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Heat Resistance of Bacillus Species Isolated From Tsire-Suya

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Abstract: *Microorganisms involved during thermal processing have dual roles in industrial setting; most industrialist and food experts give attention to havoc associated with microbes without assessing and investigating beneficial metabolites linked to them. Most of heat resistance microorganisms are thermophiles and their ability to survive certain heat treatment defined their applications in industrial development. The objective of this study was to determine the thermal resistance characteristics of Bacillus species obtained from Tsire-suya. The heat resistance for the three Bacillus species was determined by heating the tubes containing nutrient broth and the culture of the isolates with combination of time and heating temperature at 40, 45, 50,55, and 60^oC during 5, 10, 15 and 20 minutes in a water bath. A total number of ten isolates were obtained from Tsire-suya samples. The isolates were screened for heat resistance activity, 3 out of the 10 isolates survived heat treatment. Bacillus sterothemophilus F_2n, Bacillus licheniformis F_1t and Bacillus subtilis W_4m were selected and considered for further study. The result showed that Bacillus stearothermophlius (F_2n) had D-value of 14.58, 12.44, 15.20, 14.62, and 12.27 minutes at 40,45,50,55, and 60^oC respectively while D-values of 15.38, 7.99, 10.59, 6.68, and 7.12 minutes for Bacillus subtilis (w_4m) at 40 45, 50, 55, and 60^oc whereas Bacillus licheniformis (F_1t) had D-values of 16.03, 19.53, 20.93 9.17 and 8.71 minutes at 40, 45, 50, 55 and 60^oC respectively. Z-values were 1000, 65.79, and 58.82^oC, respectively, for Bacillus stearothermophilus F_2n), Bacillus subtilis W_4m), and Bacillus licheniformis F_1t. This study demonstrated that thermally resistant bacteria isolated from Tsire-suya exhibit high heat resistance for a wide range of temperature.*

Keywords: Heat Resistance, Tsire suya, *Bacillus stearothermophilus* F_2n, *Bacillus subtilis* W_4m and *Bacillus licheniformis* F_1t

1. INTRODUCTION

Meat is nutritious high protein rich source of food that serves as a good substrate for microorganism growth [1]. Tsire-suya is a roasted boneless meat of beef, goat or mutton that is grilled around a glowing charcoal fire in which the meat pieces are stacked on wood sticks, spiced with peanut cake powder, spices, vegetable oil, salt or other flavorings. Tsire-suya is currently a popular street delicacy of several countries, particularly those in West Africa. It is processed and sold along streets usually under unhygienic conditions.

The production process of Tsire involves heat treatment and despite heat involves some microbes survived heat treatment processing. Post processing handling is another source of microbial contamination of Tsire-suy. It has been observed that most ready-to-eat meat products and meat are often displayed in Nigeria markets under poor hygienic conditions and hence contaminated by various microorganisms [2].

Thermal processing is one of the widely used techniques to eliminate or reduce bacteria in food. This can has been applied in different forms such as cooking, baking, roasting, extrusion cooking, pasteurization, and sterilization. The essence of pasteurization and sterilizations is to remove 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. Generally, thermal processing is the application and use of heat in food preparation so as to destroy any harmful bacteria or microorganisms and make it available under hygienic conditions [3].

The main benefit of thermal processing is the overall improvement of food and food products quality and safety. However, certain consequences are associated with thermal processing. This includes the formation of brown pigments on food products, loss of food texture, and the degradation of vitamins, and other nutritional components. So, the overall qualities of the final food product as well as the improvement in microbial safety are yardstick for evaluation of thermal processing [4].

Thermal resistance by microorganisms is variable and reduction of microbial loads is proportional to the amount of temperature and time applied [5]. The high temperatures used in thermal processing destroy microbial cells by destabilizing the structural and functional integrity of the cytoplasmic membrane [6]. 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 [7].

There are two main categories of methods used in thermal processing: Dry heat, which involves incubation in an oven-like environment, and moist heat, which utilizes steam under pressure. The method involves in Tsire suya production process is dry

heat while moist heat was the process adopted for the heat resistance of Bacillus species in this study. 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 [6]. 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 [6].

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.

Heat treatments meant 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 [8]. Examples of the inherent resistance include differences among species and strains of bacteria, as well as the differences between spores and vegetative cells [9]. 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 menstruum (amount of carbohydrates, proteins, lipids, solutes, etc.), water activity, pH, added preservatives, method of heating, recovery procedures etc

Despite consequences attached to the resistance of microbes to heat because of spoilage of food and hazardous toxin produced by them, some of these microbes are still useful and their importance in industrial set-up is enormous. This can be links to fact that such heat treatment or survival of thermal processing will go in long way to enhance ability of microbes to produce certain metabolites, enzymes, lipids and other products that are essential bio-processing materials in industrial development. Some bacterial and other microbes are screened for their ability to survived specific heat treatment before they are selected and considered for use in industrial development.

Currently, there are limited literatures on heat resistance of microorganism despite the problems encounter in our various industries and needs to further research on few existing studies in order to improve at getting such microbes for industrial experts and overall growth and development of food industries. The current study is focus on heat resistance of Bacillus species isolated from Tsire – suya obtained from Suya spot at New Postgraduate Hall, University of Ibadan, Ibadan, Oyo state.

2. MATERIALS AND METHODS

2.1 *Sample Collection*

Tsire-suya samples were collected from suya spot at New Post graduate Hall, University of Ibadan. The latitude and longitude of the areas are 7° 23' 28.19" N and 3° 54' 59.99" E respectively. The samples were taken to laboratory for further analysis.

2.2 Isolation and Culture Methods

This was done by method described by [10]. A one in ten serial dilution was made for 'tsire'sample using peptone water. This was preceded by plating out at dilution (10^{-4} and 10^{-6}) on Nutrient agar (N.A). The plates were incubated at 37^{o}C for 24 hours. Distinct colonies were sub-cultured until pure isolates were obtained.

2.3 Maintenance of Pure cultures

This was carried out by streaking pure cultures of bacterial isolates onto nutrient agar slants and incubated at 37^{o}C for 24hrs. They were kept in refrigerator after 24 hours of incubation. The isolates were transferred into fresh nutrient agar slants oat interval of five weeks to maintain the bacterial isolates.

2.4 Screening Experiments for Heat resistance Determination

This was determined by a modified method of Casadei *et al.* [11], where effects of different temperatures on the growth of bacterial isolates were assessed. Tubes with the nutrient broth and 24 hours old culture of the inoculums were introduced into a water bath at different temperatures of 40^{o}C, 45^{o}C, 50^{o}C, 55^{o}C, 60^{o}C at different time intervals (40^{o}C for 30minutes,45^{o}C for 20minutes,50^{o}C for 10minutes,55^{o}C for 5minutes and 60^{o}C for 2minutes) and were quickly transferred into ice to cool it. Fresh nutrient broth was prepared and the heat – treated organism was re-inoculated into it and incubated at 25-30^{0}C for 48hours to recover the organisms [12] and the optical density was measured in spectrophotometer (Jenway) at a wave length of 540nm. The broth was thoroughly mixed and reading was taken according to Corry [13].

2.5 Characterization of Isolates

This was conducted by morphological and biochemical tests.

i. Morphological Characterization of Isolates

a. Colonial Morphology

The bacterial were examined on agar plates as to type of pigment, elevation, shape, surface and Edge.

b. Cellular Morphology

Simple staining

A smear of the isolates were made on a clean glass slides and heat fixed by flaming. This was followed by addition of malachite green solution and steam for 5 to 10 minutes, but stain does not allowed to dry out. The slides were then washed carefully with cold water. The stain was counter stain with safranin solution for 15 seconds. The slides were finally rinsed with water and blotted dry using a filter paper. Observation was made using oil immersion objective. Spores stain green, bacterial cells stain red for positive result while otherwise shows negative result.

c. Gram Staining Techniques

A thin smear of each of the pure 24 hours old culture was prepared on clean grease-free slides, fixed by passing over gentle flame. Each heat-fixed smear was stained by addition of 2 drops of crystal violet solution for 60 seconds and rinsed with water. The smear was again flooded with Lugol's iodine for 30 seconds and rinsed with water, decolourized with 70% alcohol for 15 seconds and was rinsed with distilled water. They were then counter stained with 2 drops of Safranin for 60 seconds and finally rinsed with water, then allowed to air dry. The smears were mounted on a microscope and observed under oil immersion objective lens. Gram negative cells appeared pink or red while gram positive organisms appeared purple [14]

ii. Biochemical Characterization

a. Catalase Test

To nutrient agar plates containing the streaked bacterial isolates (incubated for 18hours), a drop of freshly prepared 3% Hydrogen peroxide solution were added [15]. Evolution of gas (White Froth) showed a catalase positive reaction while absence of Froth indicates negative reaction.

b. Oxidase Test

This was done by means of a sterile wire loop; a few drops of oxidase reagents (1% aqueous tetra methyl-p-phenylene diamine dihydrochloride) were added to Whatman paper (Number 1) to form a spot. A sterilized wire loop was used to transfer isolates to the reagent on the Whatman paper. After 10 seconds, appearance of a very deep purple coloration showed a positive reaction while absence of deep coloration indicated negative reaction [15].

c. Starch Hydrolysis

This was conducted by addition of equimolar amount of soluble starch to nutrient agar without glucose or meat extract to give a 1% soluble starch before being poured to set in sterile plates. To dried plates, single streaks of cultures were made on them before being incubated at 30^{o}C for 48 hours. The plates were flooded with Gram's iodine after incubation. Unhydrolysed starch formed a blue colouration with iodine. Clear zones around the region of growth indicated hydrolysis by the culture [15]. While Reddish brown zones around the colony indicates partial hydrolysis of starch.

d. Voges – Proskauer

The isolates were each cultured in methyl red broth, 1ml of 6 – alpha-naphthol solution and Iml of 10% NaoH (Sodium Hydroxide) was added after two days of incubation at 30^{o}C. The test is to know whether the organisms after producing acid from glucose are capable of producing acetyl methyl carbinol from acid. Appearance of a pale pink colouration for up to 1hr to check for slow reaction in case of negative result at 5 minutes

e. Methyl Red Test

Glucose phosphate peptone broth was prepared as described by Harringan and McCance [10]. Ten milliliters of the broth was dispensed into screw cap tubes and sterilized. Inoculation with test organism was subsequently done and incubated at 30^{o}C for 2-5 days. After incubation, a few drops of methyl red indicator were added to the culture and a resultant definite red colouration was considered positive.

f. Nitrate Reduction Test

Nitrate peptone water consisting of peptone water and 0.1% potassium nitrate was used. Five milliliters portion of the medium was distributed into each of the screw-capped test tube. Each tube contents were sterilized (121^{o}C for 15minutes) and allowed to cool before inoculating with the isolates. Un-inoculated tubes serve as control. The tubes were incubated at 30^{o}C for 4days. The ability of the isolates to reduce nitrate to nitrite, ammonia or free nitrogen was determined by adding to each tube 0.5ml of 0.6% dimethyl naphthylamine in 5.0ml acetic acid. The development of a red coloration indicated a positive result and producing of nitrogen gas (Payne, 1973).

g. Citrate Test
The ability of the isolates to utilize citrate was tested for by using Koser citrate medium which contains sodium citrate 2.5g, sodium nitro-tetrahydrogen phosphate ($N_aNH_4PO_4$) 1.5g, potassium dihydrogen phosphate (KH_2PO_4) 1.0g, Magnesium Sulphate ($MgS0_4$) 0.2g, bromothymol blue 0.016g in 1000ml of distilled water. The medium was prepared and the pH was adjusted to 6.5 then sterilized. The culture was dispensed in Universal bottles inoculated with a loopful of the isolate from the broth culture of the bacterial isolate and incubated for three days at 37^{o}C. A positive test was indicated by the change in colour from green to blue.

h. Indole Test
Indole is a product of the metabolism of trytophan. This was detected by adding Kovac's reagent into the inoculated cultures inside the Maccartney bottle, allowed to stand for about 10 minutes, a dark red colouration in the amyl alcohol surface layer constitutes a positive indole test. The original colour of the reagent indicates a negative test as described by standard Methods.

i. Coagulase Test
Coagulase is an enzyme capable of coagulating certain blood plasma, notably human and rabbit plasma. This test differentiates pathogenic from non-pathogenic *Staphylococcus spp*. The test was carried out using 18-24 h old culture. A loopful of isolated bacterium was emulsified with normal saline solution on a microscope slide. A drop of undiluted plasma was added to the suspension and stirred for five seconds. A coagulase-positive result was indicated by clumping of colonies together [16].

j. Urease Test
This test detects the ability of an organism to hydrolyze urea rapidly. Organism that hydrolyzes urea rapidly will produce strong positive reactions within 1 or 6 hours of incubation. The urea broth is inoculated with a heavy inoculum from an 18-24 hour pure culture and the tube is shaken gently to suspend the bacteria. The culture medium will remain a yellowish colour if the organism is urease negative. If the organism produces urease enzyme, the colour of the slant changes from light orange to magenta.

iii Sugar Fermentation
Medium containing peptone water of 1.0% and 1.0% fermentable sugar was prepared and phenol red was added as indicator. 10ml was discharged from the medium into test tubes. This was followed by sterilizing at 121^{o}C for 20mins. 18-24 hours cultures of each isolate in broth medium were then inoculated into the test tubes containing the medium and incubated at optimal temperature for 7 days. Un-inoculated tube served as control. Colour change to yellow indicates growth and acid production.

2.6 Determination of Heat Resistance
Heat resistance of Bacillus species was determined by modified method of Yamazaki *et al.* [17]. The modification done was effected in heating temperature, 40, 45, 50, 55 and 60^{o}C were used in this study against 55, 60, 65, 70, 75 and 80^{o}C in the existing method. This experiment was performed in a test tube. Experiment used combination of time (0, 5, 10, 15, and 20 minutes) and heating temperature (40, 45, 50, 55 and 60^{o}C). Tubes with the nutrient broth and 24 hours old culture of the inoculum were introduced into a water bath at different temperature of 40^{o}C, 45^{o}C, 50^{o}C, 55^{o}C, and 60^{o}C with the following times 0, 5, 10, 15, and 20 minutes for the respective temperature used. After the heat treatment in water bath, they were quickly transferred into ice to cool it. Fresh nutrient broth was prepared and the heat treated organism was re inoculated into it and incubated at 20 to 30^{0}C for 48 hours to recover the organisms [12]. 1ml from each of the broth containing recovered *Bacillus* species was diluted into 9ml 8.5% sterile NaCl solution and homogenized to obtain sample with and 10^{-1} dilution factor. Plating was done for 10^{-1} until 10^{-6} diluton.1mL of the samples from different dilutions were moved into sterile Petri dishes and poured with sterile nutrient agar solution. Incubation was conducted at incubator with temperature of 37^{o}C for 24 hours. The colonies of each sample before and after heating were counted.

2.7 Determination of D and Z values
D values was determined by plotting heating time as an axis of the graph against logarithm value of microorganisms count after heating as ordinate, where D value represents absolute value of (1/ slope). Z value was determined by absolute value of (1/ slope) for plotting between heating temperature as an axis and D value as an ordinate , where Z value represents absolute value of (1/ slope) [18]. Decimal reduction values (D) were calculated from the best linear fit from Y= ax + b, in which x is the incubation time and y is log N. N is the number of viable spores after heat treatment. Each Z values were calculated from the slope of an individual curve of log D values against temperature by linear regression of the slopes of the plots.

2.8 Statistical Analysis
Appropriate statistics was used in this work

3. RESULT AND DISCUSSION

3.1 Screening Experiments of Isolates for Heat resistance Determination

A total number of ten isolates were obtained from tsire-suya samples. The isolates were screened for heat resistance activity, 3 out of the 10 isolates survived heat treatment. Isolate F_2n; grow best at 55^{o}C however appreciable growth was also observed at 60^{o}C. The best temperature for the growth of Isolate F_1t was at 45^{o}C, while IsolateW_4m grow better at 50^{o}C Figure 1. However, all probable Bacillus species were able to grow at certain temperatures compare to their status before heat treatment.

3.2 Identification of the Isolates

The three isolates were determined through Morphological, Sugar fermentation and biochemical characterization in accordance with Bergey's manual of determinative bacteriology [19] and by comparing their morphological and biochemical characteristics with standard reference organisms [20]. The pigment of the isolates ranges from white to yellow, light yellow to slightly yellowish and yellow to green fluorescent. The elevation of bacterial isolates were varied, majority of them are flat while few are raised and low convex About 99% of the isolates are irregular in terms of their shape while they are rarely circular in shape. The surface of bacterial isolates form agar plates varied form dull to rough, some are smooth while others have rough surface. The edges of the isolates were observed as Rhizoid, Entire and Tentate (Table 1). All the bacterial isolates are gram positive, rod shape, spore formers and motile (Table 2). It was identified that strain F_1t belonged to *Bacillus licheniformis*, strain F_2n belonged to *Bacillus stearothermophilus* and strain W_4m belonged to *Bacillus subtilis* (Table: 1, 2, 3 & 4).

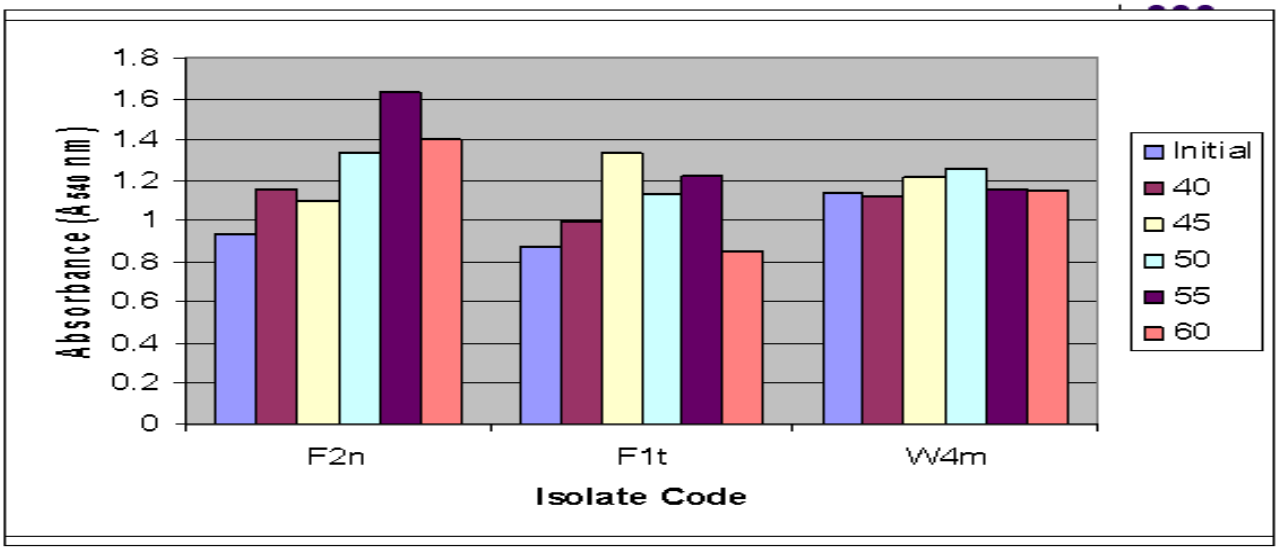

Figure 1: Effect of different temperatures on the growth of Isolate (F_2n), Isolate (W_4m) and Isolate (F_1t) from 'tsire-suya' samples.

Table 1: Cultural characterization of the screened bacterial isolates from UI 'tsire' samples

Isolates Code	Pigment	Elevation	Shape	Surface	Edge
F_1t	Light yellow	Raised	Irregular	Dull to Rough	Rhizoid
F_2n	Yellow	Low convex	Circular	Smooth	Entire
W_{4m}	Yellow green	Flat	Irregular	Smooth	Tentate flourescent

Table 2: Morphological characterization of bacterial isolates from UI "tsire" samples

Isolates Code	Gram staining	Shape	Spore formation	Motility
F_1t	+	Rod	+	+
F_2n	+	Rod	+	+
W_4N	+	Cocobacillus	+	+

Table 3: Carbohydrate fermentation of bacterial isolates from UI "tsire" samples

Isolates Code	Glucose	Lactose	Maltose	Mannitol	Sucrose	Arabinose	Xylose
F_1t	+	+	-	+	+	+	-
F_2n	-	-	+	-	+	+	-
W_4N	+	+	+	+	+	+	+

Table 4: Biochemical characterization of bacterial isolates from UI "tsire" samples

Isolates Code	Cat	Oxi	S.H	V. P	M.R	N.R	Citrate	In	Co	Ur	Probable Identity
F_1t	+	-	+	-	-	+	-	-	-	+	*Bacillus licheniformis*
F_2n	+	+	-	-	+	+	-	-	+	+	*Bacillus Stearothermophilus*
W_4n	+	+	+	-	-	+	-	-	+	-	*Bacillus subtilis*

Cat=Catalase; Oxi=Oxidase; SH=Starch Hydrolysis; VP=Voges Proskeaur; MR=Methyl Red; NR= Nitrate Reduction; Ci= Citrate; In=Indole, Co=Coagulase; Ur=Urease

3.3 Determination of Heat resistance of Bacillus species

Heat resistance of microorganisms is defined as the ability of microorganisms to survive from heating effect which is represent as D-and Z-value. The higher the D-and Z-values of a microbe, the bigger the heat resistance are. The heat resistance of *Bacillus stearothermophilus* F_2n, Bacillus subtilis W_4m and *Bacillus licheniformes* Fit were determined though the D-and Z-values.

The D-and Z-values for *Bacillus stearothermophilus* F_2n isolated from 'tsire-suya' was determined at certain temperatures and heating time Table 5. The result indicated that initial bacterial counts (7.5×10^4) prior to heating was higher than all bacterial plates counted after heat treatment. However, the highest plate count (5.3×10^4) was obtained after 5 minutes of heat-treatment at 60°C, while subsequent heating temperature showed appreciable number of recovered cells (4.2×10^4, 4.4×10^4, 4.9×10^4 and 4.6×10^4 cfu/ml) after heating time of 5 minutes. Initially, as heating temperature increases, the D-values decreases, however at 50^oC, there is sharp increase in D-values (D50°C = 15.20), this was followed by further decrease in D-values when heating temperature increases (Table 5). The decrease in D-value was as a result of the short time needed to inactivate the microorganisms.

The D-and z-values of *Bacillus stearothermophilus* F_2n isolated from 'tsire-suya' at all combinations of time and temperature of heating (Table 6) indicated that the highest D-value was reached at 50°C for 15.20 minutes of pasteurization, while the lowest D-value was at 60°C for 12.47 minutes. Z-values were calculated to be 1000^oC (Table 6).

The number of bacterial count before heat treatment was 3.2×10^4 cfu/ml, however, the highest survival cells was obtained after heating time of 5 minutes at 45°C Table 7. There was rise and falls in D-value as temperature increases from 40°C to 60°C. The Z-values was determined by constructing decimal reduction time (mean log D-value versus temperature) (Table 8). The highest D-value was reached at 40°C for 15.38 minutes, while the lowest D-value was at 50°C for 6.68 minutes. The Z-value of 65.79°C was calculated *for B. subtilis* (Table 8).

The D-and Z-values from *Baccillus licheniformis* F_1t isolated from 'tsire-suya' at various temperatures and heating time combination was shown in Table 9. The D-and Z-values were determined through line regression analysis. There was increase in D-values as temperature increases from 40°C to 50°C (D40°C =16.03, D45°C = 19.53 and D50°C = 20.92 minutes), however further rise in temperature resulted in decrease in D-values (D55 C = 9.17 minutes and D60°C = 8.71) Table 9. The highest D-values were reached at 50°C for 20.19 minutes while the lowest D-value was at 60°C for 0.94 minutes Table 10. Z-values was also calculated as the inverse of log D-value (Z-value = 58.82 C).

Table 5: Determination of D and Z Value of *Bacillus stearothermophilus* F_2n obtained from "Tsire-suya" sample at different temperatures of 40, 45, 50, 55 and 60^oC.

Heating Temperature (^0C)	Heating Time (X) (minute)	Number of Bacteria (N) (colony/ml)	Log N(Y)	Linear equation Y=ax + b	D value D=1/(a) minute
40^oC	0	7.5×10^4	4.88	Y=0.0686x+4.856 Where, Slope (a)=-0.0686 R^2=0.9500	D40°C=14.58 Log=1.16
	5	4.2×10^4	4.32		

	10	9.2×10^3	3.96		
	15	6.7×10^3	3.83		
	20	3.6×10^3	3.56		
45°C	0	7.5×10^4	4.88	Y=-0.0804x +4.894 Where, Slope (a)=-0.0804 R^2=0.9511	D45°C=12.44 Log=1.09
	5	4.4×10^4	4.64		
	10	9.6×10^3	3.98		
	15	3.3×10^3	3.52		
	20	2.7×10^3	3.43		
50°C	0	7.5×10^4	4.88	Y=-0.0658x+4.938 Where, Slope (a)=-0.0658 R^2=0.9774	D50°C=15.20 Log=1.18
	5	4.9×10^4	3.69		
	10	2.3×10^4	3.36		
	15	7.2×10^3	3.86		
	20	4.5×10^3	2.65		
55°C	0	7.5×10^4	4.88	Y=0.0684x=4.864 Where, Slope (a) =-0.0684 R^2=0.9301	D55°C=14.62 Log=1.16
	5	4.6×10^4	4.66		
	10	8.7×10^3	3.93		
	15	7.3×10^3	3.86		
	20	3.7×10^3	3.57		
60°C	0	3.2×10^4	4.88	Y=-0.0802x+4.984 Where, Slope (a) =-0.0802 R^2=0.9756	D60°C=12.47 Log=1.10
	5	2.8×10^4	4.72		
	10	2.2×10^4	4.23		
	15	1.7×10^3	3.69		
	20	1.3×10^3	3.39		

Table 6: D and Z of *Bacillus Stearothermophilus* F_2n Isolated form "Tsire-suya" Sample at various heating temperatures.

Heating Temperature (°C)	D Value (Minute)	Log D Value (Minute)	Z value (°C)
40	14.58	1.16	1000°C
45	12.44	1.09	
50	15.20	1.18	
55	14.62	1.16	
60	12.47	1.10	

Table 7: Determination of D and z values of *Bacillus subtilis* W_4m isolated from "Tsire-suya" sample at various temperatures of 40, 45, 50, 55, and 60°C.

Heating Temperature (^{0}C)	Heating Time (X) (minute)	Number of Bacteria (N) (colony/ml)	Log N(Y)	Linear equation Y=ax + b	D value D=1/(a) minute
40°C	0	3.2×10^4	4.51	Y=0.0650x+4.604 Where, Slope (a)=-0.0650 R^2=0.948	D40°C=15.38 Log=1.19
	5	2.7×10^4	4.43		
	10	7.3×10^3	3.86		

	15	5.5×10^3	3.74		
	20	1.7×10^3	3.23		
45°C	0	3.2×10^4	4.51	Y=-0.1252x +4.844 Where, Slope (a)=-0.1252 R^2=1.0711	D45°C=7.99 Log=0.90
	5	2.9×10^4	4.46		
	10	2.6×10^3	3.89		
	15	2.5×10^3	3.79		
	20	1.5×10^3	3.30		
50°C	0	3.2×10^4	4.51	Y=-0.0944x+4.336 Where, Slope (a)=-0.0658 R^2=0.9774	D50°C=10.59 Log=1.03
	5	2.9×10^3	3.46		
	10	2.6×10^3	3.41		
	15	2.5×10^3	3.40		
	20	1.5×10^2	2.18		
55°C	0	3.2×10^4	4.51	Y=0.1498x+3.874 Where, Slope (a) =-0.1498 R^2=0.8343	D55°C=6.68 Log=0.82
	5	2.7×10^2	2.43		
	10	1.8×10^2	2.26		
	15	2.5×10^1	1.40		
	20	1.9×10^1	1.28		
60°C	0	3.2×10^4	4.51	Y=-0.1404x+3.332 Where, Slope (a) =-0.1404 R^2=0.5868	D60°C=7.12 Log=0.85
	5	2.8×10^1	1.45		
	10	2.2×10^1	1.34		
	15	1.7×10^4	1.23		
	20	1.3×10^1	1.11		

Table 8: D and Z values of *Bacillus subtilis* (W_4M) Isolated from "Tsire-suya" sample at various heating temperature

Heating Temperature (°C)	D Value (Minute)	Log D Value (Minute)	Z value (°C)
40	15.38	1.19	65.79°C
45	7.99	0.90	
50	10.59	1.03	
55	6.68	0.82	
60	7.12	0.85	

Table 9: Determination of D and z values *Bacillus licheniformis* Fit Isolated from UI "Tsire-suya" sample at various temperatures of 40, 45, 50, 55 and 60°C

Heating Temperature (^{0}C)	Heating Time (X) (minute)	Number of Bacteria (N) (colony/ml)	Log N(Y)	Linear equation Y=ax + b	D value D=1/(a) minute
40°C	0	3.4×10^4	4.53	Y=0.0624x+4.544 Where, Slope (a)=-0.0624 R^2=0.6243	D40°C=16.03 Log=1.20
	5	2.5×10^4	4.40		
	10	6.5×10^3	3.81		
	15	4.2×10^3	3.62		

	20	2.0×10^3	3.30		
45°C	0	3.4×10^4	4.53	Y=-0.0512x +4.560 Where, Slope (a)=-0.0512 R^2=0.9502	D45°C=19.53 Log=1.29
	5	2.7×10^4	4.43		
	10	8.4×10^3	3.92		
	15	6.2×10^3	3.79		
	20	3.7×10^3	3.57		
50°C	0	3.4×10^4	4.53	Y=-0.0478x+4.524 Where, Slope (a)=-0.0478 R^2=0.9739	D50°C=20.92 Log=1.32
	5	2.2×10^4	4.34		
	10	8.7×10^3	3.94		
	15	6.7×10^3	3.83		
	20	3.9×10^3	3.59		
55°C	0	3.4×10^4	4.53	Y=0.109x+4.07 Where, Slope (a) =-0.109 R^2=0.8120	D55°C=9.17 Log=0.96
	5	8.5×10^2	2.93		
	10	6.5×10^2	2.81		
	15	5.5×10^2	2.74		
	20	8.0×10^1	1.90		
60°C	0	3.4×10^4	4.53	Y=-0.1184x+3.55 Where, Slope (a) =-0.1184 R^2=0.5783	D60°C=8.71 Log=0.94
	5	9.5×10^1	1.98		
	10	8.5×10^1	1.92		
	15	7.2×10^1	1.86		
	20	5.2×10^1	1.72		

Table 10: D and Z values of *Bacillus licheniformis* F_1t Isolated from "Tsire suya" sample at various heating temperature

Heating Temperature (°C)	D Value (Minute)	Log D Value (Minute)	Z value (°C)
40	16.03	1.20	58.82°C
45	19.53	1.29	
50	20.92	1.32	
55	9.17	0.96	
60	8.71	0.94	

3.4 **Thermal Death curves of Bacillus species Isolated from Tsire –suya samples**

The thermal death curves of *Bacillus stearothemiophilus* F_2n at 40°C, 45°C, 50°C, 55°C and 60°C were linear (R^2, 0.9500, 0.9511, 0.9774, 0.9301 and 0.9756, respectively). Figure 2-6. The D-values, however, determined from the slope of linear regression curves for *Bacillus stearothemiophilus* F_2n were 14.58, 12.44, 15.20, 14.62, and 12.47 minutes at 40, 45, 50, 55 and 60°C, respectively Figure 2-6. From these D-values, a Z-value of 1000°C was calculated for strain *Bacillus stearothermophilus* F_2n.

The thermal death curve were linear at all temperature tested during the experiment. *Bacillus stearothermophilus* F_2n had higher D-values at all temperatures (40, 45, 50, 55 and 60°C) considered while the highest D-value for *Bacillus stearothermophilius* was at 50°C, with D- value of 15.20 minutes (Figure 4). The Z value determined for *Bacillus stearothrmophilius* F_2n through the negative reciprocals of the line of regression of thermal death time curves was 1000°C.

However, there was general agreement between D-values cited in this work and with other published values. Published data on the thermal resistance of *Listeira monocytogenes* in meat products show D-values that range from 0.75 to 28 minutes [21]. Also, the D60°C values obtained for *Bacillus stearothrmophilus* F_2n was similar to the work of Jordan and Cogan [22] who reported a D-

value for *Lb. paracasei*, DPC 2103, isolated from cheddar cheese, at 60°C of 14.7 minutes when cells had been grown in MRS broth and heated in RSM.

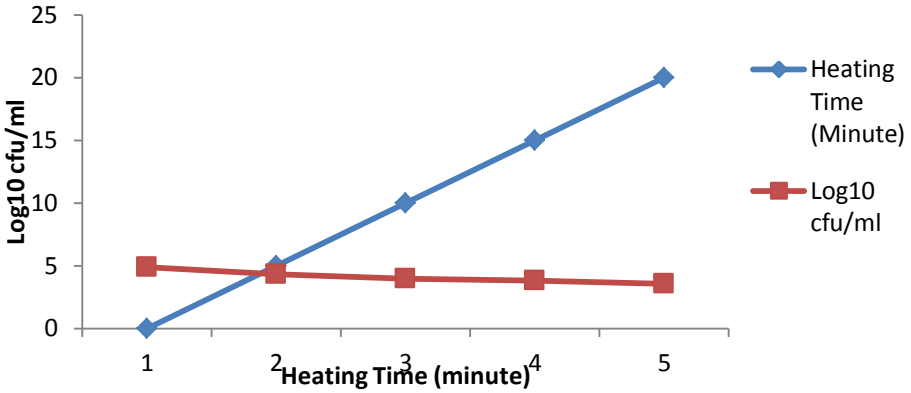

Figure 2: Thermal death curve for *Bacillus Stearothermophiius* F_2n in 'Tsire-suya' sample at 40°C

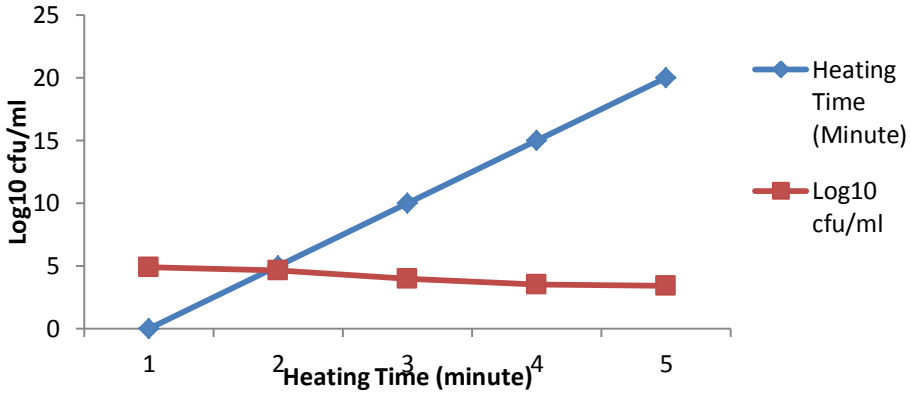

Figure 3: Thermal death curve for *Bacillus Stearothermophilus* F_2n in 'Tsire-suya' sample at 45°C

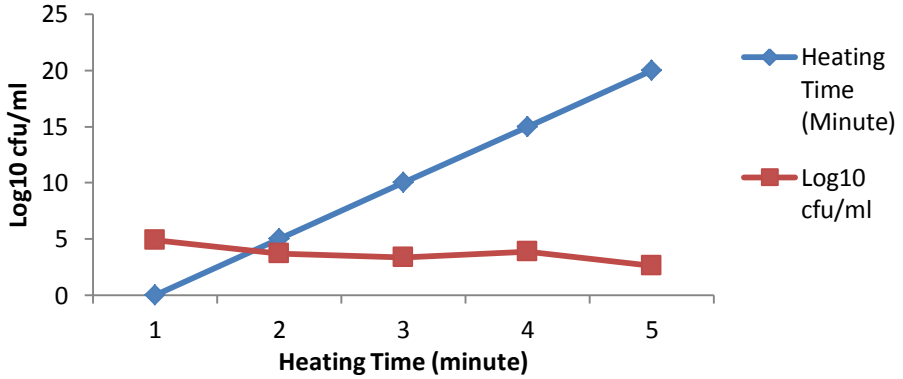

Figure 4: Thermal death curve for *Bacillus stearothermophilus* F_2n in UI 'Tsire-suya' sample at 50°C

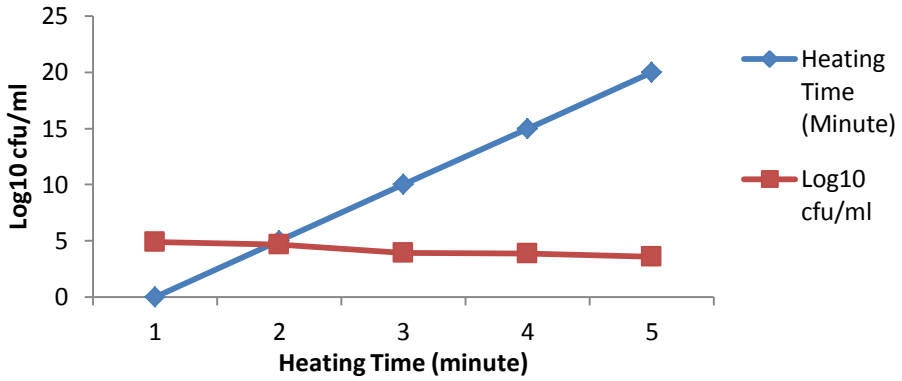

Figure 5: Thermal death curve for *Bacillus stearothermophilus* F_2n in 'Tsire-suya' sample at 55^oC

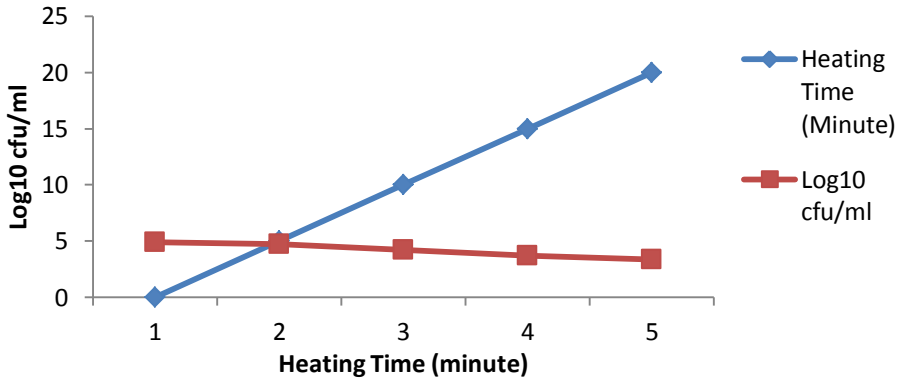

Figure 6: Thermal death curve for *Bacillus stearothermophiius* F_2n in 'Tsire-suya' sample at 60^oC

Thermal death curves for *Bacillus subtilis* W_4m in 'Tsire-suya' sample at different heating temperatures (40, 45, 50, 55 and 60°C) were shown in figure 7-11. At 40 and 45°C, the thermal death curves were linear because the results of linear regression performed gives R^2 values of > 0.90 for this two temperatures (R, 0.948 and 1.0711, respectively) figure 7 and 8. At higher temperature (heating), 45, and 50^oC (figure 8 and 9) the curves were less linear (R^2, 0.8179 and 0.8343, respectively) while at 60^oC (figure 11), thermal death curves for *Bacillus subtilis* W_4m tended to be curvilinear and better described by quadratic functions. The D-values, however, determined from the slope of linear regression curves for *Bacillus subtilis* W_4m were 15.38, 7.99, 10.59, 6.68 and 7.12 minutes at 40, 45, 50, 55 and 60°C, respectively (figure 7-11). From these D-values, a Z-value of 65.79°C was calculated for *Bacillus subtilis* W_4m.

The D-value of *Bacillus subtilis* W_4m at 40, 45, 50, 55 and 60^oC were calculated as the negative reciprocals of the slopes of the regression lines plotted with straight portion values of the survival curves. The D-values are (15.38, 7.99, 10.59, 6.68 and 7.12 minutes respectively) (Figure 7-11). At lower test temperatures, thermal death curves for *Bacillus subtilis*W_4m were linear (At 40^0C, R^2 =0.948, 45^oC, R^2=1.0711) while thermal death curves at higher temperature were less linear (At 50^oC, R2 = 0.8179, 55^oC, R^2 =0.834 and 60^oC, R^2 = 0.5868, respectively), rather tended to be curvilinear and best described by quadratic functions. However, when linear regression was applied to these data, R^2 values were invariably > 0.90. Thus, linear regression was deemed appropriate for D-value determination.

Bacillus subtilis W_4M had higher D-values at heating temperature of D40^oC =15.38 and D50^oC =10.59 minutes) respectively while D-values were low at heating temperature of 45, 55 and 60^0C, respectively (7.99, 6.68 and 7.12 minutes). However, the highest D-value for *Bacillus Subtilis* (W_4m) was at 40^0C, with D-value of 15.38 minutes (Figures 7). The Z value for *Bacillus subtilis* (W_4M) was determined (*Z value* = 65.79). However, the D-values obtained in this work is similar to that of Juneja *et al*. [23] they reported a D-value of 4.16 minutes at 55^oC in chicken broth and a Z-value for 580^oC between the temperatures of 58 to 62^oC.

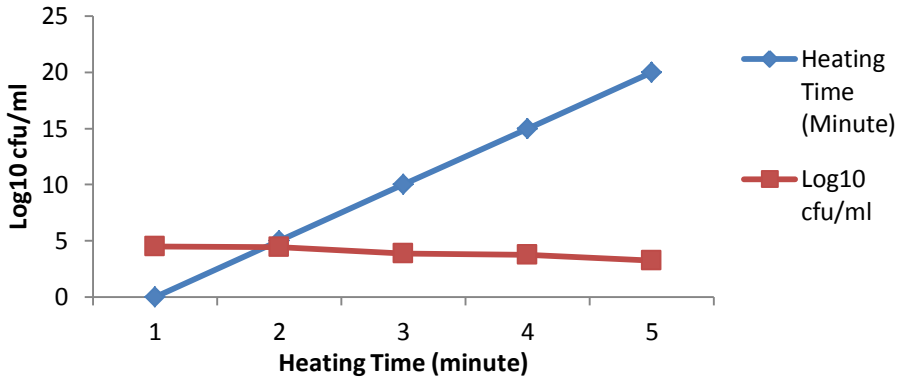

Figure 7: Thermal death curve for *Bacillus subtilis*W_4t in 'Tsire-suya' sample at 40^{o}C

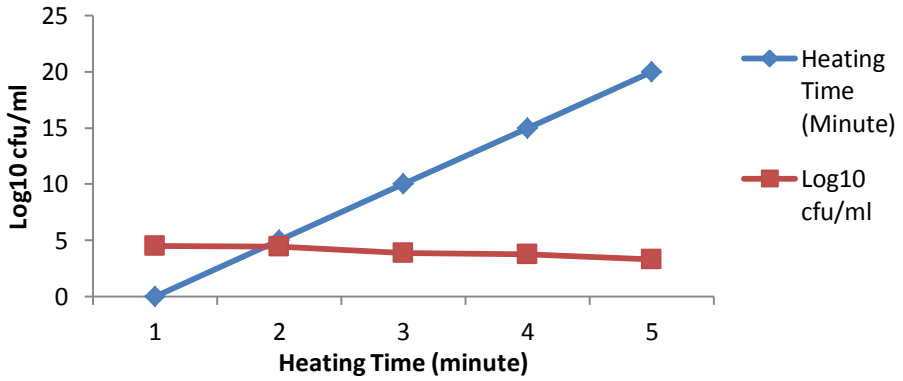

Figure 8: Thermal death curve for *Bacillus subtilis* W4m in 'Tsire-suya' sample at 45^{o}C

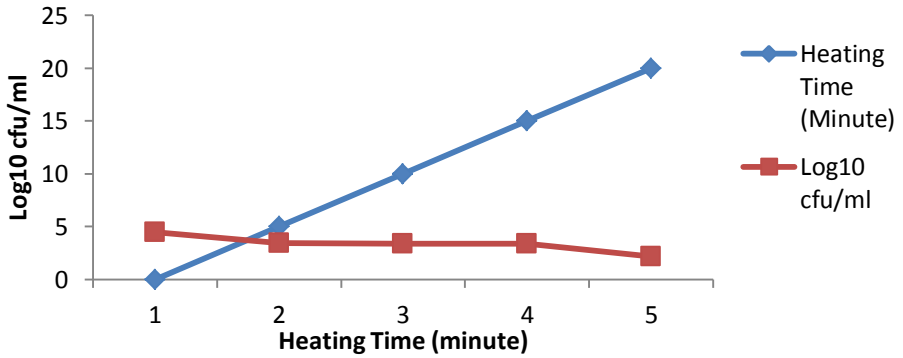

Figure 9: Thermal death curve for *Bacillus subtilis* W4m in 'Tsire-suya' sample at 50^{o}C

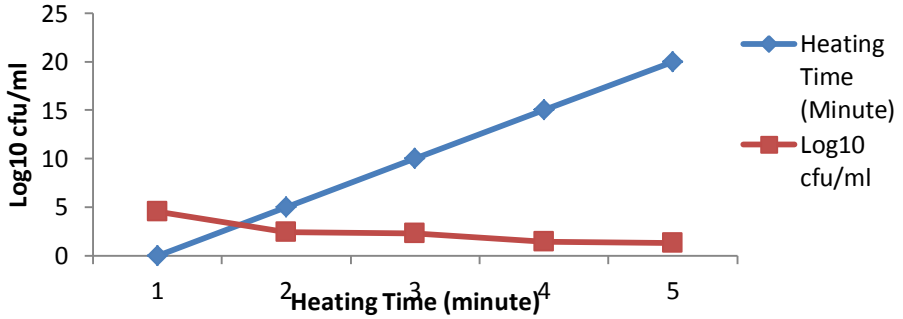

Figure 10: Thermal death curve for *Bacillus subtilis*W4m in 'Tsire-suya' sample at 55^{o}C

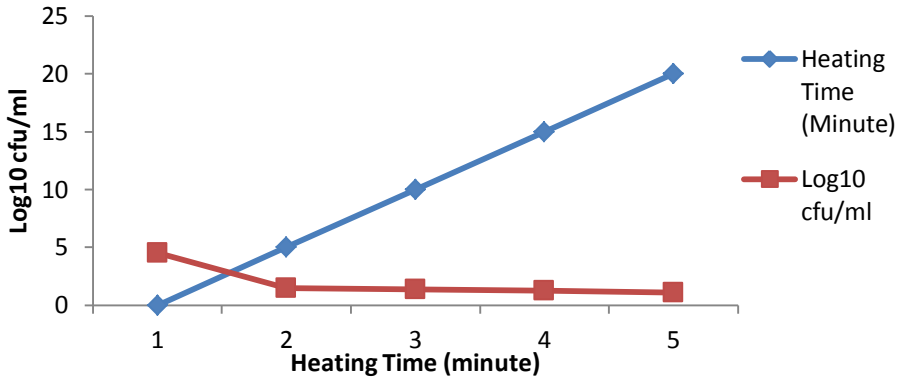

Figure 11: Thermal death curve for *Bacillus subtilis*W4m in 'Tsire-suya' sample at 60^{o}C

The thermal death curves of *Bacillus licheniformis* Fit at 40, 55 and 60°C (figure 12, 15 and 16) were less linear and tended to be curvilinear (R^2, 0.6243, 0.8120 and 0.5783, respectively) while, at 45 and 50°C (Figure 12 and 14), the thermal death curves were linear (R , 0.9502 and 0.9739). The D-values, however, determined from the slope of linear regression curves for *Bacillus licheniformis* Fit were 16.03, 19.53, 20.92, 9.17 and 8.71 minutes at 40, 45, 50, 55 and 60°C, respectively (Figure 12 -16). From these D-values, a Z-value of 58.82°C was calculated for *Bacillus licheniformis* Fit.
The D-values of *Bacillus licheniformis* Fit at 40, 45, 50 and 60^{o}C were determined (16.03, 19.53, 20.92, 9.17 and 8.71 minutes, respectively). *Bacillus licheniformis* F_1t had higher D- value at heating temperatures of 40, 45 and 50^{o}C, respectively (16.03, 19.53 and 20.92 minutes). However, at a higher heating temperatures (55 and 60^{o}C), the D-values were lowed (9.17, and 8.71 minutes, respectively). However, the highest D-vale for *Bacillus licheniformis* F_1t was at 50^{o}C, with D-value of 20.92 minutes (Figure 13). This study is in accordance with the work of Carlie *et al.* [21] that reported D-values that range from 0.75 to 28 minutes on the thermal resistance of *Listeria monocytogenes* in meat products. However, this work was in contrast with data published by Desmond *et al.* (24) who reported that D60^{o}C values of *Lb*. paracasei NFBC 338 was found to be 1.7 minutes when heated in MRS broth. The Z-values of 58.82^{o}C was calculated for *Bacillus licheniformis* F_1t.

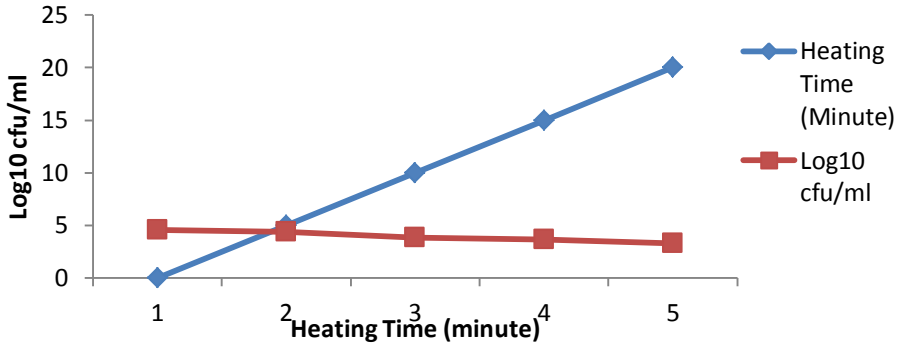

Figure 12: Thermal death curve for *Bacillus licheniformis* F_1t in 'Tsire-suya' sample at 40^{o}C

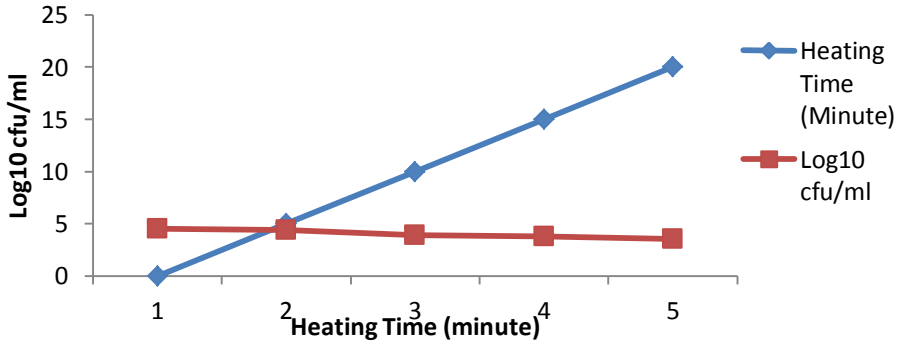

Figure 13: Thermal death curve for *Bacillus licheniformis* F_1t in 'Tsire-suya' sample at 45^{o}C

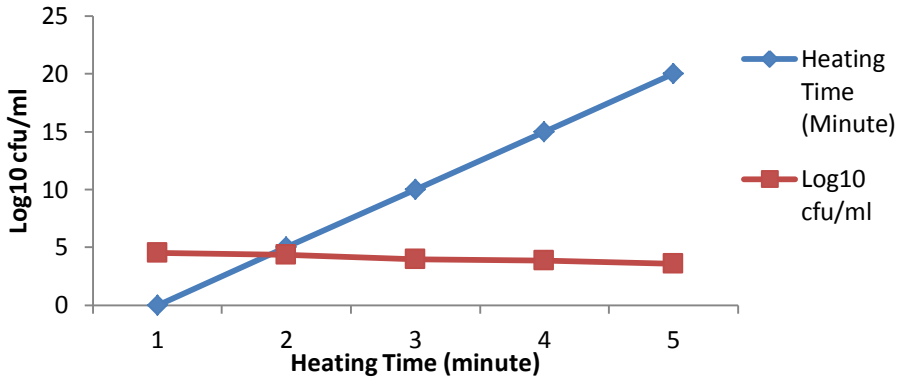

Figure 14: Thermal death curve for *Bacillus licheniformis* F_1t in 'Tsire-suya' sample at 50^oC

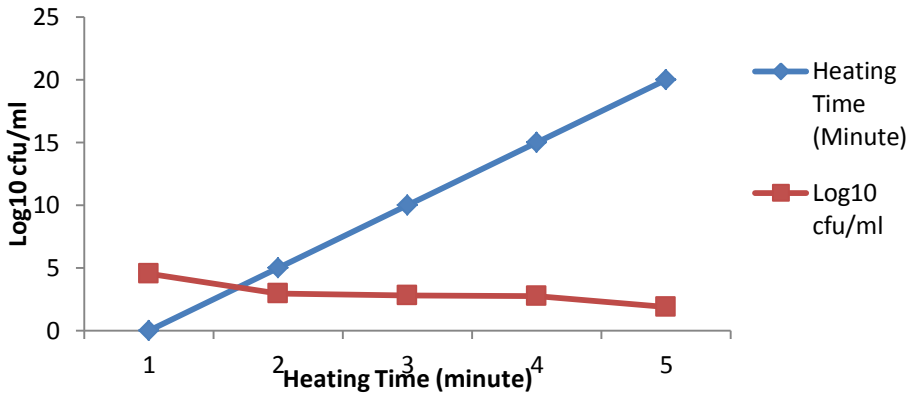

Figure 15: Thermal death curve for *Bacillus licheniformis* F_1t in 'Tsire-suya' sample at 55^oC

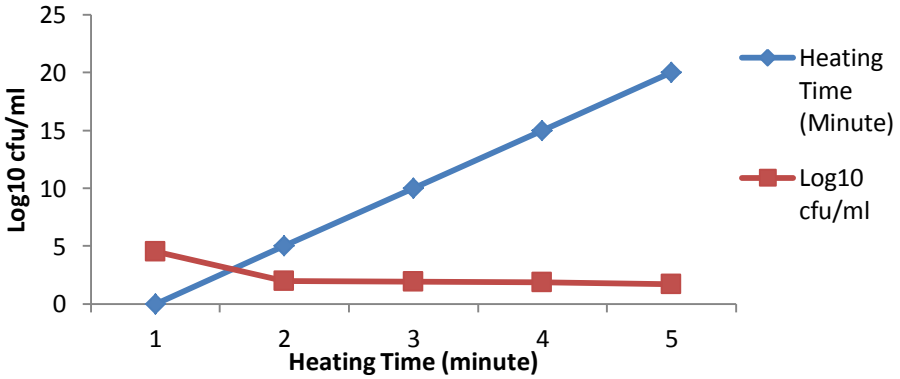

Figure 25: Thermal death curve for *Bacillus licheniformis* F_1t in 'Tsire-suya' sample at 60^oC

4. CONCLUSION

The highest D-values obtained for *Bacillus stearothermophilus* F_2n, *Bacillus subtilis* W_4m and *Bacillus licheniformis* Fit could probably be explained by the fact that those species has been heat shocked. It has been reported that thermal resistance of microorganisms such as *Listeria monocytogenes*, *Salmonella typhimurium* and *Salmonella thompson* could be significantly increase by a sub-lethal heat shock treatment [25, 26, 27]. It has also been reported that *Bacillus stearothermophilus* and *Bacillus lichenoformis* cells exposed to temperatures greater than their optimum growth temperature can lead to formation of heat shock

proteins that increase thermal tolerance. Thus, holding cells at higher than optimum temperatures for extended times can lead to the development of increased heat resistance [25, 28). D-values can also vary between different strains of the same microorganism and different growth conditions under which the bacteria were cultured [28]. The higher D-and Z-values obtained for three Bacillus species is an indication of higher heat resistance, which might be responsible for their survival in Tsire-suya despite heat involved in its processing. 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

5. ACKNOWLEDGMENT

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