive severe heat treatment often have optimal growth temperature requirements that are different from the unheated or mildly heated corresponding strains. Thus far, the optimum growth temperatures for heat treated surviving spores are well

below the growth temperatures of their unheated counterparts. The D-value for *C. botulinum* type A and B spores heat-treated in phosphate buffer or in spinach broth was larger when the surviving spores were incubated at 24 or 27°C than at 31 or 37°C (Adams 1978).

2.6.4.4 Germinants

Heat inactivation can damage pathways through one or both systems of germination by the denaturation of spore enzymes among other means, thereby inhibiting germination of the heat treated spores. The addition of certain artificial stimulants can provide enzymes needed to germinate the injured spores or bypass these pathways in germinating. Supplementing nutrient agar (NA) with Ca-DPA that is not metabolized, in NA used to recover *B. subtilis* spores heated at 113-146°C for several seconds, increased the number of colonies formed in the supplemented NA tenfold. The same treatment only slightly increased the number of germinating spores of unheated or mildly heated *B. subtilis* spores. Thus, injury due to heat treatment caused inability to germinate, particularly in NA, rather than inability to grow as Ca-DPA has no nutrient value because it is not metabolized. The L-alanine-stimulated germination system was identified as the site of damage (Adams 1978).

Egg yolk emulsion has also been added to recovery media for heated *C. perfringens* ATCC 3624 spores that resulted in increased colony formation of the heated *C. perfringens* spores. Lysozyme also increased the recovery of survivors, leading to the conclusion that it was the active germinant in the egg yolk emulsion. The addition of egg yolk emulsion or lysozyme did not increase the number of colonies of the unheated spores (Adams 1978).

2.6.4.5 Selection of growth medium

Spores that survive severe heat treatments require more nutrients. They also require nutrients that are not needed for growth by the corresponding unheated spores. Increased numbers of colonies were enumerated with heated spores of *B. subtilis*, *Bacillus cohaerans* and *Bacillus albalactis* when the NA was supplemented with glucose or blood.

Yeast and liver extracts, glucose, fructose, mannose, galactose, sucrose, maltose, soluble starch, pyruvate and glycerol-phosphatase also increased recovery of *Bacillus natto* (a variant of *B. subtilis*) spores and thereby apparent heat resistance. Biotin was also essential for colony formation in heated spores. Supplementing growth media with thiamine and pyridoxine also resulted in the highest D-values for *B. natto*. Drawing nitrogen sources from single amino acids yielded varying survival times. Therefore, the choice of recovery medium used can greatly affect the D-value (Adams 1978).

B. subtilis spores subjected to ultra-high heat treatment and grown in recovery medium supplemented with $CaCl_2$ and sodium dipicolinate resulted in increased colony counts compared to colony counts from un-supplemented media. Thus, the use of supplemented and un-supplemented media resulted in different survivor curves. The z values obtained with each of the above recovery media were also different. The z values doubled above 116-121°C from 8.9-18°C for the basal medium and above 124-129°C from 6.7-13°C for the supplemented medium (Roberts and Hitchins 1969).

2.7 Food extruders

Extrusion cooking can be described as a high temperature, short time, high pressure and high shear (dependent on the type of extruder used) process that heats, mixes, cooks

and shapes a food product (Harper 1981, 1989; Bouveresse and others 1982). In most instances extrusion is conducted under low moisture contents, less than 30%, although high moisture applications, 40-80% are also carried out. In extrusion processing the product is heated to temperatures of 150-250°C, under pressures of up to 25 MPa, within a short time, 30-120 s. It is subjected to high pressure by several restrictive mechanisms in the screw profile such as reverse pitch screws (reverse paddles), cut flight screws, dams and the discharge at the die. High shear rate is exerted by the mixing action of the screws and kneading discs (Harper 1981, 1989; Cheftel 1986).

Extrusion has been employed in food processing for over 50 years. Extruders were first used to stuff sausage casings in the meat industry. Then in the mid 1930s they were used in the pasta industry to mix and form pasta dough as well as create numerous pasta shapes. In the late 1930s the use of extruders was extended to the manufacture of ready-to-eat (RTE) cereal by shaping hot precooked cereal dough into small bite-size pieces in various shapes. General Mills was the first to use extruders in this manner to process RTE cereals. In the late 1940s extruders that both cooked and shaped the product were developed. Collet extruders that exert high shear rates and only utilize mechanical energy to heat the product being processed were used to process puffed snack products. Cooking extruders that were used to precook corn and soy used in animal feeds were also introduced. These extruders had adaptable screw, barrel and die configurations. Today cooking extruders are used in numerous applications creating a variety of products (Harper 1981; Dziezak 1989).

Some of the extruded products in the market today in addition RTE cereals include precooked and modified food starches, snacks, pet foods, confectionary products, texturized soy protein products and macaroni (Harper 1989).

Screw extruders are the main type of extruders used in food processing. Single screw extruders were developed in the 1940s. These types of extruders utilize mechanical energy required to turn the screw to heat the product being processed to over 150°C. The extruded plasticisized mass is then forced through the die. A decrease in pressure across the die as the product exits converts the moisture in the product to steam causing the product to expand (puff) (Harper 1989).

Two types of twin screw extruders are available that are designated depending on the rotation of their screws, counter-rotating or co-rotating. The degree of screw intermeshing is also a distinguishing factor within each type of twin-screw extruder. Twin screw extruders are self-wiping, which prevents product build-up on the screws, thus they have good conveying action. They have a high capacity especially co-rotating twin-screw extruders, and good mixing capability. As a result they can be used to process sticky as well as those food ingredients that are difficult to convey. As the product flows through the extruder barrel it is transferred from one screw to the other. The screw design of the conveyance screws in a twin screw extruder can be single, double or triple lead designs (the number of parallel flights along the length of the screw). Double and triple lead designs provide a more uniform shear rate than do single lead designs that enhances “processing uniformity”. In twin-screw extruders the product is heated by mechanical energy released from the turning of the screws and kneading disks

that are also used to increase mixing. Additional heat is also provided by the heated barrel wall that is in contact with the mixture (Harper 1989).

Extrusion processing confers several advantages over other food processing in that:

1) Different products including product shapes, can be produced using a variety of ingredients processed under different conditions on the same extrusion system. It has greater production capability than other cooking, forming systems.
2) High temperature short time treatments confer food safety by reducing the microbial load without depleting food nutrients. The high temperature treatment also denatures undesirable food compounds including enzymes such as trypsin inhibitors, hemagglutinins, gossypol and lipases.
3) Modification of vegetable protein, food starches and other food ingredients by extrusion processing has led to the development of new products.
4) Extruders can be used to process low moisture food thus eliminating the need for subsequent drying processes.
5) Extrusion processing is not accompanied by the discharge of any waste products that could otherwise cause pollution (Harper 1981).

2.8 Effects of extrusion on nutrition quality of food ingredients

Extrusion results in physical, chemical and nutritional changes of food products. Some of these changes are beneficial, while some changes result in reduced nutrient availability. The effect of extrusion cooking on food nutrients is dependent on extrusion parameters such as screw speed and die dimensions, process conditions and food composition.

2.8.1 Proteins

Proteins undergo structural changes when subjected to moist heat and shear including structural unfolding and/ or aggregation. These structural changes lead to denaturation as well as cause the proteins to be insoluble that increases protein digestibility and bioavailability. The digestibility and bioavailability of limiting sulfur amino acids in soy bean proteins is improved by extrusion cooking as a result of unfolding of the major seed globulins, removal of the sulfur molecules, oxidation, inactivation of trypsin inhibitors (70-95% inactivated) and growth retarding factors such as lectins. The inactivation of trypsin inhibitors was enhanced by an increase in temperature and moisture content during extrusion. Enzymes are also completely inactivated by extrusion cooking. Enzyme inactivation increases the stability of extruded foods by reducing the oxidation of ascorbic acid and unsaturated fatty acids (Bjorck and Asp 1983; Cheftel 1986).

Extrusion under high temperature conditions (> 180°C) and high shear (>100 rpm) at low moisture content (<15%) can lead to loss of lysine. The presence of reducing sugars (≥3% glucose, fructose, maltose, lactose) enhances loss of lysine under the above extrusion conditions as a result of Maillard reactions. In Maillard reactions, the NH_2 groups of the amino acid react with the carbonyl (C=O) groups of reducing sugars. In peptide chains lysine is the most reactive amino acid due to its free amino group. The decrease in protein digestibility also leads to a reduction of bioavailable lysine.

High temperature extrusion also leads to reduction of cystine at low moisture content, and to a lesser extent reduced bioavailability of arginine 21%, histidine 15%, aspartic acid 14% and serine 13% (Bjorck and Asp 1983).

2.8.2 Carbohydrates

Extrusion of starch results in physico-chemical changes that result in "rheological and textural changes", in addition to enhancing digestibility and bioavailability. Some of the changes resulting from extrusion of starch include, rapture of starch granules, modification of crystalline spectra, cold water solubility, reduced viscosity, and increased digestibility resulting from the partial to complete release of amylopectin and amylose. Complete starch gelatinization is also achieved by extrusion temperatures of 120°C, coupled with 20-30% moisture content. High shear forces coupled with lower moisture content also achieve gelatinization (Bjorck and Asp 1983; Cheftel 1986).

Amylose and amylopectin are hydrolyzed to maltodextrins by the high shear forces of extrusion processing in wheat starch. Crystalline complexes also form between amylase and polar lipids including fatty acids, mono and di-glycerides during extrusion of cereal starches. These complexes lower stickiness of snacks and lead to modified viscosity among the functional properties conferred. Alpha-galactosidases in legumes are not hydrolyzed by extrusion cooking. Extrusion of starch also leads to the production of anhydroglucose-end chains (Bjorck and Asp 1983; Cheftel 1986).

Extrusion increases the digestibility of starch by gelatinization as a result of hydration and increasing solubility of starch granules. Denaturation of endogenous α-amylase inhibitors, the disruption of the cellular structure leading to increased surface area and reduced size of starch granules, and the partial separation of bran from protein also increase starch digestibility. Increased temperature and moisture increase starch gelatinization (Bjorck and Asp 1983; Cheftel 1986).

2.8.3 Dietary fiber

It has been stated that the degradation of fiber in the colon is dependent upon the particle size of the fiber, with a reduction in particle size enhancing degradation. Bran particles are disrupted by the mechanical forces of extrusion leading to homogenized reduced particle size. Thus, extrusion increases the dietary fiber available for fermentation in the colon.

Extrusion of wheat flour also increased soluble fiber while reducing insoluble fiber. Such changes were more significant in wheat flour than in whole grain wheat flour. Extrusion of both whole grain and white wheat flour at extremely high temperatures (> 180°C), results in the formation of amylase-resistant starch fractions thereby increasing the dietary fiber content (Bjorck and Asp 1983; Cheftel 1986).

Extruded wheat bran has also been shown to result in a low glycemic index (Bjorck and Asp 1983).

2.8.4 Lipids

Extruded products have a decreased fat content. This phenomena has been observed as monoglycerides and fatty acids form complexes with amylose during extrusion cooking thus they are more difficult to extract with organic solvents. Steam distillation and thermal degradation of lipids during extrusion, could also result in the reduced fat content in extruded products. However, as extrusion cooking is such a short time process it is held that thermal destruction of lipids is unlikely (Bjorck and Asp 1983).

Extrusion cooking also results in oxidation, hydrogenation, isomerization and polymerization of lipids. Increased extrusion temperatures have been observed to

decrease the ratio of unsaturated fatty acids, while increasing saturated fatty acids as a result of hydrogenation. A moderate increase of trans fatty acids was also observed (Bjorck and Asp 1983; Cheftel 1986).

In addition, extrusion leads to the denaturation of lipase and lipoxidase reducing the susceptibility of lipids to oxidation. However, the porous nature of extruded foods as a result of expansion when exiting at the die, increases susceptibility of the extruded products to rancidity (Cheftel 1986).

2.8.5 Vitamins and minerals

Thiamine is heat labile while riboflavin is quite stable. Increasing the extrusion temperature and screw speed enhances thiamine degradation, while increasing moisture content and screw speed enhances riboflavin degradation. Niacin in a corn, soy and groundnut blend was observed to be more stable under extrusion conditions than boiling for two minutes. Pyridoxine and folic acid are also quite stable during extrusion processing (Bjorck and Asp 1983).

Ascorbic acid in a corn, soy and groundnut mixture was also determined to be more stable during extrusion, only 33% destroyed, compared to boiling for two minutes, caused 79% destruction. In extrusion the release of iron from worn screws also catalyzes oxidation of vitamin C. Vitamin C is also more resistant to oxidation and leaching with continued storage (31°C, 48% relative humidity) when added to a product after the product has been extruded (14-20% loss) than when added before extrusion processing (57-66% loss). Extrusion confers more stability to vitamin C as a result of the reduced water activity of the product. Furthermore, at high extrusion temperatures, more vitamin C was retained at lower moisture content (Bjorck and Asp 1983).

Extrusion of a corn, soy, and groundnut blend resulted in greater carotene losses (53% loss) than boiling for two minutes (25% loss). Other vitamin A compounds such as retinyl acetate, retinol and retinyl palmitate were also quite stable under extrusion conditions, especially with increasing screw speed (Bjorck and Asp 1983).

Minerals, such as Fe, Cu, Zn and Mg, are usually present in minimal amounts in cereals and legumes. These minerals are usually held in insoluble complexes with dietary fibers, phytate or proteins in cereals and legumes. Extrusion of a wheat bran, starch, gluten mixture has led to a reduction in phytate content by 13-35%. The bioavailability of Zn, Cu and Mg of extruded wheat flour into crisp bread also decreased (Cheftel 1986).

2.9 Soy protein

The reported health effects of soy resulted in an increased consumer demand for soy foods. The food industry responded with a vast assortment of soy products. The marked demand for soy foods was also driven by the FDA claim that a diet low in saturated fat and cholesterol and 25g soy protein/day would lead to reduced cardiovascular disease, (Wardlaw and Kessel 2002). Some of the health benefits imparted by soy consumption include, reduction of LDL cholesterol and total serum lipids, while increasing HDL cholesterol, (Anderson and others 1995; Potter 1996). The consumption of soy also confers a reduced risk for prostate cancer in men, reduced breast cancer risk and bone loss for premenopausal women and increased bone resorption (increased bone mineral content) in menopausal women (Potter and others 1998; Setchell and Cassidy 1999; Ikeda and others 2006).

2.9.1 Soy flour and grits

Before or after the removal of the oil fraction, soy bean flakes are ground and screened to produce soy flour and soy flakes. The resultant flour and flakes have a protein content of 40-54%. Soy flour and grits are the least refined soy products produced for human consumption. They vary in particle size distribution, fat content and extent of protein denaturation. Various forms of soy flour and grits are available in the market that vary in type and oil content. These include full-fat flour, high-enzyme flour, defatted flour, defatted grits and lecithinated / refatted flour (Khee 1994).

i. **Full-fat soy flour** is the least refined soy protein product. It contains about 40% protein. It is prepared by grinding/milling dehulled cotyledons. It is used in the baking industry and to produce soy milk.

ii. **High enzyme flour** is prepared by milling defatted flakes under conditions that result in minimum heat denaturation. It contains 52-54% protein. It is used to bleach bread (Khee 1994).

iii. **Defatted soy flour** results from finely milling defatted flakes to a particle size that can pass through No. 100 standard screen size. It contains 52-54% protein and is used where a wide solubility range of protein solubility is desired.

iv. **Defatted grits** are similar to defatted flour, except that they are milled to pass through a screen size between No. 10 and No. 80 and are, thereby, more coarse. They are mainly used as meat extenders and in bakery products.

v. **Lecithinated/refatted flour** is prepared by mixing defatted soy flour with 0.5-30% lecithin or vegetable oil. It is used in baking applications to improve dispersability and emusulfication (Khee 1994; Lusas and Riaz 1995).

2.9.2 Soy protein concentrates (SPCs)

Soy protein concentrate are prepared from dehulled and defatted soy bean flakes by extracting water soluble, non protein constituents. SPCs contain 70% protein on a dry basis. SPCs can be produced using three processes, including extraction with water at isoelectric pH 4.5, extraction with 70-90% aqueous ethanol and extraction with water after heat denaturation of the protein with moist heat (Khee 1994; Lusas and Riaz 1995).

2.9.3 Soy protein isolates (SPIs)

The soy protein is extracted from defatted flakes using sodium hydroxide or other alkaline agents approved for food use at pH 8.5-9.0, and then centrifuged to remove the insoluble residue. The protein solution is acidified to pH 4.5 by hypochloric or phosphoric acid to precipitate the protein. The protein is precipitated as a curd, which is separated by centrifugation and then washed with water. The curd is then spray dried in its acid form. The curd can also be neutralized to pH 6.5-7.0 with alkali to form Na, K or Ca-proteinates that are more soluble (Khee 1994; Lusas and Riaz 1995).

2.9.4 Soy foods

Soy products have long been utilized for food especially in East Asian countries and later on in the west. Some traditional soy products include fermented soy products such as soy sauce, tempeh, miso, tofu and unfermented products such as okara. Okara is also consumed in fermented form as okara tempeh (fermented with *Rhizopus* mold

spores) and okara onchom (fermented with *Neurospora intermedia* mold spores) (Shurtleff and Aoyagi 2004). Nowadays, soy is consumed largely in the United States in soy products such as soy milk, soy based ice-cream, yogurt and cheeses, soy pasta products, soy energy bars, and soy meat analogs among others (Morr 1990; Khee 1994; Jay 2000; Guo and Ono 2005).

In the food industry, soy proteins are used largely for their functional properties including solubility, dispersibility, water absorption and swelling, viscosity, gelation, elasticity, cohesion-adhesion, elasticity, emulsification, flavor binding and foaming properties. Thus, soy proteins are used as extenders to stabilize fat emulsions, improve viscosity, improve texture upon cooking and improve juice retention in meat and meat products such as bologna and sausages. Soy proteins are also used as beverage bases because of their solubility, in soups and gravy bases because of their dispersibility and viscosity, and in meats and baked goods to impart elasticity among others (Morr 1990; Lusas and Riaz 1995).

2.10 Wheat starch and gluten

Starch is the main component in wheat making up 63-72% of wheat. Starch is contained in the endosperm, and it's composed of the glucose polymers, amylose and amylopectin. Gluten is the main protein in wheat, which is also present in the endosperm. Gluten consists of the glutenin and gliadin protein fractions that are storage proteins contained in the endosperm. The glutenin and gliadin fractions make up 85-90% of wheat protein (Swanson 2000). Wheat flour is processed by milling the endosperm to a reduced particle size. This milling process also separates the bran and germ from the endosperm. A total of 15 processes have been presented by Kempf and Rohrmann

(1989) for the production of wheat starch from both flour and wheat kernels (Van Der Borght and others 2005). In North America four main processes are used in the industrial manufacture of wheat starch and gluten from wheat flour, including Martin (dough) process, Alfa-Laval or Raisio, Hydroclone and the High pressure disintegration (HD) processes. These processes differ in the degree of gluten agglomeration and the equipment used to separate the starch from the gluten (centrifuge, hydroclone, inclined table or screen) (Sayaslan 2004, Van Der Borght and others 2005).

2.10.1 Wet milling of wheat flour

The production of wheat starch and gluten from flour is based on the physical separation of starch granules from gluten particles formed in a neutral aqueous system. Hydrated gluten aggregates into particles that are larger than starch granules but less dense than starch granules. According to Sayaslan (2004), "the aggregation of gluten particles is accelerated by the concentration and composition of the proteins". Warmer temperatures of 40-45°C are also conducive for the aggregation of gluten particles (Sayaslan 2004). Although various methods are used to extract wheat starch and gluten from wheat flour, all the methods used coincide after the separation of gluten particles and wheat starch from flour. The separation of wheat starch and glutens from wheat flour is the first stage of the wet milling of wheat flour. Following separation, gluten is aggregated further, excluding other impurities in addition to starch, and then washed. After washing, water is removed and then it is flash dried (Sayaslan 2004).

Wheat starch is separated from gluten based on its higher density by centrifugation, tabling, in hydroclones or decanters, and based on gluten's larger particle size by sieving (screening). After initial separation, continuous centrifugation yields a dense stream

(settles at the bottom) that is the prime starch fraction, gluten fraction, squeegee starch (a gelatinous layer that consists of smaller starch granules, damaged starch and insoluble non starch particles, low levels of protein and ash) and a water extractable fraction (WEF). The prime starch fraction is washed with water in a counter current motion. The starch is then flash dried (Sayaslan 2004; Van Der Boght and others 2005).

In hydroclones, the starch/ gluten suspension is fed tangentially into the upper section of the cone. The spinning effect of the cone creates a centrifugal force that separates the starch granules that exit at the bottom of the cone, while the gluten particles and the WEF exit at the top. In starch tabling, the starch/ gluten suspension flows over an inclined table, such that the starch settles at the lower end of the table, while the gluten particles and WEF starch run off. The sedimented starch is then washed with water to remove the squeegee starch. The same principle is utilized in decanting. "Sieving retains the agglomerated gluten particles while the starch milk also containing the WEF flows through the sieve" (Van Der Borght and others 2005). Starch is then extracted from the starch milk by centrifuging or tabling (Van Der Borght and others 2005).

2.10.2 Uses of starch in the food industry

Starch imparts certain functional properties to food products as a result of crystalline structure of the granules. Upon hydration, starch granules swell and breakdown, forming gels that set when heated. Gelatinization is influenced by water absorption, temperature and time. Gelatinization occurs between 60-75°C. On cooling, the gelatinized molecules reassociate into an ordered structure known as retrogradation. Thus, starch is used in many products for its gelation properties, as well as to impart structure. Wheat starch is used in baked goods, confectionaries and pasta among other

food products. Modified wheat starches are also available (Eliasson 1989; Swanson 2000).

2.10.3 Microbiology of soy flour and wheat starch

Bacterial genera that can be found in cereals include *Bacillus*, *Lactobacillus*, *Pseudomonas*, *Streptococcus*, *Achromobacter*, *Flavobacterium*, *Micrococcus* and *Alcaligenes* (ICMSF 2005). Cereal grains pertain to wheat, corn, oats, rye, rice, barley, millet and sorghum. The spore forming bacteria are the most important group as they survive cooking temperatures and germinate under conducive conditions, such as the absence of limiting a_w. The occurrence of *B. subtilis* is of great concern especially in moist baked products. Aerobic and aerobic sporulating bacteria have been consistently found in cereals, ranging between 10^2-10^6 and 10^0-10^5 CFU/g respectively. *B. subtilis* and *B. cereus* are present in cereals in low numbers (ICMSF 2005).

Graves and others (1967) conducted a study to determine the microbial content of wheat and the resultant wheat flour processed from the wheat in 11mills in the Kansas-Nebraska region and Pacific Northwest region. They discovered several aerobic bacteria and spores growing from representative cultured samples. These grew in the following counts, 3.8×10^3 - 6×10^3 and 2.2×10^2 - 4.9×10^3 CFU/g aerobic bacteria, 0 - 7.0 and 0 - 1.2×10^2 CFU/g aerobic thermophilic spores and 0 - 4.5 and 0 - 73 CFU/g thermophilic flat sour spores in wheat and flour, respectively.

A study of Canadian flour to determine the occurrence of thermophilic bacteria yielded the following results: 1.3 $x10^4$ CFU/g thermophiles were determined to grow in low grade wheat flour, while 6.0 x 10^2 - 2.1 $x10^6$ CFU/ g aerobic thermophiles were observed in untreated soy flour. Anaerobic spore-formers from cultured plates of

untreated soy flour also grew to a level of 10^5 CFU/g. Thatcher and others (1953) studied the microbial and insect content of wheat kernels and wheat flour from 50 representative Canadian flour mills (kind of flour). They observed and enumerated various aerobic and anaerobic spores including, 0- 3.6×10^3 mesophilic spores, 0-68 aerobic thermophilic spores, 0-32 flat sour spores, and 0-23 anaerobic thermophilic spores. An assessment of the microbial content in wheat flour in nine Australian mills found 91and 81 CFU/g in the wheat kernels and 65 and 93 CFU/g in the resultant flour of *Bacillus* species and *B. cereus*, respectively (Berghofer and others 2003). Lastly, *B. cereus* and 1.6×10^3 CFU/ g mesophilic spores have been observed growing in 3 days old tofu that was vacuum packaged and refrigerated. After subjecting the tofu to high pressure treatment of 400 MPa at 5°C for 30 min, some *B. cereus*, as well as mesophilic spores, were still recovered on plating with suitable media (Pestramo and others 2000).

Although no studies on the microbial quality of wheat starch were reported in this review of literature, inferences can be made on the incidences of aerobic microorganisms, aerobic spores, aerobic flat sour spore formers and aerobic flat sour spores in wheat starch. While it is expected that the continued wet milling of wheat flour to produce wheat starch is likely to lower the microbial load, as some of the bacteria are killed in the mechanical processing steps, any remnant spores would still be of great concern to quality and safety of the final product. The remnant spores could germinate and proliferate under conducive storage conditions.

In addition, in studies to determine the microbial quality of soy beans, research have focused on the presence of molds and mycotoxins and, in particular, aflatoxins. However, spore-forming bacteria are still of great concern in soy beans and accordingly

soy flour. This is surmised as most of the processing facilities used to process soy flour also process flour from other cereals that consistently harbor *Bacillus* species such as the occurrence of *B. cereus* in rice (ICMSF2005), and consequently, rice flour.

CHAPTER 3

MATERIALS AND METHODS

3.1 Introduction

In this study, an extruded soy meat analog was used as a model to study the thermal inactivation of spores of wild-type bacilli and to compare their heat susceptibility and inactivation to the spores of *Bacillus stearothermophilus*, a spoilage organism, and *Bacillus cereus* (by comparing D-values at the given inactivation temperature), a pathogenic organism. In extrusion processing of the soy meat analog, the dry ingredients were mixed and cooked, and a product was formed in one single step. This provided ease, in addition to providing a high temperature range, as cooking is carried out under high pressure conditions. This study is composed of four main parts: 1) Isolation of wild-type microorganisms 2) Testing of selected isolated microorganisms with various sporulation media, 3) Determining of the D-value of the spores, 4) Extrusion of the inoculated soy meat analog. The aim was to isolate wild-type bacilli from soy flour, soy protein isolate and wheat gluten that could be used as indicators of the pathogenic *B. cereus* for studying the bactericidal effect of extrusion processing on the spores of these bacilli.

3.2 Raw materials

The dry mix ingredients to be used in processing the soy meat analog included soy flour and wheat starch. Soy protein isolate, soy flour and wheat starch were procured from Archer Daniels Midland (Decatur, IL) and Midwest Grain Products (Atchison, KS). These raw materials were also used in deriving the wild-type microorganisms that could

be used as indicators of pathogenic *B. cereus* during extrusion in this study. Upon receipt, all the dry ingredients were transferred from their original batch bags into large plastic white buckets, secured with tight fitting lids, and stored in a clean, cool dry room, where they could be easily retrieved for sampling as needed.

3.3 Isolation of wild-type bacilli

Microorganisms were isolated from the ingredients used in preparing the soy meat analog, in particular; soy flour, soy protein isolate and wheat gluten. The procedure used was as follows: 11 g of soy flour, soy protein isolate (SPI) and wheat gluten were quantitatively weighed into a stomacher bag, to which 99 mL of sterile peptone water was added. The contents of the stomacher bag were stomached for 2 min (Seward Stomacher 400, England), making the 10^{1} dilution. Additional dilutions in the range of 10^{-2} - 10^{-6} were prepared for each of the three ingredients that were poured in duplicate using Plate Count Agar (PCA). Three sets of plates were poured to be incubated aerobically at three different temperature settings, 45, 55 and 65°C. Enumeration of the colonies that grew on the poured plates was carried out every 48 h until 96 - 144 h had elapsed, to achieve maximum growth.

Following enumeration to determine the initial number of microorganisms in the ingredients, the various isolated microorganisms were differentiated based on colony morphology characteristics and gram stain results, and designated as strains 1a through 12b. After differentiating the wild-type isolates, 1-2 loopfuls of each were cultured in 10 mL Tryptic Soy Broth (TSB) for 48 h in incubation conditions similar to those used above, except that those isolates that had exhibited anaerobic growth (had grown beneath the agar surface) were overlaid with about 10 mm of mineral oil. Following growth, the

48-h broth culture of each isolate was streaked for isolation in duplicate on pre-poured Tryptic Soy Agar (TSA) plates. The streaked TSA plates were incubated aerobically or anaerobically depending on the wild-type isolate's growth requirements and under incubation conditions similar to those stated above. Colonies that grew individually on the streaked TSA plates were subjected to additional gram stains to verify the purity of each culture followed by catalase and oxidase tests as preliminary confirmation to differentiate bacilli from the other wild-type isolated microorganisms.

3.4 Preparation of stock cultures

After the preliminary tests described above, 1-2 loopfuls of each strain 1a-12b were cultured in 10 mL fresh TSB and incubated aerobically or anaerobically in anaerobic gas jars (BBL, Sparks, MD) at 45, 55 or 65°C, depending on the respective growth requirements, for 48 h. Each strain was then streaked onto TSA slants and grown under similar conditions, as described above for TSB, to provide stock cultures for subsequent experiments. The stock cultures were refrigerated at 4°C and re-cultured every 60 days. *B. stearothermophilus* and *B. cereus* were both from the culture collection of the Food Microbiology Laboratory, University of Missouri, Columbia.

3.5 Selection of suitable sporulation agar

Based on the results from the preliminary tests stated above, catalase and oxidase positive gram positive rods from among the wild-type strains 1a-12b were selected to be grown on a variety of sporulation agar, as these microorganisms were considered to belong to the genus *Bacillus*. By comparing the sporulation percentage yield from the

various sporulation agar used, the sporulation agar that resulted in both the highest and the most heat resistant *Bacillus* spores would be selected for use in the extrusion study.

The wild type *Bacillus* isolates designated as 3II8, 3III 1C, 4II 1, 4II 11, 4II 2, 4II 3a, 4II 3c and 4II 17, in addition to a pure culture of *B. stearothermophilus* ATCC were tested with different sporulation media. Isolates 3II8, 3III 1C, 4II 1, 4II 11, 4II 2, 4II 3c and *B. stearothermophilus* were first cultured in tryptic soy broth with 0.6% yeast (TSBY), while isolates 4II 3a and 4II 17 were first cultured in nutrient broth with 0.6% yeast (NBY). All the microorganisms were incubated aerobically for 48 h at 55°C, except for 4II 1 which was incubated at 45°C.

Following incubation and growth, 200 µL of each wild-type *Bacillus* as well *B. stearothermophilus* were spread-plated in duplicate onto plates (90 × 15 mm) pre-poured with various sporulation agar media, outlined in Table 3.1. The sporulation agar was poured into glass petri plates that were deeper with a slightly greater diameter than the conventional disposable plates to provide enough surface area for sporulation as well as to provide room to flood the plates when scraping the spores. After spreading the broth cultures onto the sporulation agar media, the plates were left to stand for 15-30 min to allow for infusion of the cultures into the agar.

After growth for 48 h, when optimum spore production had been attained, the spore lawns were flooded with 10 mL sterile distilled water and scraped using a stainless steel spatula. The resultant spore suspensions were pipetted into 15 mL centrifuge tubes and centrifuged at 600 × g (Beckman J2-21, JA 20.1 rotor, Fullerton, CA) for 15 min at 4°C. After centrifuging, the supernatants were discarded and the pellets were washed followed by re-suspension in 10 mL sterile distilled water. The suspensions were

centrifuged again at 2500 × g for 15 min at 4°C. This washing and centrifugation step was repeated once more for 20 min, followed by re-suspension of the spore pellets in 10 mL of sterile peptone water. In the event that the spore lawn was thin or sparse which resulted in a smaller spore pellet, the centrifugation speed was increased to 4640 × g.

3.5.1 Determination of sporulation percentage

The 10 mL spore suspension from above was divided into three fractions, 2 mL, 3 mL and 5 mL each. The 3 mL fraction was used to determine the initial spore count and was diluted with peptone water and pour-plated in duplicate in nutrient agar, whereas the 2 mL fraction contained in a 15 mL plastic centrifuge tube was subjected to heat treatment in a boiling (100°C) water bath for 15 min. After cooling the 2 mL heated spore suspension in an ice-bath to about 4-7°C, appropriate dilutions with peptone water were prepared and pour-plated in duplicate in nutrient agar. The remaining 5 mL fraction in a 15 mL glass test-tube was subjected to a 121.1°C heat treatment by autoclaving the spore suspension for 2 min, followed by a quick cooling in an ice-bath. Appropriate dilutions of the 5 mL heat-treated fraction were also prepared in peptone water and pour-plated in duplicate in nutrient agar. The unheated and heat-treated pour-plated spore suspensions of the various wild-type isolates and *B. stearothermophilus* were incubated in the same conditions as those used in culturing and sporulation above. The sporulation percentage yielded with the various sporulation media was determined by calculating the ratio of the number of colonies that grew from surviving spores after the 100°C heat treatment relative to the initial bacterial count as follows:

$$\frac{N}{N_o} \times 100\%$$

where N_o is the count before heat treatment and N is the count after the 100°C heat treatment.

3.5.2 Selection of heat labile and heat resistant *Bacillus* strains

After determining the sporulation media that generally resulted in the highest sporulation percentage of *B. stearothermophilus* and the wild-type *Bacillus* isolates, the next step was to narrow down the list to the least and the most thermo-resistant strain. Thermoresistance was also determined by the number of colonies that germinated from the surviving spores after the 100°C heat treatment relative to the initial microorganism count. Having determined the least heat resistant wild-type *Bacillus* isolate to be that designated as 4II 1, the isolates designated 4II 11 and 3III 1C were picked as the thermoresistant isolates.

3.6 Carbohydrate fermentation tests

The next step was to verify that 4II 1, 4II 11, 3III 1C and *B. stearothermophilus* were all different microorganisms beyond the apparent differences in colony morphology. To confirm that the four microorganisms above were all different, their ability to ferment several carbohydrates was tested. *Bacillus cereus* was also included in the carbohydrate fermentation tests as it was necessary to verify that none of the wild-type isolates selected was *B. cereus*. This is because one of the objectives of the experiment is to determine a thermoresistant wild-type isolate that can be used as an indicator of *B. cereus* in the extrusion processing experiment, since the latter is a pathogen.

Table 3.1 Sporulation media.

Constituent (g/L)	NA[1] +$MnSO_4$ +$CaCl_2$ (Hsieh et al., 1989)	NA[1]+ 1 ppm $MnSO_4$ (Maza's et al., 1995) + 0.5% yeast	FNA[2] (Mazas et al., 1995)	Angelotti medium (Mazas et al., 1995)	Milk agar (Mazas et al., 1995)	Campden sporulation agar (Casadei et al., 2000)	Sporulating agar I (Silva et al., 1999)
Beef Extract[3]	3.0	3.0	3.0	---	---	---	---
Peptone[3]	5.0	5.0	5.0	---	---	5.0	---
Nutrient Broth[3]	---	---	---	---	---	---	---
Tryptone[4]	---				---	5.0	---
Dextrose[3]	---	---	0.1	2.5	---	1.0	1.0
Casamino acids[3]	---	---	---	2.5	---	---	---
Yeast Extract[3]	---	5.0	---	5.0	---	2.0	1.0
Lab lemco[4]	---				---	1.0	---
Skim milk[3]	---				10.0	---	---
Agar[3]	15.0	15.0	20.0	30.0	20.0	15.0	20.0
Soluble starch[5]	---	---	---	---	---	---	---
NaCl[5]	---	---	8.0	---	---	---	
$CaCl_2$[5]	0.00003	---	0.06	---	---	0.056	0.25
$(NH_4)_2SO_4$[5]	---	---	0.08	---	---	---	0.2
$MnSO_4H_2O$[5]	0.00003	0.003	0.05	0.01	---	0.082	---
$FeSO_47H_2O$[5] (MC\B)[6]	---	---	0.0005	0.014	---	---	---
$MnCl_24H_2O$[5]	---	---	0.008	---	---	---	---
$MgSO_4$[5]	---	---	0.2	---	---	---	0.5
KH_2PO_4[5]	---						0.6
$CuSO_45H_2O$[5]	---	---	0.005	---	---	---	---
$ZnSO_47H_2O$[5]	---	---	0.005	---	---	---	---
pH	---	---	---	---	---	---	7.0

Table 3.1 foot notes

[1] Nutrient agar

[2] Fortified nutrient agar

[3] Difco Laboratories, Detroit, MI

[4] Oxoid Ltd, Basingtoke, Hampshire, England

[5] Fisher Scientific, Fairlawn, NJ

[6] Matheson Coleman and Bell manufacturing chemists.

The carbohydrates (0.05% v/v) to be tested, including, glucose, maltose, fructose, sucrose, lactose, rhamnose, ribose, saccharose, sorbose, xylose, cellobiose, fucose, galactose, maltotriose, mannose, melezitose, melibiose, raffinose, fructo-oligosaccharide and mannitol, were incorporated into modified TSB formulated without dextrose, to which was added 0.6% yeast and 0.003% bromcresol purple as an indicator. The modified TSBY (4.75 mL each) was dispended into 10 mL test tubes, autoclaved at 121°C for 15 min and tempered to about room temperature. One gram of each of the sugars to be tested was dissolved in 10 mL distilled water, and autoclaved and cooled separately. Just before inoculating with 100 µL of a 48-h broth culture of 4II 1, 4II 11, 3III 1C, *B. stearothermophilus* and *B. cereus*, 250 µL of each sugar solution was added to the 4.75 mL modified TSBY after it had been kept at room temperature for 1 h following refrigeration. All tubes were incubated aerobically at 55°C except for those inoculated with 4II 1, which were incubated at 45°C, and those inoculated with *B. cereus*, which were incubated at 35°C. The tubes were monitored every 24 h for 120 h for evidence of the utilization of the particular sugar for growth by a color change of the broth from purple to yellow. A four level color scale from intensely yellow, to light yellow, yellow-

purple and purple was established to accurately record the observed presence or absence of carbohydrate fermentation as outlined in Table 3.2.

Table 3.2 Carbohydrate fermentation positive and negative color scale.

Intensely yellow ++	Light yellow +	Yellow-purple +/-	Purple - -

3.7 Determination of D-value of spores

The selected wild-type isolates were further narrowed down following the carbohydrate utilization tests to include 4II 1 as the heat labile wild-type isolate, and 3III 1C as the heat resistant isolate. At this point, 4II 11 was not selected for further testing because while the carbohydrate utilization tests showed that it was different from 4II 1, its colony morphology was quite similar to that of 4II 1. Furthermore the spores of both microorganisms had a different shape, and they both had different optimal growth temperature, however, it was postulated that both 4II 1 and 4II 11 could be different variants of the same microorganism.

In performing the D-value experiments, two modes of heat treatment were used to kill the microorganisms; a boiling water bath was used to inactivate 4II 1 and *B. cereus* spores, while a 150°C oil bath was used to inactivate 3III 1C and *B. stearothermophilus* spores after heat treatment at various temperatures including the 100°C boiling water bath, failed to elicit a 90% decrease in microbial population of the germinated spores, even after 40-60min of heat treatment at the various temperatures.

3.7.1 Selection of sporulation media for use in D-value experiments

Sporulation agar I (SPA I) (Silva and others 1999) (Table 3.5) generally resulted in the highest sporulation percentage as well as the most thermo-resistant spores and, thus, was selected to be used in all subsequent sporulation of 4II 1, 3III 1C and *B. cereus*, while Campden sporulation agar (Casadei and others 2000) (Table 3.5) was selected to sporulate *B. stearothermophilus*. The fresh culture used in preparing the spores was grown by inoculating 400 µL of culture into 10 mL of TSBY broth and incubating aerobically for 48 h as per previous incubation temperatures for each microorganism.

3.7.2 D-value determination of 4II 1 and *B. cereus* spores

3.7.2.1 Sporulation

Having obtained a fresh culture for the sporulation step, 200 µL of freshly grown 4II 1 and *B. cereus* was spread in quadruplicate with a sterile bent glass rod onto pre-poured Sporulation agar I with 0.1 g $MnSO_4.H_2O$. The sporulation agar media was pre-poured in (90 × 15 mm) glass plates. The bacterial culture was allowed to stand for 15-30 min to infuse the sporulation agar, and the plates incubated aerobically at the respective optimum growth temperature for each organism (*B. cereus*-35°C and 4II 1-45°C) for 48 h. After adequate sporulation had been achieved, scraping, centrifugation, washing, and re-suspension of the spore pellets in peptone water was carried out as per the previously outlined sporulation procedure in section 3.5 above, except that the centrifugation speed was increased to 4640 × g. This higher centrifugation speed resulted in better sedimentation of the spores. The temperature and time parameters were similar to those used in section 3.5. As spore lawns were cultivated in quadruplicate, the total volume of the spore suspension was 40 mL.

3.7.2.2 Calibration of thermometers and thermocouplers

Next, the thermometers and thermo-couplers to be used in monitoring both the temperature of the 100°C distilled water bath and the control were calibrated in an ice bath, and in a boiling water bath. The 100°C water bath supplied the heat medium used to inactivate the microorganisms. As a control, 5 mL of peptone water (enough volume for accurate temperature recording) in a 30 mL screw cap test-tube was subjected to the same heat treatment as that used to inactivate the microorganisms.

3.7.2.3 Heat treatment and plotting of regression curve

After calibrating the temperature recording instruments, 2 mL of the spore suspension were transferred into 30 mL screw-cap glass test tubes that were immersed in the 100°C distilled water boiling water bath. Additionally, some of the spore suspension was also set aside for an initial spore count before the heat treatment. The spore suspensions in the glass test-tubes were subjected to heat treatment in the boiling water bath for a certain duration of time before being drawn out and cooled in an ice bath. Initially, the heat treatment was conducted from 5 - 120 min, but it was subsequently found that 100 min proved sufficient to bring about the desired inactivation level of microorganisms.

After cooling, dilutions, ranging from 10^{-1} to 10^{-9} were prepared with peptone water for both the heat-treated and unheated spore suspension fractions, and pour-plated in duplicate in nutrient agar. Plates were incubated aerobically at 35°C for *B. cereus* and 45°C for 4II 1 for 48h. Finally, enumeration of the surviving colonies was carried out and a thermal death curve plot was prepared. The D-values were interpolated from the

thermal death curve plot, whereby, D-value is the amount of time it takes to reduce the microorganism population by 90%.

3.7.3 D-value determination of 3III 1C and *B. stearothermophilus* spores

3.7.3.1 Sporulation

In the D-value determination of 3III 1C and *B. stearothermophilus* spores, ultra-high heating temperature was used to inactivate the spores, which, exponentially increased the rate of evaporation of the spore suspension. Thus, 600 µL of 48-h fresh cultures of 3III 1C and *B. stearothermophilus* broth cultures were spread in quadruplicate onto large disposable plastic Petri plates (150 × 15mm) pre-poured with Sporulation agar I with 0.1g $MnSO_4.H_2O$ and Campden agar, respectively. Thereafter, the inoculated sporulation agar media was left standing for 30-45 min to allow the culture to infuse. The inoculated plates were incubated aerobically at 55°C for 48 h.

The spores were harvested as per the protocol in section 3.5 above, except that because a larger volume of spores were being harvested, the amount of distilled water used to flood the spore plates was increased to 20 mL. Additionally, a larger size centrifuge rotor (Beckman JA 20) was used because it could hold 35 mL capacity plastic centrifuge tubes that now contained the spore wash-off to be centrifuged. The spore suspensions were centrifuged at 12100 × g for 15 min at 4^oC. The spore pellets from each of the 4 sporulated agar plates were re-suspended in 20 mL peptone water, the resultant spore-suspension combined in a glass bottle and the volume made up to 120 mL.

3.7.3.2 Calibration of thermocouplers

Once more, the thermocouplers to be used in monitoring the temperature of the 150°C oil bath as well as measure the temperature of the control, were calibrated first in an ice-bath, followed by calibration in a boiling water bath. A 150°C oil bath was used as the heat medium to inactivate the microorganisms. For the control, 5 mL of mineral oil in a 30 mL capacity screw cap glass test tube that was subjected to the same heating conditions as the spore suspensions was used. In this D-value experiment, mineral oil was used as the control because peptone water, which was used as the control in section 3.722 above evaporated at about 110°C and was, therefore, not suitable to use as a control at 150°C. A thermometer was also calibrated in the ice-bath and boiling water bath to compare with the thermocoupler temperature readings in the same media and to record the degree of any temperature deviations due to the thermocouplers.

3.7.3.3 Heat treatment and plotting of regression curve

Ten milliliters of the spore suspension was pipetted into 30 mL screw-cap glass test tubes that were immersed in the 150°C oil bath. Some of the spore suspensions were also set aside for the initial spore count before the heat treatment. The spore suspensions were subjected to heat treatment for a certain duration of time, from 0-50 min. The spore suspensions were drawn out every 5 min until 30 min had elapsed. Thereafter, they were drawn out every 10 min until 50 min had elapsed. After being drawn out, the spore suspensions were cooled immediately in an ice bath.

After cooling, dilutions were prepared with peptone water for both the heat-treated and unheated spore suspension fractions. The dilutions ranged from 10^{-1} - 10^{-8}

and depended on the amount of time each spore suspension fraction was subjected to heat treatment. Therefore, taking this into account the appropriate range of dilutions were poured in duplicate with nutrient agar and incubated aerobically at 55°C for 48 h. Lastly, enumeration of the surviving colonies was carried out and a thermal death curve plot was prepared. D-values were interpolated from the thermal death curve plot and recorded.

3.8 Residence time distribution of the twin screw extruder

For the residence time distribution, a mix of 85% soy flour (ADM) and 15% wheat starch (Midwest Grain Products) with a moisture content of 5.4% was used as the feed material. Red dye (FD & C, #40, Warner Jenkinson, St. Louis, MO) was used as the tracer to measure the residence time distribution of the soy flour and wheat starch blend in the twin screw extruder, (Hsieh and others 1994). An APV Baker MPF 50/25, 28.0kW, co-rotating, intermeshing, twin screw extruder (APV Baker, Inc., Grand Rapids, MI) was used. A volumetric feeder (Model T-35, K-tron Corp., Pitman, NJ) was used to control the feeding rate. While, there are nine temperature controlled barrel sections only five of these were used in this study. This was possible as the location of the feed port could be changed along the barrel to give five different barrel length/diameter ratios from 5:1-25:1 with 50 mm (1 diameter = 50 mm in length) equal increments (Hsieh and others 1993). The barrel length: diameter (L/D) ratio used in this research was 15:1 or 15D. The extruder barrel was divided into five zones. Zone one was unheated and remained at room temperature, 24.54°C, as this was where the product was fed in. The remaining zones, 2-5 were heated to the following temperatures: 44.49°C, 95.92°C, 151°C and 180°C, respectively. Thermocouple sensors were used to monitor the barrel and product

temperatures in different zones. In addition, a Dynisco pressure transducer inserted into the die plate was used to measure the pressure and temperature of the product at the die.

A computer data acquisition system was used to record the extrusion parameters used above, including the feed rate, barrel and product temperature, die pressure and temperature, % torque, screw speed and cutter speed. After start-up the extruder was run for 15 min to reach a steady state. The screw configuration used in this study is detailed in Table 3.3, while the extruder parameters are detailed in Table 3.4.

Table 3.3 Extruder screw profile from feed to exit.

Element	**Length/angle**	**Screw type**
1	400 mm	Spacers
2	100 mm	Single-lead feed screw
Screw profile involved in the extrusion		
3	100 mm	Twin-lead feed screw
4	50mm/ 30°	Forward paddle
5	100 mm	Single-lead feed screw
6	87.5mm/ 30°	Forward paddle
7	175 mm	Single-lead screw
8	87.5mm/ 30°	Forward paddle
9	50mm/ 30°	Reverse paddle
10	100 mm	Single-lead screw

Table 3.4 Extrusion parameters.

Screw speed (rpm)	% Torque	Die pressure (MPa)	Die temp (°C)	Feed Rate Kg/ h	Water Kg/ h
125	14.35	0.903	118.8	9.09	0.48

Barrel 5 (°C)	Barrel 6 (°C)	Barrel 7 (°C)	Barrel 8 (°C)	Barrel 9 (°C)
24.54	44.49	95.92	151	180
Product 5(°C)	Product 6 (°C)	Product 7 (°C)	Product 8 (°C)	Product 9 (°C)
23.07	37.86	94.97	115.18	146.24

3.8.1 Establishing a standard curve

In order to establish a standard curve illustrating the effect of increasing dye concentration on color values (L, a and b) in the extruded product, various amounts of red dye (by weight) were added to 100g of the soy flour and wheat starch blend and mixed in a Hobart hand mixture (Model A-200, The Hobart Mfg. Co., Troy, OH). This resulted in red dye concentrations (g red dye/ g flour blend) of 0.0%, 0.01%, 0.025%, 0.05%, 0.075%, 0.1%, 0.15%, 0.2%, 0.4% and 0.6%. These mixtures were fed into the extruder at a rate of 20lb/ hr after the extruder had reached a steady state. The moisture of the feed was adjusted to 60% by drawing water into the extruder at room temperature with a pump. As each concentration of red-dye mixture was fed into the extruder, the time clock was started consecutively and the mixture was extruded at 125 rpm. Samples with each red-dye concentration mixture were collected every 5 minutes and placed on a tray and allowed to cool at room temperature. Following, each sample was sliced into thin

slices that were laid flat on a tray. This was done to increase the surface area of the sample such that the sample surface area was large enough to cover the sensor of the colorimeter that was needed to take an accurate reading. The color values (L, a and b) were then measured and recorded using a HunterLab D25 colorimeter (Hunter Assoicates Lab Inc., Reston VA). A white tile (Standard No. C2-28656; L- 91.2, a-0.9 and b-0.7) was used to standardize the colorimeter. Three replications of the experiment were run, therefore for each red-dye concentration sample, three readings of color values were recorded. A standard curve of the averages of the color values was plotted against the red dye concentrations (g red dye/g flour blend).

3.8.2 Study of the redness color value with time during extrusion

In the second part of the residence distribution study, information on the redness of the extruded soy meat analog with time was desired. After the extruder had reached a steady state, an unknown amount of red dye was added to the feed port along with the soy and wheat starch blend. The timer was also started consecutively. Samples of the extrudate were collected every 15s until 6 min had elapsed, placed on a tray and allowed to cool at room temperature. A total of 24 samples were collected. After standardizing the HunterLab D25 colorimeter with a white tile as per 3.81, the same procedure detailed above was used to prepare the samples, measure and record the color values. A curve of the redness (a-value) versus time was plotted.

3.9 Extrusion of soy meat analog inoculated with *Bacillus* spores

3.9.1 Cultivation of spores

The spores of 4II 1, 3III 1C and *B. stearothermophilus* were grown, harvested, cleaned and re-suspended in peptone water as per the procedure outlined in 3.731 above. These spores were to be used in inoculating the soy meat analog. The cultivation of 4II 1 spores was also scaled up in a similar manner to that of 3III 1C as discussed in 3.731, and grown at 45°C, which is its appropriate growth temperature. Ten petri plates of the appropriate sporulation agar media for each microorganism as outlined in 3.71 were inoculated with each of the three microorganisms.

Harvesting from 10 sporulated agar plates, centrifuging and washing of the spores were carried out as described above. After the final centrifugation step, 5 mL of peptone water was added to the spore pellet in each of 10 centrifuge tubes and the suspension vortexed. The spore suspension in the centrifuge tubes was then pipetted into 20 mL peptone water in 30 mL capacity test-tubes, making a total volume of 25 mL of spore suspension.

3.9.2 Inactivation of vegetative cells

The spore suspension in 30 mL test tubes was then subjected to sub-lethal heat treatment to kill vegetative cells. While an 80°C distilled water bath was used to heat both 4II 1 and 3III 1C spores for 10 min to kill their vegetative cells, a 100°C distilled water bath was used to heat *B. stearothermophilus* spores for 15 min to kill *B. stearothermophilus* vegetative cells. A control of 25 mL peptone water in a 30 mL screw cap test-tube was used to verify the come-up time and heat treatment temperature.

Finally, the sublethally heat-treated spore suspension from the individual 35 mL screw cap test-tubes was combined in a 500 mL glass bottle. The volume of the spore suspension was made up to 260-270 mL. From this volume of spore suspension, 10 - 20 mL were set aside for the initial spore count, leaving 250 mL for the extrusion experiment. The spore suspension was refrigerated overnight at 4°C before use.

3.9.3 Extrusion of the inoculated soy meat analog

A blend of 85% soy flour (ADM) and 15% wheat starch (Midwest Grain Products) was used as the dry mix of ingredients for the soy meat analog. The dry mix had a moisture content of 5.4%. A 60 g sample of the dry mix was collected aseptically in a stomacher bag for the enumeration of the initial microbes in the dry ingredients. Next, 250 mL of the respective spore suspension isolate, 4II 1, 3III 1C or *B. stearothermophilus*, was added to 2.5 Kg of the soy flour and wheat starch blend in an aluminum mixing bowl and the mixture mixed for 5 min with a Hobart hand mixture (Model A-200, The Hobart Mfg. Co., Troy, OH) to evenly distribute the spores in the dry ingredients, as shown in Figure 3.1. Sixty grams of the sample of dry mix inoculated with spores was weighed out aseptically into a stomacher bag in order to determine the baseline spore count before extrusion processing was carried out to inactivate the inoculated spores.

Figure 3. 1 Dispersion of the spore suspension into the dry mix.

For the extrusion process, the exact extrusion parameters used in 3.8 above were used with the same extruder in extruding the un-inoculated and inoculated soy meat analog. After pre-heating the extruder to 180°C, the un-inoculated soy flour blend was fed into the extruder at a rate of 20 lb/h. In addition, enough water to equal 60% by weight was gradually drawn into the extruder and the mixture extruded at 125 rpm. The extruder was run for about 5 min to ensure stabilization of the product consistency and temperature before sample collection commenced. Steady state extrudates (two control soy meat analog samples) were collected 10 s apart and placed in sterile stomacher bags that were then sealed.

The feeder was then cleared of any remaining uninoculated blended soy flour. The inoculated blended soy flour was then fed into the feeder and the extruder allowed to run until all the control blended soy flour was spent and all that remained in the extruder was the inoculated soy flour blend (to prevent cross-mixing of batches). The inoculated flour blend was extruded for 5 min, to ensure consistency of the extruded sample as well

as to ensure that the maximum population of spores had been attained in the sample. Two steady state extrudate samples of the inoculated soy meat analog were then collected 10 s apart and placed in sterile stomacher bags that were then sealed.

3.9.4 Microbial investigation of extrudate

The microbial load of collected samples, beginning with those postulated to have the least number of microorganisms to those expected to have the highest spore count, was determined. Dilutions of the various samples were prepared with peptone water, beginning with the control, then the uninoculated dry mix, followed by the extruded soy meat analog and the dry mix that were both inoculated with spores of 4II 1, 3III 1C and *B. stearothermophilus* and, finally, the sublethally heat-treated spores, from section 3.9.2 above, of 4II 1, 3III 1C and *B. stearothermophilus*. Starting with a 25 g of each initial sample, dilutions ranging from 10^{-1}-10^{-6}, dependent upon the postulated initial microbial load and the given heat resistance of the specific organism, were prepared with 9.9 mL peptone water. Dilutions ranging from 10^{-2}-10^{-8} of the respective sublethally heat treated spore suspensions (as described in 3.9.2 above) of 4II 1, 3III 1C and *B. stearothermophilus* were prepared with 9.9 mL peptone water. All diluted samples were pour-plated in duplicate in three different agar media: NA, NA with 1 g starch and supplemented with 100 ppm $CaCl_2$ (NACaS), and fortified concentrated Tryptone Glucose Extract agar, containing 90% water, fortified with G_b minerals (excluding K_2HPO_4) (Cliff and others 2005), to which was added 50 mM $CaCl_2$ and 40 mM dipicolinic acid (2,6-pyridine dicarboxylic acid, 99%) per liter, dissolved in 4M NaOH in a 1:28 ratio) (CaDPA-TGE). The plated dilutions were incubated aerobically for 48 h as described above. Plates were observed for growth, and colonies enumerated.

3.10 Identifying wild type isolates

To determine the identity of the heat labile and two heat resistant isolates pooled for the extrusion study, API® 20E (bioMerieux Inc, Durham, NC) and API® 50CH kits were used. The microorganisms to be tested, 4II 1, 4II 11 and 3III 1C, were spread for isolation. Then, one or more colonies of each strain was suspended in 5 mL of 0.85% NaCl to a turbidity equivalent to 2 MacFarland standard (bioMereux, Inc.). This suspension was then used to inoculate the first 12 tubes of the API® 20 E strip, following the instructions outlined on the kit. Only the first 12 tubes of the API® 20 E kit were inoculated as the last 8 tests on the strip were carbohydrate fermentation tests that would be duplicated on the API® 50 CH strips. For the API® 50 CH strips, twice the amount of the microbial culture suspended in the 0.85% NaCl was suspended in 10 mL of API® 50 CHB/E medium so as to attain a suspension with turbidity corresponding to 2 MacFarland standard. Then, the tubes of the API® 50 CH strips were inoculated with the microbial suspension in API® 50 CHB/E medium following the directions outlined on the kit. All the strips were incubated aerobically for 48 h as described above.

The API® 50 CHB/E medium contains phenol red indicator which changes color to yellow as the pH is lowered by acid produced from carbohydrate fermentation by the microorganisms. As for the API® 20 E strips, after reading the spontaneous reactions, reagents were added to some of the tubes to test for tryptophane deaminase, hydrolysis of tryptophan to indole, acetoin production and the reduction of nitrates to nitrites (NO_2) and nitrogen gas (N_2). With the API 20 E, a 4 digit profile number was obtained for the first 12 tests of the strip that was combined with the carbohydrate fermentation results from the API® 50 CH. The numerical profile and fermentation test results were then used to

identify the microorganisms from a list of profiles in the analytical profile index, as well as, from a list of profiles detailing carbohydrate fermentation of various microorganisms.

3.11 Statistical analysis

All the data was analyzed using the Statistical Analysis System (SAS 9.1, 2004). The percentage surviving spores data was analyzed as a randomized complete block design in which the treatments were arranged by a 7 × 9 (factorial arrangement of treatments). The effect of 7 sporulation media was tested with 9 different bacteria. The variation of the means was tested using Fisher's least significant difference (LSD). The data generated from the investigation of the D-values of 4II 1, *B. cereus*, 3III 1C and *B. stearothermophilus* was also analyzed by a randomized complete block design in which treatments were arranged in a 2 × 2 (factorial arrangement of treatments). Two heat labile and two heat tolerant bacteria were tested at two different treatment temperatures. The data from the extrusion study was analyzed with a one-way ANOVA. The treatment means from both the D-value and extrusion statistical analyses were also tested with Fisher's LSD. An analysis of the means and standard deviations of the recorded values for the extruder parameters was also performed using the means procedure.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolated wild-type microorganisms

The numbers of isolated wild-type microorganisms from both soy flour and soy protein isolate ranged from the tenths to the hundredths. On the other hand, only one or two colonies were isolated from wheat gluten, appearing only in one plate of the duplicate set in the lower dilutions and in both sets of duplicate plates in the higher dilutions. Thus, while plating lower dilutions of wheat gluten did not result in any colonies, plating the higher dilutions of wheat gluten resulted in one or two colonies. Therefore, the colonies that grew in plates of higher dilutions of wheat gluten were considered to be contaminants. As illustrated in Table 4.1, wheat gluten did not generate any wild-type microorganisms and can be considered commercially sterile. In both soy flour and soy protein isolate, more microorganisms grew at 45°C than at 55 or 65°C. Thus, while the majority of the isolated microorganisms appeared to be mesophiles, the microorganisms of interest (thermophiles) in this study were those that grew at 55 and/or 65°C.

Table 4. 1 Numbers of isolated microorganisms (CFU/ mL) from raw materials.

Incubation Temperature (°C)	[a]Soy flour	[a]Soy protein isolate	[a]Wheat gluten
45	2.4×10^3	6.5×10^2	<250 (0)
55	<250 (5×10^1)	4.5×10^2	<250 (0)
65	<250 (8×10^1)	9.5×10^1	<250 (0)

[a]The average number of colonies isolated from two replications.

In examining the microorganisms isolated from soy flour, soy protein isolate and wheat gluten, preference was given to microorganisms that grew aerobically and possessed pigmented (including creamish-tan colored colonies), rough, raised or flat, serrated, irregular and spreading colony morphology that is typical of most *Bacillus*. The isolated microorganisms were obtained from two separate experiments. Two experiments were conducted primarily to verify the sterility of the ingredients. The microorganisms obtained from each experiment are represented in Table 4.2, where parts A and B in Table 4.2 represent the microorganisms isolated from the first and second replications, respectively. A selection of microorganisms isolated from A and B are also illustrated in Figure 4.1. As Table 4.2 below illustrates, all the microorganisms that possessed the above properties in part A of the Table, which represented the first run of the preliminary tests, were all except microorganisms 3 and 5. Microorganisms 3 and 5 both had cream colored, spindle shaped and cream colored, circular colonies, respectively. In part B of Table 4.2, which represented the second run of the preliminary tests, the microorganisms that did not present the properties typical of the genus *Bacillus* were 4 and 12 that formed cream and white circular colonies, respectively.

Of the microorganisms first examined, 2b, 2c and 4 in part A were gram positive (G+) cocci and were, thereby, eliminated from the list of microorganisms of interest. Circular spores were observed for 2c and, it was thought that the microorganism belonged to the genus *Sporosarcina*. As all the cocci were catalase positive, the remaining two cocci, 2b and 4, were thought to belong to the genus *Micrococcus* or *Staphylococcus*. Both *Micrococcus* and *Staphylococcus* have spherical cells that are arranged in pairs, tetrads or irregular clusters of cells, as observed in the gram stains for

2b and 4. Further, 4 formed a yellow colony on PCA, whereby *Micrococcus* typically forms pigmented colonies and *Staphyloccus aureus* usually forms yellow to orange colonies on most media. *S. aureus* is also facultatively anaerobic.

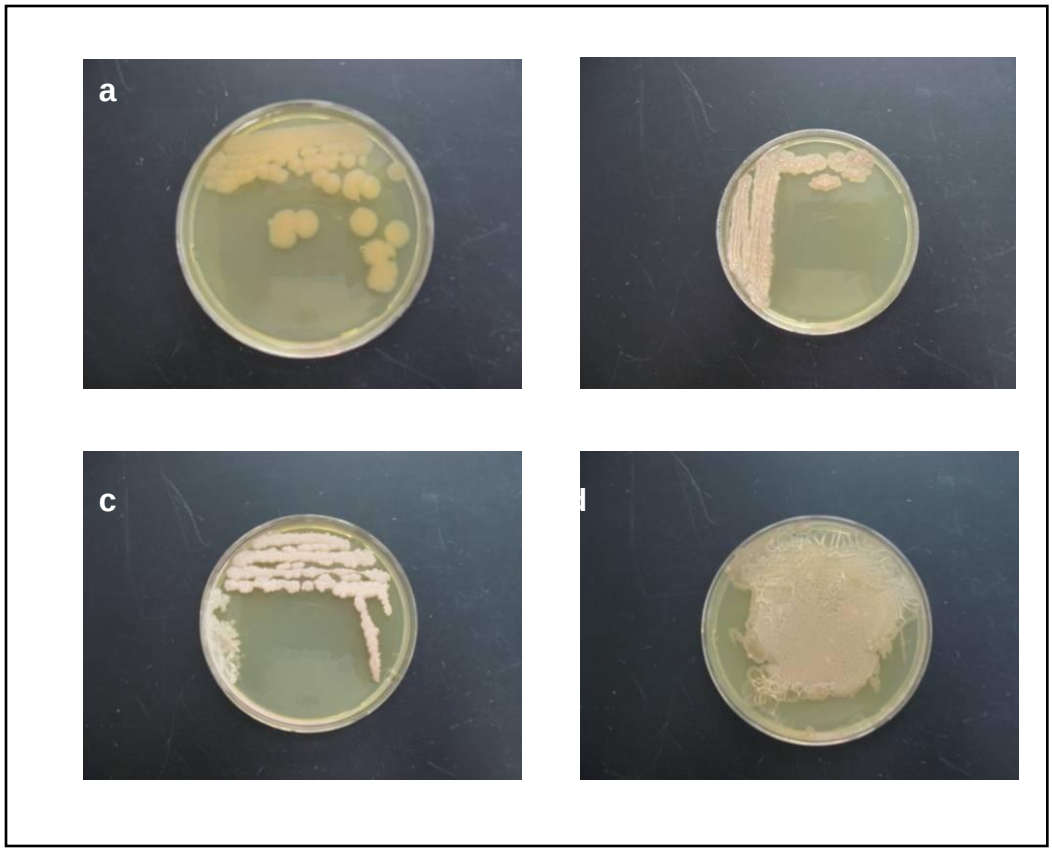

Figure 4. 1 Colony morphology of 3III 1C (a), 4II 1 (b) and 4II 11 wild-type bacilli (c, d)

Table 4. 2 Physical and biochemical characteristics of microorganisms isolated from soy flour, soy protein isolate and wheat gluten.

A Specimen/ colony morphology	Incubation Temp in °C / raw material source	Gram reaction	Catalase	Oxidase
3II 1a Medium sized, cream to tan colored, circular colonies, mucoid and spreading. More translucent towards the edges. Grow on the agar surface (aerobic), raised convex.	45 (soy flour)	Thin, short G+ rods, occur in chains of 3+ rods, thus, they look like long thin rods. The rods also occur singly and are close together. The single rods as well as the chains sometimes inter-twine. Some long chains of up to 10 rods. Central, oval spores were observed.	–	–
1b Medium to large, cream to tan colored circular colonies, mucoid and spreading. More translucent towards the edges. Grow on agar surface.	45 (SPI)	Numerous, short, thin G+ rods. Occur singly but close together-cluster like. Some in short chains of 3+ rods. Some of the chains also inter-twine.	–	–
3III 1C Medium to large, tan colored, translucent, circular colonies. Grow on agar surface.	55 (wheat gluten)	Slim, G+ rods that occur singly and in single chains of about 2, 4 and 6 rods. Central oval spores were observed.	–	+

Table 4.2 continued

2a Small, translucent, cream to tan colored circular colony that is mucoid. Also spreads. Grows on the agar surface.	45 (soy flour)	Very short G+ rods. Numerous rods clustered together. Some rods occur in short chains of 2-4 rods that both inter-twine as well as occur singly.	+	–
2b Small to medium, creamish-white (more on the white side) colored, circular colonies. Appear mucoid. They have serrated edges. Grow on agar surface.	45 (SPI)	Numerous G+ cocci that occur singly as well as in clusters of many cocci. Cocci are small to medium in size. Cocci appear to have central spores. Numerous round central spores were observed.	+	–
2c Small to medium, some large; cream to tan colored, circular colonies. They are more tan than cream. They are also mucoid. They grow on the agar surface.	45 (wheat gluten)	Small G+ cocci that occur singly as well as in clusters of many cocci; 4, 5, 6 and 8 cocci together. However, the cocci in clusters are not joined together, each is single.	+	–
3[b] Medium, cream colored, spindle shaped colonies. Grow beneath the agar surface (anaerobic).	45 (soy flour)	G+ rods	---	---

Table 4.2 continued

4[b] Medium, cream colored colony. The colony has a rough surface (the surface has small undulations-umbonate). It is also mucoid. Grows on agar surface.	45 (soy flour)	G+ cocci in clusters of numerous cocci together.	+	---
5[b] Small, cream colored, circular colonies. Grow beneath the agar surface.	55 (soy flour)	Thin, short, G+ rods that occur singly. Some occur in a single chain of about 4 rods. Some oval shaped or cocco-bacilli rods with central spores. Spores appear to be swollen.	---	---
6 Medium, cream colored, circular colonies that look dry, spreading. Grow on the agar surface.	45 (soy flour)	Slim to medium (thickness), short, G+ rods. The rods occur singly, although some also occur in chains. The rod chains vary from short 2-3 rod chains, to medium 5-rod chains to longer 8 and12 rod chains. Some chains are curved and inter-twine. One short cylindrical (rectangular-ovalish) central spore was observed.	+	–

Table 4.2 continued

7[cd] Creamish- pink, raised, spreading colonies. Look dry. Have a rough surface with vein like ridges that are criss-crossed/ inter-twined. Grow on agar surface. Smell like ammonia. Interspersed with small translucent convex globules (hill-like mounds) that are fluid growing around the edges and within the colony. The globules are also on the agar surface.	45 (SPI)	Short, slim, G+ rods, occur singly, but appear as though form paired chains of (30+), forming diamond like patterns. The G+ rods have a club shaped spore that appears centrally placed. A gram stain of the fluid filled globular mass- Thin, short G+ rod. Spores are small and circular in a short oval shaped rod.	+ ---	– ---
8[ef] Cream colored, spreading colony, raised rough. Has vein like ridges, also has fluid-like translucent raised globular mounds. The vein-like ridges are criss-crossed (inter-twine) (aerobic). (7 and 8 have similar colony morphology but for the color)	55 (SPI)	Slim, medium to long G+ rods that occur in clusters of single rods. A few short rod chains of about 3 rods. The short chains inter-twine with each other in a disarray. Fluid globular mound is observed to consist of G+ rods and what seem like G+ cocci in chains. Therefore, it is postulated that what seem like G+ cocci in chains are very short rods in chains.	+	–

Table 4.2 continued

9a[hf] Creamish-pink (more pink than cream colored) colored, irregular, raised rough colonies. The colonies have thin raised ridges that inter-twine. It is a Spreading colony as no single colonies were observed. The ridges are papery like (like nylon) (anaerobic).	45 (SPI)	Slim, long, G+ rods, not in chains. The rods are also in clusters that appear to form a hexagon-like pattern (reminiscent of #7). A central club shaped spore like that in #7 was also observed.	+	–
9b[h] Small, cream-colored, spherical, irregular with serrated edges. In addition, it has a distinguishable darker pin-point center (nucleus like).		---	---	---
10 Medium, cream colored, circular, spreading colonies. They are also dry, but not as dry #6. Grow on agar surface.	45 (SPI)	Short to medium, thin, G+ rods. Occur singly. They also form chains of about 3-5 rods. Some single rods and chains occur close together.	Weakly +	–
11[e] Creamish-pink colored, irregular colony, raised rough. Colony has a fuzzy-like appearance, also has little tiny criss-crossed ridges (vein-like network). The colony tinted tan colored TSA to pink.	45 (SPI)	Short, thin G+ rods, have a central spore that is swollen with thin ends that are not tapering (ellipsoidal).	+	–

Table 4.2 continued

Spreads over the entire agar surface. (Anaerobic).				
12a Medium, cream colored, circular, spreading colonies. They look mucoid. Also, there are some very small (tiny) cream colored, circular colonies growing over the spreading colonies. They look just like the medium, cream colored, circular colonies, and they are also mucoid. Grow on agar surface.	55 (SPI)	Short, thin, G+ rods occur singly, intertwine (criss-cross each other). Some short chains of about 3 and 6 rods.	–	+

B Specimen/ Colony morphology	**Incubation Temp in °C/ raw material source**	**Gram reaction**	**Catalase**	**Oxidase**
4II 1[bef] Creamish-pink colored, irregular, spreading colonies that have vein like network of ridges that is sparse, look dry, raised and rough. They are interspersed with hill-like mounds (umbonate) that appear to be fluid filled and are translucent and colorless. The colony spreads over the entire surface. Grows on agar surface.	45 (SPI)	Thin, short G+ rods, occur singly. Thin, short, rectangular spores were observed to occur terminally and centrally. The overall sporulated rod shape was cylindrical or oval at one end and tapering on the other (looked like a club)	+	+

Table 4.2 continued

4II 2 Large, irregular, cream colored, translucent, spreading colonies, raised rough. They have concentric ridges that surround a convex shaped globular structure (umbonate) that look as though it is fluid filled. The ridges dry with time and end up looking like vein-like structures that are paper thin and nylon like. Grow on the agar surface.	55 (SPI)	Medium length and thickness G+ rods, in chains. The chains occur singly although they intertwine. The chains are curved and spiraled. The spores are very short and swollen (almost circular, although they are oval spores), cocco-bacilli spores. These are central spores.	+	+
3a Medium and large, circular tan colored colonies (creamish-tan, more cream than tan). Have some translucency. Grow on the agar surface.	55 (SPI)	Thin, short-medium G+ rods that occur singly. Rod chains of medium length, about 4 rods, occur. Also some form long rod chains of more than 8 rods that are all single strand chains. These chains curve. Numerous chains and rods occur close together, and some chains intertwine.	–	–
3b[d] Spreading tan colored translucent colony on PCA agar. Has a smooth surface. Grows on the agar surface.	65 (SPI)	Medium length, medium thickness G+ rods. Rods form long curving chains that intertwine. Spores are short oval spores that look like cocco-bacilli and occur singly.	---	---

Table 4.2 continued

3c Large, circular tan colored, spreading colonies. They are opaque and have a smooth surface. Colonies in 3c have a darker tan color than #3a. Grow on agar surface.	55 (SPI)	Very short, thin G+ rods, many of the rods occur close to each other (en-mass). Some of the rods form short chains of about 2 rods. Numerous extremely thin chains of rods that intertwine were observed. Numerous short central spores were also observed. The spores are short, thin, oval spores.	+	+
4[c] Small and medium, circular, cream colored colonies. They are mucoid. Some of the colonies are very small (tiny). Grow on the agar surface when incubated anaerobically.	45 (soy flour)	Small numerous G+ cocci, in clusters that are in very distinct patterns. Within each cluster of numerous cocci (en-mass) is a smaller cluster of about 11 and 13 cocci in distinct patterns.	–	–
6[d] Small, cream colored, circular colonies, with serrated edges, have a more opaque darker pin-point center that looks like a nucleus. Grows beneath agar surface.	55 (soy flour)	Thin, short G+rods, occur singly. The rods form chains of about 2 rods. Short, cylindrical (square-rectangular-ovalish) spores. Also some G+ cocci, but cocci are the minority. (Probably have G+ rods with contamination or coccobacilli that look like cocci)	---	---

Table 4.2 continued

8[d] Large, cream colored, irregular colony. It has a smooth surface, although it looks fuzzy. Grows on agar surface.	45 (SPI)	G+ rods. The rods are short to medium and thin. These rods occur singly. Quite short central oval spores that look like cocco-bacilli. Also some short, terminal club shaped spores. Some cylindrical spores were also observed. The gram stain probably presents a number of strains as a result of contamination especially with respect to the fuzzy strain that may have been a second colony growing on the first.	---	---
9[d] Cream to tan colored, spreading, smooth, dry colony. As the colony spread over the entire agar surface it was more like a thick lawn. Grows on agar surface.	65 (SPI)	Very short G+ rods. Occur singly. Numerous rods were observed. Short oval spores. Both rods and spores are of medium thickness.	–	–
10a Large, cream colored, irregular colonies that are spreading. They have a smooth surface. They grow on the agar surface.	45 (SPI)	Medium, slim, G+ rods, occur singly, many occur together (en-mass). A few short chains of 2-3 rods.	–	+

Table 4.2 continued

10b[g] Large, cream colored (milky cream and somewhat translucent), irregular shaped colonies. The colonies are raised rough. They have fluid filled translucent globules (umbonate). Could have been #2 growing on # 10.		**10b**-Thin, very short G+ rods that occur singly. They have thin rectangular terminal spores. Some spores are a little more swollen (thicker) than others, but they still have the same shape and are also terminally located.	+	+
4II 11[e] Light pink colored, irregular, spreading colony. The colony has globules/ hill-like mounds (umbonate) interspersed with vein like network of ridges that are sparse, raised rough and look dry. The raised globules/ hill-like mounds are translucent and colorless, and appear to be filled with fluid that is mucoid. The colony spreads over the entire plate. Grows on the agar surface.	55 (SPI)	Thin, short G+ rods. The rods have terminal spores that occupy half the rod, hence, they appear to be central (para-central), but are placed on one half of the rod that tapers on the other end.	+	+
12 Tiny, small and medium, white, circular colored colonies. They look mucoid. Grow on agar surface.	55 (soy flour)	Small G+ cocci. Numerous cocci were observed, some occur singly, some in pairs, while others were in clusters of 3, 5, 8 and more cocci.	–	–

Table 4.2 continued

13 Creamish-tan colored spreading colonies. The colonies are flat in some areas and smooth others. The rough section predominates the colony. Both the rough and smooth section appear to be the same microorganism, (rough variant and smooth variant). Both rough and smooth colonies grow on the agar surface when they are grown under anaerobic conditions.	45 (soy flour)	**13 smooth**- numerous, extremely short G+ rods. Occur singly. Cylindrical shaped spores that are centrally placed, (also, observed some terminal spores; club-shaped rods with terminal spores, although the spore takes up half the rod, hence, it looks like a centrally placed spore (para-central). **13 rough**- numerous extremely short, slim G+ rods. Central cylindrical shaped spores (rectangular-like) are majority. Some terminal spores.	+ +	– +
14 Small to medium, light tan, circular, spreading colony. Grows on agar surface.	65 (SPI)	Short to medium thin G+ rods. Some rods form short chains of 3-4 rods as well as longer chains that appear thinner. In the long chains, the rod delineation is not clear. Short, oval spores like cocco-bacilli were observed. Some of the spores are quite swollen. (Could be 3b above).		

[a]The characteristics of microorganisms 5^b and 7^b in B are not shown as they are the anaerobic and exact strains of previously mentioned microorganisms.

Table 4.2 continued

[d]Isolated from PCA agar but did not grow at the isolation growth temperature upon further culturing in TSB.

[e]Microorganisms thought to be variants of the same strain, each similar letter represents a common strain.

[f]The globular fluid filled translucent colonies observed always occurring concomitantly to the pinkish-cream, irregular, rough, raised, colonies that have intertwining vein like structures, are thought to either be secretions or a competitive contaminant of the pinkish microorganism. This is thought so as with prolonged storage or incubation, the globular structures dry out and only the rough raised venular like colony is left behind.

[g]Both 10a and 10b grew on the same TSA plate streaked for isolation, but 10b appeared 48-96hrs after 10a had grown. Therefore, 10b is thought to be a contaminant, as *Bacillus* spores present in the air could have been deposited on the agar and germinated when the plate was open. It could be microorganism 2 from B growing on microorganism 10 from B.

[h]Initially 9a and 9b had similar colony morphology, except, while 9b had a nucleus like center, 9a did not. It is postulated that while transferring the colony into fresh broth, contamination occurred as the microorganism being transferred had anaerobic growth requirements. Therefore, the inoculating needle may have touched an aerobic microorganism of similar colony morphology to 9a that was more competitive during incubation and growth.

In naming microorganisms selected for sporulation, D-value experiments and Extrusion studies from A and B, those selected from A are designated **3II** except for 1C that is 3III 1C, while those from B are designated **4II** followed by the identification numbers and/ or letters, respectively.

All the other microorganisms first tested in A were all G+ rods, showing a positive catalase test or a positive oxidase test as shown in Table 4.2. In B, all the microorganisms first tested were G+ rods, and most presented positive reactions for the catalase and oxidase tests. The exceptions were that 17 was catalase negative, while 3a and 9a were both catalase and oxidase negative. Spores were also observed in some of the G+ stained rod cells in A and B above. Therefore, all the G+ rod cells tested, thus far, would be included in the pool of microorganisms from which select microorganisms would be drawn to be further tested with different sporulation media.

Next, the microorganisms that did not portray the typical colony morphology of the genus *Bacillus*, such as 3 and 5 in A and 4 and 12 in B were examined. Although 3 was a G+ microorganism, it grew anaerobically and had cream colored spindle shaped colonies, thus, it was tentatively considered to belong to the lactic acid bacteria family and was of no further interest. Although 5 in A had G+ rod shaped cells, it grew anaerobically and as its capability to grow aerobically was not immediately apparent, it was not considered in further experiments. In B, microorganisms 4 and 12 that formed cream and white colored circular colonies, respectively, had spherical G+ stained cells. These microorganisms, like 2b and 4 in A, were thought to belong to the genera *Micrococcus* or *Staphylococcus* that grow aerobically or facultative anaerobically for microorganisms of the genus *Staphylococcus*. In addition, the cells of microorganism 4 were arranged in clusters of numerous cells in a unique pattern, while the cells in 12 occurred singly, in pair, tetrads or smaller clusters in patterns. Such cellular arrangements have been observed in both micrococci and staphylococci, as detailed

above. Some micrococci as well as staphylococci are catalase negative just like 4 and 12 as illustrated in Table 4.2.

Of the microorganisms tested in A, 7 and 9a appeared to be the same microorganism that could grow facultatively as both colony morphology and gram stain including the shape and location of the spores were identical. 8 and 11 appeared to be variants of 7, as although 8 had a colony morphology very similar to 7, the cells in 7 have a very unique arrangement. The cells in 7 appeared to be in long paired chains arranged in a diamond like pattern. While the colony morphology in 11 was quite similar to that in 7, the shape of the spores of both 11 and 7 differed greatly as those in 11 had tapering ends, while those in 7 are club shaped. Thus, it was hypothesized that 8 and 11 were both variants of 7. It was also hypothesized that 1 and 11 in B were variants of the same strain as they both have the pink, venular like structures that are raised rough, irregular and spreading as 7 in A above. However, the spores of 7 in A and 1 and 11 in B possessed a different shape. Spores of microorganism 1 from A were thin, short, rectangular spores that are terminally or centrally located, in a rod that is oval on one end and tapered on the other end, and some were also club-shaped. The spores in 11 were oval terminal spores that are centrally placed in a rod that that tapers on the other end. Finally, an anaerobic variant of 1 in B above with the exact colony morphology and spore shape, size and location was observed. Therefore, it was likely that the pinkish colored, rough raised colonies that had a distinctive ammonia odor emanating from them could all be species of the same variant. A microorganism designated 7, with the exact colony morphology and gram stain reaction, including cell shape and size as observed in the gram stain of

microorganism 4 in A that grew anaerobically was also isolated, and therefore, not listed in Table 4.2.

To summarize the pre-liminary tests in A and B, some of the microorganisms did not grow on TSA agar. Hence, pure cultures could not be determined even though they had been observed growing on PCA agar. The colonies that did not grow on TSA agar included 3 and 9b in A and 3b, 6, 8 and 14 in B above. Later on, it was observed that both 9b in A and 6 in B also had similar colony morphology to recovered colonies of 4II 11that grew anaerobically. These anaerobic colonies were recovered from heat-treated spores of 4II 11in the experiment to determine % spore survival. However, while 6 in B is both catalase and oxidase negative, 4II 11 is both catalase and oxidase positive. Furthermore, the shapes of the spores of 6 and 4II 11 were very different. It is still possible that they could be variants of a similar strain, although further tests would be needed to prove this. These microorganisms may have died as the various incubation temperatures used were not optimal for growth even in TSA, which is a very nutritious growth media.

4.2 Fermentation of select carbohydrates by *B. cereus, B. stearothermophilus* and some isolated microorganisms

The aim of the biochemical sugar tests was to determine if the microorganism was capable of using the given carbohydrate as the sole carbon source. Hence, a modified TSBY that had no other carbon sources, except for the sugar to be tested, was formulated. When the microorganism was capable of using the sugar, it fermented the sugar, and acid was produced as a by-product of the fermentation. The reduced pH resulting from the acidic conditions changes the color of the bromcresol purple indicator dye from purple to

yellow. Therefore, depending on the range of carbohydrate fermentation, and thereby, the amount of acid produced, the purple indicator was observed to range from purple when no fermentation took place, to yellow-purple when a little acid was produced, to yellow when fermentation progressed fairly, and finally intensely yellow when fermentation was advanced as is shown in Table 4.3.

4.3 Sporulation

Sporulation for most *Bacillus* strains are carried out with Nutrient agar supplemented with 10-50 mg of Mn. The use of standard sporulation media in sporulation studies is of great importance, as the components present in the media and, thereby, the sporulation environment, determine the resistance, activation and germination of the spores produced (Gould 1971). In this study, seven sporulation media were tested with wild-type microorganisms suspected to belong to the genus *Bacillus*. All the media tested were solid media which have been shown to support good sporulation, as well as aid in water retention, which is important at higher temperatures of incubation.

Table 4. 3 Biochemical tests of select sugars with *B. cereus, B. stearothermophilus,* 4II 1, 3III 1C and 4II 11 spores.

Sugar	*B. cereus*	4II 1	*B. stearothermophilus*	3III 1C	4II 11
Glucose	++[a]	++[a]	++[a]	++[a]	++[a]
Maltose	++[a]	++[a]	++[a]	++[a]	++[b]
Fructose	++[a]	++[a]	++[a]	++[a]	++[a]
Sucrose	++[a]	++[a]	++[a]	++[a]	++[b]
Lactose	– –	– –	– –	– –	– –
Rhamnose	– –	– –	– –	– –	– –
Ribose	+–[b]	+[b]/ ++[c]	+–[c]	+–[c]	+–[b]
Saccharose	++[a]	++[a]	++[a]	++[a]	+[b]
Sorbose	– –	– –	+–[d]	– –	– –
Xylose	– –	– –	+–[d]	+–[e]	– –
Cellobiose	+[d]	++[a]	– –	++[a]	+[d]
Fucose	– –	– –	– –	– –	– –
Galactose	– –	+–[c]	– –	– –	– –
Maltotriose	++[a]	++[a]	++[a]	– –	+–[d]
Mannose	– –	++[a]	++[b]	+[b]	– –
Melezitose	– –	– –	++[b]	– –	– –
Melibiose	– –	– –	++[b]	– –	– –
Raffinose	– –	– –	++[b]	– –	– –
Fructo-oligosaccharide	+[a]	++[a]	++[a]	+ [b]	+[d]
Mannitol	– –	++[b]	– –	+–[b]	+ –[e]
Control	– –	– –	– –	– –	– –

++ intensely yellow
+ light yellow
+ – yellow- purple
– – purple

[a] Refers to results after 24 h. [b] Refers to results after 48 h.
[c] Refers to results after 72 h. [d] Refers to results after 96 h.
[e] Refers to results after 120 h.

Some of the nutrient factors affecting sporulation include the presence of carbon and nitrogen compounds, and the concentration of these compounds. The presence of minerals and in particular cations also enhances sporulation, as well as heat resistance of the resultant spores. Non-nutrient factors affecting sporulation include temperature of growth, pH of the medium and aeration (Gould 1971).

In this study, after sporulation on the various media of each of the microorganisms had taken place, the resultant spore suspension was subjected to a 100°C heat treatment for 15 min. With the exception of *Bacillus stearothermophilus*, a heat treatment of 70-80°C for 10 min was found to be the best to kill all the vegetative cells for most of the *Bacillus* strains tested. As a result, the spores that germinated upon cultivation on nutrient agar were likely an underestimation of the actual sporulation that took place because some of the spores may have been injured or even killed, with respect to the heat-labile spores. Thus, the surviving spores were expressed as the percentage surviving spores, as shown in Table 4.4, and not percent sporulation.

Table 4. 4 Percentage surviving spores of microorganisms on various media.

	Surviving spores %								
	Isolated wild type microorganisms								
Sporulation medium	**3II 8**	**3III 1C**	**4II 1**	**4II 11**	**4II 2**	**4II 3a**	**4II 3c**	**4II 17**	**B. st[E]**
NA[A]	$_{x}0.079^{a}$	$_{x}0.035^{a}$	$_{x}0.19^{c}$	$_{x}0.058^{a}$	$_{x}0.20^{d}$	$_{x}0.48^{ab}$	$_{x}0.075^{a}$	$_{x}0.14^{b}$	$_{x}0.037^{ab}$
FNA[B]	$_{x}0.21^{a}$	$_{x}3.80^{b}$	$_{x}0.20^{d}$	$_{x}7.50^{c}$	$_{x}0.55^{b}$	$_{x}0.46^{b}$	$_{x}1.50^{b}$	$_{x}2.10^{ab}$	$_{x}0.03^{ab}$
Angelotti agar	$_{xy}32.67^{a}$	$_{y}12.70^{ab}$	$_{x}0.005^{a}$	$_{y}10.0^{a}$	$_{x}2.41^{b}$	$_{y}5.79^{ab}$	$_{y}33.75^{a}$	$_{y}8.33^{ab}$	$_{xy}41.54^{c}$
Milk agar	$_{x}0.50^{ad}$	$_{x}0.62^{b}$	$_{x}0.86^{ab}$	$_{y}6.18^{ab}$	$_{x}1.00^{d}$	$_{xy}13.33^{c}$	$_{x}2.13^{ab}$	$_{x}0.08^{b}$	$_{x}0.31^{b}$
NAY[C]	$_{y}9.13^{a}$	$_{y}21.11^{a}$	$_{x}0.09^{a}$	$_{x}1.15^{b}$	$_{y}51.56^{c}$	$_{xy}61.33^{a}$	$_{x}0.12^{a}$	$_{xy}16.94^{d}$	$_{x}0.06^{a}$
SPA I[D]	$_{xy}21.11^{a}$	$_{y}90.91^{d}$	$_{x}4.00^{e}$	$_{xy}100^{c}$	$_{y}100^{b}$	$_{xy}100^{c}$	$_{y}97.30^{b}$	$_{xy}53.64^{b}$	$_{xy}81.58^{b}$
Campden agar	$_{x}1.56^{a}$	$_{x}1.44^{b}$	$_{x}0.31^{b}$	$_{y}9.23^{b}$	$_{y}37.1^{b}$	$_{xy}27.78^{b}$	$_{x}1.63^{a}$	$_{x}2.77^{d}$	$_{y}10.00^{c}$

[A] Nutrient agar enriched with manganese sulfate and calcium chloride
[B] Fortified nutrient agar
[C] Nutrient agar with 1ppm $MnSO_4.H_2O$ and 0.5% yeast extract
[D] Sporulation agar I
[E] *B. stearothermophilus*
^{abcde}Same letter within the same row indicates no significant difference at $P \leq 0.05$
$_{xy}$Same letter within the same column indicates no significant difference at $P \leq 0.05$

As shown in Appendix C.1.8, the media that generally resulted in the highest percentage of surviving spores and, thereby, the most heat resistant spores for all the strains tested was Sporulation agar I (SPA I). Next, in chronological order was Nutrient agar with 1 ppm $MnSO_4.H_2O$ and 0.5% yeast extract (NAY) and Angelotti agar (both NAY and Angelotti did not differ significantly [$P \leq 0.05$] in the mean % surviving spores recovered), followed by Campden agar. Milk agar (MA), Fortified nutrient agar (FNA) and Nutrient agar enriched with manganese sulfate and calcium chloride (NA) produced the least heat resistant spores. MA, FNA and NA did not differ significantly in the % mean surviving spores recovered at $P \leq 0.05$.

4.3.1 Nutritional requirements for sporulation

As mentioned, most sporulation studies have been carried out with Nutrient agar (beef extract -3g, peptone -5g, agar -15g/L) supplemented with 10-50 mg Mn. Additionally, for strains that did not form spores on the aforementioned basic sporulation media, calcium chloride ($CaCl_2.2H_2O$, not exceeding 100 mg/L) and magnesium sulfate ($MgSO_4.7H_20$, not exceeding 500 mg/L) was frequently added to enhance sporulation. Halvorson (1962) also stated that potassium and zinc are necessary for sporulation. The media tested in this study varied from what is considered the basic media, Nutrient agar with manganese, calcium or magnesium cations to media containing a plethora of nitrogen and carbon sources, as well as minerals.

4.3.1.1 Nitrogen and carbon sources

SPA I did not contain beef extract or peptone but contained yeast extract as the nitrogen source and dextrose (D-glucose) as the carbon source (Table 3.5). It also

contained inorganic nitrogen in the form of Ammonium sulfate $(NH_4)_2SO_4$. Angelotti agar contained casamino acids and yeast extract as the nitrogen source and dextrose as the carbon source. NAY also had readily available sources of nitrogen in beef extract, peptone and yeast but no additional carbon sources except for those provided by the agar. Campden agar appeared to be a highly conducive media for sporulation with multiple nitrogen sources from yeast, peptone, tryptone (a trypsin hydrosylate of casein) and lab lemco (a purified form of meat extract), in addition to dextrose as a carbon source. However, this did not translate into the most heat resistant spores. Although Campden agar yielded 10^8-10^{10} spores similar to the range of sporulation for all the media tested for all the strains, it resulted in spores of relative heat resistance for only two of the wild-type isolates. Milk agar was the simplest to prepare and it contained skim milk as the sole nitrogen source and agar as the sole carbon source. FNA and NA had the basic nitrogen sources for general sporulation media, beef extract and peptone, although FNA also contained inorganic nitrogen, $(NH_4)_2SO_4$.

4.3.1.2 Ionic components in sporulation media

With respect to the cations present and their role in enhancing sporulation, SPA I had magnesium, calcium and potassium cations (Table 3.5), all of which were considered absolutely necessary for sporulation. Angelotti agar had manganese and iron cations. NAY only had manganese ions, Campden agar had manganese and calcium cations, while Milk agar did not contain any minerals. FNA had numerous cations, including two sources of manganese ions, calcium, magnesium, iron, potassium, copper and zinc. While FNA contained all the minerals necessary for sporulation, it would appear that the minerals either did not work synergistically with other components in the media to

enhance sporulation or produce the most heat resistant spores. NA only had manganese and calcium cations.

4.3.2 The effect of nitrogen, carbon and ionic components on spore heat resistance

For the purposes of this study, sporulation media that contained yeast and one other nitrogen source enhanced the formation of heat resistant spores. This was shown by both SPA I and Angelotti agar which contained yeast and one other nitrogen source. The presence of a carbon source other than agar in addition to yeast also enhanced the formation of these heat resistant spores as both SPA I and Angelotti agar also contained dextrose. It would seem that fewer nitrogen sources in the presence of yeast even with no additional carbon sources other than agar still resulted in relative heat resistant spores for the purposes of this study. This is shown in that spores grown in NAY were generally more heat resistant than those grown in Campden agar. Both NAY and Campden agar had peptone and a form of meat extract, in beef and lab-lemco respectively, but Campden agar also had tryptone as an additional nitrogen source and dextrose as a carbon source. Hence, simpler formulations resulted in spores that had increased heat resistance. Milk agar only resulted in relative heat resistant spores for 4II 3a that was the most heat resistant strain ($P \leq 0.05$). FNA and NA that had the same basic nitrogen sources as NAY, except that NAY also had yeast, resulted in heat labile spores. In addition to the presence of yeast, the cations present were the next determining factor influencing the heat resistance of the spores produced.

Angelotti agar and NAY had one or two of the cations stated to greatly enhance sporulation, while SPA I had three. In addition, the concentration of these cations also played a role in eliciting increased heat resistance in the spores produced. SPA I had 250

mg/L Ca^{2+}, 500 mg/L Mg^{2+} cations, and 600 mg K^{2+} cations, Angelotti had 10 mg/L Mn^{2+}, while NAY had 3 mg/L Mn^{2+} cations. A combination of Ca^{2+} and Mg^{2+} cations as demonstrated in SPA I, resulted in spores of the greatest heat resistance. A combination of Ca^{2+} and Mn^{2+} cations moderately increased spore heat resistance, as illustrated in Campden agar. Having only Mn^{2+} cations may not have influenced heat resistance in any way (Russell 1971). As stated above, the presence of cations did not seem to influence the heat resistance of the spores as much as did the presence of yeast. This is because except for FNA that contained a plethora of ions, only two of the sporulation media used, SPA I and Campden agar, contained cations that greatly or moderately enhanced the heat resistance of the spores. All the other sporulation media either contained Mn^{2+} cations that did not seem to affect heat resistance, or did not contain any cations. NAY, in addition to yeast, had the same exact nitrogen and carbon sources as NA and FNA. Furthermore, although NAY only had Mn^{2+} ions while FNA contained numerous cations (Table 3.5), FNA produced some of the most heat labile spores.

Finally, a more readily available nitrogen source in the media also increased the heat resistance of the spores. SPA I, which had readily available inorganic nitrogen in the form of $(NH)_4SO_4$ produced the most heat resistant spores as opposed to Campden agar that did not have a readily available source of nitrogen. Although Campden agar contained more nitrogen sources in peptone, tryptone and lab lemco, the nitrogen was not as readily available as in $(NH)_4SO_4$ as it was bound to molecules. Thus, Campden agar resulted in spores with relative heat resistance for only strains 4II 2 and 4II 3a.

4.3.3 The effect of various media on the heat resistance of the different spore strains

Although heat resistance of bacterial spores is a genetic property, it is also influenced by the surrounding environment such as sporulation media. Mazas and others (1995) reported variations in spore heat resistance in spores produced on different media in thermal death rate and sporulation studies with bacterial spores conducted by Yokoya and York (1965) and Duncan and Strong (1968). As shown in Table 4.4, each of the media tested produced spores of varying heat resistance with the same strain. In 3II 8, Angelotti agar produced the most heat resistant spores followed by SPA I. In the rest of the microorganisms, SPA I elicited the most heat resistant spores, followed by NAY for strains 3III 1C, 4II 2, 3a and 17. Angelotti agar also resulted in the second most heat resistant spores for 4II 3c and *B. stearothermophilus*, while Milk agar elicited the second most heat resistant spores for 4II 11.

4.3.4 Effect of incubation temperature on heat resistance of the spores

Finally, a non-nutrient factor also affecting the heat resistance of spores is the incubation temperature. Studies have shown that spores produced at higher temperatures have greater heat resistance (Gonzalez and others 1999). In this study, only two incubation temperatures, 45 and 55°C were used. Only one microorganism, 4II 1, was grown at 45°C. For all the media tested, 4II1 had a similar range of spore count or had the highest count of spores compared to the other microorganisms tested. However, it also registered the highest percentage drop in CFU/mL after the 100°C heat treatment. Such results were observed after 4II 1 was grown with SPA I, Angelotti, NAY and Campden agar, as illustrated in Appendices C.1.1-C.1.8, where it had the highest count and also the least number of surviving spores after heat treatment. Consequently, the

spores of 4II 1 were the least heat resistant spores of the isolated microorganisms. 4II 1 also registered the most significant drop in percentage surviving spores when grown in FNA. Thus, it can be clearly shown that although it sporulated readily on all the media tested, the spores formed were also more susceptible to sub-lethal heat treatment than the spores of the other strains isolated in the study. The decreased heat resistance of 4II 1 spores was thereby attributed to its growth and sporulation temperature.

4.4 D-value of *B. cereus, B. stearothermophilus* and isolated spores

Madsen and Nyman (1907) are credited for discovering that microorganisms, when subjected to lethal treatments, followed a logarithmic order of destruction. These findings were cemented by Chick in 1910, who postulated that in order for microorganisms to follow this first-order of kinetics pattern, destruction took place as individual cells reacted with the disinfecting substance or cellular molecules become activated by heat. Esty and Meyer (1922) also found that spores of *Clostridium botulinum* inactivated by moist heat followed a linear destruction pattern (Cerf 1977; Josyln 2001). Numerous research and findings on the inactivation of microorganisms by chemicals or heat have led to the now concretely held position that the death of microorganisms is exponential or follows a logarithmic order.

In conducting research on the destruction of microorganisms, whether the mode of lethal treatment is heat, radiation, chemicals, or a combination of all three, the results arising from such research are presented on semi-logarithmic graphs, plotted with the log of surviving colonies against the time of treatment called survival curves. The decimal reduction time, designated as the D-value that is the reverse of the slope is then

calculated. The D-value is the time taken in minutes to reduce the number of microorganisms by one log cycle at a given temperature (Gould 1971).

While heat destruction models are not perfect, to obtain results that fit the model with any given microorganism, several factors have to be met: 1) the use of a homogenous inoculum, 2) the lethal treatment must have a uniform effect regardless of treatment time, 3) the recovery medium must provide optimal growth conditions for both heated and unheated spores, and 4) there must be no interfering environmental factors that cause deviation in the ideal reaction of the spores to the treatment (Pflug and others 2001).

In this study, the spores of 4II 1 and *B. cereus*, as well as those of 3III 1C and *B. stearothermophilus*, were subjected to 100°C and 150°C heat treatment using a boiling water bath and an oil bath, respectively, to determine their D-values. After cooling the spore suspensions to about 4-7°C, they were recovered on Nutrient agar. Then, the logarithm of the survivor colony counts were plotted against time. The best fit regression curve was run for each set of data. Lastly, the time taken for the microbial count to traverse one log cycle, the D-value was then calculated in minutes. The survivor curves for each of the microorganisms are illustrated in Figures 4.2 - 4.13. The D-values of the microorganisms tested are shown in Table 4.5.

It has been shown by numerous researchers, including Pflug and Smith (1977), that variation in the shape of the semi-logarithmic survivor curve from the classic linear survivor curve occurs. Variation is dependent upon the spore crop, substrate, temperature and recovery medium used. In reality, the linear semi-logarithmic survivor curves constitute approximately 40% of spore survivor curves, such as the heat inactivation of *C.*

sporogenes in phosphate buffer, (Pflug and others 2001). There are two common deviations from the logarithmic curves. These are concave downward curves that have a shoulder or lag and concave upward curves as well as the tails of biphasic curves (Cerf 1977; Pflug and others 2001). Figures 4.2 through 4.13 present a series of shapes of survivor curves frequently encountered in experiments involving the inactivation of spores.

4.4.1 D-values of *B. cereus* and 4II 1 spores

Figures 4.2 and 4.3 represents the survival curves of two replications of the inactivation of *B. cereus* and 4II 1 spores, respectively, in a 100°C boiling water bath. Heat treatment in both replications of *B. cereus* was conducted for 100 min. While heat treatment of 4II 1 for the first replication was carried out for 100 min and then for 80 minutes in the second replication, that was also adequate to bring about a satisfactory drop in the microbial population to determine the D-value. The second step was to extrapolate the curves. According to Cerf (1977) the first seven decimal reductions are more than adequate in deducing the shape of the survival curve and, thereby, also adequate in extrapolating the D-value. As illustrated in Figures 4.2 and 4.3, in all four curves, there were no more logarithmic reductions in the number of spores after 40 min of heat treatment, and tailing occurred and became more pronounced past 50 min of treatment. Thus, only inactivation of the spores during the first 40 min was considered in determining the D-values in both experiments. The tailing observed after 40 min could be as a result of several factors, including clumping of the spores, as the spores are not equally heated. According to Cerf (1977), a spore suspension with a heterogeneous population, in addition to cell-to-cell differences in a homogenous spore suspension,

could also result in tailing. Heat adaptation resulting in increased heat resistance as treatment progresses, in addition to the activation of germination systems that are usually dormant by prolonged heat exposure, also causes tailing. Faulty equipment and experimental procedures are another factor that result in tailing (Pflug and others 2001). Additionally, some of the spores in the test tubes could have adhered to the walls of the tubes because the suspending liquid splashed during treatment where they dried, thus protecting the spores from being killed (Gould 1971).

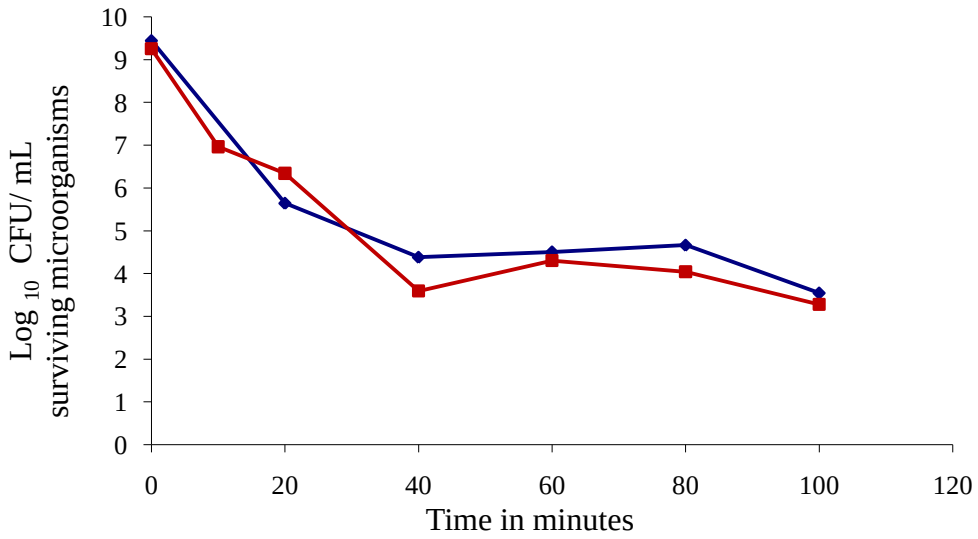

Figure 4.2 ***B. cereus*** **survival curves after 100°C heat treatment; (♦) replicate 1, (■) replicate 2.**

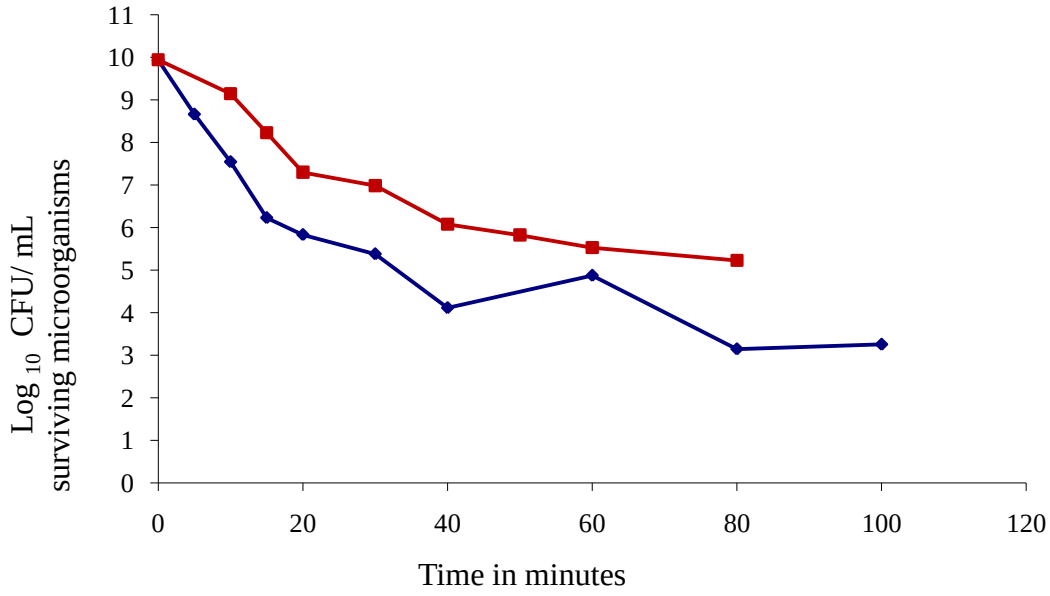

Figure 4. 3 4II 1 survival curves after 100°C heat treatment; (♦) replicate 1, (■) replicate 2.

In examining the four inactivation curves of *B. cereus* and 4II 1 spores, respectively, Figure 4.4 represents a concave upward biphasic curve. Figures 4.5 and 4.6 are biphasic curves, with the latter portion of the biphasic curve showing differing resistance, hence, appearing concave downward. Figure 4.7 looks sigmoid, or has an initial lag followed by a biphasic curve.

In a gently sloping concave upward curve, a high initial destruction rate of microorganisms occurs that is followed by a lower constant rate of microbial destruction. Concave upward curves account for 25% of survivor curves. The curve in Figure 4.4 represents a biphasic concave upward curve. Rahn (1945) noted that a concave upward curve results when a suspension contains microorganisms of varying resistance.

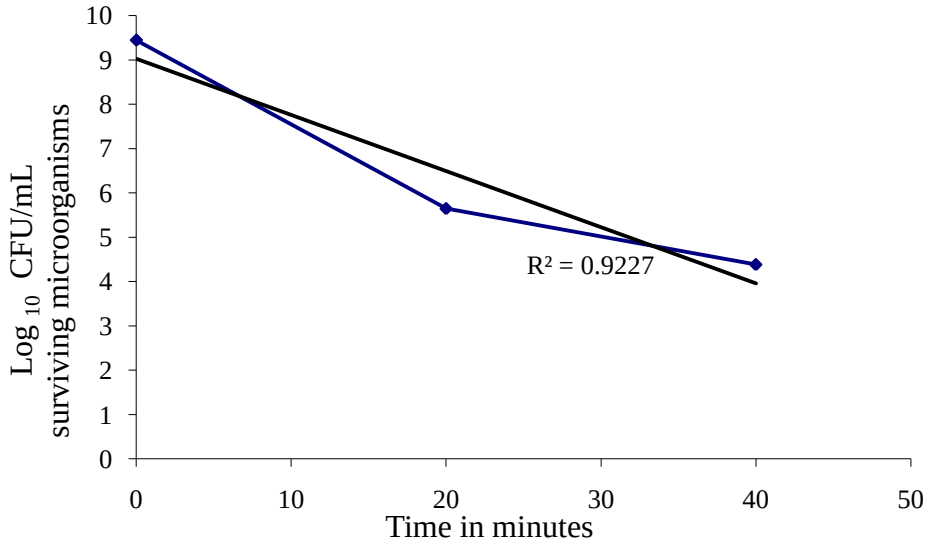

Figure 4. 4 ***B. cereus*** **survival curve after 100°C heat treatment for 40 min; (♦) replicate 1.**

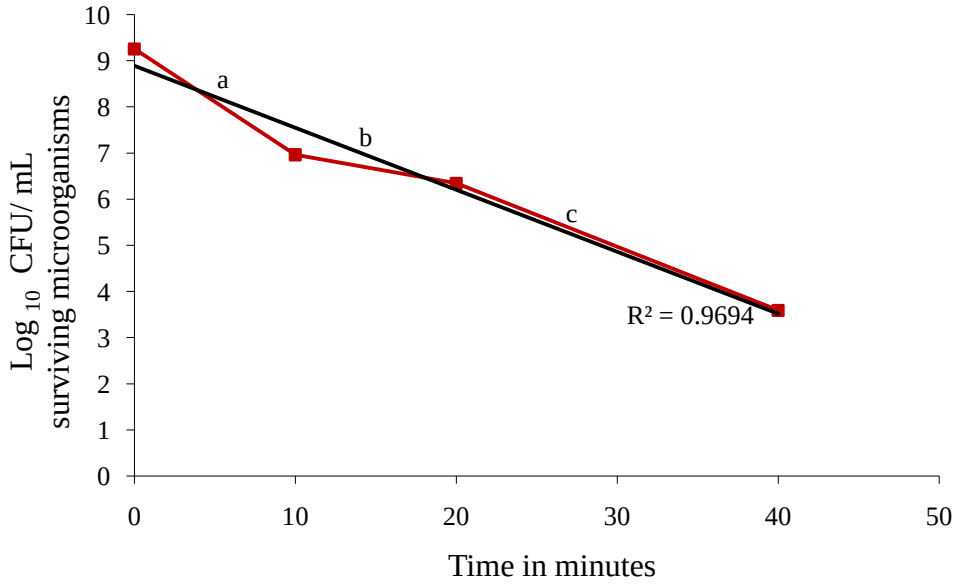

Figure 4.5 ***B. cereus*** **survival curve after 100°C heat treatment for 40 min; (■) replicate 2.**

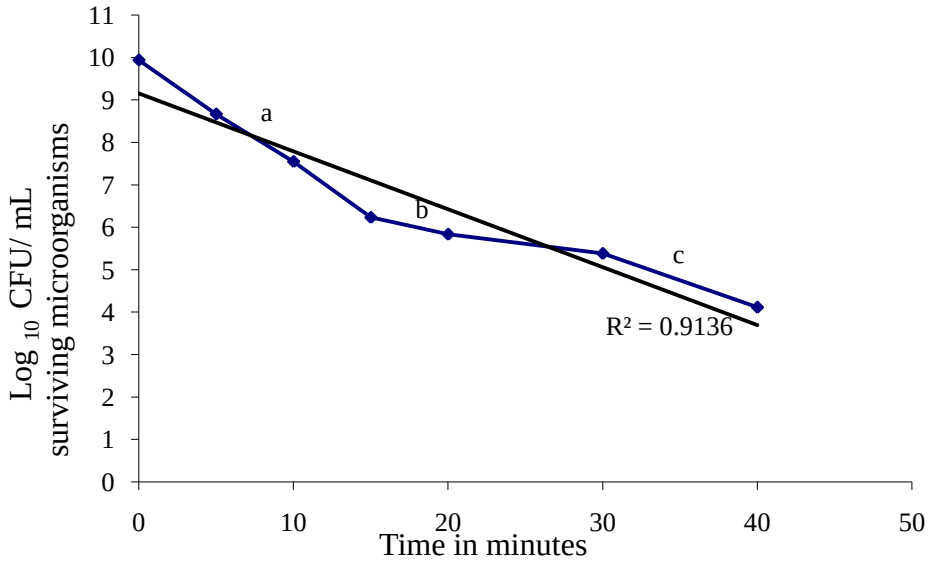

Figure 4. 6 **4II 1 survival curve after 100 °C heat treatment for 40 min; (♦) replicate 1.**

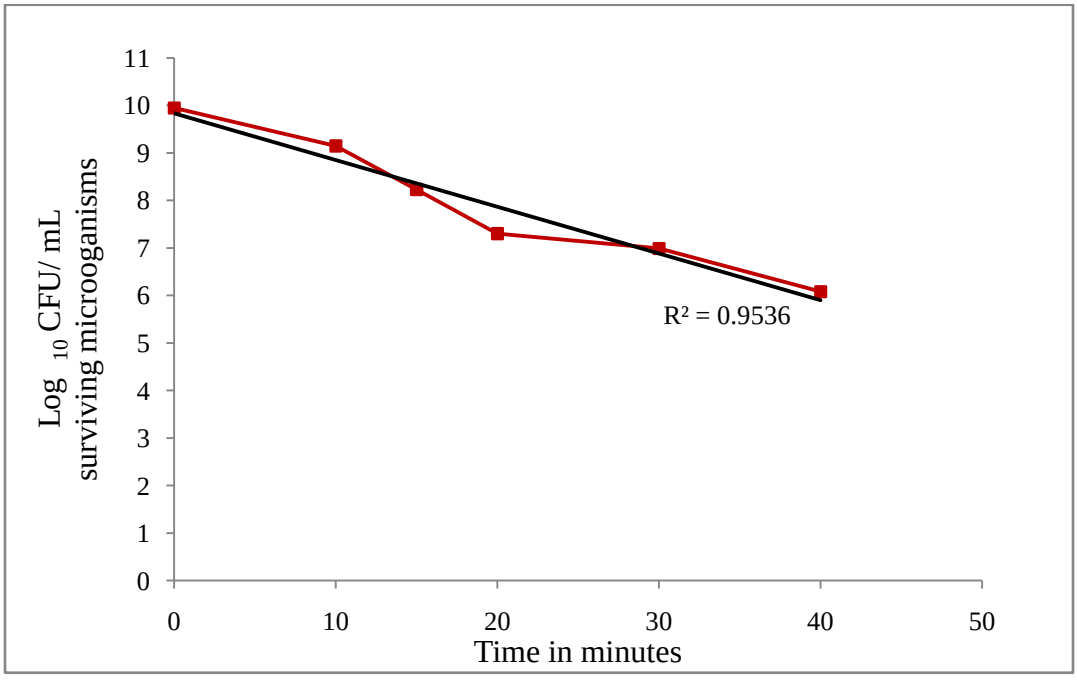

Figure 4. 7 **4II 1 survival curve after 100 °C heat treatment for 40 min; (■) replicate 2.**

Cerf (1977) also attributes a biphasic curve to the presence of two homogenous populations with varying heat resistance, such as the biphasic curve that was generated in a study conducted with *B. stearothermophilus* spores that possessed both R and S variants (rough and smooth) (DeGuzman and others 1972).

While the above explanations largely attribute biphasic curves to two different microbial strains, the vitalistic approach, promulgated by Lee and Gilbert (1918), holds that the individual microorganisms in a pure culture are not identical and, thereby, could posses varying degrees of resistance to a lethal agent such as heat treatment. Thus, the survival curves of such microorganisms are sigmoidal or concave upward, as the majority of the population posses an average degree of resistance, while the minority of the population posses the maximum and minimum degrees of resistance. A phenomenon that has been noted in concave upward curves, as illustrated in Figures 4.4-4.7, is that when concave upward curves and biphasic curves are extrapolated, the Y-intercept of the regression line is less than the initial count of the microorganisms being tested (Pflug and others 2001).

Figures 4.5 and 4.6 present biphasic curves in which the surviving microorganisms in the latter portion of the curve appeared to possess varying heat resistance. Figures 4.5 and 4.6 are typical survivor curves of spore suspensions in which flocs (flocullate) are formed during heating. According to Stumbo (1965) "Solids in liquiform food products are known to flocculate during heating" in a phenomenon referred to as curdling. Examples of products that form flocs (clumps) include cream of mushroom soup and meat homogenates.

In this study, curdling of *B. cereus*, *B. stearothermophilus* and 4II 1 spore suspensions during heat treatment was observed part of the time. Curdling was especially observed with 4II 1 spores that required vigorous mixing with a vortex to form a homogenous suspension. Moreover, once 4II 1 spores were homogenously suspended, they did not always remain dispersed in the suspension medium during heat treatment. Once flocs form, they are difficult to break up, even by dilution or vigorous shaking as "gentle agitation promotes flocculation" (Stumbo 1965).

Flocculation may cause errors in the enumeration of viable spores in thermoresistivity studies where spore suspensions are subjected to inactivation by heat for a certain duration of time, then withdrawn and cultured. This happens as spore suspensions are likely to be well dispersed at the beginning of heat treatment. Therefore after heat treatment, culturing will yield colonies that arise for the most part from single cells. However, as heating proceeds and flocculation increases with continued heating, more and more of the colonies recovered from the heated spores will grow from multi-cellular clumps, until the flocculation reaction comes to an end. Thus, as flocculation comes to an end, the survivor curve levels off as the number of survivors per clump reduces to one cell per clump (Stumbo 1965). Finally, the survivor curve attains a logarithimic death rate when remaining clumps with viable cells have only one viable cell per clump. Survivor curve shapes, like those in Figures 4.5 and 4.6 result as the flocs that are formed prevent effective and uniform heating of the spores trapped in the floc needed to inactivate the spores.

In Figures 4.5 and 4.6, part "a" represents a mixture of flocculation and death, and part "b" represents the reduction in number of viable cells per clump. At this point, some

cells are inactivated singly. However, some flocculation may still continue to exist, while part "c" represents the reducing colony count (increased death rate) with time as each colony arises from one viable cell. Part "c" represents the actual survivor curve. Therefore, in the event that sampling does not continue past part "b", the true survivor curve would not be observed (Stumbo 1965).

In Figure 4.7, the survival curve has a lag followed by a curve shape similar to that illustrated in Figures 4.5 and 4.6. The initial lag, or what is known as a shoulder portion of the curve, is attributed to heat activation of the spores (Curran and Evans 1945; 1946; 1947; Busta and Ordal 1964). Activation is needed to induce germination of spores, thereby ending their dormant state and increasing their susceptibility to lethal agents and inactivation. As a result, activation increases the microbial count during the early stages of heating as dormancy comes to an end and the spores germinate upon culturing in recovery media (Gould 1971; Josyln 2001; Pflug and others 2001). The latter section of the curve, as in Figures 4.5 and 4.6, is typical when flocculation occurs during heat treatment of the spores as explained above.

As the cultures used in this study were pure cultures of *B. cereus* and 4II 1 respectively, it is postulated that the biphasic nature of the curves may have been as a result of the mode of treatment used in the study. This was surmised as the spore suspensions in the test tubes were heat-treated in an open water bath. Although more than three-quarters of the test tube was immersed in boiling water bath, the remaining exposed sections of the tube, thought to be cooler, may have provided a protective effect on spores that dried on the walls of the tube.

4.4.2 D-values of *B. stearothermophilus* and 3III 1C spores

Figures 4.8-4.13 represent the survival curves obtained in determining the D-values of *B. stearothermophilus* and 3III 1C spores heat-treated in a 150°C oil bath. In experiments with both *B. stearothermophilus* and 3III 1C spores, heat treatment at 150°C for 20 min significantly reduced the microbial counts from 10^8-10^9 spores to zero in both *B. stearothermophilus* replications and 6 and 24 colonies, respectively, with 3III 1C spores. With respect to *B. stearothermophilus*, a close to linear survival curve with a steep downward sloping shoulder was obtained in the first replication, as illustrated in Figure 4.8, while the second replication resulted in a survival curve with a very gently sloping, almost flat shoulder, hence a pronounced lag, as shown in Figure 4.9. The survival curves illustrating the inactivation of 3III 1C spores were contrasting, whereby, Figure 4.12 detailed a lag, as spores were activated before the ensuing inactivation could take place. Therefore, in 4.12, as in 4.9, a downward sloping shoulder was observed. Figure 4.13 presented a biphasic curve. As previously mentioned, some of the factors affecting the shape of survival curves include the spore suspension, the heat treatment, the recovery medium, and any environmental factors that may cause deviation in the reaction of the spores to the lethal treatment. As there was no deviation in the preparation procedure of the spores or in the preparation and plating with the recovery medium, it was surmised that the differences in the resultant curves within each strain may have resulted from the effect of some environmental factors during the heat treatment process. Accordingly, Figure 4.10 was used to extrapolate the D-value for the second replication with *B. stearothermophilus*, by eliminating the data point at 10 min that was thought to be an outlier. Figure 4.11 shows the surviving microorganisms of

both replicates of 3III 1C spores beyond 20 minutes. As Figure 4.11 illustrates, after heat treating 3III 1C for 20 min, only 6 and 24 colonies were recovered after 25 min of heat treatment, while 10 and 0 colonies were recovered after 30 min of heat treatment, refer to Appendix C.2.

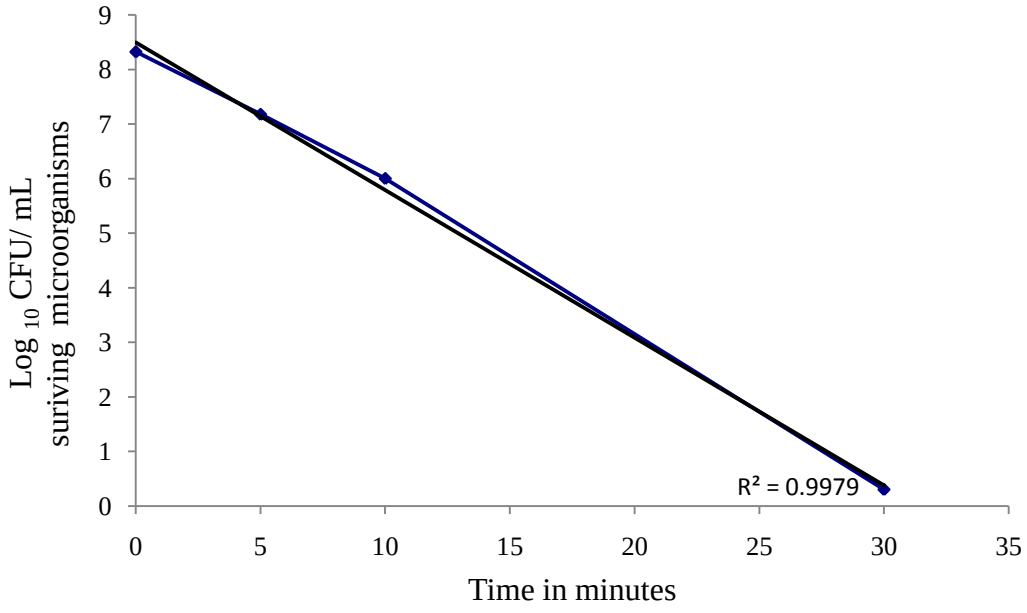

Figure 4.8 ***B. stearothermophilus*** **survival curve after 150°C heat treatment for 30 min; (♦) replicate 1.**

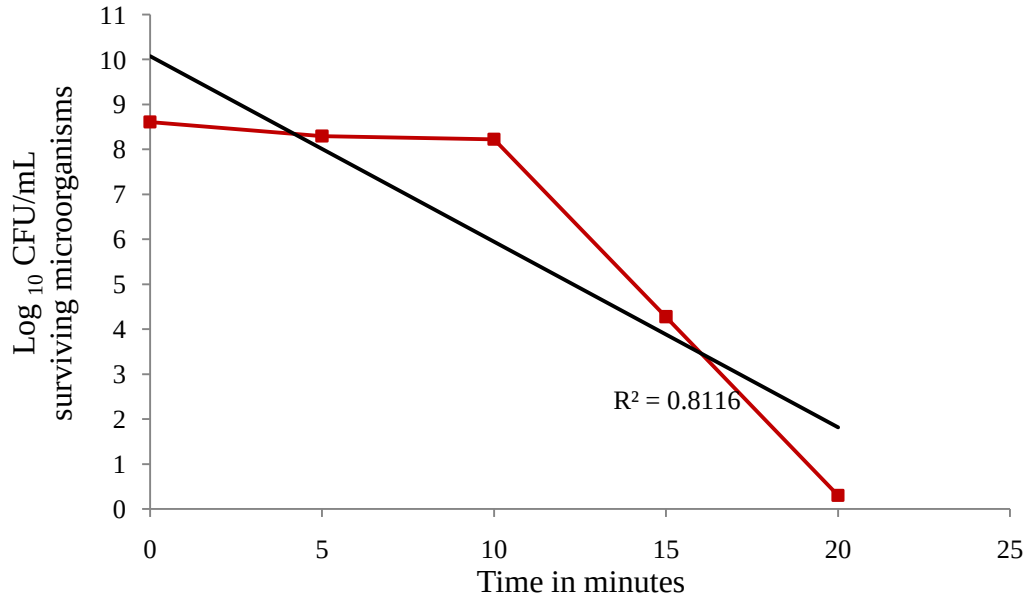

Figure 4. 9 ***B. stearothermophilus*** **survival curve after 150°C heat treatment; (■) replicate 2.**

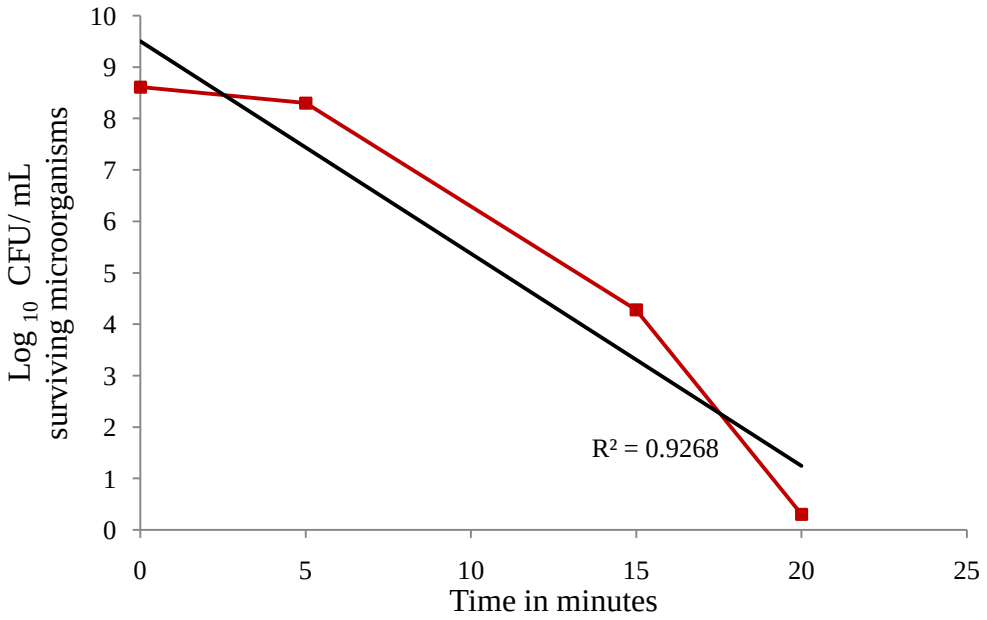

Figure 4. 10 ***B. stearothermophilus*** **survival after 150°C heat treatment for 20 min; (■)replicate 2 (without data point 10).**

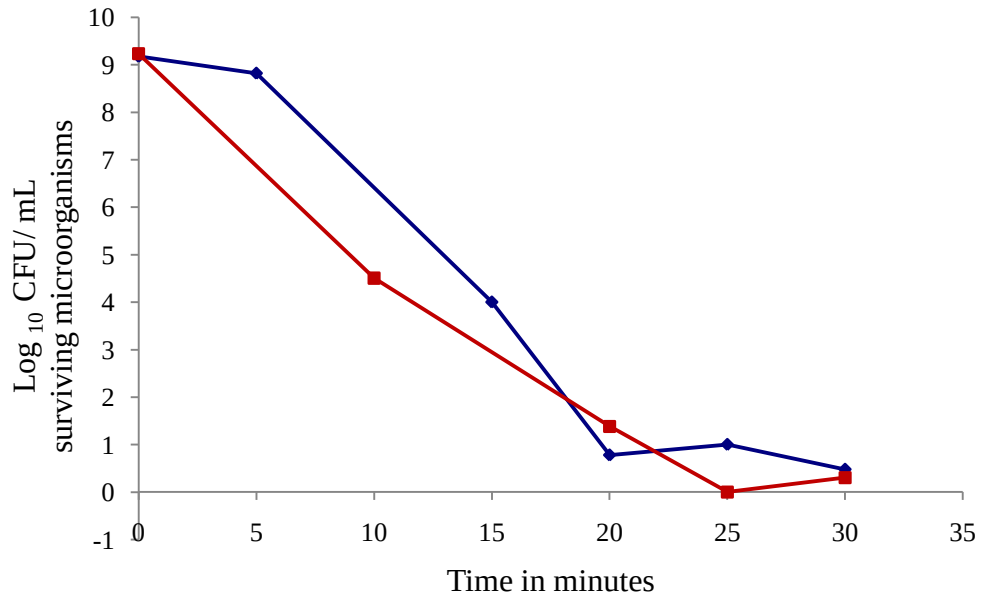

Figure 4.11 **3III 1C survival curves after 150°C heat treatment; (♦)replicate 1, (■) replicate 2.**

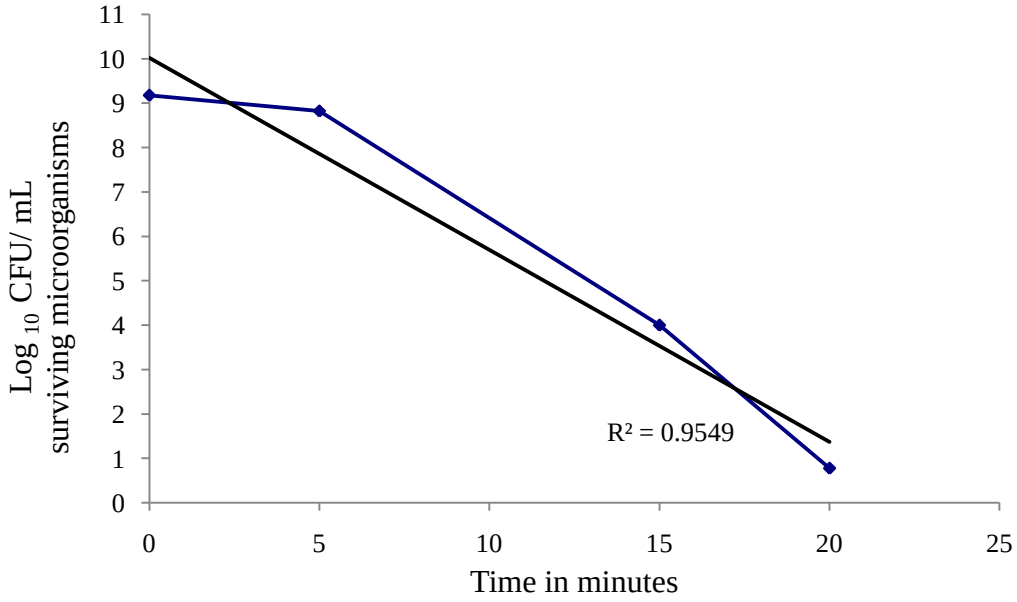

Figure 4.12 **3III 1C survival curve after 150°C heat treatment for 20 min; (♦) replicate 1.**

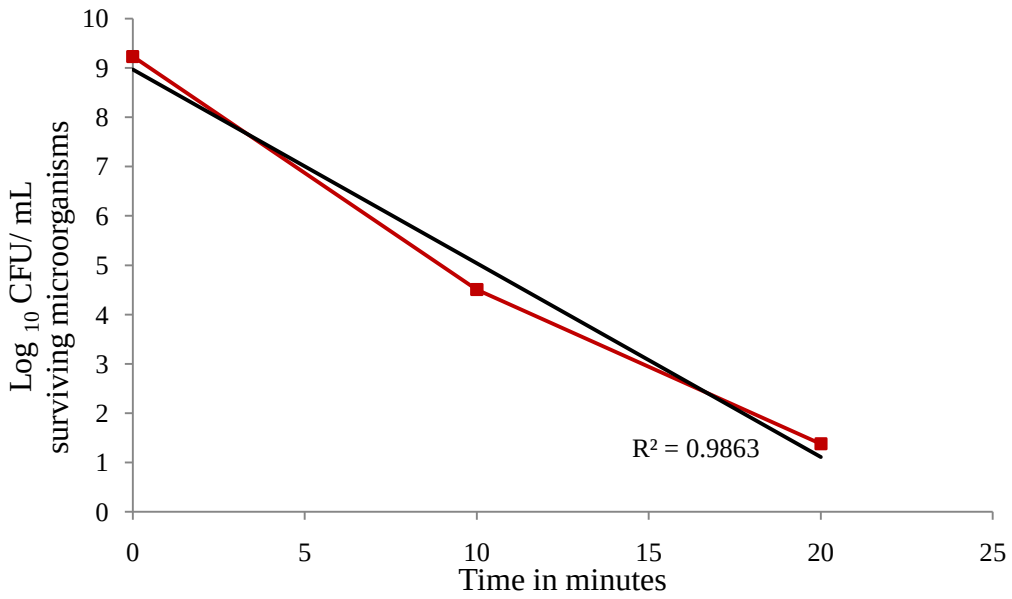

Figure 4. 13 3III 1C survival curve after 150°C heat treatment for 20 min; (■) replicate 2.

Heat inactivation studies with *B. stearothermophilus* have resulted in survivor curves with a lag, demonstrating activation that precedes inactivation. The presence of a shoulder is, therefore, typical of the survival curve for the inactivation of *B. stearothermophilus* spores, but perhaps the lag may have been more pronounced as it stretches on for 10 min in both replications even with heat treatment at 150°C. It has been shown that as the treatment temperature is raised, the activation time is reduced, the shoulder becomes higher, and the lag time less pronounced as to be undetectable. The lag time becomes undetectable as it becomes difficult to observe the population increase in short time increments (Josyln 2001). However, Figure 4.9 presents a shoulder that is almost horizontal, which occurs when activation and death of the microbial spores occur at the same rate. Moreover, the shoulder in Figure 4.9 is gently sloping downward, which means that the rate of inactivation is only slightly higher than the activation rate.

On the other hand, in Figure 4.8, the lag is steeply downward sloping as inactivation is much greater than activation, thus, initial inactivation of the spores proceeds at a greater rate in 4.8 than in 4.9. The initial microbial count in the second replication was more or less double that in the first replication, as demonstrated in Appendix C.2, accounting for the slightly more energy, converted to time, as the same inactivation temperature is used in both experiments. Gilbert (1966) has postulated that while the initial number of microorganisms influences the amount of time needed to inactivate microorganisms, it does not change the shape of the survival curve (Russell 1971). Therefore, in the experiment detailed by Figure 4.9 where the initial microbial load was greater, while activation took the same time in both 4.8 and 4.9, the lag in 4.9 is gently sloping, unlike in 4.8 where the lag is a steep downward slope. Due to the increased microbial load in the second replication in the inactivation of *B. stearothermophilus* spores, it may be surmised that activation proceeded at a much slower rate than in the first replication, thereby accounting for the gently downward sloping shoulder.

Also, in all the heat inactivation experiments, temperature was carefully monitored. There were no deviations in the treatment temperature (beyond ±2°C, a slight drop in temperature, consistent upon the immersion of the screw-capped test tubes containing the spore suspension) to account for the pronounced lag, as lower treatment temperature is associated with an initial pronounced lag. Thus, mistakes in enumeration of the survivors are thought to account for the pronounced lag in Figure 4.9, in addition to the increased initial microbial load, compared to that in the first replication that is illustrated by the survival curve in Figure 4.8.

Spores of 3III 1C, like those of *B. stearothermophilus*, demonstrated high thermal resistance. Therefore, it very reasonable that survival curves of 3III 1C spores would possess a shoulder, as activation may be required to induce germination of the heat resistant spores necessary for inactivation to proceed. Figure 4.12 demonstrates a low downward sloping shoulder like that shown in the inactivation of *B. stearothermophilus* spores in Figure 4.9. As a result, activation proceeded at a rate slightly lower than the inactivation rate. Furthermore, after activation, inactivation proceeds, for the most part, in a logarithmic order. The alteration in the shape of the curve as the experiment draws to the end could have been as a result of the experiment protocol. An extremely hot oil bath of 150°C was used to inactivate spores suspended in peptone water that had a boiling point of 108-110°C (this information was gleaned by observing the recorded temperature, as peptone water evaporated, on thermocouplers and thermometers in preliminary inactivation runs with peptone water as the control) contained in screw cap test-tubes. Thus, as the suspending medium evaporated, increased clumping of the spores may have resulted in a slower death rate as time went on, in addition to some of the previously mentioned factors affecting heat resistance, such as heat adaptation of the spores.

The effect of treatment variation on the shape of a curve has been demonstrated. The same strain of spores were subjected to two heat treatment techniques, in the first method, the spores were placed in an open tube partly submersed in a water bath and in the second method the spores were sealed in ampoules. The first treatment method resulted in a concave upward curve while the second method yielded a logarithmic survivor curve (Cerf 1977). It was concluded that some spores could have adhered in

droplets of the suspending medium in higher levels of the tube that were not submerged in the water bath. Therefore, in examining Figure 4.13, representing the second run of the inactivation of 3III 1C spores, the concave upward survival curve is, in part, due to the treatment method used. On the other hand, its biphasic nature could be alluded to varying cell-to-cell differences in heat resistance as per the vitalistic theory, as well as heat adaptation of the spores, resulting in progressive resistance as the heating continues.

4.4.3 Effect of peptone water on D-value

The D-values obtained from the best fit regression lines of the survival curves in Figures 4.3-4.6 for *B. cereus* and 4II 1, respectively, and Figures 4.7, 4.9, 4.11 and 4.12 for *B. stearothermophilus* and 3III 1C values, respectively, are given in Table 4.5.

Table 4. 5 D-values of spores of *B. cereus*, *B. stearothermophilus* and isolated wild-type bacteria thought to belong to the genus *Bacillus*

Microorganism	Heat treatment temperature °C	D-value in minutes	
		Replicate 1	Replicate 2
B. cereus	100	$_{x}7.75^{a}$	$_{x}7.37^{a}$
4II 1	100	$_{x}7.19^{a}$	$_{x}10.12^{a}$
B. stearothermophilus	150	$_{y}3.7^{b}$	$_{y}2.4^{b}$
3III 1C	150	$_{y}2.31^{b}$	$_{y}2.69^{b}$

^{ab}Same letter within the same row indicates no significant difference at $P \leq 0.05$.
$_{xy}$For each treatment, 100°C and 150°C same letter within the same column indicates no significant difference at $P \leq 0.05$.

The D-values obtained in this study were generally higher than those cited in literature. For example, the D-values reported by Feehery and others (1987) and Lopez and others (1997) at 121.1 and 120°C for *B. stearothermophilus* ATCC 12980 suspended in Mcllvaine and phosphate buffers, respectively, were 3.33 and 2.36 min. Additionally, a D-value of 3.9 min at 121.1°C in phosphate buffer of a smooth variant of *B. stearothermophilus* NCA-FS 1518D was obtained by Bouveresse and others (1982). It has been reported that spores of the smooth variant of *B. stearothermophilus* are more heat resistant than those of the rough variant. The range of D-values at 100°C conducted in two studies by Mazas and others (1995) and Gonzalez and others (1999) for three strains of *B. cereus* grown at 30 and 35°C, respectively, in Mcllvaine buffer are as follows: - 0.23 and 0.35 min for *B. cereus* ATTC 7004, 1.56 and 3.91 min for *B. cereus* ATTC 4342 and 7.18 and 6.21 min for *B. cereus* ATTC 9818.

While most heat inactivation experiments with spores have been carried out using sterile distilled water, phosphate buffer or Mcllvaine buffer as the suspending medium, in this study, peptone water was used as the suspending medium. The isolated spores in this study were to be used in heat inactivation studies in a soy meat analog. Accordingly, peptone water was used as the suspending medium, as it was believed that peptone water would provide the most accurate information on the range of spore heat resistance relative to the protective effect of the soy meat analog. This information would then be used to determine the processing temperature in the extrusion of the soy meat analog.

Previously, it was held that peptone water enhanced the heat resistance of *B. stearothermophilus* spores, as the average D-value of *B. stearothermophilus* spores in peptone water at 150°C was 3.05 min. As shown above with *B. cereus* ATTC 9818, D-

values$_{100°C}$ of *B. cereus* within the range of 7.56 min (where *B. cereus* average D-value$_{100°C}$ = 7.56 min in this study) have been recorded. It was thought that peptone water had increased the heat resistance of the spores, because, the addition of peptone would have decreased the A_w in the suspending medium. Spores have been observed to gain additional heat resistance by the addition of solutes into the suspending medium, in the inactivation of microorganisms by dry heat, which decreases A_w, (Pflug and others 2001). The increased heat resistance is as a result of plasmolysis, hence, the cell is dehydrated and has reduced pores in the cell wall that also decrease the loss of cell components. Dehydration also confers stability to cell proteins. Pflug and others (2001) noted that Cory, in 1976, was able to show that the degree of protection conferred by various solutes correlated with the level of plasmolysis that they brought about, thus proving that the increased heat resistance was a result of dehydration of the cell. However, in this experiment, the amount of peptone water contained in the suspension medium, 1 g/ L peptone, is too minimal to greatly reduce A_w and thereby enhance the heat resistance of the heat treated spores. Therefore, it hypothesized that the mode of heat treatment is responsible for the apparent increased heat resistance. Spore suspensions in peptone water, contained in screw-cap test-tubes, were immersed in a hot (150°C) oil bath. At this elevated temperature, some of the liquid suspending medium evaporated, resulting in a dried layer (coating) of spores adhering to the inner sides of the tube. This may have occurred as the suspended spores were in direct contact with the heating medium, the extremely hot oil in the oil bath were rapidly heated, evaporating the suspending medium. This coating of spores protected the spores that were still suspended

in the peptone water from being effectively heated and inactivated, which would account for the apparent increased heat resistance.

There are various factors affecting the heat resistance of spores, including the media used to grow and sporulate the bacteria, growth temperatures, and inherent resistance that is also affected by the source of the microorganism. Furthermore, while the media used to grow the survivors does not affect heat resistance, it does influence the final rate of heat resistance in as far as the colonies that are enumerated. Therefore, the enumeration media should not be too rich or minimal for the recovery of injured microorganisms. Various media and temperatures are used in thermoresistivity studies in growing and sporulating the microorganisms, as well as in recovery of the microorganisms. Therefore, it is expected that the most optimal media and growth temperature that will result in the most heat resistant spores are those used in cultivating the microorganisms. It is also expected that the recovery media used is that which will repair the damage caused by the heat treatment and enable the spores to regain viability. Consequently, in this study, the mode of heat treatment, especially at the elevated treatment temperature (150°C), resulted in what was initially thought to be the enhanced heat resistance of *B. stearothermophilus* spores by the suspending medium used, peptone water.

4.5 Residence time distribution

The residence time distribution serves the purpose of elucidating the mixing conditions as well as the chemical kinetics taking place in the extruder. Thus, it is used to improve the extrusion parameters for blending, dispersion and polymerization in

extrusion applications. Residence time can also be used to verify the efficacy of the extrusion process in the destruction of microorganisms (Hsieh and others 1994).

With respect to the standard curve, the color values L, a, and b versus the percentage color concentration (g red dye/ g flour) were plotted as illustrated in Figure 4.14. The "L" value represents lightness. "L" ranges from 0- darkness to 100-lightness, "a" represents redness when positive and greenness when negative, while "b" represents yellowness when positive and blueness when negative. Both "a" and "b" indicate grayness when they are zero (Francis 2003).

L-values decreased from 50.81 to 30.73, and b-values varied from 16.62 to 12.01 as the concentration of the red dye increased from 0 to 0.6%, while a-values increased from 5.63 to 35.60 as the red dye concentration was increased. Therefore, it was concluded that the extrudate became darker and redder as the concentration of the red dye was increased. In addition, the most significant change in color values were those recorded for the a-values. The color values for b hardly changed. Although, there was a comparative change in L-values, the most significant change was that observed for the a-values. As a result, the curve for the a-color value was selected as the standard curve.

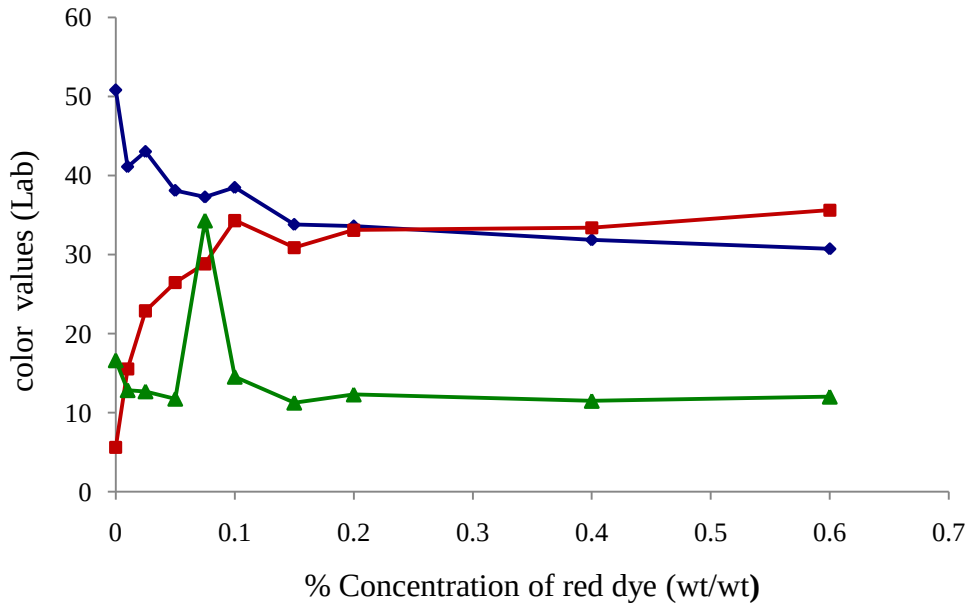

Figure 4. 14 Standard curve of color values L (♦), a (■) and b (▲) (concentration approach).

The second approach used in determining the residence time distribution in this study involved using an unknown amount of dye in the extrudate to determine the redness color value with time during extrusion. Figure 4.15 illustrates the redness color values that were recorded between 0 and 330 s.

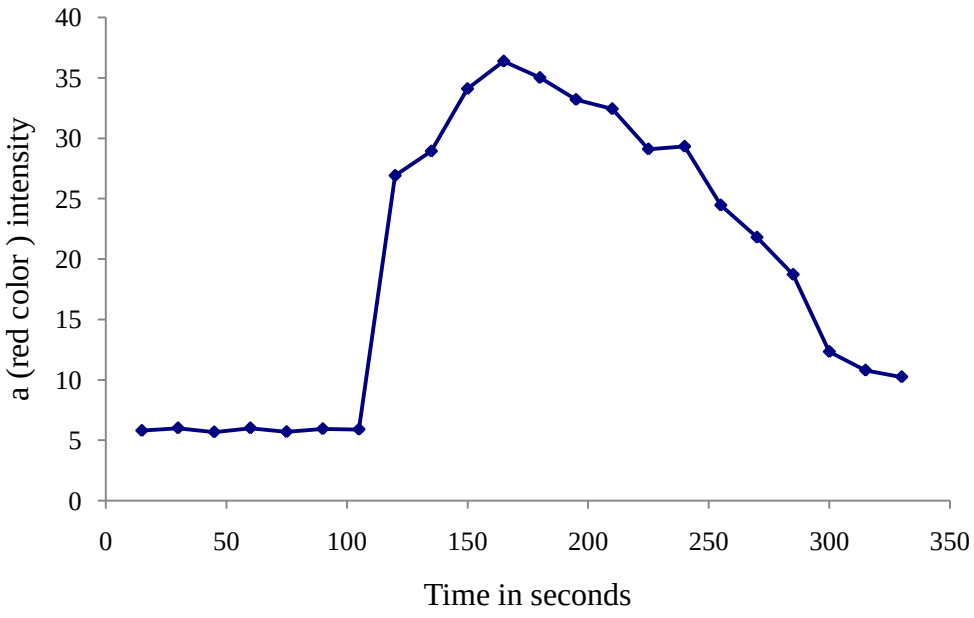

Figure 4.15 Redness a-color (♦) value with time during extrusion (color approach).

As Figure 4.15 illustrates, maximum redness was recorded at 165 s. After 165 s, the redness color value (a) consistently fell. Hsieh and others (1994) conducted a colorimetric analysis using the color dye method to determine if there was a difference in two common ways used to determine residence time distribution. They compared the mean residence time ($\bar{t}$) and spread of residence time distribution (σ) using both redness color values as measured from a HunterLab colorimeter (color approach), and also converted the redness color values to concentrations using a standard curve similar to the "a"-color curve in Figure 4.14 (concentration approach). Hsieh and others (1994) determined that the $\bar{t}$ and σ from the color approach was significantly higher than that determined using the concentration approach for the given extruder parameters (screw speed and feed rate). It was also concluded that the color approach overestimated the $\bar{t}$

and σ. As a result, the concentration approach was recommended to be used in determining $\bar{t}$ and σ in residence time studies.

As previously observed, a maximum a-value of 36.36 was recorded at 165 s, using the color approach, followed by a consistent drop in the a-value with time. In the concentration approach, the highest a-value, 35.60, was determined using 0.6% red dye concentration within 300 s. The a-value also varied from 30.87-35.60 with 0.1-0.6% red dye concentration.

In this experiment, the resultant intensity of the red color was used to model the efficacy of the extrusion process for the given parameters in maximally activating the spores inoculated in the product before inactivation commenced. As mentioned, the color approach overestimates the $\bar{t}$. Thus, it can be surmised that a $\bar{t}$ of less than 165 s would be sufficient to maximally activate the spores in the inoculated meat analog before inactivation could commence. In addition, increasing the red dye concentration beyond 0.1 did not seem to significantly increase the a-value, whereby the treatment time is kept constant. Similarly, increasing the spore concentration beyond 10^7, to10^9 spores/mL (10^7-10^9 spores/mL is the spore range used in most research in inactivation studies) would not significantly increase the $\bar{t}$. Therefore, a residence time of 5 min was determined to be more than sufficient to activate the spores, followed by inactivation by extrusion cooking.

4.6 Extrusion

4.6.1 Control

The majority of the microorganisms recovered from the dry mix without spores using all three media were those from spores of 4II 1, 4II 2, followed by what appeared

like *B. cereus*, then those from spores of 3II 8, 3II 10 and 4II11 spores. Soy flour and wheat starch that had been recently purchased and thereby, not stored for a long duration was used to prepare the dry mix. Thus, the dry mix was quite clean with microbial numbers in the dry mix ranging from 10^1-10^2, as illustrated in Table 4.6. Therefore, owing to this very minimal initial microbial load, the extrusion process rendered the soy meat analog commercially sterile as the microbial counts were reduced to zero or single digit numbers. The exception was with *B. stearothermophilus*, where 50-70 colonies on average were recovered. However, these colonies all grew in the second run of the experiment where it is postulated that the spatula used to collect the extruded sample was probably contaminated with *B. stearothermophilus* spores. The above conclusion was made as only *B. stearothermophilus* colonies were observed to have grown in the cultures of the extruded control in the experiment conducted with *B. stearothermophilus* spores, as shown in Table 4.7.

4.6.2 Inoculated soy meat analog

As shown in Table 4.6, the initial microbial load of the spore suspension ranged from 10^7-10^9. Although *B. stearothermophilus* consistently formed over 80% heat resistant spores, first with SPA I, and later with Campden agar, as per Table 4.4 above, it still did not form as many spores as 3III 1C or 4II 1which formed about 10^8 and 10^9 spores, respectively.

However, as seen from the extrusion study, *B. stearothermophilus* spores were the most heat resistant. The spore load decreased by about two log cycles on being dispersed into the dry mix. After extruding the dry mix (Table 4.6), extrusion decreased the spore load of 4II 1 and 3III 1C spores by about 7 and 6 log cycles, to 0 spores/g of the

inoculated dry mix within 5 min. Although samples were collected both after 5 min and then after 5 min and 10 s (for 0 and 10 s collection points after the extruder had stabilized for 5 min), as per 3.9.3, both samples yielded 0 spores on being plated with each of the three media used in this experiment. Extrusion at 180°C of *B. stearothermophilus* spores that proved to be the most heat resistant fraction of the spores tested, reduced the number of spores in the inoculated dry mix by about 4 log cycles in the first run, and 2-3 log cycles in the second run. The average spore-load reduction was also by 2-3 logs.

Several factors played into effect in the above variation of results between the first and second replication of the extrusion of the soy meat analog inoculated with *B. stearothermophilus* spores. The first was that while in the first run, sample collection was at 30 and 40 s, respectively, in the second run, sample collection was at 0 and 10 s. In the first run, the samples collected initially were too small to be considered representative of the extruded product, thus sampling at 30 and 40 s was carried out. Therefore, the additional extrusion time further reduced the viable spores.

In addition, it is thought that the spatula used to collect the extruded samples in the second run was contaminated with *B. stearothermophilus* spores, such that only strains possessing *B. stearothermophilus* colony morphology were observed growing in cultured dilutions of the inoculated extruded soy meat analog. Moreover, while at the first glance, there may have appeared to be a significant variation between the viable spores recovered in the second run with *B. stearothermophilus* at 10 s versus those recovered at 0 s, as illustrated in table 4.7. Even though the viable spores/g recovered at 10 s were slightly more than those at 0 s, this variation was not significant.

4.6.3 Effect of recovery media

Neither of the three media used, NA, NACaS and CADPA-TGE, appeared to be superior over the other in enhancing recovery of any injured spores, as lethal heat treatment (180°C at 1.0MPa pressure) was used for inactivation in the extrusion process. Nutrient agar that contains beef extract and peptone as the nitrogen sources and agar as the carbon sources was used for the routine cultivation of both unheated and heat-treated *Bacillus* spores in many studies and can be considered a basic media.

In NACaS, 0.1% starch was added to absorb long-chain fatty acids. It has been determined that *Bacillus* spores surviving heat treatment have increased sensitivity to unsaturated fatty acids. Increased recovery of heat-treated *B. stearothermophilus* spores grown in media to which starch or charcoal was added has been reported (Adams 1978). Therefore, while starch does not provide a nutrient source, it mediates the sensitivity of heat-treated bacilli spores to growth inhibitors, such as long chain fatty acids.

CaDPA also plays a non-nutritive role in enhancing the recovered colonies of heat-treated spores because it is not metabolized. A tenfold increase was determined in the number of *B. subtilis* colonies that germinated with the addition of CaDPA into nutrient agar after heat treatment at 113-146°C. On the other hand, there was only a slight increase in the number of observed colonies for the same unheated or mildly heated *B. subtilis* strain grown in nutrient agar with CaDPA. Thus, it was concluded that CaDPA mitigated spore injury, enabling the injured spores to germinate (Adams 1978).

None of the media appeared to influence, in any way, the recovery of any of the heat-treated spores, except with *B. stearothermophilus.* CaDPA-TGE resulted in reduced number of viable *B. stearothermophilus* spores that germinated and formed colonies,

compared to the number of recovered colonies with NA and NACaS, by one log cycle. According to Adams and Busta (1972), CaDPA alleviates the damaged L-alanine stimulated germination system in the spore. *B. stearothermophilus* is said to undergo spore injury with heat treatment, especially ultra-high heat treatment, such as 180°C that includes increased sensitivity to sodium chloride (NaCl) (Foegeding and Busta 1981). Thus, perhaps a different system rather than *B. stearothermophilus*' L-alanine stimulated germination system is adversely affected, resulting in increased NaCl sensitivity.

Table 4. 6 Number of *Bacillus* colonies recovered from the uninoculated dry mix, extruded control, inoculated dry mix, and extruded sample, in comparison to the initial spore count (spores/g)[1].

Recovery media	Dry mix without spores	Microorganism/ Control	Initial spore count	Dry mix with spores	Extruded sample 0 s	Extruded sample 10 s
4II 1						
[2]**NA**	$_{x}$ <250 (6.5 × 10) [a]	$_{x}$<25 (5) [ab]	5.3 × 10^{9}	$_{x}$2.4 × 10^{7} [c]	$_{x}$<25 (0)[b]	$_{x}$<25 (0)[b]
[3]**NACaS**	$_{x}$1.5 × 10^{2a}	$_{x}$<25 (0)[b]	6.0 × 10^{9}	$_{x}$2.2 × 10^{7c}	$_{x}$<25 (0) [b]	$_{x}$<25 (0)[b]
[4]**CADPA-TGE**	$_{x}$ <250 (3.5 × 10)[a]	$_{x}$<25 (0)[a]	5.0 × 10^{9}	$_{x}$1.4 × 10^{7b}	$_{x}$<25 (0)[a]	$_{x}$<25 (0)[a]
3III 1C						
NA	$_{x}$1.4 × 10^{2a}	$_{x}$<25 (0)[b]	7.8 × 10^{8}	$_{x}$4.5 × 10^{6c}	$_{x}$<25 (0)[b]	$_{x}$<25 (0)[b]
NACaS	$_{x}$1.4 ×10^{2a}	$_{x}$<25 (0)[b]	7.1 × 10^{8}	$_{x}$4.1 × 10^{6c}	$_{x}$<25 (0)[b]	$_{x}$<25 (0)[b]
CADPA-TGE	$_{x}$9.0 × 10^{1a}	$_{x}$<25 (0)[b]	6.5 × 10^{8}	$_{x}$5.4 × 10^{6c}	$_{x}$<25 (0)[b]	$_{x}$<25 (0)[b]
B. stearothermophilus						
NA	$_{x}$1.2 × 10^{2a}	$_{x}$6.5 × 10^{a}	3.1 × 10^{7}	$_{y}$1.2 × 10^{5b}	$_{yz}$7.0 ×10^{2a}	$_{yz}$8.6 ×10^{2a}
NACaS	$_{x}$1.1 × 10^{2ab}	$_{x}$7.0 × 10^{a}	2.8 × 10^{7}	$_{y}$1.3 × 10^{5c}	$_{y}$9.4 ×10^{2b}	$_{y}$1.8 ×10^{3b}
CADPA-TGE	$_{x}$1.3 × 10^{2a}	$_{x}$5 × 10^{a}	3.0 × 10^{7}	$_{y}$1.2 × 10^{5b}	$_{xz}$8.0 ×10^{a}	$_{xz}$1.4 ×10

[1]Data are averages of duplicate experiments.

[2]Nutrient Agar.

[3]Nutrient Agar with 1 g starch supplemented with 1 ppm $CaCl_2$.

[4]Fortified concentrated Tryptone glucose extract agar with $CaCl_2$ and dipicolinic acid.

[abc]Same letter within the same row indicates no significant difference at $P \leq 0.05$

$_{xyz}$Same letter within the same column indicates no significant difference at $P \leq 0.05$.

Table 4. 7 Number of *B. stearothermophilus* spores in uninoculated dry mix, control, inoculated dry mix and extruded sample in comparison with the initial number of spores (spores/g)[1].

Recovery media	Dry mix without spores	Control	Initial spore count	Dry mix with spores	Extruded sample	
1.1					**30 s**	**40 s**
[2]NA	1.0×10^2	<25 (0)	1.2×10^7	4.3×10^4	<250 (1×10)	<250 (3×10)
[3]NACaS	1.0×10^2	<25 (0)	1.3×10^7	5.2×10^4	<250 (7×10)	<250 (9×10)
[4]CaDPA-TGE	1.2×10^2	<25 (0)	1.2×10^7	6.0×10^4	<250 (0)	<250 (0)
1.2					**0 s**	**10 s**
NA	1.4×10^2	1.3×10^2	4.9×10^7	2.0×10^5	1.4×10^3	1.7×10^3
NACaS	1.2×10^2	1.4×10^2	4.2×10^7	2.1×10^5	1.8×10^3	3.5×10^3
CaDPA-TGE	1.4×10^2	9×10	4.7×10^7	1.8×10^5	1.6×10^2	2.7×10^2

[1]Data are averages of duplicate experiments.
[2]Nutrient Agar.
[3]Nutrient Agar with 1 g starch supplemented with 1 ppm $CaCl_2$.
[4]Fortified concentrated Tryptone glucose extract agar with $CaCl_2$ and dipicolinic acid.

4.6.4 Effect of suspending medium

Additional factors influencing the heat resistance of spores include the suspending medium. In this study, the spores were suspended in a high protein (soy flour) and a low carbohydrate (wheat starch) medium. The microorganisms were isolated from high protein sources, 4II 1 from soy protein isolate, a derivative of soy flour and 3III 1C from wheat gluten, a form of protein in wheat flour. Thus, it was hypothesized that the soy

flour, as well as wheat starch, would have conferred some protection because these microorganisms are naturally occurring in these ingredients. In addition, soy flour would have provided a good nitrogen source for continued dormancy of the spores, as peptone that is added to various sporulation media, enhances sporulation and heat resistance of resultant spores. Also, high carbohydrate concentrations increase heat resistance as they enhance plasmolysis. Although the concentration of starch used was quite low, it was thought that it would still influence heat resistance to some degree. In addition, organic matter, such as peptone, albumin, sugars and starch, have been determined to increase the heat resistance of spores at certain concentrations (Josyln 2001).

However, due to the ultra high temperature used to inactivate the spores, coupled with the shape and size of the extruded product that generally had a high surface area and was thin, heating was very effective in inactivating the spores. Thus, no protective effect was observed to be conferred by the soy flour or wheat starch. Figure 4.16 depicts the high surface area and reduced thickness of the extruded soy meat analog.

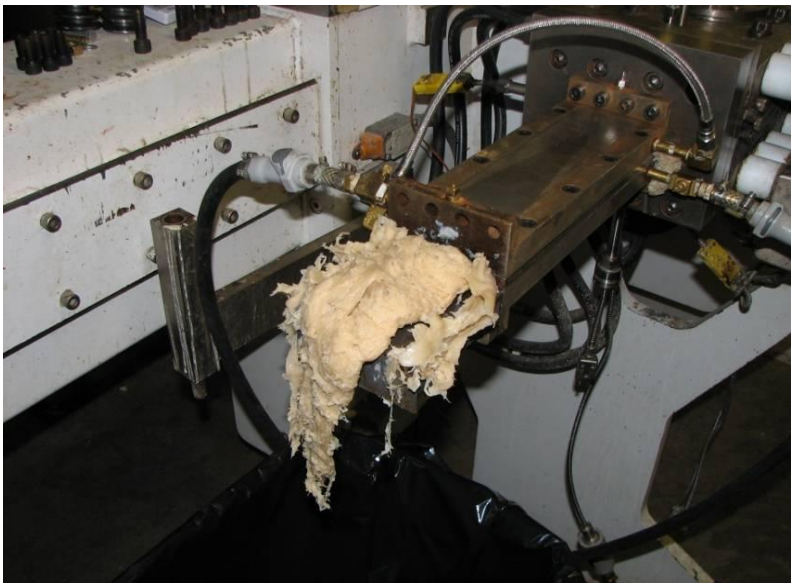

Figure 4.16 Extruded soy meat analog as it flows from the extruder barrel.

4.6.5 Effect of extruder parameters

The shearing action of the screws as they turn, mixing the plasticized mass (soy meat analog mixture), coupled with the high pressure conditions in the extruder and high temperature also worked synergistically to inactivate the spores. It was thought that the mechanical stress of the shearing action on the spores played a role in inactivating the spores. The feed had 60% moisture content; therefore, it remained quite viscous even with the higher temperature conditions that could cause increased gelatinization of the protein, thereby, reducing viscosity. Thus, it was held that the shearing action of the screws in mixing and, thereby, agitating the spores was involved in inactivating the spores. This was believed as spores in aqueous solutions have been inactivated by rapid agitation with glass beads or abrasive particles (Roberts and Hitchins 1969). However, the destruction of spores by shear action during extrusion is negligible owing to their reduced size (Likimani and others 1990b).

Accordingly, perhaps it was the increased pressure coupled with high temperature under high moisture conditions that were responsible for sterilizing the product. A combination of hydrostatic pressure and heat reduces the heat needed to kill spores (Russell 1982). The ultra-high temperature effectively destroys the spores that have been sensitized by the high pressure conditions in the extruder. Still, the pressure conditions in the extruder are extremely low, compared to the pressure conditions used in inactivation studies with high hydrostatic pressure. In this experiment, the pressure treatment is not the primary lethal treatment (the ultra high temperature is the primary lethal treatment), but it still served the purpose of sensitizing the spores. The moist conditions in the mix also increase the spore's sensitivity to heat as the spore core is hydrated.

A study in the destruction of *Bacillus globigii* spores in a corn/soy mixture (Likimani and others 1990a) revealed that increasing the moisture content, while keeping the feed mass and treatment temperature constant reduced the destruction rate of the spores. The increased moisture content resulted in a lowered mass temperature, hence, the decreased spore destruction. Furthermore, the heat generated due to enhanced viscosity of the drier feed also enhanced the destruction of the bacterial spores.

In our study, a high protein mixture was used to suspend the spores, whereas in the study above a high starch mixture (70:30%, corn:soy gruel w/w) was used to suspend the spores. A single screw extruder was used in the study above. Temperatures ranging from 110-130°C and a screw speed ranging from 100-140 rpm were used. The feed was contaminated with a pellet (18-22% moisture content), also different from the process used to inoculate the ingredients in our study. Thus, owing to the significant differences in treatment variables in that study and our study, it is difficult to compare the mechanism

of inactivation and the role of the treatment parameters in the inactivation process for each study.

Bouveresse and others (1982) determined that the number of spores of the smooth variant of *B. stearothermophilus* NCA-FS 1518 spores extruded in a starch-protein-sucrose mix with 14% moisture content at 182°C, (under 11.8-13 MPa pressure, screw speed-78 rpm), decreased by more than 7-11 log cycles. The method used to contaminate the mix (a liquid inoculum inserted at the extruder opening), the model in which the spores were suspended in as well as other experiment variables differed from those used in our study. Less moisture was used in this study, thereby enhancing spore resistance, as spores are more resistant to dry heat than to moist heat by 30°C (Setlow 2006). A lower screw speed was also used. However, this treatment was still effective in significantly reducing the spore load.

Likimani and others (1990b) have concluded that temperature, screw speed, the type of extruder and the microbial species and strain are the determining factors in the inactivation of microorganisms by extrusion processing.

4.7 Identification of wild-type isolates 4II1, 4II 11and 3III 1C

At the end of the study, the identity of the sporulating microorganisms whose spores were used in this study were determined. The identities of the microorganisms are listed in Table 4.9. Although, 4II 11 was not included in the extrusion study, it had been pooled to as an alternative in the extrusion study, thus it was desired to determine its identity.

Table 4. 8 Identification of the wild-type *Bacillus* isolates pooled to be used in the extrusion study

Bacteria reference/ Colony morphology	Gram stain	Catalase/ Oxidase	Identity
4II 1 Creamish-pink colored, irregular, spreading colonies that have vein like network of ridges that is sparse, look dry, raised and rough. They are interspersed with hill-like mounds (umbonate) that appear to be fluid filled and are translucent and colorless. The colony spreads over the entire surface. Grows on agar surface.	Thin, short G+ rods, occur singly. Thin, short, rectangular spores were observed to occur terminally and centrally. The overall sporulated rod shape was cylindrical or oval at one end and tapering on the other (looked like a club)	+ / +	*Bacillus licheniformis*
4II 11 Light pink colored, irregular, spreading colony. The colony has globules/ hill-like mounds (umbonate) interspersed with vein like network of ridges that are sparse, raised rough and look dry. The raised globules/ hill-like mounds are translucent and colorless, and appear to be filled with fluid that is mucoid. The colony spreads over the entire plate.	Thin, short G+ rods. The rods have terminal spores that occupy half the rod, hence, they appear to be central (para-central), but are placed on one half of the rod that tapers on the other end.	+ / +	**Significant taxa** *Geobacillus thermoglucosidasius* (91.3%) *B. subtilis* / *Bacillus amyloliquefaciens* (7.2%)
3III 1C Medium to large, tan colored, translucent, circular colonies.	Slim, G+ rods that occur singly and in single chains of about 2, 4 and 6 rods. Central oval spores were observed	– / +	**Low discernation** *Bacillus non reactive* (79.1%) *Brevibacillus non reactive* (20.3%)

B. licheniformis rods are often in chains. They also form opaque colonies with a dull and rough surface that have hair-like outgrowths. They are usually strongly attached to the agar, which was also observed in 4II 1colonies on TSA and TSAY agar. The colonies have mounds and lobes consisting of slime. A red pigment (pulcherrimin) is formed by many strains on carbohydrate media containing sufficient iron. Freshly isolated strains grow with ammonia as the sole source of nitrogen (Sneath 1984). *B. licheniformis* has been implicated in food poisoning although it is not known to be pathogenic. *B. licheniformis* also produces a lantibiotic that is active against a wide range of gram-positive bacteria, (Dischinger J and others 2008).

Bacillus thermoglucosidasius (Geobacillus thermoglucosidasius) is strictly aerobic. It is an obligate thermophile with an optimum growth temperature at 61-63°C. It produces heat-stable glucosidase. It has not been implicated in any food-borne illness (Sneath 1984).

B. subtilis rods rarely occur in chains. *B. subtilis* colonies are cream colored or brown, round or irregular, with a dull surface, they also become opaque with time. They are wrinkled, and spread on agar with a moist surface. A disk of reddish pigment (pulcherrimin or melanin) may also form below the growth (as observed with 4II 11 colonies, where it was thought the colony was cream colored, with a red pigment forming beneath the growth, hence the observed light pink colored colonies). They are aerobic, with restricted anaerobic growth in complex media containing glucose and nitrate. *B. amyloliquefaciens* is very similar to *B. subtilis*. It was separated from *B. subtilis* as it has a slightly higher molecular % G + C (43.5-44.9) than *B. subtilis* (42-43). Strains of these

two species also differ in other properties. *B. subtilis* has also been implicated in food poisoning although it is not considered to be pathogenic (Sneath 1984).

With respect to *Bacillus* non-reactive, this wild-type microorganism was determined to belong to the *Bacillus* species using the API profile index, but the API profile system could not be used to determine its particular species. Henceforth, *Bacillus non-reactive* is referred to simply as *Bacillus sp.*

CHAPTER 5

CONCLUSIONS AND FUTURE RESEARCH

In this study, wild-type bacilli were isolated from ingredients to be used in processing a soy meat analog product. The isolated bacilli were tested with several sporulation media to determine the media that would ensure the production of the most heat resistant spores. Sporulation Agar I was determined to be the media that resulted in the most heat resistant spores. Next, two wild-type bacilli, a heat labile and a heat tolerant strain, respectively, to be used in conducting thermoresistivity tests were selected. D-values of *Bacillus cereus* and *Bacillus stearothermophilus* spores in peptone water were demonstrated to be generally higher than those reviewed in most literature. However, it was concluded that 1 g/L solution peptone water was to minimal to confer increased heat resistance of the spores. Thus, it was thought the apparent increased heat resistance, especially that of the *B. stearothermophilus* and 3III 1C spores, was due to the mode of heat treatment used in inactivating the spores. In the extrusion study, several media were used to recover the extruded spores. None of the media used were observed to enhance the recovery of the extruded spores. Additionally, the food matrix did not affect the resistivity of the spores to the treatment. *B. stearothermophilus* spores proved to be the most resistant to extrusion cooking. However, extrusion at 180°C, 1 MPa pressure and 125 rpm, significantly reduced the numbers of *B. stearothermophilus*, 3III 1C and 4II 1 spores.

The D-values of *B. cereus*, *B. stearothermophilus* and 3III 1C spores revealed that *B. cereus* was inactivated at a lower temperature than the isolated wild-type 3III 1C

spores. There was no significant difference ($P \leq 0.05$) in the D-values between *B. stearothermophilus* and 3III 1C. Consequently, *B. stearothermophilus* and 3III 1C can be used as indicators in thermal food processing applications to study the heat inactivation parameters of pathogenic bacilli, specifically *Bacillus cereus.* *B. cereus* is of great concern in food processing applications due to its heat resistance and pathogenicity. This pathogenic *Bacillus* is also found consistently in cereal ingredients and their end products and, thus, poses a risk as they could germinate under favorable growth conditions.

The exact mechanism by which spores are inactivated by extrusion cooking is not clearly understood, although the factors influencing inactivation of spores in extrusion processing have been outlined. In addition, a procedure to examine bacterial spore destruction in extrusion processing is lacking. Likimani (1988) proposed a method to examine bacterial spore destruction in extrusion processing. He determined the 'D' and 'Z' values for the destruction of *Bacillus globigii* spores in a corn/soybean mixture by extrusion cooking. Furthermore, the use of extrusion processing in developing new products continues to advance, thereby, compounding the importance to develop a method of analysis in the inactivation of spores as well as other microorganisms by extrusion cooking. Additionally, variable machine geometry exists with respect to screw configuration among others, in single screw vs. twin screw extruders, in addition to the uses of different extrusion parameters including screw speed and temperature. Therefore, a precise methodology of examining the potency of extrusion cooking would enhance the adaptability in assessing and using extrusion processing in microbial inactivation studies.

In light of this, having determined the identity of 3III 1C, 3III 1C spores can be cultured and contaminated into products whose raw ingredients are known to harbor

Bacillus, *Clostridium* and their respective spores. Examples of such products include pasta, RTE cereals, crispbreads, dry pet foods and many other cereal products that are extruded. A combination of various treatments, with respect to moisture content, screw speed, temperature and pressure, can be used in the inactivation of the inoculated spores by extrusion processing. The results can then be analyzed to determine the most optimal extrusion parameters for inactivation. In addition, extrusion 'D' and 'Z' values can be established. This would allow ease of scaling up production of the afore-mentioned products, in addition to modeling the extrusion parameters even with varying screw configuration, and perhaps even in single vs. twin screw extruders.

APPENDIX A

Analysis of Variance (ANOVA) Program for SAS

A. 1 Percentage surviving spores

```
options ls=100 ps=70;
data one; *infile cards dsd missover;
input Bac$ Trt$ Rep1 Rep2;
cards;

…

...

...

proc print;
data one; set one;
cnt=rep1; rep=1; output;
cnt=rep2; rep=2; output;
proc glm; classes rep bac trt;
model cnt=rep bac|trt;
means bac trt/lsd lines;
means bac|trt;
lsmeans bac|trt/s p;


run;

run;
```

A. 2 *B. cereus, B. stearothermophilus,* 4II 1 and 3III 1C D-values

```
data two; infile cards dsd missover;

input Bac$ Trt$ Rep1 Rep2;
cards;
B. cereus, 1(100°C),7.75 ,7.37 ,,,,,
4II 1, 1(100°C),7.19  ,10.12 ,,,,,
B. stearothermophilus, 2 (150°C),3.7 ,2.4,,,,,
3III 1C, 2 (150°C),2.31,2.69 ,,,,,
proc print;
data three; set three;
cnt=rep1; rep=1; output;
cnt=rep2; rep=2; output;
proc glm;  classes rep bac;
model cnt=rep bac;
means bac;
```

```
lsmeans bac/s p;


run;
```

A. 3 Extrusion of inoculated soy meat analog

```
data three; infile cards dsd missover;

length bact$ 20 samp$ 20 trt$ 40;
input Bact$ Trt1$ Samp$ Rep1a Rep2a Rep1b Rep2b Rep1c Rep2c Rep1bact Rep2bact
Avg;
trt2=1;rep=1;cnt=rep1a;lcnt=log10(cnt+1);
! trt=compress(bact||'_'||substr(trt1,1,1)||'_'||samp||'_'||trt2);output;
! trt=compress(bact||'_'||substr(trt1,1,1)||'_'||samp||'_'||trt2);output;
trt2=2;rep=1;cnt=rep1b;lcnt=log10(cnt+1);
! trt=compress(bact||'_'||substr(trt1,1,1)||'_'||samp||'_'||trt2);output;
trt2=2;rep=2;cnt=rep2b;lcnt=log10(cnt+1);
! trt=compress(bact||'_'||substr(trt1,1,1)||'_'||samp||'_'||trt2);output;
trt2=3;rep=1;cnt=rep1c;lcnt=log10(cnt+1);
! trt=compress(bact||'_'||substr(trt1,1,1)||'_'||samp||'_'||trt2);output;
trt2=3;rep=2;cnt=rep2c;lcnt=log10(cnt+1);
! trt=compress(bact||'_'||substr(trt1,1,1)||'_'||samp||'_'||trt2);output;
trt2=4;rep=1;cnt=rep1bact;lcnt=log10(cnt+1);
! trt=compress(bact||'_'||substr(trt1,1,1)||'_'||samp||'_'||trt2);output;
trt2=4;rep=2;cnt=rep2bact;lcnt=log10(cnt+1);
! trt=compress(bact||'_'||substr(trt1,1,1)||'_'||samp||'_'||trt2);output;

drop rep1a--rep2bact;

cards;

proc print;


proc glm; classes trt;
model cnt lcnt=trt;
means trt/lsd lines;
lsmeans trt/s p;


run;
```

A. 4 Extruder parameters

```
data four;
title1 'extruder parameters';
options ps=52;
infile 'f:\extruderdata\parameters.prn' missover;
input day  brl9 brl8 brl7 brl6 brl5 die_t p9 p8 p7 p6 p5 rpm torque psi ;
proc sort; by day;
proc means; by day;


run;
```

APPENDIX B

GLM Analysis of Variance and Fishers Least Significant Difference Output

B. 1 Percentage Surviving Spores

The GLM Procedure

Class Level Information

Class	Levels	Values
Rep	2	1 2
Bac	9	3II8 3III1C 4II1 4II11 4II17 4II2 4II3a 4II3c B.st
Trt	7	I II III IV V VI VII
Number of observations Read 126		
Number of observations Used 126		

Dependent Variable: cnt

Source	DF	Sum of squares	Mean square	F Value	Pr >F
Model	63	102752.5036	160.9921	92.71	<0.0001
Error	62	1090.6816	17.5916		
Corrected Total	125	103843.1852			

R-square	Coeff Var	Root MSE	Cnt Mean
0.989497	24.36149	4.194239	17.21668

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Rep	1	21.58969	21.58969	1.23	0.2722
Bac	8	8961.76411	1120.22051	63.68	<0.0001
Trt	6	65875.54481	10979.25747	624.12	<0.0001
Bac*Trt	48	27893.60499	581.11677	33.03	<0.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Rep	1	21.58969	21.58969	1.23	0.2722
Bac	8	8961.76411	1120.22051	63.68	<0.0001
Trt	6	65875.54481	10979.25747	624.12	<0.0001
Bac*Trt	48	27893.60499	581.11677	33.03	<0.0001

t Tests (Least Significance Difference) for cnt

Alpha	0.05
Error Degrees of Freedom	62
Error Mean Square	17.59164
Critical Value of t	1.99897
Least Significant Difference	3.1689

Means with the same letter are not significantly different

t Grouping	Mean	N	Bac
A	30.111	14	4II3a
A	27.681	14	4II2
B	19.208	14	4II11
B	19.038	14	B.st
B	18.871	14	3III1C
B	17.644	14	4II3c
C	12.008	14	4II17
C	9.551	14	3II8
D	0.838	14	4II1

t Tests (Least Significance Difference) for cnt

Alpha	0.05
Error Degrees of Freedom	62
Error Mean Square	17.59164
Critical Value of t	1.99897
Least Significant Difference	3.1689

Means with the same letter are not significantly different

t Grouping	Mean	N	Trt
A	70.853	18	VI
B	18.143	18	V
B	16.260	18	III
C	10.472	18	VII
D	2.800	18	IV
D	1.836	18	II
D	0.153	18	I

B. 2 *B. cereus*, *B. stearothermophilus*, 4II 1 and 3III 1C D-Values

The GLM procedure

Class Level Information

Class	Levels	Values
rep	2	1 2
Bac	4	3III 1C 4II 1 B. cereu B. stear
Number of Observations Read 8		
Number of Observations Used 8		

Dependent Variable: cnt

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	58.70475000	14.67618750	8.90	0.0157
Error	3	4.94973750	1.64991250		
Corrected Total	7	63.65448750			

R-Square	Coeff Var	Root MSE	cnt Mean
0.922241	23.60651	1.284489	5.441250

Source	DF	Type I SS	Mean Square	F Value	Pr > F
rep	1	0.33211250	0.33211250	0.20	0.6841
Bac	3	58.37263750	19.45754583	11.79	0.0362

Source	DF	Type III SS	Mean Square	F Value	Pr > F
rep	1	0.33211250	0.33211250	0.20	0.6841
Bac	3	58.37263750	19.45754583	11.79	0.0362

B. Extrusion of Inoculated Soy Meat Analog

The GLM Procedure

Class Level Information

Class	Levels	Values
trt	60	3III1C_1_After_1 3III1C_1_After_2 3III1C_1_After_3 3III1C_1_After_4 3III1C_1_Before_1 3III1C_1_Before_2 3III1C_1_Before_3 3III1C_1_Before_4 3III1C_2_A-1(0sec)_1 3III1C_2_A-1(0sec)_2 3III1C_2_A-1(0sec)_3 3III1C_2_A-1(0sec)_4 3III1C_2_A-2(10sec)_1 3III1C_2_A-2(10sec)_2 3III1C_2_A-2(10sec)_3 3III1C_2_A-2(10sec)_4 3III1C_2_Before_1 3III1C_2_Before_2 3III1C_2_Before_3 3III1C_2_Before_4 4II1_1_After_1 4II1_1_After_2 4II1_1_After_3 4II1_1_After_4 4II1_1_Before_1 4II1_1_Before_2 4II1_1_Before_3 4II1_1_Before_4 4II1_2_A-1(0sec)_1 4II1_2_A-1(0sec)_2 4II1_2_A-1(0sec)_3 4II1_2_A-1(0sec)_4 4II1_2_A-2(10sec)_1 4II1_2_A-2(10sec)_2 4II1_2_A-2(10sec)_3 4II1_2_A-2(10sec)_4 4II1_2_Before_1 4II1_2_Before_2 4II1_2_Before_3 4II1_2_Before_4 B.stearothermophilu_1_After_1 B.stearothermophilu_1_After_2 B.stearothermophilu_1_After_3 B.stearothermophilu_1_After_4 B.stearothermophilu_1_Before_1 B.stearothermophilu_1_Before_2 B.stearothermophilu_1_Before_3 B.stearothermophilu_1_Before_4 B.stearothermophilu_2_A-1(30/0sec)_1 B.stearothermophilu_2_A-1(30/0sec)_2 B.stearothermophilu_2_A-1(30/0sec)_3 B.stearothermophilu_2_A-1(30/0sec)_4 B.stearothermophilu_2_A-2(40/10sec)_1 B.stearothermophilu_2_A-2(40/10sec)_2 B.stearothermophilu_2_A-2(40/10sec)_3 B.stearothermophilu_2_A-2(40/10sec)_4 B.stearothermophilu_2_Before_1 B.stearothermophilu_2_Before_2 B.stearothermophilu_2_Before_3 B.stearothermophilu_2_Before_4
Number of Observations Read 120		
Number of Observations Used 120		

Dependent Variable: cnt

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	59	3.2420981E15	5.4950815E13	1.51	0.0573
Error	60	2.184088E15	3.6401466e13		
Corrected Total	119	5.426186E15			

R-Square	Coeff Var	Root MSE	cnt Mean
0.597491	360.7174	6033363	1672601

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	59	3.2420981E15	5.4950815E13	1.51	0.0573

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	59	3.2420981E15	5.4950815E13	1.51	0.0573

Dependent Variable: lcnt

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	59	641.8933600	10.8795485	21.93	< .0001
Error	60	29.7668936	0.4961149		
Corrected Total	119	671.6602536			

R-Square	Coeff Var	Root MSE	cnt Mean
0.955682	35.32290	0.704354	1.994044

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	59	641.8933600	10.8795485	21.23	< .0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	59	641.8933600	10.8795485	21.23	< .0001

t Tests (Least Significance Difference) for cnt

Alpha	0.05
Error Degrees of Freedom	60
Error Mean Square	3.6E13
Critical Value of t	2.00030
Least Significant Difference	1.21E7

Means with the same letter are not significantly different

t Grouping	Mean	N	trt
A	24150000	2	4II 1_2_Before_1
A	22100000	2	4II 1_2_Before_2
A	20450000	2	4II 1_2_Before_3
B A	14550000	2	4II 1_2_Before_4
B C	5350000	2	3III 1C_2_Before_3
B C	4650000	2	3III 1C_2_Before_4
B C	4500000	2	3III 1C_2_Before_1
B C	4100000	2	3III 1C_2_Before_2
C	131000	2	B. stearothermophilu_2_Before_2
C	126000	2	B. stearothermophilu_2_Before_4
C	121500	2	B. stearothermophilu_2_Before_1
C	120000	2	B. stearothermophilu_2_Before_3
C	1795	2	B. stearothermophilu_2_A-2 (40/10sec)_2
C	935	2	B. stearothermophilu_2_A-1(30/0sec)_2
C	930	2	B. stearothermophilu_2_ A-2 (40/10sec)_4
C	865	2	B. stearothermophilu_2_ A-2 (40/10sec)_1
C	705	2	B. stearothermophilu_2_ A-2 (30/0sec)_1
C	570	2	B. stearothermophilu_2_ A-2 (30/0sec)_4
C	145	2	3III 1C_1_Before_2
C	145	2	4II 1_1_Before_2
C	140	2	3III 1C_1_Before_1
C	135	2	B. stearothermophilu_2_ A-2 (40/10sec)_3
C	130	2	B. stearothermophilu_1_Before_3
C	125	2	3III 1C_1_Before_4
C	120	2	B. stearothermophilu_1_Before_1
C	120	2	B. stearothermophilu_1_Before_4
C	110	2	B. stearothermophilu_1_Before_2
C	90	2	3III 1C_1_Before_3
C	82	2	4II 1_1_Before_4
C	80	2	B. stearothermophilu_2_A-1(30/0sec)_3
C	70	2	B. stearothermophilu_2_After_2
C	65	2	B. stearothermophilu_2_After_1
C	65	2	4II 1_1_Before_1
C	60	2	B. stearothermophilu_2_After_4
C	45	2	B. stearothermophilu_2_After_3
C	35	2	4II 1_1_Before_3
C	5	2	4II 1_1_After_1
C	2	2	4II 1_1_After_4
C	0	2	4II 1_2_A-1_(0 sec)_3
C	0	2	4II 1_2_A-1_(0 sec)_2
C	0	2	3III 1C_1_After_1
C	0	2	3III 1C_1_After_2
C	0	2	3III 1C_1_After_3
C	0	2	3III 1C_1_After_4
C	0	2	4II 1_2_A-1_(0 sec)_1
C	0	2	3III 1C_2_A-2_(0 sec)_2
C	0	2	3III 1C_2_A-2_(0 sec)_3

C	0	2	4II 1_2_A-1_(0 sec)_4
C	0	2	4II 1_2_A-2_(10 sec)_1
C	0	2	4II 1_2_A-2_(10 sec)_2
C	0	2	4II 1_2_A-2_(10 sec)_3
C	0	2	4II 1_2_A-2_(10 sec)_4
C	0	2	3III 1C_2_A-2_(10 sec)_3
C	0	2	4II_1_After_2
C	0	2	4II_1_After_3
C	0	2	3III 1C_2_A-2_(10 sec)_4
C	0	2	3III 1C_2_A-1_(0 sec)_1
C	0	2	3III 1C_2_A-1_(0 sec)_2
C	0	2	3III 1C_2_A-1_(0 sec)_3
C	0	2	3III 1C_2_A-1_(0 sec)_4

t Tests (Least Significance Difference) for lcnt

Alpha	0.05
Error Degrees of Freedom	60
Error Mean Square	0.496115
Critical Value of t	2.00030
Least Significant Difference	1.4089

Means with the same letter are not significantly different

t Grouping	Mean	N	trt
A	7.2113	2	4II 1_2_Before_1
A	7.1535	2	4II 1_2_Before_2
A	7.0796	2	4II 1_2_Before_4
A	6.7038	2	3III 1C_2_Before_3
A	6.6674	2	3III 1C_2_Before_4
A	6.6443	2	3III 1C_2_Before_1
A	6.5966	2	3III 1C_2_Before_2
B A	6.2312	2	4II 1_2_Before_3
B	5.0191	2	B. stearothermophilu_2_Before_2
B	5.0167	2	B. stearothermophilu_2_Before_3
B	5.0085	2	B. stearothermophilu_2_Before_4
B	4.9673	2	B. stearothermophilu_2_Before_1
C	2.7516	2	B. stearothermophilu_2_A-2 (40/10sec)_2
D C	2.5534	2	B. stearothermophilu_2_A-1(30/0sec)_2
D C	2.5204	2	B. stearothermophilu_2_ A-2 (40/10sec)_4
D C E	2.3610	2	B. stearothermophilu_2_ A-2 (40/10sec)_1
D C E	2.3273	2	B. stearothermophilu_2_ A-1 (30/0sec)_4
D F C	2.1641	2	4II 1_1_Before_2
D F C	2.1620	2	3III 1C_1_Before_2

D F C	2.1448	2	3III 1C_1_Before_1
D F C	2.1160	2	B. stearothermophilu_1_Before_3
D F C	2.1000	2	3III 1C_1_Before_4
D F C	2.0939	2	B. stearothermophilu_2_A-1(30/0sec)_1
D F C	2.0801	2	B. stearothermophilu_1_Before_4
D F C	2.0768	2	B. stearothermophilu_1_Before_1
D F C	2.0436	2	B. stearothermophilu_1_Before_2
G D F C E	1.9101	2	4II 1_1_Before_4
G D F C E	1.8810	2	3III 1C_1_Before_3
G D F C E	1.8183	2	4II 1_1_Before_2
G D F E	1.2165	2	B. stearothermophilu_2_ A-2 (40/10sec)_3
G D F H E	1.1034	2	B. stearothermophilu_2_ A-21 (30/0sec)_3
G D F H E	1.1076	2	B. stearothermophilu_1_After_2
G D F H E	1.0586	2	B. stearothermophilu_1_After_1
G D F H E	1.0414	2	B. stearothermophilu_1_After_4
G D F H E	0.9795	2	B. stearothermophilu_1_After_3
G F H	0.9256	2	4II 1_1_Before_3
G H	0.5207	2	4II 1_1_After_1
H	0.3184	2	4II 1_1_After_4
H	0.0000	2	4II 1_2_A-1_(0 sec)_3
H	0.0000	2	4II 1_2_A-1_(0 sec)_2
H	0.0000	2	3III 1C_1_After_1
H	0.0000	2	3III 1C_1_After_2
H	0.0000	2	3III 1C_1_After_3
H	0.0000	2	3III 1C_1_After_4
H	0.0000	2	4II 1_2_A-1_(0 sec)_1
H	0.0000	2	3III 1C_2_A-2_(10 sec)_2
H	0.0000	2	3III 1C_2_A-2_(10 sec)_3
H	0.0000	2	4II 1_2_A-1_(0 sec)_4
H	0.0000	2	4II 1_2_A-2_(10 sec)_1
H	0.0000	2	4II 1_2_A-2_(10 sec)_2
H	0.0000	2	4II 1_2_A-2_(10 sec)_3
H	0.0000	2	4II 1_2_A-2_(10 sec)_4
H	0.0000	2	3III 1C_2_A-2_(10 sec)_1
H	0.0000	2	4II 1_1_After_2
H	0.0000	2	4II 1_1_After_3
H	0.0000	2	3III 1C_2_A-2_(10 sec)_4
H	0.0000	2	3III 1C_2_A-1_(0 sec)_1
H	0.0000	2	3III 1C_2_A-1_(0 sec)_2
H	0.0000	2	3III 1C_2_A-1_(0 sec)_3
H	0.0000	2	3III 1C_2_A-1_(10 sec)_4

APPENDIX C

TABLES IN RESULTS AND DISCUSSION

C. 1 Determination of percentage surviving spores.

C.1. 1 Nutrient Agar enriched with Manganese Sulfate and Calcium Chloride

Microorganism	Incubation temp °C	Spore count before heat treatment (trt) (CFU/mL)	[A]Spore count after heat treatment (CFU/mL)	[B]Spore count after heat treatment (CFU/mL)	Sporulation %
3II8	55	1.8×10^9	1.4×10^6	<25 (0)	.079
3IIb 1C	55	6.3×10^9	2.2×10^6	<25 (0)	0.035
4II 1	45	3.5×10^9	6.5×10^6	<25 (0)	0.19
4II 11	55	1.2×10^{10}	6.9×10^6	<25 (0)	0.0575
4IIb 2	55	2.2×10^9	4.4×10^6	<25 (0)	0.20
4IIb 3a	55	2.1×10^8	1.0×10^6	<25 (0)	0.48
4IIb 3c	55	2.8×10^9	2.1×10^6	<25 (0)	0.075
4IIb 17	55	6.5×10^9	9.0×10^6	<25 (0)	0.14
B. stearothermophilus	55	2.6×10^9	9.5×10^5	$3.2x10^4$	0.037

[A]100°C heat treatment for 15 min [B]121°C (autoclave) heat treatment for 2 min

C.1. 2 Fortified Nutrient Agar.

Microorganism	Incubation temp °C	Spore count before heat trt (CFU/mL)	[A]Spore count after heat trt (CFU/mL)	[B]Spore count after heat trt (CFU/mL)	Sporulation %
3II8	55	8.7×10^9	1.8×10^7	<25 (0)	0.21
3IIb 1C	55	2.5×10^9	9.5×10^7	<25 (0)	3.8
4II 1	45	9.6×10^9	1.9×10^7	<25(0)	0.20
4II 11	55	1.6×10^9	1.2×10^8	24	7.5
4IIb 2	55	1.5×10^9	8.3×10^6	<25(0)	0.55
4IIb 3a	55	5.2×10^9	2.4×10^7	<25 (0)	0.46
4IIb 3c	55	1.5×10^{10}	2.2×10^8	<25(0)	1.5
4IIb 17	55	1.0×10^{10}	2.1×10^8	<25 (0)	2.1
B. stearothermophilus	55	6.1×10^8	1.6×10^5	<25 (12)	0.03

[A]100°C heat treatment for 15 min [B]121°C (autoclave) heat treatment for 2 min

C.1. 3 Angelotti Agar.

Microorganism	Incubation temp °C	Spore count before heat trt (CFU/mL)	[A]Spore count after heat trt (CFU/mL)	[B]Spore count after heat trt (CFU/mL)	Sporulation %
3II8	55	1.5×10^9	4.9×10^8	<25(2)	32.67
3IIb 1C	55	7.4×10^9	9.4×10^8	$1.2x10^2$	12.70
4II 1	45	2.2×10^{11}	1.0×10^7	<25 (7)	0.0045
4II 11	55	1.0×10^{10}	1.0×10^9	30	10
4IIb 2	55	5.8×10^9	1.4×10^8	<25 (5)	2.41
4IIb 3a	55	1.9×10^9	1.1×10^8	<25 (2)	5.79
4IIb 3c	55	1.6×10^9	5.4×10^8	< 25(4)	33.75
4IIb 17	55	1.8×10^{10}	1.5×10^9	<25 (6)	8.33
B. stearothermophilus	55	1.3×10^{10}	5.4×10^9	0	41.54

[A]100°C heat treatment for 15 min [B]121°C (autoclave) heat treatment for 2 min

C.1. 4 Milk Agar.

Microorganism	Incubation temp °C	Spore count before heat trt (CFU/mL)	[A]Spore count after heat trt (CFU/mL)	[B]Spore count after heat trt (CFU/mL)	Sporulation %
3II8	55	4.8×10^8	2.4×10^6	<25 (0)	0.5
3IIb 1C	55	1.1×10^9	6.8×10^6	<25 (0)	0.62
4II 1	45	2.8×10^9	2.4×10^7	<25 (0)	0.86
4II 11	55	1.1×10^9	6.8×10^7	<25 (0)	6.18
4IIb 2	55	2.1×10^9	2.1×10^7	<25 (0)	1
4IIb 3a	55	5.4×10^8	7.2×10^7	<25 (0)	13.33
4IIb 3c	55	1.6×10^9	3.4×10^7	<25 (0)	2.13
4IIb 17	55	6.7×10^9	5.6×10^6	<25 (0)	0.08
B. stearothermophilus	55	3.0×10^9	9.4×10^6	<25 (0)	0.31

[A]100°C heat treatment for 15 min [B]121°C (autoclave) heat treatment for 2 min

C.1. 5 Nutrient Agar with 1ppm $MnSO_4.H_20$ and 0.5% yeast extract.

Microorganism	Incubation temp °C	Spore count before heat trt (CFU/mL)	[A]Spore count after heat trt (CFU/mL)	[B]Spore count after heat trt (CFU/mL)	Sporulation %
3II8	55	2.3×10^9	2.1×10^8	24	9.13
3IIb 1C	55	9.0×10^9	1.9×10^9	<25 (0)	21.11
4II 1	45	2.1×10^{10}	1.8×10^7	<25 (0)	0.09
4II 11	55	1.3×10^{10}	1.5×10^8	<25 (3)	1.15
4IIb 2	55	6.4×10^8	3.3×10^8	$9.4x10^1$	51.56
4IIb 3a	55	7.5×10^8	4.6×10^8	<25 (0)	61.33
4IIb 3c	55	1.3×10^{10}	1.5×10^7	10	0.12
4IIb 17	55	3.6×10^9	6.1×10^8	<25 (0)	16.94
B. stearothermophilus	55	1.2×10^{10}	7.0×10^6	<25 (1)	0.06

[A]100°C heat treatment for 15 min [B]121°C (autoclave) heat treatment for 2 min

C.1. 6 Sporulation Agar I.

Microorganism	Incubation temp °C	Spore count before heat trt (CFU/mL)	[A]Spore count after heat trt (CFU/mL)	[B]Spore count after heat trt (CFU/mL)	Sporulation %
3II8	55	1.8×10^{10}	3.8×10^{9}	<25 (0)	21.11
3IIb 1C	55	6.6×10^{9}	6.0×10^{9}	<25 (0)	90.91
4II 1	45	6.0×10^{10}	2.6×10^{9}	<25 (0)	4
4II 11	55	7.8×10^{9}	1.0×10^{10}	$1x10^{2}$	100
4IIb 2	55	2.0×10^{9}	1.3×10^{10}	$1.1x10^{4}$	100
4IIb 3a	55	1.9×10^{9}	7.0×10^{9}	<25 (0)	100
4IIb 3c	55	7.4×10^{9}	7.2×10^{9}	<25 (0)	97.3
4IIb 17	55	1.1×10^{10}	5.9×10^{9}	<25 (0)	53.64
B. stearothermophilus	55	7.6×10^{8}	6.2×10^{8}	$5.4x10^{5}$	81.58

[A]100°C heat treatment for 15 min [B]121°C (autoclave) heat treatment for 2 min

C.1. 7 Campden Sporulation Agar.

Microorganism	Incubation temp °C	Spore count before heat trt (CFU/mL)	[A]Spore count after heat trt (CFU/mL)	[B]Spore count after heat trt (CFU/mL)	Sporulation %
3II8	55	6.4×10^{9}	1.0×10^{8}	<25 (0)	1.56
3IIb 1C	55	9.7×10^{9}	1.4×10^{8}	<25 (1)	1.44
4II 1	45	1.6×10^{10}	4.9×10^{7}	<25 (8)	0.31
4II 11	55	2.6×10^{9}	2.4×10^{8}	<25 (8)	9.23
4IIb 2	55	2.1×10^{8}	8.0×10^{7}	<25 (0)	37.1
4IIb 3a	55	5.4×10^{8}	1.5×10^{8}	<25 (0)	27.78
4IIb 3c	55	8.0×10^{9}	1.0×10^{8}	<25(2)	1.63
4IIb 17	55	9.4×10^{9}	2.6×10^{8}	<25(0)	2.77
B. stearothermophilus	55	2.0×10^{9}	2.0×10^{8}	$1.6x10^{6}$	10

[A]100°C heat treatment for 15 min [B]121°C (autoclave) heat treatment for 2 min

C.1. 8 Inherent strain effect and media effect on percentage surviving spores.

	Percentage surviving spores									
	Isolated wild type microorganisms									
Sporulation agar media	**3II 8**[c]	**3III 1C**[b]	**4II 1**[d]	**4II 11**[b]	**4II 2**[a]	**4II 3a**[a]	**4II 3c**[b]	**4II 17**[c]	[E]**B. st**[b]	**Average %**
[z]NA[A]	0.079	0.035	0.19	0.058	0.20	0.48	0.075	0.14	0.037	0.153
[z]FNA[B]	0.21	3.80	0.20	7.50	0.55	0.46	1.50	2.10	0.03	1.836
[x]Angelotti	32.67	12.70	0.005	10.00	2.41	5.79	33.75	8.33	41.54	16.260
[z]Milk agar	0.50	0.62	0.86	6.18	1.00	13.33	2.13	0.08	0.31	2.800
[x]NAY[C]	9.13	21.11	0.09	1.15	51.56	61.33	0.12	16.94	0.06	18.143
[w]SPA I[D]	21.11	90.91	4.00	100	100	100	97.3	53.64	81.58	70.853
[y]Campden	1.56	1.44	0.31	9.23	37.1	27.78	1.63	2.77	10.00	10.472
Average %	9.55	19.04	0.84	19.21	27.68	30.11	17.64	12.01	19.04	

[A] Nutrient agar enriched with manganese sulfate and calcium chloride
[B] Fortified nutrient agar
[C]Nutrient agar with 1ppm $MnSO_4.H_2O$ and 0.5% yeast extract
[D]Sporulation agar I
[E]*B. stearothermophilus*

[abcd]Same letter within the same row indicates no significant difference at $P \leq 0.05$
[wxyz]Same letter within the same column indicates no significant difference at $P \leq 0.05$

C. 2 Number of survivors in CFU/ mL of *B. cereus*, *B. stearothermophilus* and isolated spores after heat treatment used to determine D-values.

Time of heat treatment at 100°C (min)	*B. cereus*		4II 1	
0	2.8×10^9	1.8×10^9	8.6×10^9	8.8×10^9
5	---*	---*	4.6×10^8	---
10	---	9.2×10^6	3.5×10^7	1.4×10^9
15	---*	---*	1.7×10^6	1.7×10^8
20	4.4×10^5	2.2×10^6	6.8×10^5	2.0×10^7
30	---*	---*	2.4×10^5	9.7×10^6
40	2.4×10^4	3.9×10^3	1.3×10^4	1.2×10^6
50	---*	---*	---*	6.7×10^5
60	3.2×10^4	2.0×10^4	7.5×10^4	3.4×10^5
80	4.6×10^4	1.1×10^4	1.4×10^3	1.7×10^5
100	3.5×10^3	1.9×10^3	1.8×10^3	---*
120	3.1×10^5	---*	---*	---*

Time of heat treatment at 150°C (min)	*B. stearothermophilus*		3III 1C	
0	2.1×10^8	4.1×10^8	1.5×10^9	1.7×10^9
5	1.5×10^7	2.0×10^8	6.6×10^8	---
10	$>4.5 \times 10^3$	1.7×10^8	$<2.5 \times 10^5$ (4×10^4)	3.2×10^4
15	1.0×10^6	1.9×10^4	1.0×10^4	---
20	<25	<25 (2)	<25 (6)	<25 (24)
25	3.5×10^3	<25 (0)	<25 (10)	<25 (0)
30	<25 (2)	<25 (0)	<25 (3)	<25 (2)
40	<25 (0)	<25 (0)	<25 (0)	<25 (0)
50	<25 (0)	---*	<25 (0)	<25 (2)

*Data not collected in some initial and subsequent runs as sampling at these times was not included in all experiments

C. 3. 1 Color values (L, a and b) determined from extruded soy meat analog with different red dye concentrations.

Color concentration (%)	a			a - avg	b			b - avg	c			c - avg
0	51.08	52.27	49.07	50.8067	5.68	5.37	5.83	5.62667	17.19	16.52	16.14	16.6167
0.01	40.31	42.57	40.44	41.1067	14.74	14.99	16.89	15.54	13.18	11.89	13.45	12.84
0.025	40.52	44.22	44.33	43.0233	21.28	21.19	26.16	22.8767	11.5	11.14	15.42	12.6867
0.05	39.31	37.74	37.26	38.1033	25.93	27.39	26.03	26.45	11.33	12.07	11.86	11.7533
0.075	37.25	38.54	36.04	37.2767	27.76	31.53	27.22	28.8367	34.63	36.44	31.82	34.2967
0.1	39.77	40.16	35.51	38.48	34.63	36.44	31.82	34.2967	15.11	15.68	12.81	14.5333
0.15	33.54	33.18	34.7	33.8067	30.19	32.2	30.24	30.8767	10.89	12.42	10.45	11.2533
0.2	33.25	33.82	33.77	33.6133	29.77	33.24	36.23	33.08	10.67	12.4	13.81	12.2933
0.4	34.6	30.95	30.03	31.86	41.64	28.33	30.18	33.3833	15.12	9.1	10.23	11.4833
0.6	30.38	31.76	30.04	30.7267	37.58	39.6	29.63	35.6033	12.98	13.75	9.31	12.0133

C. 3. 1 Color values (L, a and b) of extruded soy meat analog with an unknown amount of red dye.

Time in minutes	L	a	b
0.25	43	5.8	13.97
0.5	41.57	6.01	13.77
0.75	44.31	5.68	14.14
1	43.82	6.01	14.55
1.25	47.31	5.7	15.09
1.5	44.52	5.94	14.5
1.75	47.2	5.9	15.18
2	33.46	26.9	10.76
2.25	32.79	28.92	11.86
2.5	31.08	34.08	12.01
2.75	30.11	36.36	12.79
3	31.51	35	13.06
3.25	31.63	33.19	12.9
3.5	33.15	32.42	13.04
3.75	36.9	29.09	13.57
4	36.78	29.31	13.7
4.25	40.19	24.45	14.02
4.5	43.69	21.79	14.51
4.75	49.28	18.71	15.52
5	47.12	12.33	15.22
5.25	48.54	10.79	15.52
5.5	45.7	10.24	15.09

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