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# Heat inactivation of thermo-resistant bacteria isolated from poultry offal

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HEAT INACTIVATION OF THERMO-RESISTANT BACTERIA  
ISOLATED FROM POULTRY OFFAL

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A Thesis  
Presented to  
the Graduate School of  
Clemson University

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In Partial Fulfillment  
of the Requirements for the Degree  
Master of Science  
Food, Nutrition, and Culinary Sciences

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by  
Lina Marcela Ramirez-Lopez  
December 2006

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Accepted by:  
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## ABSTRACT

The presence of thermo-resistant bacteria in products from the rendering industry is a concern problem in the United States because only about 80% of the rendering industries use thermal processing to eliminate bacteria. The objective of this study was to determine the thermal inactivation characteristics of thermo-resistant bacteria from raw rendering materials. Ground beef with 21% protein, 19% fat, and 60% moisture was used as a model media to simulate raw rendering material. Raw animal co-products were heated at 91, 95, 96°C for 90 min, then surviving bacteria were recovered. These heat resistant isolates were inoculated into meat samples then thermally treated using a dry bath incubator. Calculations for D and Z values were determined for thermo-resistant bacteria subjected to thermal treatment at 91°C, 94°C, and 96°C.

This study demonstrated that thermally resistant bacteria isolated from raw rendering materials exhibit high heat resistance for a wide range of temperature, suggesting that use of thermal processing at an optimized temperate (> 96°C) is required for an efficient removal of microbes.

## DEDICATION

I dedicate this work to my Mom, my Dad, my Brothers, and Edgar, whose supported and motivated me during the completion of my thesis.

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## TABLE OF CONTENTS

|  | Page |
|--|------|
| TITLE PAGE .....   | i    |
| ABSTRACT .....   | ii   |
| DEDICATION .....   | iii  |
| ACKNOWLEDGEMENTS .....                                       | iv   |
| LIST OF TABLES .....   | vii  |
| LIST OF FIGURES .....  | viii |
| CHAPTER  |      |
| I. INTRODUCTION .....  | 1    |
| Thermal inactivation .....                                   | 5    |
| The Rendering process.....                                   | 7    |
| II. LITERATURE REVIEW .....                                  | 11   |
| PART 1: MICROBIOLOGY OF THERMOPHILIC<br>BACTERIA.....        | 11   |
| Classification of bacteria based on temperature growth.....  | 12   |
| Sources of thermophilic bacteria .....                       | 13   |
| Resistance of spores to the environment.....                 | 15   |
| Enumeration of spores .....                                  | 16   |
| Possible mechanism of survival.....                          | 18   |
| Thermophilic spoilage .....                                  | 23   |
| Sporeformers causing food-poisoning .....                    | 24   |
| Sporeformers causing spoilage .....                          | 32   |
| PART 2: THERMAL DESTRUCTION OF THERMOPHILIC<br>BACTERIA..... | 36   |
| Steam sterilization.....                                     | 37   |
| Dry and Moist heat treatment .....                           | 38   |

Table of Contents (Continued)

|                                      | Page |
|--------------------------------------|------|
| <i>D</i> and <i>z</i> values .....   | 39   |
| III. MATERIALS AND METHODS.....      | 43   |
| Sampling .....                       | 43   |
| Spore stain.....                     | 44   |
| Preparation of inoculum.....         | 45   |
| Model media .....                    | 45   |
| Thermal inactivation studies .....   | 46   |
| Statistical Analysis.....            | 46   |
| IV. RESULTS AND DISCUSSION.....      | 50   |
| Microbiological analyses .....       | 50   |
| Genus <i>Bacillus</i> .....          | 51   |
| Thermo-spore observations.....       | 52   |
| Heating profile of model media ..... | 54   |
| Thermal inactivation models.....     | 55   |
| Shape of the survival curve.....     | 63   |
| V. CONCLUSIONS.....                  | 80   |
| Future Research .....                | 82   |
| VI. REFERENCES .....                 | 83   |

## LIST OF TABLES

| Table  | Page |
|--|------|
| 1. Classification of bacteria accord to temperature required for growth.....   | 13   |
| 2. Some characteristics about of bacteria studied .....  | 50   |
| 3. Summary of heat treatments applied to spores and vegetative cells of heat resistant bacteria in a meat medium ..... | 54   |
| 4. <i>D</i> - and <i>z</i> values of heat resistant bacteria studied in meat .....                                     | 61   |
| 5. Results of second model applied to 90.88°C .....  | 65   |
| 6. Results of second model applied to 94.84°C .....  | 67   |
| 7. Results of second model applied to 95.90°C .....  | 69   |



## LIST OF FIGURES

| Figure   | Page |
|--|------|
| 1. What happens in a rendering plant.....  | 7    |
| 2. Typical curve of the thermal death time .....   | 40   |
| 3. Decimal reduction curve .....   | 40   |
| 4. Standard curve for the change temperature required for the<br>thermal destruction curve to transverse one log cycle ..... | 41   |
| 5. Spore and vegetative cell preparation .....   | 51   |
| 6. Surviving heat resistant bacteria at 90.88°C.<br>First replication .....  | 55   |
| 7. Surviving heat resistant bacteria at 91.02°C.<br>Second replication.....  | 56   |
| 8. Surviving heat resistant bacteria at 90.88°C.<br>Third replication.....   | 56   |
| 9. Surviving heat resistant bacteria at 94.77°C.<br>First replication .....  | 57   |
| 10. Surviving heat resistant bacteria at 94.54°C.<br>Second replication.....   | 57   |
| 11. Surviving heat resistant bacteria at 94.94°C.<br>Third replication.....  | 58   |
| 12. Surviving heat resistant bacteria at 95.89°C.<br>First replication .....   | 58   |
| 13. Surviving heat resistant bacteria at 95.98°C.<br>Second replication.....   | 59   |
| 14. Surviving heat resistant bacteria at 95.89°C.<br>Third replication.....  | 59   |

List of Figures (Continued)

| Figure  | Page |
|---|------|
| 15. Plot of $\log_{10}$ of D-values of heat resistant bacteria at different temperatures z value..... | 60   |
| 16. Sample of survivor curve for heat resistant bacteria in meat medium.....                          | 62   |
| 17. Second model parameters .....   | 64   |
| 18. Survivors versus time curve for heat resistant bacteria at 90.88°C. ....                          | 65   |
| 19. Survivors versus time curve for heat resistant bacteria at 94.84°C. ....                          | 67   |
| 20. Survivors versus time curve for heat resistant bacteria at 95.90°C. ....                          | 69   |
| 21. Prediction model at 90.88°C .....   | 73   |
| 22. Prediction model at 94.84°C.....  | 75   |
| 23. Prediction model at 95.90°C.....  | 77   |

## CHAPTER I

### INTRODUCTION

Thermal processing is one of the most extensively used methods to eliminate or reduce bacteria in food. It has been applied in many forms such as cooking, baking, roasting, extrusion cooking, pasteurization, and sterilization. The main objective of pasteurization and sterilizations is to eliminate microorganisms and prevent enzymatic reactions that can spoil food. Thermal processing involves the controlled use of heat and can be applied in food process to increase or reduce bacterial populations depending on what is desired. Many authors, dictionaries, books, articles, etc. have defined thermal processing. In general, thermal processing is the application of heat to food in order to destroy any harmful bacteria or microorganisms (Richardson, 2002) allowing foods to be preserved and available out of season. Destruction of microorganisms is important for food preservation since microorganisms can be pathogenic or cause rapid spoilage. Thermal processing can also inactivate natural heat-labile toxins and enzyme systems that cause degradation in food, preserving desirable texture, flavor, and odor.

The study of thermal processing for microbial safety is required to determine under what conditions harmful microorganisms will be destroyed (Hawley, 1971). Again, this study can (and should) also be extended to the conditions at which natural spoilage reactions caused by enzymes are inactivated. A major benefit of thermal processing is the overall improvement of product quality and safety. However, thermal processing can also give rise to negative consequences. Examples of this is include the formation of

brown pigments on food products, loss of food texture, and the degradation of vitamins, and other nutritional components. So, thermal processing should always be evaluated in terms of the overall quality of the final product as well as the improvement in microbial safety (Arnoldi, 2001).

Thermal resistance by microorganisms is variable and reduction of microbial loads is proportional to the amount of temperature and time applied (Sale, 1970). The high temperatures used in thermal processing destroys microbial cells by destabilizing the structural and functional integrity of the cytoplasmic membrane (Hoover, 1993). The ability of microorganisms to survive high temperatures can be greatly increased by the nutritional composition of the media, which may contain substances that can provide protection against damage, or nutrients essential for repair (Hoover, 1989). In practice, several factors can affect the accuracy of the data obtained from bacterial thermal inactivation studies for food preservation and care must be taken in interpreting results from different research groups (Mazas, 1997).

Microbial spores are often the most heat resistant form of bacteria in food products. A spore is a structure that contains the absolute minimum of genetic material and associated components required to allow sporulation into a vegetative cell under the proper conditions (Piggot, 1976). Spores are highly refractile bodies, consisting of a central core surrounded by five layers, plasma membrane, germ cell wall, cortex, coats, and exosporium. The genetic material (DNA) and other indispensable substances are packaged into a dry, heavily shielded coat that is able to resist high temperature, drying, UV light, deleterious chemicals, heat, poor nutrient supply and other harmful conditions. In their dormant state, all metabolic activity ceases and spores appear "dead" or in deep

hibernation (stasis) (Piggot, 1976). However, when activated, spores give rise to vegetative cells and microorganism growth (Levinson, 1970).

After thermal treatment, spores can produce new vegetative cells that in turn generate new colonies of microbes. The only way to estimate if spores present after thermal processing are viable or dead is to force those surviving spores to form vegetative cells that can produce colonies when plated onto a nutrient agar medium (colony count). The colony count methodology is effective provided that all the viable spores germinate and grow simultaneously to form colonies. A delay in germination of even a few hours will result in delayed formation of the microorganism colony with the subsequent associated error in colony counting.

Spore characteristics, such as wet heat resistance and germination properties, are determined by the conditions under which spore formation or sporulation takes place (Melly, 2002). Once formed, spores can give rise to vegetative cell growth by a reversible process. To form a vegetative cell, a spore has to undergo three distinct and successive stages: activation, germination, and outgrowth (Keynan & Evenchick, 1969). Inactivated spores will not germinate spontaneously until activation occurs.

**Spore activation** signals the initial stage of spore germination. It is usually a reversible process and the activated spore retains nearly all its spore properties (refractility, nonstability, heat resistant etc.). Activation can take place in different ways: thermal stimulation, sublethal injury, or cell death, although the latter may or may not be accompanied by cell lysis (Desrosier, 1956).

**Germination** is the period where spores sprout and begin to grow. Germination is irreversible and involves loss of all spore properties, especially heat resistance (Foerster, 1983).

**Outgrowth** is the period where the embryotic vegetative cell emerges from the spore coat and forms the vegetative organism by macro molecular synthesis (Levinson, 1970; Leuschner, 1999). Outgrowth occurs during post-germinative development (Keynan, 1965).

These processes occur over temperature ranges that include most temperatures at which thermal discontinuities occur in water structure (Fitz-James, 1960). Spores are formed by bacteria to survive during periods of deprivation or environmental stress, such as the loss of nutrient or water supply.

### Thermal Inactivation

There are two main thermal processing methods to treat inoculated meat: Dry heat, which involves incubation in an oven-like environment, and moist heat, which utilizes steam under pressure. The former was used in this study since it better resembles conditions used in rendering thermal processing. Heat resistance of bacteria is normally superior in dry-heat than in moist or wet heat conditions (Larson, 1918). The application of moist heat is more effective in thermal processing of foods because it more effectively denatures proteins, which results in microbial cell death (Hoover, 1993). In addition, the application of dry heat requires higher temperatures and longer contact times than the application of moist heat process, making dry heat more expensive and affording a lower quality product (Hoover, 1993). The heat resistance of bacteria is described by two

parameters, the D and z values. The D value is defined as the heating time required at a specific temperature to kill 90% of the viable cells or spores of a specific organism. The z value is defined as the change in heating temperature needed to change the D value by 90% (1 log cycle). The z value provides information on the relative resistance of an organism to different destructive temperatures in a given substrate. D and z values are invaluable tools in developing heat-processing requirements for destruction of microorganisms in a specific target food product.

The goal of food safety processes is the destruction of pathogenic microorganisms below the concentration of their ability to produce disease (Richardson, 2002). Heat treatments designed to achieve a specific lethality of microorganisms is influenced by many factors, some of which are due to inherent resistance of microorganisms, while others are due to environmental influences (Brown, 1994). Examples of the inherent resistance include differences among species and strains of bacteria, as well as the differences between spores and vegetative cells (Tomlins, 1976). Environmental factors include those affecting the microorganisms during growth and formation of cells or spores (e.g., stage of growth, growth temperature, growth medium, previous exposure to stress, etc.) and those affecting during exposure to heat, such as the composition of the heating medium (amount of carbohydrates, proteins, lipids, solutes, etc.), water activity, pH, added preservatives, method of heating, recovery procedures etc. (Smelt, 1994).

Rendering is a classical example of effective heat treatment to destroy microorganisms under controlled and specific processes. Rendering is an industrial process that converts inedible animal tissue into stable, value added materials. The majority of tissue

processed comes from slaughterhouses but may also include restaurant grease and butcher shop trimmings (Clemen, 1978). This material can include the fatty tissue, bones and offal, as well as whole animal carcasses disposed at slaughterhouses, and those that have died on farms (dead stock). The most common animal species are bovine, porcine, ovine, and poultry. Rendering processes correlate quite closely to those used by the food industries (Prokop, 1992). Rendering involves crushing animal by products (e.g, internal organs), heating them to drive off the water (which can be as high as 65 per cent by weight) and then separating the residue into fat (generally called tallow) and solids (known as greaves). During the rendering process at atmospheric pressure, the temperature remains at 100°C for the majority of the cycle, gradually rising to approximately 120°C once the bulk of the water has evaporated. European regulations require a period of heating under pressure, where the objective is to ensure that the products are sterilized. However, it is well established that dry lipid environments will protect bacterial spores against thermal inactivation (Senhaji, 1977). As water is driven off the rendering material during the process, conditions become more favorable for spore survival. The rendering process simultaneously dries the material and separates the fat from the bone and protein. A rendering process yields a fat commodity (yellow grease, white grease, bleachable tallow, etc.) and a protein meal (meat & bone meal, poultry by product meal etc). Rendering plants often also handle other materials, such as slaughterhouse blood, feathers, and hair; but do so using processes otherwise distinct from the main rendering stream.



## The Rendering process

In most systems, raw materials are ground to a uniform size and placed in continuous cookers or batch cookers, which evaporate moisture and free fat from protein and bone. A series of conveyers, presses, and a centrifuge continue the process of separating fat from solids. The finished fat (e.g, tallow, lard, yellow grease) goes into separate tanks, and the solid protein (e.g, MBM (meat and bone meal), poultry meal) is pressed into cake for processing into feed. Other rendering systems include those that recover protein solids from slaughterhouse blood or that process used restaurant grease.

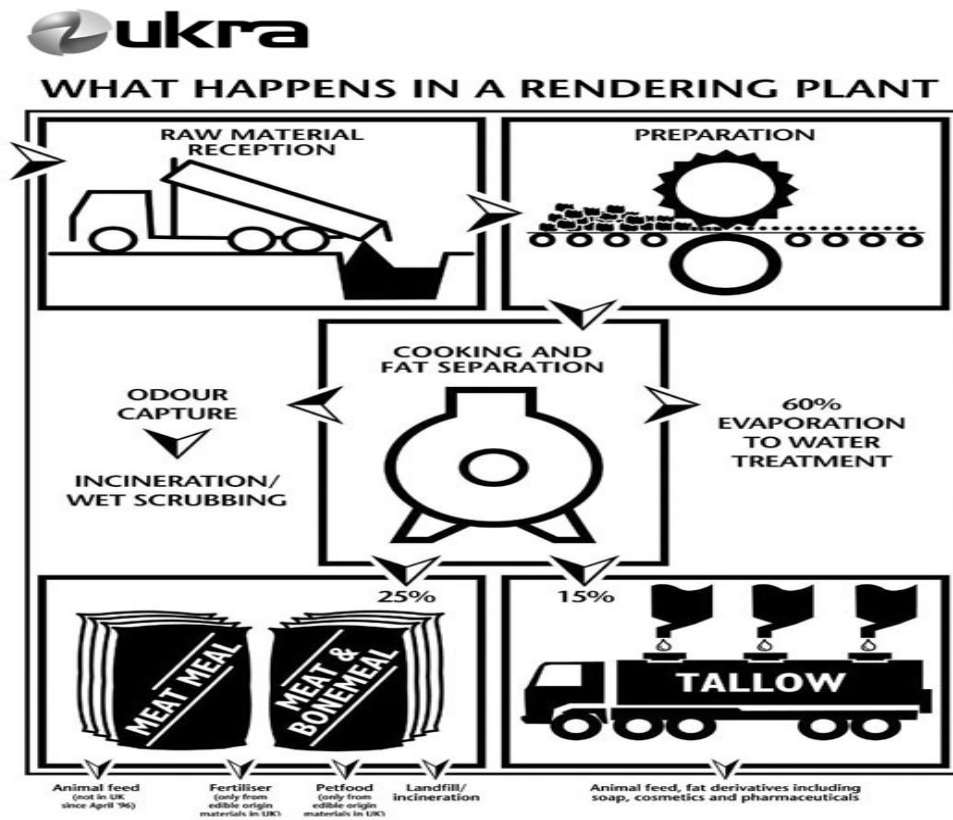


Figure 1. What happens in a rendering plant.

The principal raw material in rendering is low-grade (green) offal and condemned material obtained from slaughterhouses, and other low grade material which may contain fallen stock (or parts thereof) from knackers and hunt kennels (Clemen, 1978).

### Raw Materials

Incorporated rendering plants normally process only one type of raw material, whereas independent rendering plants often handle several materials that require either multiple rendering systems or significant modifications in the operating conditions for a single system (Taylor, 1997).

### Edible Rendering

Fat trimmings, usually consisting of 14 to 16 percent fat, 60 to 64 percent moisture, and 22 to 24 percent protein, are ground and then belt transmitted to a melt tank. The melt tank heats the materials to about 43°C (110°F), and the melted fatty tissue is pumped to a disintegrator, which ruptures the fat cells. The proteinaceous solids are separated from the melted fat and water by a centrifuge (Schreuder, 1998). The melted fat and water are then heated with steam to about 93°C (200°F) by a shell and tube heat exchanger. A second stage centrifuge then separates the edible fat from the water, which also contains any remaining protein fines (Companies, 2001). The water is discarded as sludge, and the polished fat is pumped to storage. Throughout the process, direct heat contact with the edible fat is minimal and no cooking vapors are emitted.

## Inedible Rendering

There are two processes for inedible rendering: the wet process and the dry process. Wet rendering is a process that separates fat from raw material by boiling in water. This involves addition of water to the raw material and the use of live steam to cook the raw material and achieve separation of fat. Dry rendering is a batch or continuous process that dehydrates raw material in order to release fat (Taylor, 1995). Following dehydration in batch or continuous cookers, the melted fat and protein solids are separated. At the present, only dry rendering is used in the United States (Taylor, 1997). The wet rendering process is no longer widely used because of the high cost of energy and of an adverse effect on the fat quality.

## Batch Rendering Process

The raw material from the receiving bin is conveyed to a crusher where it is reduced to 2.5 to 5 centimeters (1 to 2 inches) in diameter to improve cooking efficiency. Cooking normally requires 1.5 to 2.5 hours, but adjustments in the cooking time and temperature may be required to process the various materials (Companies, 2001). A typical batch cooker is a horizontal, cylindrical vessel equipped with a steam jacket and an agitator. To begin the cooking process the cooker is charged with raw material, and the material is heated to a final temperature ranging from 121° to 135°C (250° to 275°F). Following the cooking cycle, the contents are recovered in a percolator drain pan. The percolator drain pan contains a screen that separates the liquid fat from the protein solids. From the percolator drain pan, the protein solids, which still contain about 25 percent fat, are conveyed to the screw press. The screw press completes the separation of fat from solids,

and yields protein solids that have a residual fat content of about 10 percent. These solids, called cracklings, are the ground, and screened to produce protein meal (Clemen, 1978).

### Blood Processing and Drying

Whole blood from animal slaughterhouses, containing 16 to 18 percent total protein solids, is processed and dried to recover proteins as blood meal. At the present time, less than 10 percent of the independent rendering plants in the U.S. process whole animal blood (Companies, 2001). The blood meal is a valuable ingredient in animal feed because it has high lysine content. Continuous cookers have replaced batch cookers that were initially used in the industry because of the improved energy efficiency and product quality provided by continuous cookers. In the continuous process, whole blood is introduced into a steam injected, inclined tubular vessel in which the blood solids coagulate (Companies, 2001). The coagulated blood solids and liquid (serum water) are then separated in a centrifuge, and the blood solids dried in either a continuous gas-fired, direct contact ring dryer or a steam tube, rotary drier (Trout, 2001).

Due to the high number of and mixture of bacteria associated with raw rendering material, reduction of bacterial loads is important. The mixture is likely to contain both spore and non-spore forming bacteria, the research objective was to determine the thermal inactivation characteristics of thermo-resistant bacteria from rendering materials (poultry offal).

## CHAPTER II

### LITERATURE REVIEW

#### PART 1: MICROBIOLOGY OF THERMOPHILIC BACTERIA

“Thermophilic bacteria” have been studied for many years and have special importance to food preservation processes. Their high heat resistance has been studied to determine the probable mechanisms of survival under conditions which most bacteria are killed. Some investigators have designated thermophilic bacteria as those capable of growth at 50°C (122°F) while others have designated bacteria with a minimum and maximum temperature over 50°C (122°F) as “thermo tolerant” (Morrison, 1921; Williams, 1954). According to Peleg (2006), thermophilic bacteria are those that grow at temperatures above the maximum for most bacteria, especially the pathogenic forms. The maximum growth temperature for most pathogenic bacteria is about 45°C (113°F) and their optimum growth temperature is about 37.5°C (99.5°F) (Bergey, 1919). Most thermophiles belong to the *Archaea* and are able to grow at temperatures around the boiling point of water; with the upper temperature limit for survival between 110 (230°F) and 130°C (266°F) (Brock, 1986).

Bacterial species vary widely as to what conditions are conducive for growth. Conditions that are optimum for one organism may be lethal for another. Nutrient availability, moisture, pH, the presence, or absence of oxygen, the presence of inhibitors and temperature can all influence the growth of bacteria. In most cases, these are not independent variables but are interactive.

## Classification of bacteria based on temperature growth

Bacteria have been classified based on temperature growth and they have been divided into categories based on their optimal growth temperature ranges. The optimum temperature for growth for a bacterium is the temperature where the generation time is shortest or the bacterium grows the fastest. Each bacterium has a minimum and a maximum temperature for growth, which will vary between species and strains and with other environmental conditions. Microorganisms in nature have been divided into several temperature growth classes. Temperature affects the growth and activity of all living cells. Microorganisms have been classified into three distinct categories according to their temperature preference. These are not rigid ranges as some bacterial species may overlap into adjacent groups. General groupings of bacteria are as follows:

**Psychrophiles** are microorganisms that live and grow best between -10 to 20°C (14 to 68°F). Psychrophilic bacteria are found in the Arctic and Antarctic Oceans, which remains frozen most of the year. Nutrients needed by psychrophiles reside inside frozen glaciers and seawater, but flows in tiny streams in between cracks and layers of ice.

**Psychrotrophs** are cold tolerant organisms capable of growth at temperatures at or below 7°C (44.6°F), regardless of their optimum temperature. Psychrotrophs are of primary concern to the refrigerates food industry since they can grow and cause spoilage in raw or processed products commonly held under refrigeration.

**Mesophiles** thrive at mid-range temperatures, 20-45°C (68-113°F), and include in any human pathogens. They are found in soil and water environments. Most human diseases

caused by bacteria and viruses come from the mesophile group. Some of the most dangerous mesophiles are *Staphylococcus aureus*, *Salmonella* sp., *Proteus vulgaris*, and *Yersinia enterocolitica*.

**Thermophiles** thrive above 45°C (113°F), and some survive exposure to the temperatures at or even above the boiling point of water. Thermophiles live in either natural geothermal habitats, or can be found in environments that artificially create heat. (See table 1).

*Temperature required for growth°C*

| Type of bacteria | Minimum | Optimum | Maximum | General sources of bacteria                |
|------------------|---------|---------|---------|--|
| Psychrophiles    | 0       | 15      | 25      | Water and frozen foods                     |
| Psychrotrophs    | 0       | 25      | 40      | Dairy foods                                |
| Mesophilic       | 10      | 35      | 45      | Pathogenic and non-pathogenic bacteria     |
| Thermophilic     | 45      | 55      | 70      | Spore forming bacteria from soil and water |

Table 1. Classification of bacteria accord to temperature required for growth

Sources of thermophilic bacteria

Even though thermophilic aerobic sporeforming bacteria are widely distributed in nature, it is evident that they are capable of surviving under and extreme conditions. These bacteria survive in thermal springs, in the sands of the Sahara desert; in the surface layers of the soil in the temperate and torrid zones even frequency at elevated temperatures (Bergey, 1919). Thermophilic bacteria are naturally distributed in hot springs, tropical soils, compost heaps, excrement, hot water heaters (both domestic and industrial), and garbage (Brock and Thomas, 1986). Composted or manured garden soils may contain 1-10 percent thermophilic types of bacteria, while field soils may have only

0.25% or less. Under simple conditions, the colonies of this group of bacteria develop the capacity to create heat resistant spores which can be recovered in pure culture by transplanting on to tubes of slanted agar. They have been isolated from air (Sames, 1900), freshly fallen snow (Golikowa, 1926), seawater (MacFadyen and Blaxall, 1894), sewage, feces of humans, and various animals (Black and Tanner 1928). The temperature conditions are of course favorable in hot springs, but the contribution of organic matter in most thermal water is low and autotrophic thermophilic sporeformers are rare. Milk is other source of thermophilic bacteria. Thermophilic bacteria development during the heating process; some spores are activated when the milk is cooled (Eckford 1927, Robertson 1927). In the fermentation of feed, temperatures have been found as high as 55°C (131°F), but more commonly the temperature ranges between 35 (95°F) and 45°C (113°F). A common characteristic of thermophilic microorganisms is the capacity to form spores at high temperatures. These spores can vary in size of rods, location, and to a less degree in their biological characteristics depending of the species (Bergey, 1919). As a prerequisite for their survival, thermophiles contain enzymes that can function at high temperature. Some of these enzymes are used in molecular biology (for example heat-stable DNA polymerases for polymerase chain reaction), and in washing agents (Morrison, 1921).

#### Resistance of spores to the environment

Bacterial spores are extremely resistant to heat, cold and chemical agents. Some bacterial spores can survive in boiling water (100°C, 212°F) for more than 16 hours. The



same organisms in the vegetative state and the non-sporeforming bacteria will not survive heating in boiling water.

Spores that successfully resist heat are also highly resistant to destruction by chemicals. Bacterial spores have been found survive more than three hours in sanitizing solutions normally used in food processing plant (Larson, 1918). On the other hand, these sanitizing agents readily destroy vegetative cells. The purpose of sanitizing is not to sterilize surfaces, so the survival of spores under appropriate sanitation practices is not a concern if they are present in low numbers and can be inactivated or controlled in the final product. Smelt (1994) reported that bacteria with a relatively high content of diphosphatidylglycerol are more susceptible to inactivation. Some studies have indicated that denaturation of enzymes, such as membrane-bound ATPases, plays an important role in the pressure induced injury and inactivation of microorganisms (Mackey, 1995). For spores, a combination of high pressure and high temperature is necessary for inactivation. Under high pressure, bacterial spores germinate to vegetative cells and are then inactivated due to effect of temperature (Collier, 1956). High pressure weakens or denatures protein molecules in the food components because the hydrophobic and ion-pair bonds are disrupted (Juneja, 1996), while covalent bonds are not affected. However, changes in the tertiary structure from the breaking and reformation of chemical bonds can alter the coagulation or gelation characteristics of some foods, giving them a unique and novel texture. The flavor or nutrient content of a food is generally not altered.

## Classification of thermophilic bacteria based on the temperature at which the spores germinate and grow

Bacterial spores have been divided into groups based on the temperature at which spores germinate and grow. Investigators have studied different strains of thermophilic bacteria, trying to classify them into categories to better understand the heat resistance properties of these microorganisms. The thermophilic bacteria have been classified into obligate and, facultative organisms: obligate thermophiles (also called extreme thermophiles) require high temperatures for growth, while facultative thermophiles (also called moderate thermophiles) can thrive at high temperatures but also at lower temperatures (below 50°C, 122°F). Hyperthermophiles are particular extreme thermophiles of which optimal temperatures are above 80 °C (176°F). The strains of thermophilic bacteria have been identified with optimum temperatures ranging from 55°C (131°F) to an unbelievable 105°C (221°F) (above the boiling point of water), and many temperatures in between (Bergey, 1919).

## Enumeration of Spores

There are several methods used for counting bacterial spores.

These include two methods, depending on whether spore density is low or high.

- I. Direct count (low density) Spore density can be determined by counting their number in a unit volume. The simplest technique is to use a special counting chamber similar to a haemocytometer, which is used for counting blood cells (Hamdy, Harper and Weiser 1955). The counting chamber is simply a ruled slide

with a supported glass tube that holds a definite volume of fluid. When there are more than 3-4 in spores some fields of view and no spores in others (25-50% of fields examined), then the method above cannot be used because it will overestimate total number of spores in the sample. In this case, spores often are few enough to be counted directly. This method involves the follow steps.

- a) Transfer spore suspension to a test tube, vortex, and transfer 1 ml to a watch glass. Perform this step three more times to count spores in four replicates.
- b) Swirl water in watchglass (clockwise or counterclockwise) to concentrate spores in the center. Expand field of view to see all spores and make a direct count. Average the counts from four watchglasses and multiply result by dilution factor ( $1 / x$  total mls in test tube).
- c) If number of spores in watchglass is too many to count comfortably, then increase the dilution and recount.

II. Ocular field (high density) this method should not be used for extracted spores that have been stores more than 24 hr because aggregates will form. If there are less than 30-40 spores per field, the spores are counted in 40 fields randomly chosen over the area of the dish. The following steps are involved in this method.

- a) Using a fine ruler, determine the diameter of the ocular field of the stereomicroscope at a magnification where spores can be easily distinguished from mineral particles and organic debris (some of which can be easily mistaken for spores by the inexperienced person). Then calculate the area of the spherical field (circle) at that magnification.

- b) Plastic petri dishes are preferred to count spores because the base of the plate is flat. Because the dish also is hydrophobic, enough water must be added to have complete coverage of the base. Dishes vary in diameter.
- c) Spore extraction. Add the spore suspension to a petri dish and then randomly rotate the dish to spread out spores evenly as possible. Calculate average number of spores per field and multiply this number by 289 (# fields/ dish). If there are more than 30-40 spores per field, then place spore extract in a test tube, dilute 1:1 with water. While vortexing, remove a specific volume (10ml is the recommended amount), transfer to petri dish, and recount. Make sure to keep track of each dilution to calculate spores in total sample.

#### Possible mechanisms of survival

There are several mechanisms proposed to explain the survival of thermophilic bacteria at high temperatures. All of these proposed mechanisms have been developed around the following three concepts: (i) stabilization may be completed through lipid interaction; (ii) heat denatured cellular compounds may be rapidly resynthesized and (iii) thermophilic organisms may possess macromolecules complexes with inherent heat stability. All these factors are explained as follows.

#### Lipids

Heilbrunn and Belehradec studied thermal stable organisms and noted that they had lipids with higher melting points than did thermally labile organisms. They noted that the

temperature at which cell lipids melted might place an upper limit for cellular growth (Heilbrunn, 1924; Belehradek, 1931). Other studies have noted that as the growth temperature increased, the percentage of saturated and branched-chain fatty acid increased (Daron, 1970; Ray, 1971). All these changes could cause a higher melting point and greater flexibility in the lipid membrane. In 1967 Brock suggested, that these changes might provide the organism with a more stable membrane. This hypothesis was supported with a study of differences in the membranes and cell walls of thermophiles and mesophiles using electron microscopy technique (Novistsky, 1972). Wisdom and Welker (1973) reported that the alkaline phosphatase of *Bacillus sterothermophilus* was more thermostable inside the cell membrane than in lysed cells.

### Rapid Resynthesis

Smaller cells should have a higher metabolic rate due to a greater surface to volume ratio, which facilitates the rapid transport of substrates and dissipate products into and out of the cell (Allen, 1953). Based on these and other observations (Allen, 1953) postulated that the growth at elevated temperatures is simply the result of a rapid resynthesis of heat denatured cellular compounds. Allen (1953) has revised evidence supporting this hypothesis. Nevertheless, Koffler (1957) suggested that if thermophily is simply a kinetic function of rapid resynthesis, the mesophiles organisms should be able to grow at elevated temperatures (and equally, the thermophiles should be able to grow at low temperatures). He postulated that an organism should be any where from 16 to 81 times as active at 70°C (157.999°F) as it is at 30°C (85.99°F). It should be noted that the

number of data points for this determination were few, and the relationships noted may not be statistically valid (Koffler, 1957).

### Thermally Stable Macromolecules

Numerous mechanisms have been proposed to explain the survival of thermophilic organisms on the molecular level. These have been summarized into three general mechanisms: a) thermophiles may contain factors, which increase the stability of their cell components with respect to heat; b) mesophiles may contain factors, which increase the lability of their components with respect to heat; c) cellular compounds of thermophiles may have inherent heat stability, independent of exogenous factors (Koffler, 1957). Hypotheses like these were tested (Koffler, 1957; Amelunxen, 1968) by incorporating cell free extracts from thermophilic microorganisms with extracts prepared from mesophiles and by testing for protein denaturation after heating. This experiment concluded that the thermophilic extracts were found to possess a degree of heat stability, which was not transportable to the mesophilic extract. In addition, in other experiments Amelunxen and Lins (1968) analyzed numerous enzymes from *B. stearothermophilus* and *Bacillus cereus* in crude lysates. From these experiments, they concluded that thermophilic enzymes were considerably more thermo stable than mesophilic enzymes (Amelunxen and Lins, 1968). Howell found similar results with several glycolytic enzymes from thermophilic and mesophilic *Clostridia spp.* (Howell, Akagi and Himes, 1969).

After finding a number of similarities with respect to thermophilic enzymes, investigators located an example of a thermophilic enzyme (catalase) being associated

with a stabilizing factor (other than a substrate) (Nakamura, 1960). The crude enzyme was observed to possess a high degree of heat stability that decreased upon purification. Another term used in the concept of thermal stable macromolecules is S factor(s). Primary S factors are essential proteins required for vegetative growth, whereas alternative S factors mediate transcription in response to various stimuli. In the experiment described before, the S factor was removed from the crude enzyme by charcoal treatment and could be isolated by boiling the crude enzyme. The optimum temperature for the isolated enzyme was 60°C (139.99°F), while the optimum temperature in the presence of S factor was 65°C (148.99°F). Consequently, still in the absence of the factor, the enzyme was heat stable. The isolated enzyme was found to have kinetic behavior similar to other catalases. The physical properties of the enzyme and the nature of S factor have not been reported.

#### Cellular proteins associated with heat resistance

The capacity of thermophilic aerobic sporeformers to survive when exposed to elevated temperatures is associated with the nature of their proteins. Microbial life has been found at temperatures approaching or slightly exceeding the boiling point of water (Brock, 1970). The proteins are localized in the cell of thermophilic bacteria. Without the presence of thermally stable biosynthetic systems, the organisms could not survive. At this time, approximately 20 proteins have differentiated from many thermophilic microorganisms (Ayde, 1957). These proteins often show high stability at normal temperatures, a feature that has attracted commercial interest (e.g., the proteins have a long shelf life) and have been carefully purified and analyzed biologically, physically and

chemically. Farrell and Campbell (1961) proposed an enzyme classification from thermophilic bacteria as:

Class I Enzymes that are stable at the temperature of synthesis (generally 55 to 65°C (131°F to 149°F), but are inactivated at higher temperatures.

Class II Enzymes that are inactivated at the temperature of synthesis, except when in the presence of substrate.

Class III Enzymes that are highly heat resistant and are heat stable at temperatures above the temperature of synthesis.

The Farrell and Campbell (1961) hypothesis was rejected for several reasons. One reason was an elevated temperature optimum is directly related to thermo stability. However, the proposed system proposed relates the stability of the enzyme to the growth temperature of organisms; this did not relate the necessity of the enzyme for the survival of the organism. Finally, this scheme of classification avoids a significant class of proteins for thermophilic microorganisms; it makes no provision for those proteins, which are not stable under any conditions at the optimal temperature of growth. The disagreement for the presence or absence of stabilizing factors was not seriously challenged until Campbell and his co-workers isolated and studied a crystalline, heat stable  $\alpha$ -amylase from *Bacillus stearothermophilus* (Campbell, 1969). The conclusion of this work was that the amino acid composition data of thermophilic bacteria had an abnormally high proline content (Campbell, 1969). The conclusion has been made that the thermo stability of the protein molecule may be a consequence of the fact that the



enzyme exists in an open configuration in the initial or native state, and therefore heat has relatively little further effect upon denaturation (Manning, 1970). Although, this conclusion could not serve as a universal mechanism, there are studies that heat stable highly purified flagellar proteins obtained from thermophiles did not contain large amounts of proline (D. Abram and H. Koffler, Abstr. Proc. Intern. Congr. Microbiol. 8<sup>th</sup>, Montreal, 1962, p21). Amelunxen and Lins (1968) noted that pyruvate kinase and glutamic transaminase in crude lysates of *B. stearothermophilus* were inactivated by temperature near the maximum growth temperature for the organism (Amelunxen and Lins, 1968).

### Thermophilic Spoilage

The distribution of thermophilic aerobic sporeforming bacteria in food is of interest to food microbiologists because of their potential importance as spoilage organisms in retort-processed foods (Cameron, 1926). Generally, the higher the temperature at which a sporeforming organism can grow, the greater the heat resistance of its spore. Thus, the spores of thermophilic bacteria usually have a greater heat resistance than the spores of mesophilic bacteria. The spores of thermophilic bacteria are so resistant to heat that heat processes designed to kill the mesophilic bacteria may not be adequate to destroy thermophilic bacteria. In order to prevent thermophilic spoilage, the product must be properly cooled, preferably below 41°C (105°F), after thermal processing and held below 35°C (95°F). Thermophiles can grow on food processing equipment if the temperature is within their growth range. As a result, product should always be held at 77°C (170°F) or above and below of room temperature to prevent the growth of thermophiles. For meat

and poultry products containing ingredients known to be a source of thermophiles (sugar, starch, and spices) and where thermophilic spoilage may be a problem, prudent processors will use ingredients that are guaranteed to be free of thermophilic bacteria or that meet specifications for thermophiles for canning processes.

### Sporeformers causing food poisoning

Bacterial spores are much more resistant to heat, chemicals, irradiation and desiccation than their vegetative cell counterparts. The major food poisoning sporeformers are *Clostridium botulinum*, *Clostridium perfringens*, *Bacillus cereus*, *Bacillus subtilis*, and *Bacillus licheniformis* (ICMSF, 1996). Food contamination with thermophilic bacteria has come from a variety of natural sources including water, soil and gastro-intestines tracks of chicken. Hence, preventive measures should be taken the control these bacteria in food.

There are several nonpathogenic sporeformers including butyric and thermophilic anaerobes that cause considerable economic losses to food producers. These microorganisms are not pathogenic but can spoil food. Spoilage is an indication that has been a mistake in the process or a lack of maintenance of hygiene.

### *Bacillus* spp.

*Bacillus* represents a genus of Gram-positive bacteria which are ubiquitous in nature (soil, water, and airborne dust), while other species are natural flora in the human intestine. However, it is generally accepted that the primary habitat of aerobic endospore-forming bacilli is soil. Russian microbiologist Winogradsky considered them

"normal flora" of soil. When grown on blood agar, *Bacillus* produce large, spreading, gray-white colonies with irregular margins. A unique characteristic of this bacterium is its ability to produce endospores when environmental conditions are stressful. Although most species of *Bacillus* are harmless saprophytes, two species are considered medically significant: *B.anthraxis* and *B. cereus*. Early attempts at classification of *Bacillus* species were based on two characteristics: aerobic growth and endospore formation. This resulted in tethering together many bacteria possessing different kinds of physiology and occupying a variety of habitats. Hence, the heterogeneity in physiology, ecology, and genetics, made it difficult to categorize the genus *Bacillus* or to make general assumption about it. Most *Bacillus* species are versatile chemoheterotrophs capable of respiration using a variety of simple organic compounds (sugars, amino acids, organic acids). In some cases, they also ferment carbohydrates in a mixed reaction that typically produces glycerol and butanediol. A few species, such as *Bacillus megaterium* may require amino acids, B-vitamins, or both. The majority are mesophiles, with optimum growth temperature between 30 (85.99°F) and 45 degrees (112.99°), but the genus also contains a number of thermophilic species with optimum growth as high as 65 degrees (149°F). In the laboratory, under optimal conditions of growth, *Bacillus* species exhibit generation times of about 25 minutes.

#### 1. *Bacillus cereus*

*Bacillus cereus* is a Gram positive, facultative aerobic sporeformer, with morphology of large rods and spores do not enlarge the sporangium. This organism is a common cause of food poisoning (Erlendur, 2000). *Bacillus cereus* is found in the soil, on grains

and vegetables, in flour, and in many raw and processed foods. The organism grows rapidly in moist, cooked protein or inadequately refrigerated starch foods. When present in relatively high numbers ( $10^6$  or greater per gram), *Bacillus cereus* may cause several types of food poisoning. Two recognized types of illness are caused by two separate metabolites (Mazas, 1997). The first type is characterized by nausea, vomiting and abdominal cramps and has an incubation period of 1 to 6 hours. The second type is manifested primarily by abdominal cramps and diarrhea with an incubation period of 8 to 16 hours (Todar, 2006). *Bacillus cereus* food poisoning usually occurs because heat-resistant endospores survive cooking or pasteurization and then germinate and multiply when the food is inadequately refrigerated. Toxins produced in the food during bacterial growth, principally a necrotizing enterotoxin and potent haemolysins (especially cereolysin); cause the symptoms of *B. cereus* food poisoning. The conditions that favors the growth of *B. cereus* include cooking procedures that activate the spores followed by slow cooling and storage of food at 10-50°C (50°F -122°F) (ICMSF, 1996). The spores of *B. cereus* appear to vary widely in heat resistance. Nevertheless, when the available data are plotted as log decimal reduction or D value against temperature, most spores group as a particular heat resistant strain (Brown, 1992). A D value of 2.35 min at 121.1°C (249.98°F) was reported for this strain (Bradshaw, Peeler and Tweet, 1975). Under dry heat conditions, spores of *B. subtilis* can be extremely resistant with D values at 160°C (320°F) of 0.1-3.5 min being reported by various researchers (Brown 1994; (Kramer, 1989; Brown, 1992; Moir, 1994).

## 2. *Bacillus Subtilis*

*Bacillus subtilis* is a gram positive, non-pathogenic bacterium found in soil and rotting plant material and is non-pathogenic (Holdsworth, 1997). This organism was one of the first bacteria studied, and was named *Vibrio subtilis* in 1835 and renamed *Bacillus subtilis* in 1872 (ICMSF, 1996). It is one of the most well characterized bacterial, and is a model system for cell differentiation and development (Brown, 1992). One feature that has attracted a lot of interest in *B. subtilis* is its ability to differentiate and form endospores. This bacterium can divide asymmetrically, producing an endospore that is resistant to environmental factors such as heat, acid, and salt, and which can persist in the environment for long periods (Brown, 1992). The endospore is formed at times of nutritional stress, allowing the organism to persist in the environment until conditions become favorable (Stringer, 1985). Prior to endospore formation, the bacterium might become motile through the production of flagella, and take up DNA from the environment through the competence system (Kramer, 1989). The sporulation process is complex and involves the coordinated regulation of hundreds of genes in the genome. This initial step results in coordinated asymmetric cellular division and endospore formation through multiple stages that produces a single spore from the mother cell. This cascade of gene regulation has been intensively studied. *B. subtilis* forms colonies that are dull in appearance, may be wrinkled, are cream to brown in color and when grown in broth have a coherent pellicle; usually with a single arrangement (Stumbo, 1973).

### 3. *Bacillus licheniformis*

*Bacillus licheniformis* is a Gram-positive, motile, spore-forming, facultative anaerobic rod belonging to the *B. subtilis* group of Bacilli. This apathogenic soil organism is mainly associated with plant and plant materials in nature but can be isolated from nearly everywhere due to its highly resistant endospores that are disseminated with the dust (ICMSF, 1996). *B. licheniformis* spores are similar in resistance to typical *B. cereus* spores with D values at 100°C (212°F) around 4-8 min (ICMSF, 1996).

#### *Clostridium* spp.

The bacterial cells of *Clostridium* spp are large, Gram-positive, rod-shaped bacteria. All *Clostridia* form endospores and have a strictly fermentative mode of metabolism. Most *Clostridia* will not grow under aerobic conditions and vegetative cells are killed by exposure to Oxygen, but their spores are able to survive long periods of exposure to O<sub>2</sub> (Hatheway, 1990). These organisms are found in soil as well as in normal intestinal flora of man and animals and uncooked meat. There are both gram-positive and gram-negative species, although the majority of isolates are gram-positive. Exotoxin(s) play an important role in disease pathogenesis.

#### 1. *Clostridium botulinum*

*Clostridium botulinum* is a Gram-positive, endosporeforming, anaerobic, rod, that produces neurotoxins. This organism is found in soil and aquatic and marine muds. Most foods originating from the soil or aquatic environments contain some cells or

spores. Neither the spore nor the vegetative cell itself is toxic, only the toxin, which is formed by the vegetative cell after sporulation during growth (Doyle, 1989).

Seven types of *C. botulinum* are recognized, based on the antigenic nature of the toxin produced. These are types A, B, C, D, E, F, and G; a number of subtypes exist. All type A and some B and F strains are proteolytic with growth and toxin production optimal at 35°C (95°F). All type E, the remaining B and F strains, C and D strains are non-proteolytic, with optimal growth a toxin production at 26°C (78.8°F) (Odling 1978). Foodborne disease caused by *C. botulinum* is referred to as botulism. It is caused by the ingestion of a neurotoxin produced by the microorganism in food (Segner, 1971). Infant botulism can also occur which is thought to occur from the ingestion of *C. botulinum* spores with honey being the major food implicated (Arnon, 1990). In Infant botulism, spores in the gastrointestinal tract will germinate and multiply, producing the toxin. *C. botulinum* can grow and produce toxin with ease in many foods. It is important to ensure that not only are the correct thermal processes and representative regimens followed, but also that all parts of the food are under control (ICMSF, 1996). Generally, it is thought that any low acid foods (pH above 4.6) can support the growth of *C. botulinum* and its subsequent production of toxin (Segner, 1971). Despite this, an outbreak of botulism occurred in canned tomatoes, which had a low pH. It was thought that the production of toxin occurred due to the growth of mold increasing the pH of the product. There is now an antidote to the toxin, which has reduced the mortality rate somewhat (Segner, 1971). Cultural methods can be used to detect the presence of the microorganism in foods, which can take up to 10 days. Mouse bioassay can then be used to test for the presence of the toxin (Hocking, 1997).

## 2. *Clostridium perfringens*

*Clostridium perfringens* are Gram-positive, anaerobic sporeforming rods. They are widely distributed in the environment and frequently occur in the intestines of humans and many domestic and feral animals (Stringer, 1985). The spores are capable of surviving in soil, sediments, and areas subject to fecal contamination. The spores are also extremely heat resistant and have been reported to survive boiling for several hours (Brown, 1992). *C. perfringens* can grow at temperatures ranging from 15 to 50°C (59 to 122°F), and pH values of between 5.0 and 8.0 (Stringer, 1985). The illness caused by *C. perfringens* is referred to as perfringens food poisoning. The illness is caused by toxin being produced in the stomach by large numbers of the microorganisms ( $10^7$  cells per gram). A more serious but rare illness is also caused by a certain strains of *C. perfringens*, Type C strain. This is known as enteritis necroticans. The most common cause of *C. perfringens* food poisoning is temperature abuse of prepared foods. Small numbers of organisms or spores are often present after cooking. After cooking, spores germinate and cells multiply to levels capable of causing food poisoning during cooling foods and warm storage. Meats, meat products, casseroles, and gravy are foods most frequently implicated, but any food cooled to slowly could pose a hazard (Doyle, 1989). Spores of *C. perfringens* Type A are widespread in the environment and are present in a wide variety of foods including meat, fish, poultry, vegetables, dairy products and dried foods (ICMSF, 1996). Cooking and cooling meat in smaller portions, which heats and cools food more rapidly, would significantly reduce the risk of *C. perfringens* food poisoning (Brown, 1992). A temperature above 70°C (158°F) is necessary to destroy vegetative cell before consumption (Labbe, 1989).



*Sporolactobacillus* spp.

The genus *Sporolactobacillus* (Kitahara and Suzuki, 1963) is comprised of five species of catalase-negative, Gram positive, facultative anaerobic or microaerophilic endosporeformers. Originally proposed as a component of the genus *Lactobacillus*, *Sporolactobacillus* was subsequently elevated to genus status in the family *Bacillaceae* (Kitahara and Toyota, 1972). DNA-to-DNA hybridization studies by Dellaglio and others (1975) showed its distinction from the genus *Lactobacillus*. The type species for *Sporolactobacillus* is *Sporolactobacillus inulinus* (Kitahara and Suzuki, 1963; Kitahara and Lai, 1967). It produces a negative result for lactic acid, but is unable to ferment lactose. Optimal growth temperature is 35 °C (95°F). Fatty acid configuration and isoprenoid quinone cell components are consistent with the *Bacillus* group and differ from those of *Lactobacillus* (Uchida and Mogi, 1973). The habitats of the members of the genus *Sporolactobacillus*, apart from the original isolation from chicken feed, are believed to be the soil, milk products, and pickle (as contaminants).

The incidence of these sporeformers in the environment is low. Doores and Westhoff in 1983 used a selective method specific for *Sporolactobacillus*, examined samples of food, beverages, plant, and animal material. Only 2 out of 699 samples examined were positive for *Sporolactobacillus*, documenting the rarity of this species in these environments. Strains of *Sporolactobacillus* have been found to survive exposure to low pH (Hyronimus and others, 2000), although the procedure used to assay this resistance did not allow discrimination between spores and vegetative cells.

### Sporeformers causing spoilage

These groups of microorganism have not been associated with food poisoning but which can produce considerable food spoilage of groceries (Russell, 1982).

#### *Clostridium butyricum*, *C. beijerinckii* and *C. pasteurianum*

*Clostridium butyricum*, *C. beijerinckii*, and *C. pasteurianum* gram positive, produce gas and butyric odors in canned foods, predominantly those with pH values between 3.9 and 4.5 (e.g tomatoes and pears) (Hersom, 1980). During the storage and ripening of hard cheeses such as Gouda, Edam and Emmentaler, *Clostridium butyricum* and *Clostridium tyrobutyricum* can cause spoilage and gas production, the spores often occurring in milk from cows fed silage during winter months (Russell, 1982). Spores of *C. butyricum* have been reported to have D values as high as 23 min at 85°C (185°F) and pH 7 (Russell, 1982). At pH 4.4, the thermal death time may be 10-15 min at 100°C (212°F) (Hersom, 1980). For destruction of spores of *C. pasteurianum*, it has been suggested that a core temperature of 95°C (203°F) should be reached for products with a pH between 4.2, 4.5, and a core temperature of 84°C (183.2°F) for products with a pH below 4.2 (Hersom, 1980).

*C. beijerinckii* is a close relative of *C. butyricum* with D values of 2-4 min at 85°C (185°F) and pH 7 (Brown, 1990). Control of butyric anaerobes requires thorough washing of the raw material together with pH and process temperature control. Failure to control sporeformers in products with pH values 3.9-4.5 may result not only in spoilage but also a rise in pH which could allow spores of *C. botulinum*, which had survived pasteurization, to germinate and produce toxin.

### *Clostridium sporogenes*

*C. sporogenes* is gram positive, closely related to proteolytic strains of *C. botulinum* but produces spores which are approximately 5 times as heat resistant with D values up to 1.5 min at 121°C (249.8°F) (Van Rijssel, 1992). Spoilage from this organism produces typically blown or burst packs with a strong putrefactive odor. If spoilage from *C. sporogenes* is experienced, all suspect packs should be recalled and investigations into the cause of spoilage undertaken. A process fault that allows *C. sporogenes* to survive and proliferate may also have been serious enough to allow spores of *C. botulinum* to survive, germinate and produce toxin.

### *Clostridium thermosaccharolyticum*

This organism is gram positive, has the most heat resistant spores of any bacterium, and is a common food spoiler. D values as high as 195 min at 121°C (249.8°F) have been recorded (Xezones, 1965). The author investigated a spoilage outbreak in canned mushrooms caused by heat resistant spores of *C. thermosaccharolyticum* that had grown in the composted forest bark used on the mushroom beds (Brown, 1983). D values of these spores were 68 min at 121°C (249.8°F). Spoilage from this organism manifests itself by blown or burst packs with a strong butyric or cheesy odor. The spores survive thermal processing to germinate and grow when the product is stored at elevated temperatures around 30-60°C (86°F-140°F).

### *Clostridium putrefaciens*

Studies by Roberts and Derrick demonstrated that this organism was able to grow in 4% NaCl +100ppm of NaNO<sub>2</sub> at pH 7.0 even at 5°C (41°F). The spores were not particularly heat resistant, with D values of 8-14 min at 80°C (176°F). Modern processing trends for cured meat are to use lower levels of salt and nitrite, increased pH levels of 6.8-7.0 and chill storage, which would tend to favor the growth of *Clostridium putrefaciens* (Roberts, 1975).

### *Bacillus sporothermodurans*

This is a mesophilic sporeformer, which produces highly heat resistant spores. Peterson (1996) reported spores surviving the heat process and multiplied to a maximum of about 10<sup>5</sup>/ml in milk during incubation at 30°C (86°F) for 5 days, but caused no noticeable spoilage and were none pathogen. Raw milk must be autoclaved to enrich the spores and eliminate competitive microflora. According to Meier (1995), the spores of this species are more resistant than the spores of many thermophiles.

### *Bacillus stearothermophilus* and *B. coagulans*

*Bacillus stearothermophilus* is gram-positive organism. This is a common thermophilic spoilage organism that normally produces no gas in spoiled packs that have been held at elevated temperatures around 50-55°C (122-131°F). If readily fermentable sugars are in limited supply and this has been found that can elevate pH (Kramer, 1989). The minimum pH for growth is around 5.3. The D values at 120°C (248°F) can be as high as 16.7 min (Davies, 1977). Prevention of spoilage is achieved by holding product

below 30-60°C (86-140°F) because it is often impracticable to try to process product for long enough to destroy spores. Under dry heat conditions at 121°C (249.8°F), the D value of spores of *B. stearothermophilus* can be as high as 936 min (Collier 1956). *B. coagulans* is also gram positive thermophile but differs from *B. stearothermophilus* in being able to grow at pH values down to 4.0 (Hersom, 1980). It is less resistant having a D value at 98.9°C (210.02°F) of 3.1 min (Hersom, 1980). It produces off flavors and souring of product during spoilage.

*Desulfotomaculum nigrificans*

Gram-positive organism. This organism causes sulphur stinker spoilage often resulting in blackened product when the steel in cans reacts with the H<sub>2</sub>S produced. D values as high as 55 min at 121°C (249.8°F) have been recorded (Donnelly, 1980). An unusual outbreak of spoilage caused by this organism in Japan was reported (Matsuda, 1982). The spoilage occurred in canned coffee and “Shiruko” (a soft drink made from red beans and cane sugar) produced for retail in hot vending machines at temperatures above 50°C (122°F).

## PART 2: THERMAL DESTRUCTION OF THERMOPHILIC BACTERIA

Thermophilic bacteria growth can be controlled by keeping food at temperatures below the minimum or above the maximum for the organism to grow. Bacterial spores are resistant to extremes of temperatures and pH, to desiccation, UV irradiation, enzyme action organic chemicals and may remain dormant for long periods (Moir, 1994). As was mentioned before, heat resistant spores are found among several species, mainly those belonging to the genera *Bacillus* and *Clostridium* (Hyung, 1983; Fernandez, 2001). Most of the research into heat resistance of spores has been done using mesophilic species in food industrial processes. Spores from thermophilic bacteria are more heat resistant than spores from mesophilic species (Warth, 1978). Heat resistant spores from thermophilic bacteria can be troublesome in research laboratories and routine autoclaving protocols of culture media and materials might be insufficient to inactivate bacterial spores. On the contrary, normal autoclaving procedures (20 min., 121 °C) may even activate and increase the apparent heat resistance of spores (Hyung, 1983; Byrer, 2000). Germination can be induced by nutrient germinant (Johnstone, 1994). Germination involves sequences of events, which result in a breakdown of the spore structure. Consequently, spores lose their resistant properties and become hydrated, which can be observed as a phase change from bright to dark to phase contrast microscopy (Moir, 1994). The first high decimal reduction value DT (the incubation time at temperature T necessary for a 90% decrease in the viability of the spores. For thermophilic species was reported in 1965 for *Clostridium thermoaceticum* with a  $D_{124}$  of more than 72 minutes (Xezones, 1965). More recently, extremely heat resistant spores have been detected in other thermophilic *Clostridium* and *Moorella* species, i.e. *Cl. thermohydrosulfuricum* ( $D_{120} =$

11 min.) (Hyung, 1983), *Cl. thermoautotrophicum* ( $D_{120} = 70$  min.) (Van Rijssel, 1992), and *Moorella thermoacetica* JW/DB2 and JW/DB4 ( $D_{120} = 84$  min., 111 min.) (Byrer, 2000), respectively. What determines the spore heat resistance is not known with certainty and multiple factors are involved, such as the composition of the proteinaceous spore-coat (Henriques and Moran, 2000), the dipicolinic acid concentration, thickness, and  $Ca^{++}$  content of the spore-cortex (Beaman and Gerhardt, 1986), the dehydration and mineralization state of the spore (Popham, 1999), and specialized DNA-binding proteins termed  $\alpha/\beta$  type small acid soluble spore proteins (SASP) (Setlow and Setlow, 1998).

### Steam sterilization

Steam sterilization has long been used in hospitals as well as in the pharmaceutical, aseptic processing, and food industries. In many ways, it has been a product of its own successes. For example, steam is most often characterized by its overkill. It uses extremely high temperatures to inactivate highly resistant nonpathogenic thermophile spores and, more recently, extremely resistant prions that other sterilization methods cannot destroy (Pflug., 2001). Steam sterilization is generally carried out at 121°C (250°F) for 15 minutes or at 134°C for 3 to 4 minutes. Temperatures can be reduced to 115°C, and even as low as 105°C, depending upon the integrity, heat resistance, and characteristics of the material being sterilized (Perkins, 1970). Steam is readily available, delivers high heat condensation, and it is an activating agent. Before a dormant spore can begin germinate and grow, it must be activated (Pflug, 2001). However, at higher temperatures, steam becomes sporicidal. Sterilization, by definition, destroys or eliminates resistant microbes, including bacterial spores. More resistant virulent agents

(e.g., prions) cannot be eliminated using most standard methods. Extended and high steam sterilization, however, can at least reduce the resistance of these organisms. Using the classical definition of sterilization, it is an absolute principle (Pflug, 2001), a method of destroying or eliminating all forms of life. In practice, however, sterilization is best defined as a process that is capable of delivering a certain probability that a treated product or material is free from viable microorganisms, including resistant agents, such as *Bacillus anthracis* and *smallpox*, and prions.

#### Dry and Moist heat treatment

Heat can result in death or injury. In addition, many food conditions can alter the heat resistant of microorganism. There are several heat treatments used to kill microorganisms in food. Including, incineration, boiling, and autoclaving. Dry and moist heat have been applied to destroy microorganisms. Dry heat is rarely used and may be different physiologically from moist heat destruction (Pflug, 1960). Moist heat is defined as heating in a medium where the relative humidity is 100%. Dry heat has been less defined, but refers to an environment where the relative humidity is less than 100%. Heat resistance of bacteria is normally higher in dry heat conditions as contrasted to moist heat conditions (Holdsworth, 1997). Moist heat has shown to act on the denaturation of different proteins causing the bacterial death of the cell (Farkas, 1997). Nevertheless, foods that are higher in fat and oil (beef) require enhanced thermal processing due to the increased thermal resistance of microorganisms found in these foods (Farkas, 1997). The diverging heat resistance of similar microorganisms is directly related to the varying foods in which they are found.



### Thermal destruction, Z and D value definitions

Numerous methods are offered to calculate thermal processes for the inactivation of bacterial spores in foods. The kinetics calculated from the reaction rate is proportional to the concentration of the number of microorganisms (Holdsworth, 1997).

The thermal death time (TDT) (time necessary to kill a given number of organisms at a specific temperature) is based on an empirical approach to the temperature dependence of the first order destruction of microorganism. Ramaswamy (1989) stated that the Arrhenius theory contradicts the TDT technique. The TDT method affirms that kinetic parameters are reciprocal to temperature (Ramaswamy, 1989). Pflug (1982) analyzed the effectiveness of each method and concluded that the TDT method could be used in the laboratory and manufacturing plants to validate and monitor sterilization processes (Ramaswamy, 1989). The TDT is used to define the temperature sensitivity of the kinetic parameters involved in thermal inactivation (Ramaswamy, 1989). The thermal resistance of bacterial cells has shown that exponentially growing cells are more sensitive to heat than stationary growing phase cells. The death of bacterial cells exposed to thermal treatments at a specific temperature is believed to occur at a constant rate (Farkas, 1997) and the recovery of cells after thermal processing allows the construction of survivor curves. The curve is a scheme of  $\log N$  (axis X), which is the number of colony forming units (cfu), in opposition to time at a specific temperature (Holdsworth, 1997). Figure 2.

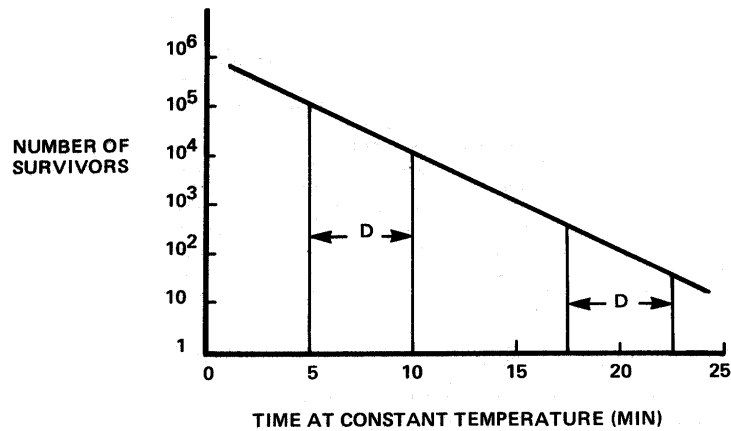


Figure 2. Typical the thermal death time curve.

This is based over the assumption that the logarithm of the first order rate constant (D-value) is directly proportional to temperature (Ramaswamy, 1989). Decimal reduction time (D-value) is the time required to destroy 90% of the organisms at a specific temperature. Thus after an organism is reduced by 1 D, only 10% of the original organisms remain. In addition, this has been defined as the time in minutes required to reduce a bacterial population by one  $\log_{10}$  (Holdsworth, 1997). D value is a measure of the heat sensitivity of an organism. D value can be calculated for a particular microorganism at a specific temperature.

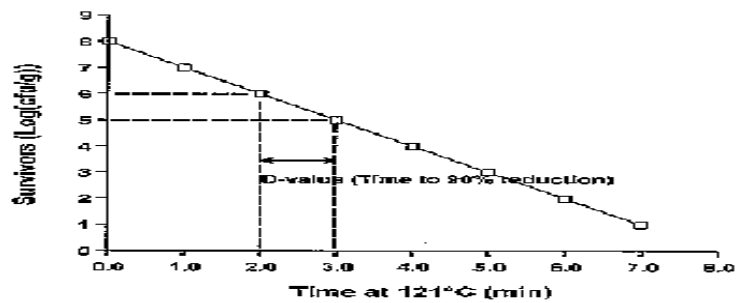


Figure 3. Decimal reduction curve.

The *D*-values of microbial spores are greater in dry heat than in moist heat (Collier, 1956). *D* value is determined by inoculating the target bacteria in food substrate then heating to a specific temperature and taking samples for enumeration timed intervals. Survivors are measured by counting cells capable of growing after recovery. The  $\log_{10}$  of survivors are plotted against time in minutes. The inverse of the slope of the semi logarithmic curve ( $-1/D$ ) is the *D* value, and it is important to remember that most *D* values are calculated using this method (Peleg, 1999). The *Z* value refers change in temperature ( $^{\circ}\text{C}$  or Fahrenheit) required for the thermal destruction curve to transverse one log cycle. It is determined from a plot of the logarithm of *D* values against their particular temperature (Holdsworth, 1997). *Z* values can be calculated for any two values of *D* and the corresponding temperatures (Holdsworth, 1997). Nevertheless, the suspending medium plays an important role in this aspect when these studies are made.

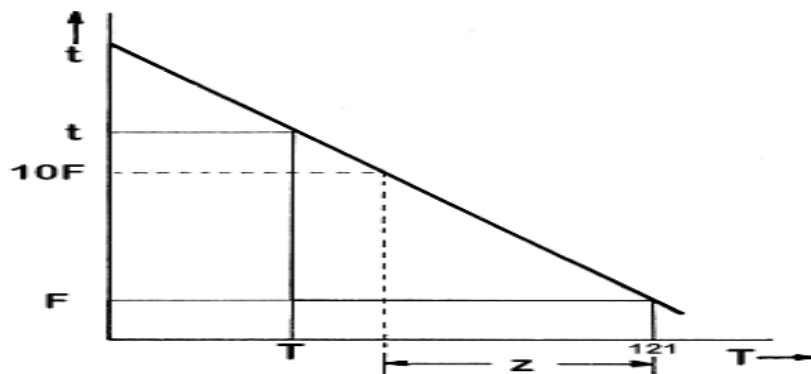


Figure 4. Standard curve for the change in temperature required for the thermal destruction curve to transverse one log cycle.

While the D value gives the time needed at a certain temperature to kill a certain percentage of a population, the Z value relates the resistance of an organism to different temperatures. Therefore, the Z value allows calculating a thermal process of equivalency, if there are one D value and the Z value (Holdsworth, 1997). Thus, if it takes an increase of  $-12.22\text{ }^{\circ}\text{C}$  ( $10^{\circ}\text{F}$ ) to change the log of D value 1 unit, then Z value is 10. Therefore, if the D value at  $65.55^{\circ}\text{C}$  ( $150^{\circ}\text{F}$ ) is 4.5 min, we can calculate D values for  $71.1^{\circ}\text{C}$  ( $160^{\circ}\text{F}$ ) by reducing the time by one log. Therefore, the D value for  $71.1^{\circ}\text{C}$  ( $160^{\circ}\text{F}$ ) is 0.45 minutes. This means that each  $10^{\circ}\text{F}$  ( $-12.22^{\circ}\text{C}$ ) increase in temperature will reduce the D value by one log. Conversely, a  $10^{\circ}\text{F}$  ( $-12.22^{\circ}\text{C}$ ) decrease in temperature will increase the D value by one log. Consequently, the D value for a temperature of  $60^{\circ}\text{C}$  ( $140^{\circ}\text{F}$ ) would be 45 minutes. Reactions that have small Z values are highly temperature dependent, whereas those with large Z values require larger changes in temperature to reduce the time. A Z value of  $10^{\circ}\text{C}$  ( $49.99^{\circ}\text{F}$ ) is typical for a spore forming bacterium.

## CHAPTER III

### MATERIALS AND METHODS

#### Sampling

The organism used in this study was isolated from raw poultry offal obtained from a poultry processing plant, which is normally destined for rendering. The bacterial isolates were obtained from Dr. Annel Greene's Lab at Clemson University, SC. who subjected raw ground offal to two treatments: 1) Autoclaved cycle for 30 minutes 121°C (250°F) under pressure 15 psi and 2) Heated at 127°C (260°F) for 15 minutes.

Bacteria surviving each treatment were isolated on Brain Heart Infusion + 1% yeast, then two separate colonies from each treatment were isolated on Tryptic Soy Broth (TSB) tube, and marked as A<sub>1</sub> and A<sub>2</sub> (for autoclave treated) and H<sub>1</sub> and H<sub>2</sub> (for heat treated). Tubes were incubated at 37°C overnight with vigorous shaking. A sample of both treatments (heat and autoclave) were isolated separately in Tryptic Soy Agar and Trypticase Soy Agar w/5% sheep blood at the same time to determine the best growth medium. To simulate conditions prior to receiving and hold offal by rendering facilities, samples from both mediums (TSA and TSA w/5% sheep blood) were held at 37°C (98.59°F) from 2-4 days for spore formation. The A<sub>1</sub> treatment was selected for this study because of its clearer appearance on the plate after streaking compared to isolates from other treatments. The colony population in both agars differed in appearance.

### Spore stain (Schaeffer and MacDonald 1933)

Spore preparation was performed according to the work of Laurent (1999). In brief, vegetative cells and spores were prepared by plating 0.5 ml of a 48 h TSB (Difco Laboratories, Detroit, Mich.) vegetative culture onto BBL Trypticase Soy Agar (TSA) w/ 5 % Sheep Blood plates. After 2 days at 37°C, spores and vegetative cells were harvested by depositing 3 ml of peptone solution on the surface of each plate and rubbing gently with a sterile plastic spreader. The suspension of spores and vegetative cells was collected in a sterile tube. Pooled suspensions were centrifuged at 3000 x g for 20 min (IEC HN-SII Centrifuge, International Equipment Company, Inc. Needham Heights, MA), and the supernatant liquid was discarded. The resulting pellet was suspended in 4 ml of peptone solution (0.1%) to obtain a concentration of  $10^7$ - $10^9$  suspension/ml and the solution was vortexed (Fisher Brand® Vortex Genie 2).

The suspension was smeared on a glass slide and fixed with a Bunsen flame. Slides were flooded with 5% aqueous malachite green (Fischer Scientific Co. Fair Lawn, NJ, USA). Slides were intermittently heated with a Bunsen flame for approximately five min, to ensure that the dye remained hot but not boiling. Slides were rinsed with tap water, and then counterstained with 0.5% Safranin-O (Sigma Chemical Co., St. Louis, MO, USA) for 1 min. After drying, the slides were examined using the oil immersion power of a light microscope (Olympus BH-2, Tokyo, Japan) and found to contain few vegetative cells. This statement was based on the color difference after spore stain. See Figure 5.

Tentative identification of isolates were made by the Microbiology Department at Clemson University. Plates of TSA w/5 % Sheep blood were prepared using the streak method. A loopful of a 48h culture in TSB (Difco Laboratories, Detroit, Mich) was transferred onto the surface of TSA w/5 % Sheep blood agar which were incubated at 37°C (98.59°F) for 48 h. Spore formation occurred after two days. The staining method was used to verify the presence of spores using a microscope. The process was repeated twice.

The isolate was grown on PEMBA (polymyxin pyruvate egg yolk mannitol bromothymol blue agar) which is selective for *Bacillus cereus*.

#### Preparation of Inoculum

To prepare inoculums, 0.1 ml of isolated bacteria from 10 ml of BHI broth + 1% yeast was pipetted into 10mL of TSB (Difco Laboratories, Detroit, Mich.) under a Germfree® Bioflow Chamber. The TSB tube was placed in water bath (Bellco, Sciera, Vineland, New Jersey, USA) and aerobically incubated at 37°C for 16-18 hours with continuous shaking.

#### Model Media

Ground beef with 21% protein, 19% fat, and 60% moisture was used as a model media to simulate raw rendering material. Typically the proximate composition of raw poultry offal is about 10% fat, 25% protein, and 65% moisture (Prokop 1992), depending upon the raw mixture of co-products. The meat/fat mixture was irradiated with  $\gamma$  <sup>60</sup>Co rays to ensure sterility prior to incubation (Physics Department, Auburn University, AL). A bacterial suspension containing approximately 10<sup>9</sup> CFU/ml was poured evenly inside 50 grams of the sterile ground beef /fat contained in a sterilized bag and distributed

throughout by kneading for 5 min. After mixing, 1.5 g with approximately  $3 \times 10^8$  CFU/ml of the inoculated ground beef /fat mixture was weighed and added to a Pyrex tube (test tube).

#### Thermal inactivation studies

Sterile model media samples were aseptically removed from the freezer and thawed in the refrigerator one day before use. Preliminary testing was performed at 87°C, 95°C, 96°C, 98°C, and 115°C to determine the best temperatures for determining the thermal inactivation parameters (including D and z values). D values (in min) were determined from survivor curves plotted using linear regression analysis.

Model media samples were thermally treated using dry bath incubator (Fischer Scientific 711, Pittsburgh, P.A). Pyrex tubes with the model medium were placed in the heating block (Fischer Scientific, Pittsburgh, Pa.) simultaneously. Each tube was covered with heavy duty aluminum foil to prevent moisture loss. This procedure was developed to simulate heating during rendering. Model media temperature was measured using thermocouples (6 inches, Omega, Engineering, Inc.) oriented at the center of each sample. Time and temperature heating data were recorded using a channel datalogger and thermal processing software (CALPlex 32, TechniCal, New Orleans, LA). Once the treatment temperature was reached, samples were removed at intervals of 5 or 10 min. After removal from heat, samples were placed into an ice bath for 15 s to minimize any further thermal effects. Each sample was homogenized with 20 ml of sterile 0.1% peptone solution and the big meat particles inside the test tubes were broken up. Homogenates were then serially diluted and surface plated on TSA w/ 5% Sheep Blood. Plates were incubated at 37°C for 48 h before enumerating colonies.



## Statistical Analysis

Three separate replications were conducted to illustrate the variation between replications, using bacteria at each temperature 91, 95, and 96°C, resulting in nine different examples. The total bacterial counts (TBCs) were expressed as CFU/ gram of model media. The TBCs were then transformed into  $\log_{10}$  cfu/g values because logarithms convert a multiplicative relation to additive one.

The data were fitted to three models. The first model was a linear model to relate TBC to time in each of the nine experiments. The form of this model was:

$$Y = a_0 + a_1X + E$$

Where,

**Y** is LCFU (log of colony forming per unit).

**a<sub>0</sub>** is the intercept of the model for log cfu/g vs. time

**a<sub>1</sub>** represents the slope of the model for log cfu/ g vs. time.

**X** represents time in minutes

**E** represents error.

Estimates of **a<sub>1</sub>** could be used to then estimate D and z values.

The second model involved fitting a step function with different segments to further understand the relationship of TBC and time in each of the nine experiments. The form of this model was:

$$Y = (a_0 + a_1X)Z_1 + (b_0 + b_1X)Z_2 + (c_0 + c_1X)Z_3 + \dots + (g_0 + g_1X)Z_8 + E.$$

Where,

**Y** is LCFU.

**a0, b0, c0, d0,e0, f0, and g0** are the intercepts for the different segments of the model for log cfu/g vs. time

**a1, b1, c1, d1,e1, f1, and g1** are the slopes for the different segments of the model for log cfu/g vs. time

Z1, Z2, Z3, Z4, Z5, Z6, Z7, and Z8 are the dummy variables indicating which segment of the model for log cfu/g vs. time is being considered. For example Z1 = 1 for the segment of the model with parameters a0 and a1, and Z1=0 for all other segments of the model.

Not all of the nine temperature and replication combinations required all of the eight segments. To find an appropriate number of segments to use in modeling log cfu/g vs. time, we used a hypothesis test and an F-statistic. For example, to determine a model with two segments was better than a model with only one segment (the linear model), the hypotheses were

Ho:  $b_0 = b_1 = 0$

Ha:  $b_0 \neq 0$  and/or  $b_1 \neq 0$

and the F-statistic was

$$F = \frac{[SSE(a_0, a_1) - SSE(a_0, a_1, b_0, b_1)]/2}{SSE(a_0, a_1, b_0, b_1)/n-4}$$

If we failed to reject Ho, we concluded that the one segment (the linear model) was appropriate. If we rejected Ho, we concluded that at least two segments were appropriate and tested to determine if a model with three segments was better than a model with only one segment (the linear model).

The third model involved fitting a three-segment function to all nine experiments. Comparing the model parameter estimates among the nine experiments allowed us a

quantitative method to contrast the relationship of log cfu/g vs. time among the experiments. The form of this model was:

$$Y = (a_0 + a_1X)Z_1 + (b_0 + b_1X)Z_2 + (c_0 + c_1X)Z_3 + E.$$

Where,

**Y** is LCFU.

**a<sub>0</sub>, b<sub>0</sub>, and c<sub>0</sub>** are the intercepts for the three different segments of the model for log cfu/g vs. time

**a<sub>1</sub>, b<sub>1</sub>, and c<sub>1</sub>** are the slopes for the three different segments of the model for log cfu/g vs. time

**Z<sub>1</sub>, Z<sub>2</sub>, and Z<sub>3</sub>** are the dummy variables indicating which segment of the model for log cfu/g vs. time is being considered. For example  $Z_1 = 1$  for the segment of the model with parameters  $a_0$  and  $a_1$ , and  $Z_1=0$  for all other segments of the model.

All calculations were performed using procedure NLIN of SAS.

CHAPTER IV  
RESULTS AND DISCUSSION

Microbiological analyses

Strain characteristics: cell morphology

The isolate was Gram-positive, motile, usually catalase positive, 1.2  $\mu\text{m}$  wide, and 5.3  $\mu\text{m}$  long. It occurred singly or in chains and in monomorphic forms. Spores were subterminal to terminal, and ellipsoidal in shape. Predominantly unswollen spore cases were also seen. The isolate grew well aerobically on TSA w/5 % sheep blood at 37°C for 48 h, but it did not grow anaerobically under the same conditions. On nutrient agar, the colonies usually were circular with smooth edges, bright, and convex. Their size varied from 1mm to 4 mm in diameter. There was not more than one spore per cell, and sporulation is not repressed by exposure to air.

| <b>Characteristics A<sub>1</sub></b>          | <b><i>Reaction</i></b> |
|---|------------------------|
| Rod-shaped in young cultures                  | +                      |
| Aerobic                                       | +                      |
| Diameter over 2.5 $\mu\text{m}$               | -                      |
| Filaments                                     | -                      |
| Rods or filaments curved                      | -                      |
| Endospores produced                           | +                      |
| Motile  | +                      |
| Stain Gram positive at least in young culture | +                      |
| Catalase test                                 | +                      |

Table 2. Some characteristics about of Bacteria studied

## Genus Bacillus

A unique characteristic of this genus is its ability to produce endospores when environmental conditions were stressful, as shown in Figure 5. (Picture taken during study under microscopy in the Microbiology department at Clemson University).

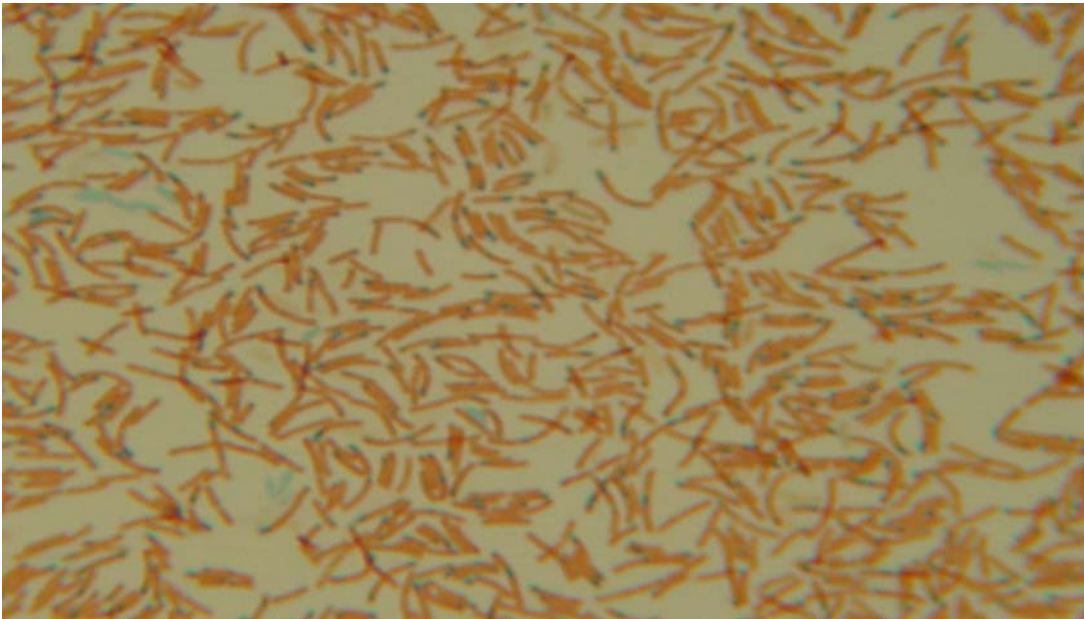


Figure 5. Spore and vegetative cell preparation

There was a strong similarity in appearance between the two replications for samples from each isolation medium (TSA and TSA w/5% Sheep blood). In addition, the following characteristics were found for each sample based on visual observations under magnification.

### Conditions:

Temperature: 37°C

Duration: 4 days

Agar: Tryptic Soy Agar w/ 5% Sheep Blood

Thermo-spore observations

- **A<sub>1</sub>**: thermo-spores. (A lot spores) There was not more than one spore per cell.
- **A<sub>2</sub>**: Has a few spores. “It was not the same bacteria than A<sub>1</sub>”
- **H<sub>1</sub>**: Many spores. “Possibly different culture”.
- **H<sub>2</sub>**: Had a fewer spores than A<sub>1</sub> but more than A<sub>2</sub>.

The results after growth on the PEMBA found not positives colonies for *Bacillus cereus* bacteria.

The bacterial strain and growth phase also determines a microorganism thermal resistance. Cells in stationary phase growth are generally more resistant than cells in log phase growth (Doyle, 2001). The culture used in this research were grown under aerobic conditions for 16-18 hours prior to use in an attempt to achieve stationary phase growth (Table 2). The duration of incubation was chosen for visual observations after preliminary studies, and these observations were used by other researchers on sporeformer microorganisms (Iciek, 2005). *D*- values can also vary between different strains of microorganisms, and different growth conditions under which the bacteria are cultured (Doyle, 1989). Based on the assumption of an exponential rate of death, higher initial thermal death rates have been attributed to the presence of endospores of different

innate resistances in a given population (Ball and Frank, 1957). When a population is heated, in the first seconds or minutes of exposure to high temperatures, the process of the spore activation and vegetative cell destruction begins. (Figures 6, 7, 8, 9,10, 11,12, 13, and 14). Under certain thermal processing conditions, bacterial spore destruction slows with a “tail” in the last stage of the thermal death curve resulting from the presence of extremely heat resistant spores that are in a state of deep dormancy. To be inactivated they must be exposed to elevated temperatures. Temperatures above 60°C will generally inactivate vegetative cells, although the presence of salts or fat in the surrounding medium can increase resistant of cells to heat (Doyle, 1989). Previous research has shown conditions with growth at a higher temperature and a heat shock of 55°C for 30 minutes increase heat resistance (Fernandez, 2001). Spores are also more heat resistance when tested in water compared to phosphate buffer and are even more resistant in meat (Doyle, 1989).

The experiment utilized bacterial cells from raw animal co-products that survived heat treatments approaching those used in the commercial rendering process. Aerobic plate counts of heat resistant poultry offal isolate after come up time were reduced from 6.21 to 4.93 logs cfu/g at 91°C for 90 minutes, at 95°C were from 6.62 to 4.76 logs cfu/g for 165 minutes and at 96°C from 6.23 to 2.7 logs cfu/g for 90 minutes. The ranges of log reduction were 1.28, 1.86, and 3.53 respectively. Counts of colonies were highest at 91°C ( $1.05 \times 10^5$  cfu/g) and lowest at 96°C ( $1.40 \times 10^1$ ). Based on these results the log reduction was faster at higher temperature than the lower temperatures as would be expected.

### Heating profile of model media

Come up times (the time for the thermocouple temperature in the model medium to equal the heating block temperature setting) of bacteria for the three replications at each temperature were 3, 4:30 and 8:40 minutes at 96°C, at 94°C the come up times were 10:20, 9:30, and 7:20 minutes. For 91°C the come up times for replications were 4, 4:50, and 6:20 minutes, respectively. The come up time can affect the overall killing effect of the thermal treatment. The rate of the temperature increase was slower at 95°C compared to the other temperatures. The heating times (averaged for the 14 test tubes at 91°C and 96°C, and 17 test tubes for 95°C) are shown in the Table 3.

| Experiment no. | Replication No | Target Temperature | Actual Temp. (°C) | Come-up time (min) <sup>a</sup> |
|----------------|----------------|--------------------|-------------------|---------------------------------|
| 1              | 1              | 91                 | 90.76             | 4:00                            |
|                | 2              | 91                 | 91.02             | 6:20                            |
|                | 3              | 91                 | 90.88             | 4:50                            |
| 2              | 1              | 94                 | 94.77             | 10:20                           |
|                | 2              | 94                 | 94.55             | 9:30                            |
|                | 3              | 94                 | 94.94             | 7:20                            |
| 3              | 1              | 96                 | 95.80             | 3:00                            |
|                | 2              | 96                 | 95.98             | 4:30                            |
|                | 3              | 96                 | 95.89             | 8:40                            |

<sup>a</sup> Time required for the temperature at the center of the meat medium.

Table 3. Summary of heat treatments applied to spores and vegetative cells of heat resistant bacteria in a meat medium.



## Thermal Inactivation Models

Temperatures used in this study were 91°C, 95°C, and 96°C. The temperatures were chosen in this range because preliminary studies shown a lot population at 87°C and preliminary studies show that at 98°C the bacteria population did not survive. Linear regression were established for each temperature and replicate combination, and the  $D$ -values for the individual experiments were obtained as the inverse negative of the slope (a0) of the linear regression line. The line was used for the first 65 minutes of the curve (Figures 6, 7, and 8) because until this time all replications shown a linear behavior.  $D$ -values are reported in minutes, and are defined as the time required to achieve a 1-log reduction in the bacterial population a set temperature. The  $z$ -value was determined by plotting the logarithmic of the  $D$ - values versus temperature at 91°C, 95°C, and 96°C (Holdsworth, 1997).

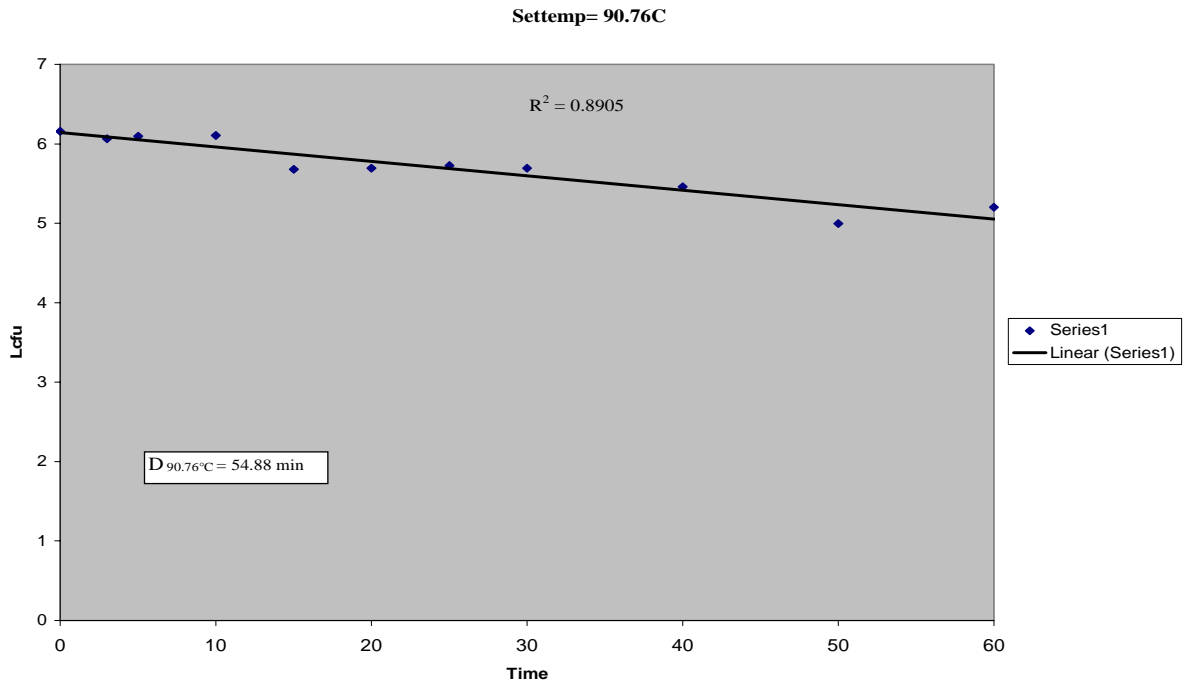


Figure 6. Surviving heat resistant bacteria at 90.88°C. First replication.

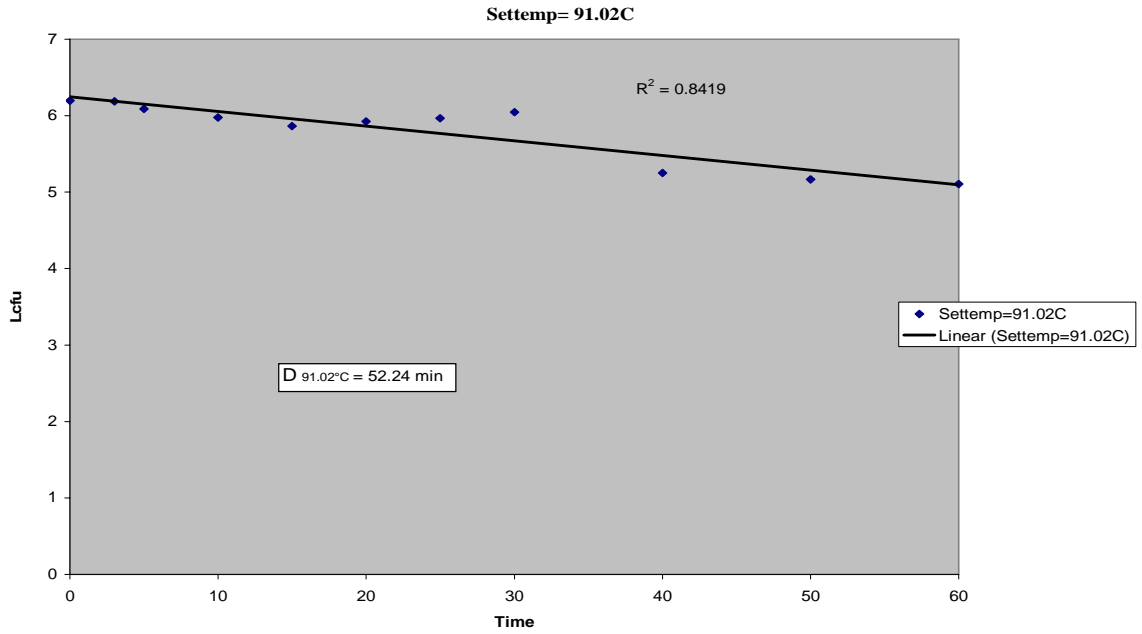


Figure 7. Surviving heat resistant bacteria at 91.02°C. Second replication.

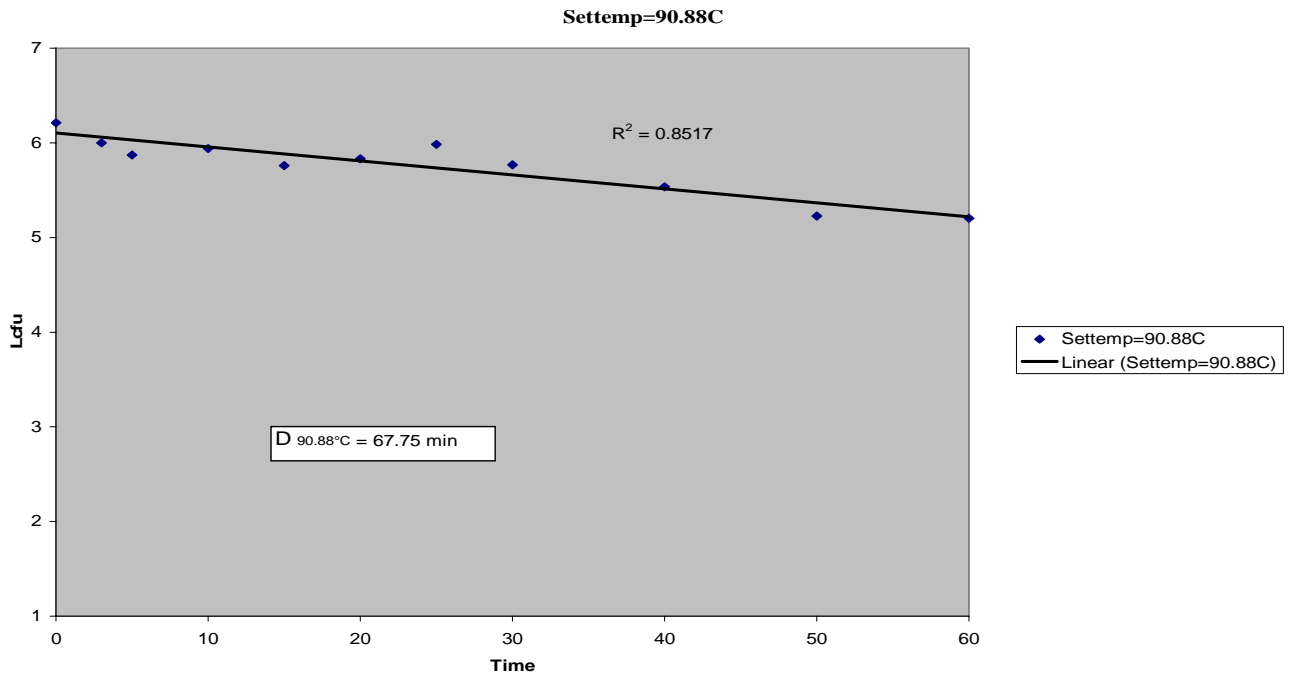


Figure 8. Surviving heat resistant bacteria at 90.88°C. Third replication.

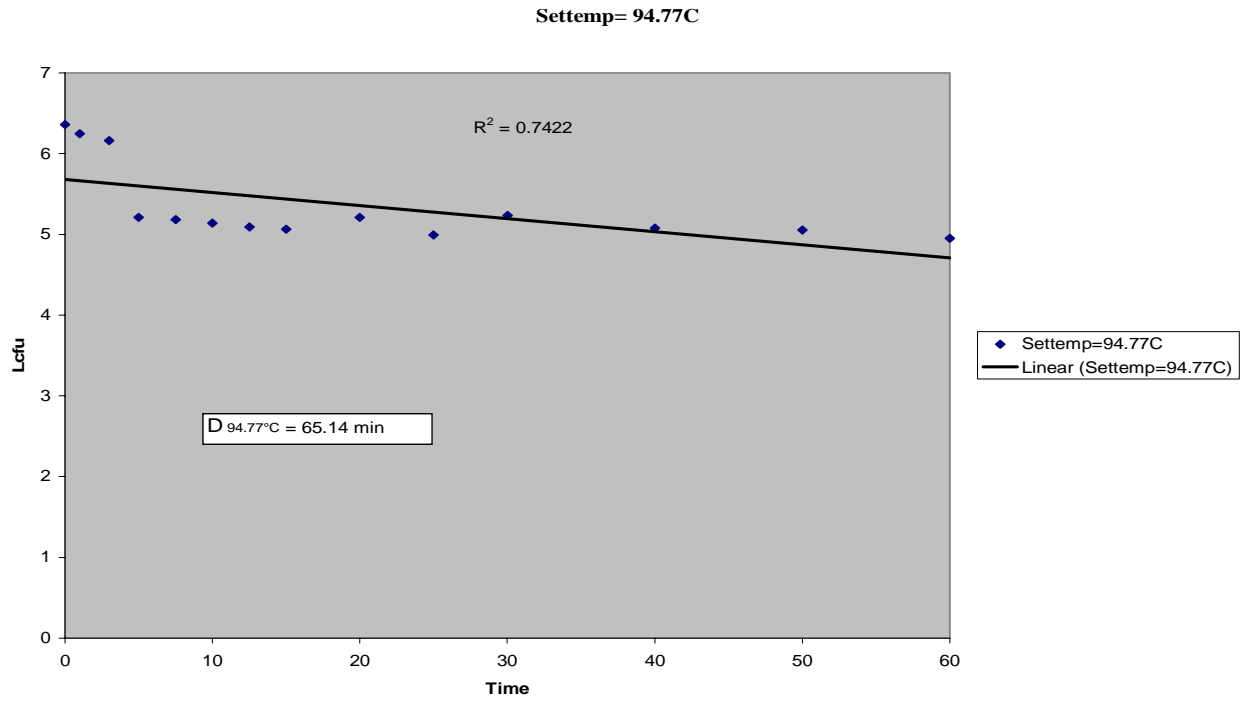


Figure 9. Surviving heat resistant bacteria at 94.77°C. First replication.

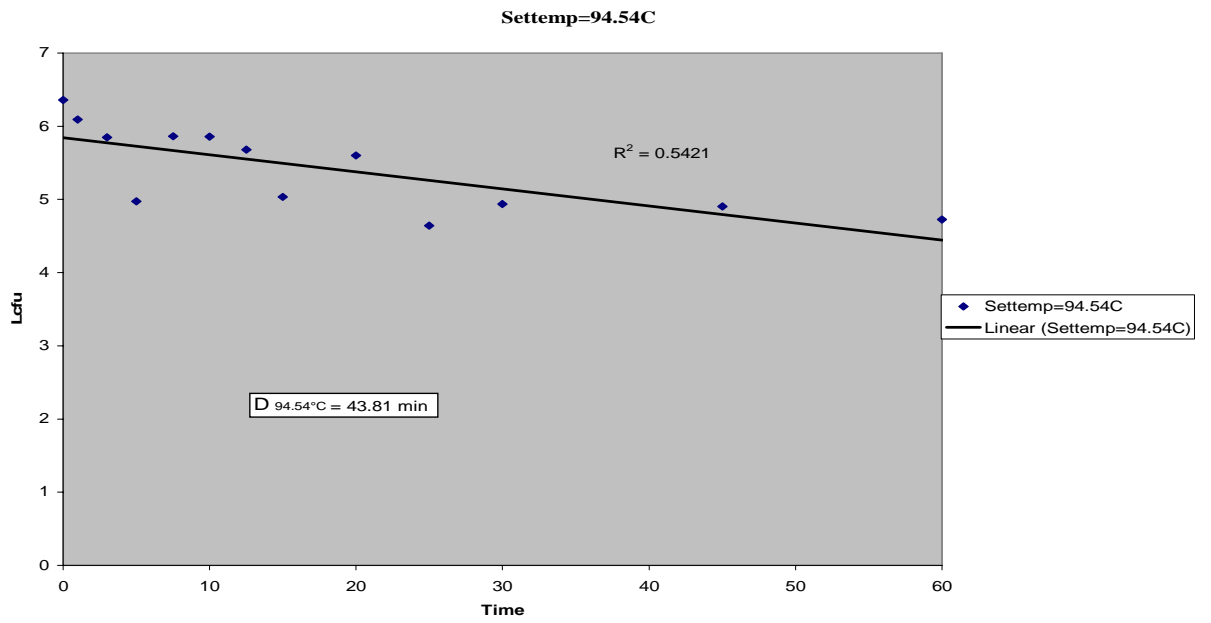


Figure 10. Surviving heat resistant bacteria at 94.54°C. Second replication.

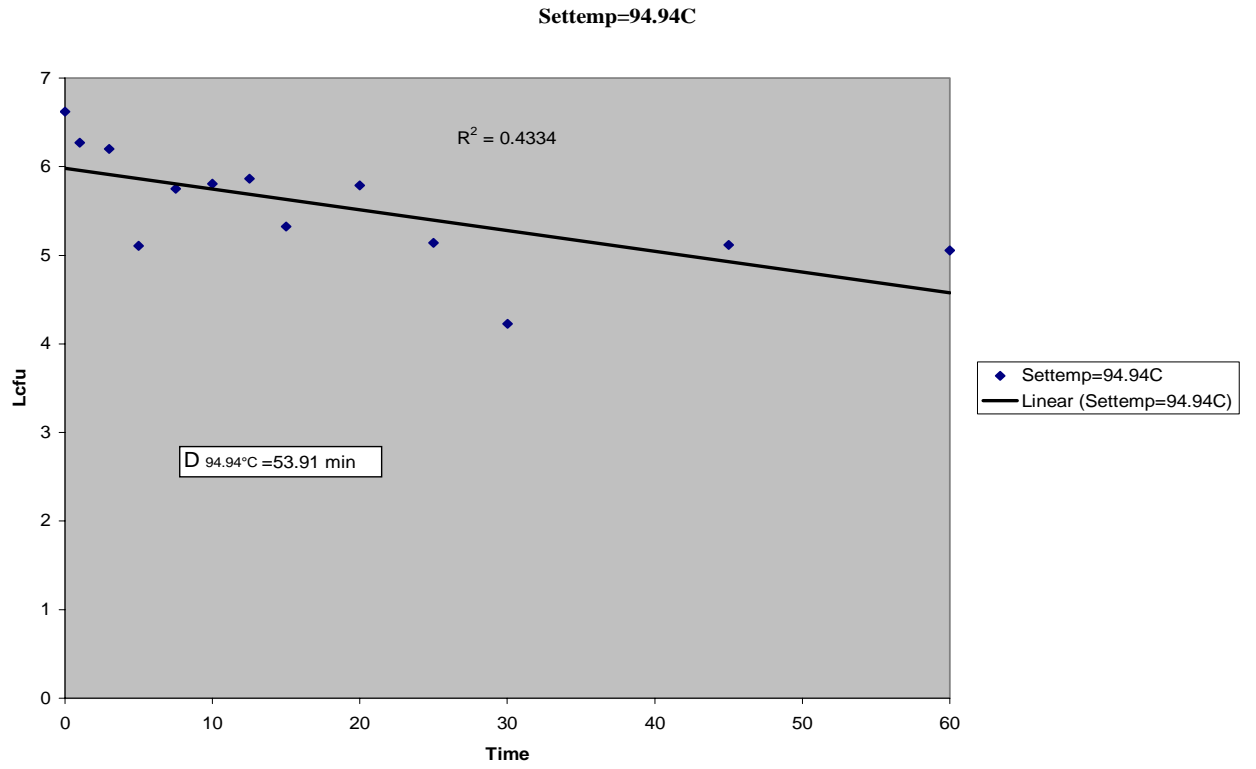


Figure 11. Surviving heat resistant bacteria at 94.94°C. Third replication.

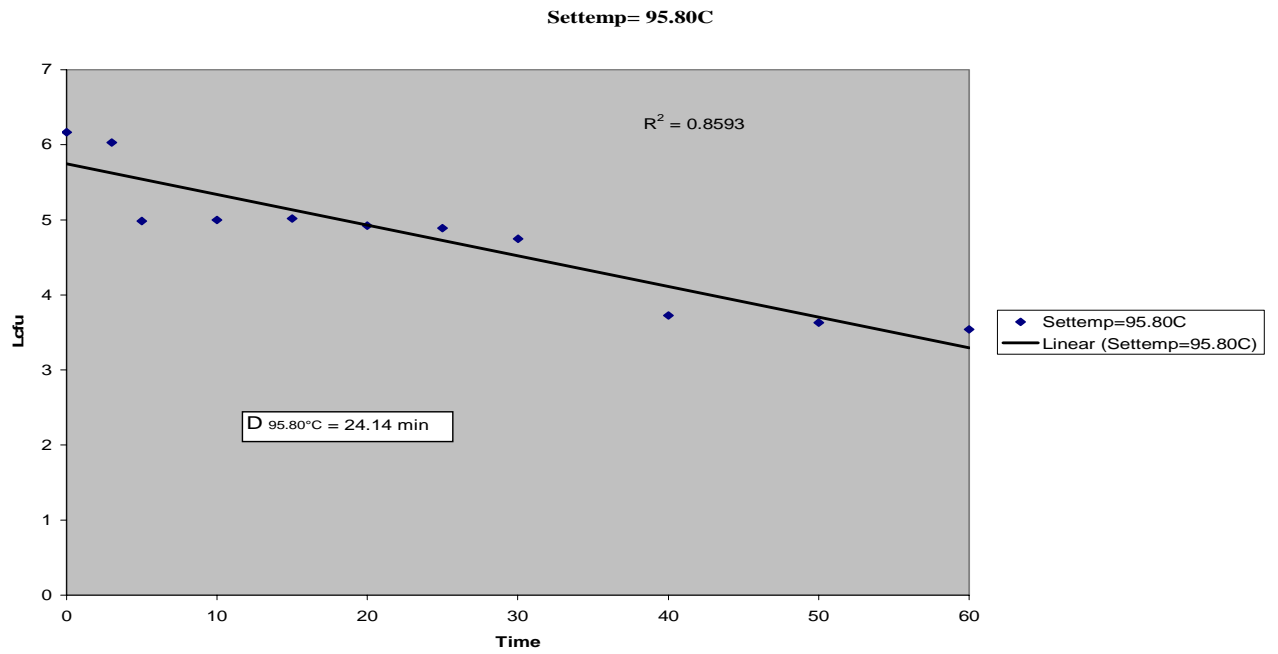


Figure 12. Surviving heat resistant bacteria at 95.89°C. First replication.

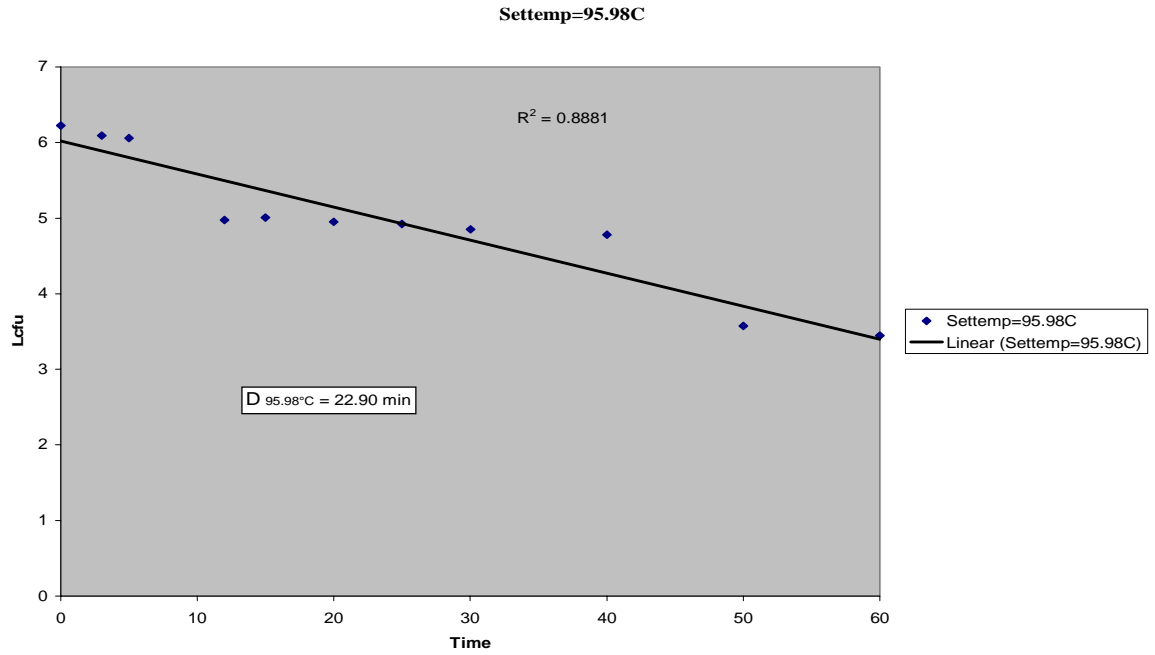


Figure 13. Surviving heat resistant bacteria at 95.98°C. Second replication.

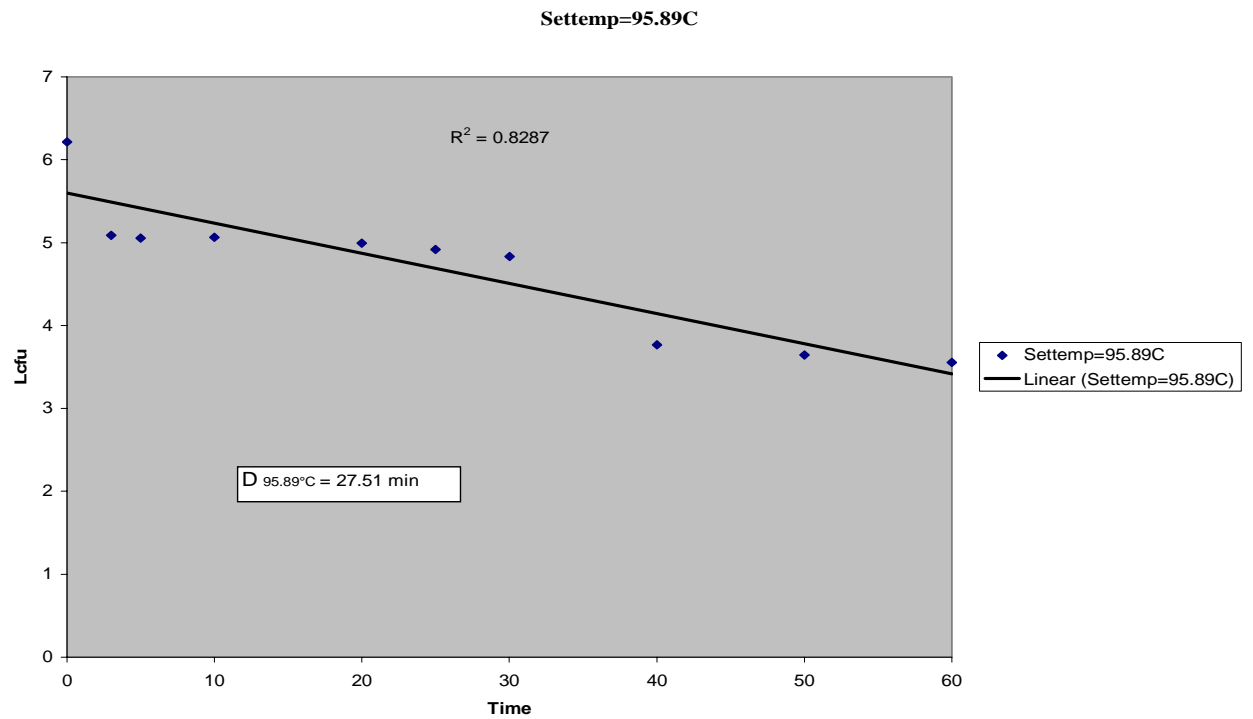


Figure 14. Surviving heat bacteria at 95.89°C. Third replication.

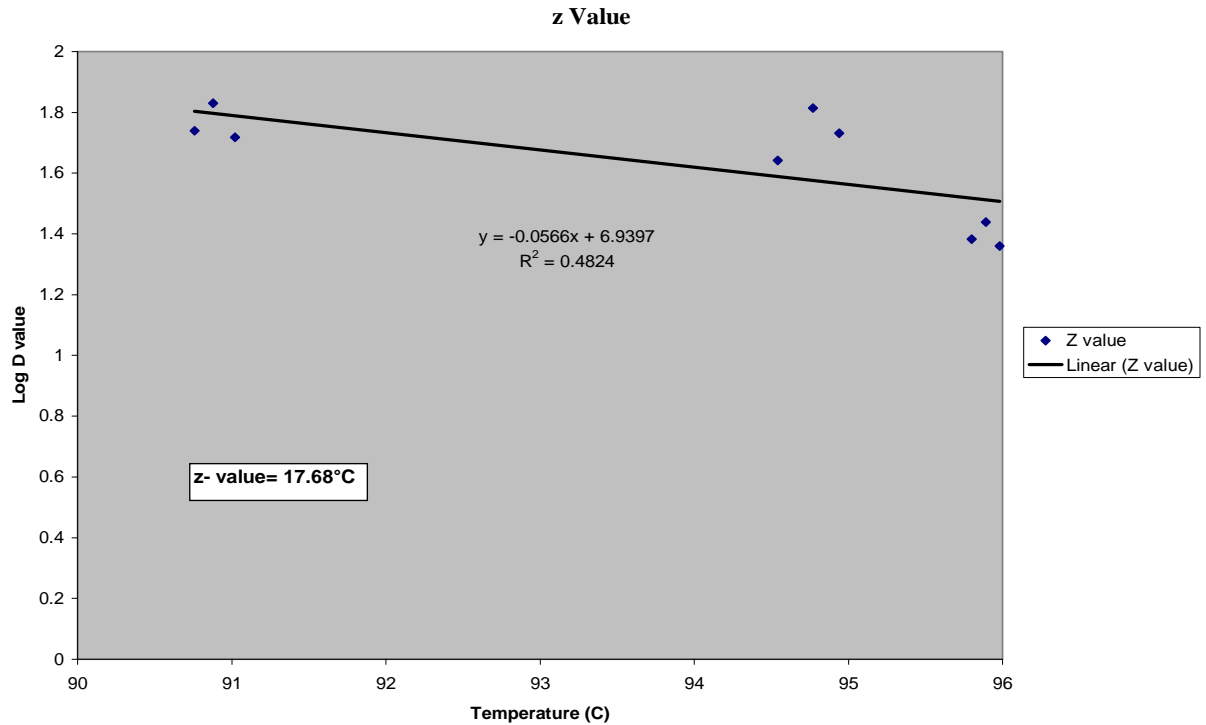


Figure 15. Plot of  $\text{Log}_{10}$  of D-values of heat resistant bacteria at 90.76°C, 91.02°C, 90.88°C, 94.77°C, 94.54°C, 94.94°C, 95.80°C, 95.98°C, and 95.89°C.

Heat treatment of the model medium containing both spores and vegetative cells of the poultry offal isolate at 90.88°C for 90 minutes resulted in a reduction in viable spore and cells by a factor of  $10^{3.6}$ . A reduction by a factor  $10^{4.0}$  cells in viable spores and vegetative cells occurred at 94.98°C for 148 minutes. At 95.91°C for 90 minutes, the reduction in the number of viable spores and vegetative cells was by a factor  $10^4$

The *D*- value for the offal isolate at 90.88°C was 57.55 minutes, at 94.98°C the *D*- value decreased to 56.4887 minutes, while at 95.91°C the *D*- value decreased to 25.1284 minutes (Table 4). The *z*- value was calculated using the logarithm of *D*- value versus the temperature at 90.88°C, 94.84°C, and 96°C. The *z*- value was 5.55°C (Figure 9).

| Replication | Temperature °C | D- value (minutes) |
|-------------|----------------|--------------------|
| 1           | 90.76          | 54.88 ± 5.8038     |
| 2           | 91.02          | 52.24 ± 6.8266     |
| 3           | 90.88          | 67.75 ± 8.5236     |
| 1           | 94.77          | 65.15 ± 23.8798    |
| 2           | 94.54          | 43.81 ± 13.0668    |
| 3           | 94.94          | 53.91 ± 17.8324    |
| 1           | 95.80          | 24.14 ± 3.0251     |
| 2           | 95.98          | 22.89 ± 2.4498     |
| 3           | 95.89          | 27.51 ± 3.9546     |
| <i>z</i> °C |                | 17.68 ± 0.5429     |

Table 4. *D*- and *z* values of heat resistant bacteria studied in model media (ground beef).

The *D*- values and *z*- value (Table 4) can be affected by a wide array of variables, which should be considered when discussing *D* values. Fat content can impact thermal resistance of spore-former bacteria, as *D* value in foods high fat (Figure 6) content have been reported to be four to eight times higher than in a low fat medium (Doyle, 2001). At 94.84°C, *D* value was higher compared to the other temperatures. An explanation for that could be that the come up time at 94.84°C was slower than at the other temperatures. The longer come up time may have resulted in greater destruction of cells and spores of lower heat resistance leaving more heat resistant cells in the medium. There is also some evidence that spores or vegetative cells suspended in lipid medium (ground beef) might survive exposure to temperatures used in the conventional heat sterilization of foods

(Jensen, 1954; Ingram, 1955; Hersom, 1980). If heat penetration is assumed to take place at about the same rate, and the composition of the meat medium is accurate then there are two possible alternative explanations. 1. Meat medium used in this study contained 19% of fat and the faster death rate phases observed in sections of the survival curves could be due to microorganisms passing from the aqueous to oil phase. If the number of spores transferred to the aqueous phase is accepted to be a function of time, then this explains the observed results, at higher temperatures, the inactivation approaches that in oil (water evaporation), while at low temperatures it approaches that in water. 2. A second explanation to the broken on death curve could be a direct phasic effect of free water present in the system. Solubility of water in oil increases with increasing temperature (Parsons, 1937; Mills, 1949), thus at higher temperatures, there would be a more homogeneous mixture than at lower temperatures resulting in a concurrent none homogenous or even heating of the medium. Based on analyses performed, it was confirmed that the resistance of the spores and vegetative cells were inactivated at three or more stages depending of temperature. Figure 16 shows the typical shape of the survivor curves obtained in this study.

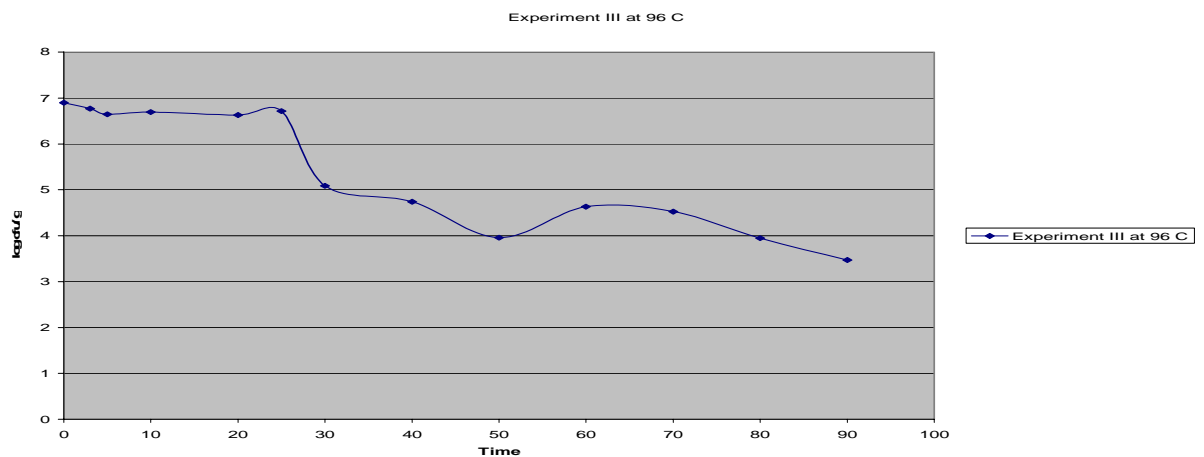


Figure 16. Sample of survivor curve for heat resistant bacteria in meat medium at 96°C.



### Shape of the survival curve

Thermal death of microorganism is often considered to be a first order reaction, but many authors have observed deviations in practice (Roberts, 1969; Rusell, 1971). Survival curves of sporeformer bacteria in meat medium showed deviations on the initial parts of the curves. These deviations could have resulted from activation of the spores (increased germination rate) (Shull & Ernest, 1962), a transition period from a heat resistant to a heat sensitive spore form (Levinson, 1971) or attributed to the presence of spores of differing heat resistance within population (Stumbo, 1965). The survival curves shown triphasic and quatrphasic results. The discrepancy between theoretical and experimental results seems at first surprising, but can be explained by the fact that a transfer of spores and vegetative cells occur from the aqueous to the oily phase which was proposed by Daron (Daron, 1970). Study of this transfer proved difficult, but it appeared to depend on the temperature of the system, the type of bacteria, the transfer surface and the contact time of the two phases, i.e. it was more important for treatment at 94.84°C when heating times were long. Most spores are destroyed in the aqueous phase and not in the oil, which may explain the variation in experimental results for some replications at 94.84°C and 95.89°C (Figures from 9 to 14). The activation of a bacterial spore is generally considered to be the first in a series of events involved in the transformation of a dormant spore into a vegetative cell (Murrell, 1961). The optimal temperature for germination appears to be 30°C, although germination has been reported to occur at a wide the range of temperatures (Doyle, 1989). The bacterial cells exposed to temperatures greater than their optimum temperatures can lead to formation of heat shock proteins that increase thermal tolerance. Times of 165 and 90 minutes (Figure 16)

were used in thermal treatments, which could induce the production of heat shock proteins. Furthermore, variable heating times between temperature treatments indicate that holding cells at higher than optimum temperatures for extended times can lead to the development of increased heat resistance (Mackey, 1990). Bacteria naturally present in raw animal co-products survived heat treatments similar to those used commercially (130° C [260° F] for 15 minutes). The objective in fitting to create models 2 and 3 was developed in an attempt to predict the behavior of thermo-resistant bacteria in meat medium which appeared to be stepwise rather than a linear relationship between time and temperature. The second statistical was a “best-fit” for each replication. The best-fit model had the following results for replications at different temperatures (Tables 5, 6, and 7; Figures 11, 12, and 13). The form model is:  $Y = (a_0 + a_1X)Z_1 + (b_0 + b_1X)Z_2 + (c_0 + c_1X)Z_3 + \dots + (g_0 + g_1X)Z_8 + E$ . The figure 17 shows the significance of each parameter from SAS program.  $a_0$  represents the intercept of the model for log cfu/g vs. time. The other parameters ( $b_1$ ,  $c_1$ ,  $d_1$ ,  $e_1$ ,  $f_1$  and  $g_1$ ) represent the partial slope of each portion of the curve. SE is the standard error estimated for each slope.

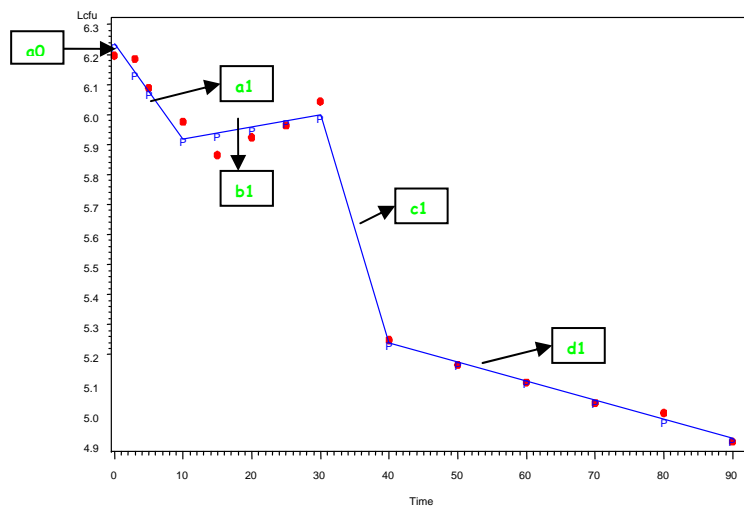


Figure 17. Second model parameters.

| Replication | Parameter | Estimate ± SE          |
|-------------|-----------|------------------------|
| 1           | $a0$      | $6.1432 \pm 0.1286$    |
|             | $a1$      | $-0.0136 \pm 0.0382$   |
|             | $b1$      | $-0.00639 \pm 0.0361$  |
|             | $c1$      | $-0.1950 \pm 0.0883$   |
|             | $d1$      | $-0.0138 \pm 0.00199$  |
| 2           | $a0$      | $6.1802 \pm 0.1053$    |
|             | $a1$      | $-0.0119 \pm 0.00999$  |
|             | $b1$      | $-0.00381 \pm 0.0192$  |
|             | $c1$      | $-0.0319 \pm 0.00752$  |
|             | $d1$      | $-0.0104 \pm 0.00459$  |
| 3           | $a0$      | $6.2068 \pm 0.0599$    |
|             | $a1$      | $-0.0652 \pm 0.0135$   |
|             | $b1$      | $-0.00580 \pm 0.00908$ |
|             | $c1$      | $-0.0254 \pm 0.00242$  |
|             | $d1$      | $-0.00338 \pm 0.00421$ |
|             | $e1$      | $-0.0196 \pm 0.00868$  |

Table 5. Results of second model applied to 90.88°C.

The death rate of the log concentration of the spores, vegetative cell mixture, and time do not have a linear relationship. Three replications at 91°C of survivors versus time curves of heat resistance bacteria at 91°C are shown in the Figure 18.

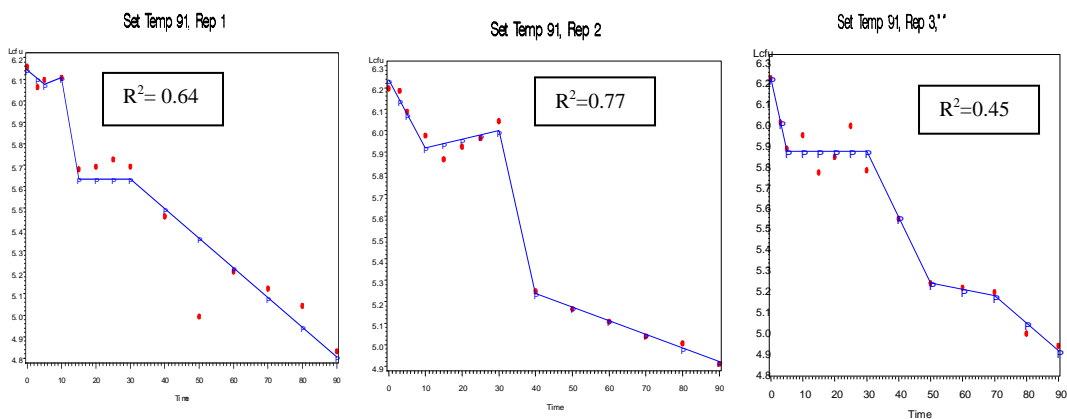


Figure 18. Survivor versus time curve for heat resistance bacteria at 91°C.

F test was calculated for each replication and the results were compared to the linear regression model. A null hypothesis for this situation is as follow:

F test for 90.76°C

Ho:  $b_1=c_1=d_1=e_1=0$

Ha: Ho is not true

F value= 0.925.

The tabled value  $F_{0.05}$  for 4 and 8 degrees of freedom is 3.84. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept Ho.

F test calculated for second replication at 91.02°C.

Ho:  $b_1=c_1=d_1=e_1=0$

Ha: Ho is not true

F value= 0.009.

The tabled value  $F_{0.05}$  for 4 and 8 degrees of freedom is 3.84. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept Ho.

F test calculated for a third replication at 90.88°C

Ho:  $b_1=c_1=d_1=e_1=0$

Ha: Ho is not true

F value= 0.003.

The tabled value  $F_{0.05}$  for 4 and 8 degrees of freedom is 3.84. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept  $H_0$ .

Results of second model applied at 94.84°C are shown in the Table 6.

| Replication | Parameter | Estimate ± SE      |
|-------------|-----------|--------------------|
| 1           | $a0$      | 6.5246 ± 0.1082    |
|             | $a1$      | -0.2650 ± 0.0247   |
|             | $b1$      | -0.00671 ± 0.0181  |
|             | $c1$      | -0.00861 ± 0.00215 |
| 2           | $a0$      | 6.3138 ± 0.0825    |
|             | $a1$      | -0.1504 ± 0.0332   |
|             | $b1$      | -0.00381 ± 0.0192  |
|             | $c1$      | -0.0449 ± 0.00410  |
|             | $d1$      | -0.00244 ± 0.00146 |
| 3           | $a0$      | 6.4660 ± 0.1018    |
|             | $a1$      | -0.0790 ± 0.0148   |
|             | $b1$      | -0.00580 ± 0.00908 |
|             | $c1$      | -0.0235 ± 0.00613  |
|             | $d1$      | -0.00230 ± 0.00316 |
|             | $e1$      | -0.00919 ± 0.00648 |

Table 6. Results of second model applied to 94.84°C.

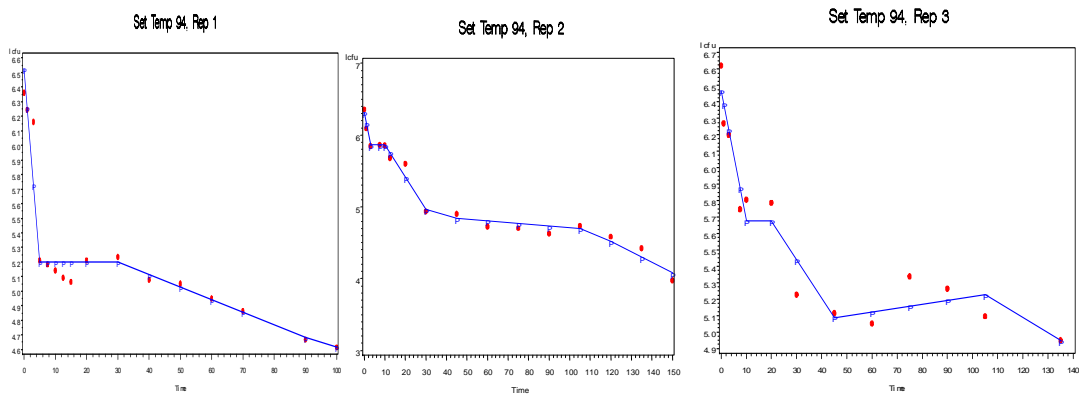


Figure 19. Survivors versus time curve for heat resistance bacteria at 94.84°C.

F test at 94.77°C

Ho:  $b_1=c_1=d_1=e_1=0$

Ha: Ho is not true

F value=1.86.

The tabled value  $F_{0.05}$  for 3 and 11 degrees of freedom is 3.59. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept Ho.

F test at 94.54°C

Ho:  $b_1=c_1=d_1=e_1=0$

Ha: Ho is not true

F value=1.23

The tabled value  $F_{0.05}$  for 5 and 10 degrees of freedom is 3.33. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept Ho.

F test for 94.94°C

Ho:  $b_1=c_1=d_1=e_1=0$

Ha: Ho is not true

F value= 0.044.

The tabled value  $F_{0.05}$  for 3 and 8 degrees of freedom is 4.07. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept Ho.

Results of second model applied at 96°C are shown in the table 7.

| Replication | Parameter | Estimate SE           |
|-------------|-----------|-----------------------|
| 1           | $a_0$     | $6.3532 \pm 0.1892$   |
|             | $a_1$     | $-0.2642 \pm 0.0429$  |
|             | $b_1$     | $-0.0477 \pm 0.0224$  |
|             | $c_1$     | $-0.0860 \pm 0.0129$  |
|             | $d_1$     | $-0.0131 \pm 0.00881$ |
|             | $e_1$     | $-0.0582 \pm 0.0132$  |
| 2           | $a_0$     | $6.4603 \pm 0.1399$   |
|             | $a_1$     | $-0.1512 \pm 0.0167$  |
|             | $b_1$     | $-0.00947 \pm 0.0397$ |
|             | $c_1$     | $-0.1376 \pm 0.0197$  |
|             | $d_1$     | $-0.0125 \pm 0.0259$  |
|             | $e_1$     | $-0.1092 \pm 0.0259$  |
|             | $f_1$     | $0.00609 \pm 0.0303$  |
| 3           | $a_0$     | $6.9210 \pm 0.3158$   |
|             | $a_1$     | $-0.0719 \pm 0.0732$  |
|             | $b_1$     | $-0.1054 \pm 0.0262$  |
|             | $c_1$     | $-0.1488 \pm 0.0210$  |
|             | $d_1$     | $-0.0123 \pm 0.0151$  |
|             | $e_1$     | $-0.0547 \pm 0.0221$  |

Table 7. Results of second model applied to 96°C.

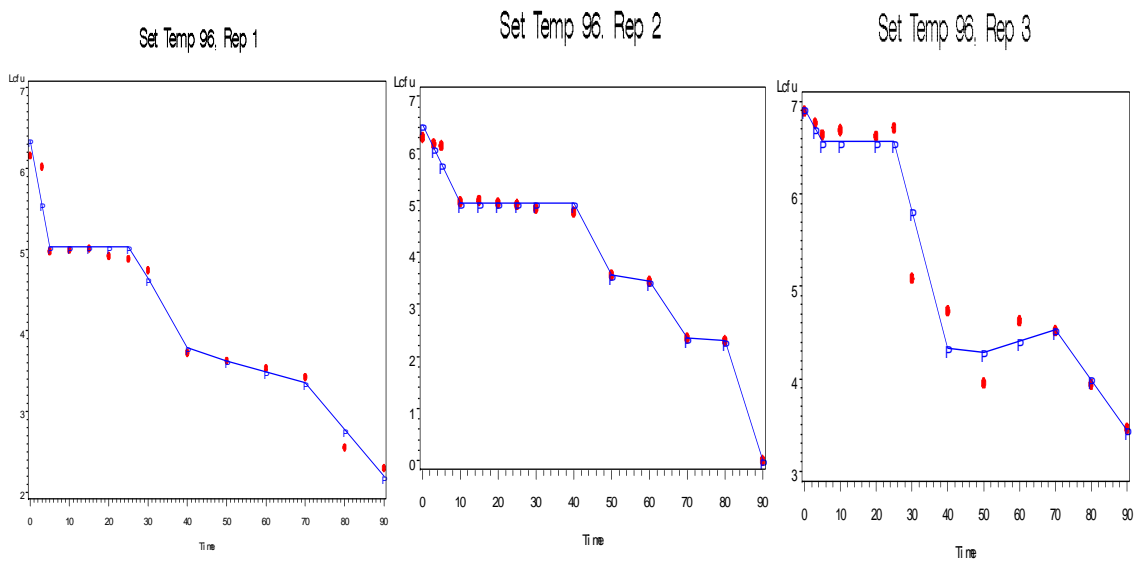


Figure 20. Survivors versus time curve for heat resistant bacteria at 96°C.

F test at 95.80°C

Ho:  $b_1=c_1=d_1=e_1=0$

Ha: Ho is not true

F value= 0.290.

The tabled value  $F_{0.05}$  for 3 and 8 degrees of freedom is 4.07. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept Ho.

F test for 95.98°C

Ho:  $b_1=c_1=d_1=e_1=0$

Ha: Ho is not true

F value= 3.34.

The tabled value  $F_{0.05}$  for 5 and 6 degrees of freedom is 4.39. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept Ho.

F test for 95.89°C

Ho:  $b_1=c_1=d_1=e_1=0$

Ha: Ho is not true

F value= 2.81.

The tabled value  $F_{0.05}$  for 3 and 7 degrees of freedom is 4.35. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept Ho.

A third and final model was applied to predict the behavior most appropriate for all temperatures used in this study. This model was chosen since there appeared to be a



trend for all temperatures and replications for a “standard” pattern of cell death. This model was the follow form  $Y = (a_0 + a_1X)Z_1 + (b_0 + b_1X)Z_2 + (c_0 + c_1X)Z_3 + E$ .

F-tests were calculated for the first model (Linear), second model (involved fitting a step function with different segments), third model (involved fitting a three-segment), and these were compared among them.

The follow are the results obtained after the comparison at 90.76°C.

F value calculated for first model and second model was 1.5.

The tabled value  $F_{0.05}$  for 3 and 8 degrees of freedom is 4.07. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept  $H_0$ .

F value calculated for the first model and the third model was 1.64.

The tabled value  $F_{0.05}$  for 3 and 8 degrees of freedom is 4.07. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept  $H_0$ .

F value calculated for the second model and the third model was 0.84.

The tabled value  $F_{0.05}$  for 1 and 8 degrees of freedom is 5.32. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept  $H_0$ .

The follow are the results obtained after the comparison at 91.02°C.

F value calculated for the first model and the second model was 1.76.

The tabled value  $F_{0.05}$  for 3 and 8 degrees of freedom is 4.07. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept  $H_0$ .

F value calculated for the first model and the second model was 3.22.

The tabled value  $F_{0.05}$  for 3 and 8 degrees of freedom is 4.07. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept  $H_0$ .

F value calculated for the second model and the third model was 4.35.

The tabled value  $F_{0.05}$  for 1 and 8 degrees of freedom is 5.32. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept  $H_0$ .

The follow are the results obtained after the comparison at 90.88°C.

F value calculated for the first model and the second model was 1.5.

The tabled value  $F_{0.05}$  for 3 and 8 degrees of freedom is 4.07. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept  $H_0$ .

F value calculated for model I and model III was 1.64.

The tabled value  $F_{0.05}$  for 3 and 8 degrees of freedom is 4.07. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept  $H_0$ .

F value calculated for the second model and the third model was 0.84.

The tabled value  $F_{0.05}$  for 1 and 8 degrees of freedom is 5.32. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept  $H_0$ .

The follow are the results obtained after the comparison at 90.88°C.

F value calculated for the first model and the second model was 4.03.

The tabled value  $F_{0.05}$  for 4 and 7 degrees of freedom is 4.12. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept  $H_0$ .

F value calculated for the first model and the third model was 0.44.

The tabled value  $F_{0.05}$  for 3 and 8 degrees of freedom is 4.07. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept  $H_0$ .

F value calculated for the second model and the third model was 0.010.

The tabled value  $F_{0.05}$  for 1 and 7 degrees of freedom is 5.59. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept  $H_0$ .

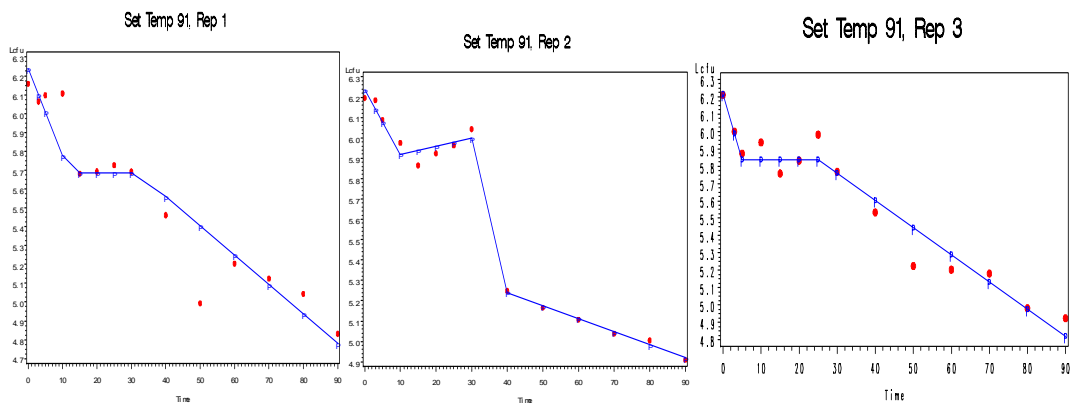


Figure 21. Prediction model at 90.88°C.

The follow are the results obtained after the comparison at 94.77°C.

F value calculated for the first model and the second model was 3.04.

The tabled value  $F_{0.05}$  for 2 and 11 degrees of freedom is 3.98. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept  $H_0$ .

F value calculated for the first model and the third model was 3.04.

The tabled value  $F_{0.05}$  for 2 and 11 degrees of freedom is 3.98. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept  $H_0$ .

F value calculated for the second model and the third model was 0.0009.

The tabled value  $F_{0.05}$  for 1 and 11 degrees of freedom is 4.84. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept  $H_0$ .

The follow are the results obtained after the comparison at 94.54°C.

F value calculated for the first model and the second model was 1.26.

The tabled value  $F_{0.05}$  for 3 and 10 degrees of freedom is 3.71. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept  $H_0$ .

The follow are the results obtained after the comparison at 94.94°C.

F value calculated for the first model and the second model was 1.62.

The tabled value  $F_{0.05}$  for 3 and 7 degrees of freedom is 4.35. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept  $H_0$ .

F value calculated for the first model and the third model was 1.00.

The tabled value  $F_{0.05}$  for 3 and 7 degrees of freedom is 4.35. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept  $H_0$ .

F value calculated for the second model and the third model was 3.43.

The tabled value  $F_{0.05}$  for 1 and 7 degrees of freedom is 5.59. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept  $H_0$ .

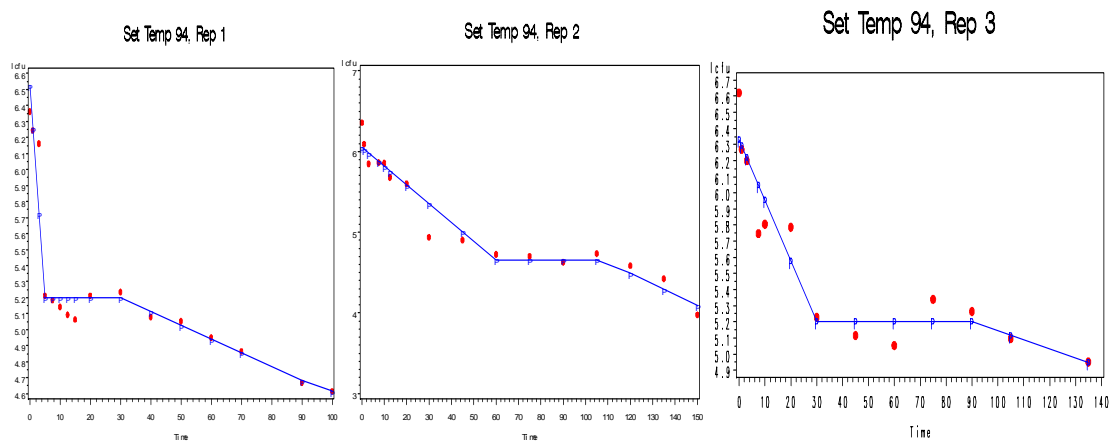


Figure 22. Prediction Model at 94.84°C.

The follow are the results obtained after the comparison at 95.80°C.

F value calculated for the first model and the second model was 0.35.

The tabled value  $F_{0.05}$  for 4 and 7 degrees of freedom is 4.12. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept  $H_0$ .

F value calculated for the first model and the third model was 0.29.

The tabled value  $F_{0.05}$  for 3 and 8 degrees of freedom is 4.07. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept  $H_0$ .

F value calculated for the second model and the third model was 0.6.

The tabled value  $F_{0.05}$  for 1 and 7 degrees of freedom is 5.59. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept  $H_0$ .

The follow are the results obtained after the comparison at 95.98°C.

F value calculated for the first model I and the second model was 1.6.

The tabled value  $F_{0.05}$  for 5 and 6 degrees of freedom is 4.39. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept  $H_0$ .

F value calculated for the first model and the second model was 2.64.

The tabled value  $F_{0.05}$  for 3 and 8 degrees of freedom is 4.07. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept  $H_0$ .

F value calculated for the second model and the third model was 1.87.

The tabled value  $F_{0.05}$  for 2 and 6 degrees of freedom is 5.14. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept  $H_0$ .

The follow are the results obtained after the comparison at 95.89°C.

F value calculated for the first model and the second model was 4.20

The tabled value  $F_{0.05}$  for 3 and 7 degrees of freedom is 4.35. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept  $H_0$ .

F value calculated for the first model and the third model was 2.8.

The tabled value  $F_{0.05}$  for 3 and 7 degrees of freedom is 4.35. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept  $H_0$ .

F value calculated for the second model and the third model was 2.02.

The tabled value  $F_{0.05}$  for 1 and 7 degrees of freedom is 5.59. The value of the test statistic is much smaller than the tabled value, so there is conclusive evidence to accept  $H_0$ .

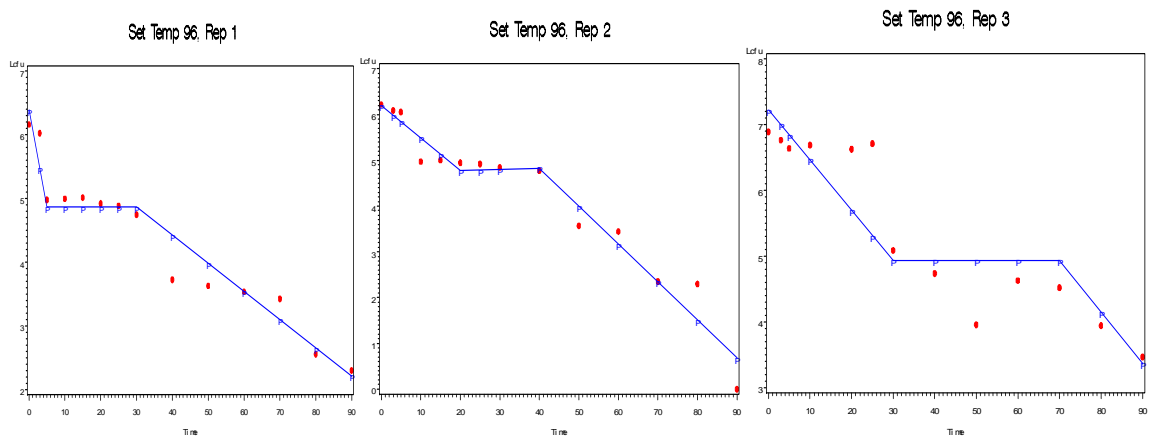


Figure 23. Prediction model at 96°C.

The process of spore activation that can begin during pasteurization facilitates their inactivation during sterilization. However, at pasteurization temperatures, the spores in deep dormancy can be activated. This can occur only at temperatures above to 110°C

(Iciek, 2005). In the experiments reported here, the time and temperature combinations used reduced the presence of spores and vegetative cells of heat resistant bacteria by a factor of approximately  $10^6$ . Surviving spores and vegetative cells after heat treatment were observed in all temperatures tested (91°C, 95°C, and 96°C). An initial lag or lower initial rates of thermal destruction can most often be explained by experimental error, for example, lags in heat penetration, clumping of cells, protection of spores by vegetative cells, and contamination by spores of a different bacterial type. Several explanations have been proposed for initially slower rates when observed experimental errors have been eliminated. Initial lag times in thermal death have been attributed to such causes as the activation of germination of endospores by heat (Morrison, 1930; Curran, 1945; Finley, 1962). Thermal destruction of heat resistant bacterial spores and vegetative cells used in this study did not follow first-order kinetics, indicating that some spores were more heat resistant than others are (Murrell, 1966).

Significant variation in heat resistance has been observed for sporeforming bacteria at different temperatures. Spores and vegetative cells of some strains of *Bacillus* are completely destroyed after exposure to 90°C for several minutes, while other isolates associated with foodborne outbreaks survived boiling for six hours (Walker, 1975). Experiments with *B. cereus* have demonstrated that inactivation of spores was less and germination of spores was greater when media were heated non-isothermally as would occur during cooking or processing (Fernandez, 2001). The effects of heat on spores and vegetative cells in laboratory media have been studied for many years. Bacterial spores are very resistant to heat and survive many thermal processes. Transformation of these spores to actively growing and potential toxin-producing vegetative cells is a three stage



process: activation, germination, and outgrowth. Heat often serves to activate spores, and they will subsequently germinate and grow if the conditions are favorable (Gaillard S., 1998). High temperatures can also injure spores, and depending on available nutrients, pH, temperature, and inhibitory substances, injured spores may or may not be able to recover and grow (Johnson, 1984). A review of spore injury cites numerous research papers with data on recovery of injured spores (Foegeding, 1981). The heat resistance of the bacteria surviving the thermal process that approached commercial conditions was relatively high. While the high heat resistance and non-linear behavior of the bacteria inactivation cannot be explained because the added organisms in the vegetative state were killed during heating but is more likely due to several factors, including water-oil phase interactions, and variation in heat resistance within of the environmental isolate population.

## CHAPTER V

### CONCLUSIONS

Growth conditions of cells prior to sporulation affect the thermal resistance of spores of heat resistant sporeforming bacteria grown at a higher temperature. Exposure to heat during thermal processing may activate spores but can also injure them however germination will occur if the damage is repaired. A nutrient rich medium such as the meat used in this study, is helpful to spore recovery and vegetative cell repair. As with growth, germination depends upon the presence or absence of a variety of chemicals and environmental conditions. Unlike the *Clostridia*, *B. cereus* requires aerobic conditions for sporulation and not all strains require heat activation for spores to germinate.

Bacterial spores are not only very heat resistant but heat actually stimulates spores to initiate growth. Generation times as short as 7 minutes have recorded; therefore, it is important to cool meat quickly to temperatures below the minimum that allows germination and growth of sporeformers. The critical temperature range for growth of *B. cereus*, *C. perfringes*, and some *C. botulinum*, strains is approximately 10-50°C although some psychrotrophic *C. botulinum* strains can grow at refrigeration temperatures. The heat resistance of bacterial cells is related to the maximum temperature of growth. In the case of bacterial spores a similar relationship exists. The inexact relationship of maximum growth temperature to thermal resistance of spores is interpreted to mean that factors in addition to those which determine maximum

temperature of growth are involved in thermal resistance. The fat protection effect would not occur in most food emulsions because of the small size of the droplets in suspensions. However, attention should be paid to their stability before sterilization.

The determination of the most heat resistant bacteria and their heat inactivation characteristics will allow the rendering industry to establish and document process controls to ensure the final rendered product is free from bacteria that can reduce the quality of the product.

In summary, results from laboratory experiments and pathogen growth models can provide estimates of the times and temperatures required to inactivate vegetative cells and spores. Since, there are many other factors that affect heat resistance of bacteria, validation of a processing method should be undertaken under realistic conditions using the meat or meat product as the growth medium. The variation in animal type, body component composition, and physical size and shape of the raw materials, which occurs in commercial operations, would result in non-uniform heat transfer, so in practice the process vary more than the experimental rendering in eliminating spores. It is clear that spores that survive the initial drying phase of rendering may be protected against thermal deactivation, thus temperatures employed for rendering may be ineffective. It is unlikely that such rendered products have ever been completely free of viable spores, so the necessity for routine sterilization of these products is open to question. Nevertheless, if sterilization is deemed to be necessary, the raw material must be heated under pressure while the moisture content is still high enough to ensure that the spores are not protected against thermal inactivation.

## Future Research

As an agenda for future work on the heat inactivation of the heat-resistant bacteria isolated from poultry offal, we now summarize the issues we left open in this study.

Two parameters need to be specified for detection and control: bacteria identification and change the medium of bacterial growth.

We have argued that heat resistant bacteria used in this study could be affected by the percentage in fat used in the medium, germination or activation of spores and vegetative cells due to temperature.

However, it has been shown that temperature versus time affects the spore former bacteria growth. It would be interesting to develop an inexpensive and easy method to obtain the bacteria identification and the behavior under these parameters. In addition, other parameters should, in the future, be selected in this study such as amount of water activity on the medium, pH, sodium, etc.

Finally, a complete investigation of the detection of thermo-resistant bacteria and heat inactivation methods presented in this study requires a more thorough performance evaluation. First, the methods should be compared experimentally with those previously proposed in the literature. Second, the method should be tested for a large number and more diverse rendering conditions.

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