Proteolytic Activity of Bacillus Species Isolated from Tsire-Suya, A West African Stick Meat

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***Abstract* —** Proteases are enzyme of various industrial applications. The current sources of proteases are limited and importance of the available one cannot meet various enormous applications. There is search for scarce thermo stable proteases, despite several efforts at meeting the high industrial demands; little success has been recorded. Proteases from bacteria are essential because they grow rapidly, can be maintained easily and are accessible for genetic manipulations, the aforementioned characteristics are germane and will be of advantages to expectations and much needed proteases parameters for our industrial use. This study is focus on isolation and screening of bacteria isolates from tsire for proteolytic activity. Two samples of 'tsire' were collected at the suya spot in Abudulsalam Abubakar Post-graduate Hall, University of Ibadan within seven days interval. The first sample was stored for seven days while the second sample is fresh sample. Isolation was carried out according to the method of Harrigan and McCance. Characterization of isolates was carried out using cultural, morphological, sugar fermentation and biochemical tests. Screening experiment for choice of protease-producing isolates was carried out on skim milk agar. Protease activities (assay) was determined by adding 1ml of supernatant into 1ml of casein solution and incubate for 1 hour at 350C Effects of temperature, pH and incubation time on proteases produced by isolates were carried out using standard protocols. Nineteen bacterial isolates were obtained from fresh and state tsire samples; these isolates were identified to be Bacillus sterothemophilus (F2n), Bacillus licheniformis (F1t), Bacillus subtilis (W4m), Bacillus brevis (W2n), Bacillus cereus (W1n), and Bacillus pumilus (W4n). Eight isolates were positive on skim milk agar, of which three isolates identified to be Bacillus sterothemophilus (F2n), Bacillus licheniformis (F1t) and Bacillus subtilis (W4m), were selected and considered for further study. For the three bacillus species, protease production was carried out in a culture medium maintained at 30oc for 24 hours in shaking incubator 150rpm. The three bacillus species had optimum protease activity at temperature 55oC and incubation period of 1 hour. Bacillus stearothemophilus (F2n) had maximum protease activity at alkaline PH of 8.5, Bacillus subtilis (W4m) at slightly alkaline PH of 8.0 and Bacillus licheniformis (F1t) at neutral PH of 7.0. The result showed that Bacillus species under study are good producers of extracellular protease at high temperature. This is an indication that protease produced would be thermostable.

***Keywords*—** Enzyme, Protease, Bacillus subtilis, Bacillus licheniformis, Bacillus stearothermophilus, Tsire.

I. INTRODUCTION

Enzymes are biocatalysts that speed up the rate of biochemical reaction. They are produced mainly by cells of living organisms in order to enhance specific reaction in cell metabolic processes (Mohammad et al., 2013). Enzyme plays a vital role in industrial development. Protease is one of the known enzymes, ranked among the three groups of enzymes that account for close to 60% world-wide sale of enzymes (Nurullal et al., 2011). They catalyze the hydrolysis of peptide bonds of proteins to form polypeptides or free amino acids and also made up of 59% of the global market of enzymes expected to exceed $2.9 billion by 2012 (Deng et al., 2012).

One of the versatile, most essential with several industrial applications that are easily assessable is microbial enzyme. Production of protease from microorganisms is the leading and best form of protease ever produced because of their rapid growth rate, easy and simple cultivation, limited space required for cultivation and ease with which they can be genetically altered to multitude production as well as manipulated to generate new enzymes with altered properties that are desirable for their various applications (Kocher and Mishra, 2009). Bacillus is one of the microorganisms linked with production of an array of extracellular proteases. B. cereus, B. sterothermophilus, B.

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mojavensis, B. megaterium and B. Subtilis are Bacillus species involved in protease production (Shumi et al., 2004)

There are three common categories of protease type, alkaline, neutral and acidic proteases. Alkaline and neutral proteases are produced from bacterial while acid protease is obtained from fungi. Food, pharmaceutical, agricultural and medical industries have been taking maximumadvantage of using Bacillus sp. due to their wide range of physiological characteristics and ability to produce enzymes and other essential metabolites (Voigt et al., 2004). Bacillus subtilis , Bacillus stearothermophilus and Bacillus licheniformis species are recognized as GRAS (generally recognized as safe), because of their growth rates resulting into shorter period of fermentation and ability to secrete extracellular protein (Parado et al., 2014).

Several applications of proteases physiological and pharmaceutical includes, vital role in immune system of humans by defending against pathogens (Raju et al., 1994), proteolytic steps in tumor invasion or in infection cycle of a number of pathogenic microorganisms (Haq et al., 2006). Alkaline protease has been used in different industries such as detergent where they help to remove the protein stain, leather industry as dehairing agent (Barindra et al., 2006). They are used to remove the stiff and dull gum layer of sericine from the raw silk fiber to achieve improved softness in textile industry (Sharma et al., 1980). Protease is also used in pharmaceutical products like contact lens proteins cleaner. It also helps in natural healing process of skin by removal of necrotic tissue.

Despite numerous application of industrial proteases, the major barriers is unavailability of thermostable proteases, most proteases needed in our industrial set-up required several heat processes and heat reactions. This is an indication of prowess attributed to thermostable proteases in industrial development. Search for thermostable enzyme especially proteases is a continuous exercise as a result high demand and loss suffers by several industries due to its scarce and deficient on part of non-thermostable protease .

In a bid to enhance and fast track the availability of thermostable proteases, this study targeted the source of isolates for protease production. The tsire-suya was focus for the main source of the isolates based on the fact that a lot of bacterial are implicated in pre and post processing, the final process in its production involved heat and there is possibility that isolates obtained from post processing samples will be heat stable as well as heat resistant which will be of advantageous in the production of thermostable enzyme, especially thermostable proteases as a result of high protein content of meat used in tsire production.

Thermostable proteases are advantageous in some industrial applications(Cottage or Large scale) because higher processing temperatures can be employed, resulting in faster reaction rates as well as increase in solubility of non-gaseous reactants and products. The objective of this study includes Isolation and screening of bacteria from tsire for proteolytic activity, Cultural, morphological, sugar fermentation and biochemical identification of screened isolates and Effect of temperature, pH and incubation time on protease production.

II. MATERIALS AND METHODS

A. SAMPLE COLLECTION

Two samples of suya were collected at the suya spot in Abudulsalam Abubakar Post-graduate Hall, University of Ibadan within seven days interval. The area was within the latitude and longitude of 7° 23' 28.19" N and 3° 54' 59.99" E respectively. The first sample was stored for seven days while the second sample is fresh sample.

B. ISOLATION AND CULTURE METHODS

I. ISOLATION FROM TSIRE SAMPLES

This was carried out by method described by Harrigan and McCance (1966). A one in ten serial dilution was made for each stale and fresh ‘tsire’sample, peptone water was used instead of distilled water. This was followed by plating of appropriate dilution (10-4 and 10-6) on Nutrient agar (N.A), Mannitol salt agar (M.S.A) for probable Staphylococcus aureus, Tryptone soy agar (T.S.A) for Bacillus and Clostridium and Eosin Methylene Blue agar (E.M.B) for E coli . The plates were incubated at 37oC for 24hours. Developed colonies were counted to obtain total bacterial counts. Distinct colonies were sub-cultured until pure isolates were obtained.

II. MAINTENANCE OF PURE CULTURES

The pure cultures of bacterial isolates were streaked onto Nutrient agar slants and incubated at 37oC for 24hrs. After incubation period of 24hrs, they were kept refrigerated. At 5 weeks intervals, the isolates were transferred into fresh nutrient agar slants of the same composition to maintain the bacterial isolates.

C. CHARACTERIZATION OF ISOLATES

Characterization of isolates was carried out using morphological and biochemical tests.

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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 thin 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.

GRAM STAINING TECHNIQUES

A thin smear of each of the pure 24 hrs 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 secs and rinsed with water. The smear was again flooded with Lugol's iodine for 30 secs and rinsed with water, decolourized with 70% alcohol for 15 secs and was rinsed with distilled water. They were then counter stained with 2 drops of Safranin for 60 secs 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 (Fawole and Oso, 2004).

II. BIOCHEMICAL CHARACTERIZATION

A. CATALASE TEST

Nutrient agar plates containing the streaked bacterial isolates were each incubated for 18hours. A drop of freshly prepared 3% Hydrogen peroxide solution was added to each plate (Seeley and Van Demark, 1972). Evolution of gas (White Froth) indicates a catalase positive reaction. Absence of Froth indicates negative reaction.

B. OXIDASE TEST

Whatman (Number 1) filter paper was used for this test. By means of a sterile wire loop, a few drops of oxidase reagents (1% aqueous tetra methyl-p-phenylene diamine dihydrochloride) were applied on the Whatman paper to form a spot. The wire loop was sterilized again and used to touch colony of the test isolates and then transferred on the reagent on the Whatman paper. Formation of a very deep purple colouration within 10 seconds indicated a positive reaction while absence of deep colouration indicated negative reaction (Seeley and Van Demark, 1972).

C. STARCH HYDROLYSIS

Equimolar amount of soluble starch was prepared and added to nutrient agar without glucose or meat extract to give a 1% soluble starch before being poured to set in sterile plates. Single streaks of cultures were made on the dried plates before being incubated at 300C for 48hours. 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 (Seeley and Van Demark, 1972). While Reddish brown zones around the colony indicates partial hydrolysis of starch.

D. VOGES – PROSKAUER

The isolates were each cultured in methyl red broh7, 1ml of 6 – alpha-naphthol solution and Iml of 10% NaoH (Sodium Hydroxide) was added after two days of incubation at 300C. 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 (1966). Ten milliliters of the broth was dispensed into screw cap tubes and sterilized. Inoculation with test organism was subsequently done and incubated at 300C 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 (1210C for 15minutes) and allowed to cool before inoculating with the isolates. Un-inoculated tubes serve as control. The tubes were incubated at 30oC 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

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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 (NaNH4PO4) 1.5g, potassium dihydrogen phosphate (KH2PO4) 1.0g, Magnesium Sulphate (MgS04) 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 370C. 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 (Olutiola et al., 2000).

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 121oC for 20mins. 18-24hours 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 (Demman et al., 1960).

D.SCREENING EXPERIMENT FOR CHOICE OF PROTEASE-PRODUCING ISOLATES

The bacterial isolates were inoculated onto the casein agar plates. Casein agar was prepared as described by Larone (1993) but modified by Mosca et al., (2003). 10g of skim milk was dissolved in 90ml of sterile distilled water and 3g of Agar agar was dissolved in 97ml distilled water. Both solutions were sterilized separately. The skimmed milk was sterilized in water bath at 80oC for 10mins while Agar agar was sterilized at 121oC for 20mins. Both were allowed to cool between 45 and 50oC before mixed together and dispensed in 25ml amounts into 90mm diameter Petri dishes. Inoculation was done according to Azokpota et al. 2006. Well of 8mm diameter were made in the middle of the agar in Petri-dishes after solidification, using a sterilized cork borer and 1ml of inoculum from isolates were transferred to the wells followed by incubation at 37oC for 24 hours. The clearance zone around each well was measured as an indicator of protease producing organisms (proteolytic activity of the isolates).

E. PROTEASE PRODUCTION

Protease production was carried out using modified methods of Odunfa (1985). The culture medium contained 10g/L glucose, 5g/l casein, 2g/l peptone, 2g/L KH2PO4 and 1g/L MgSO4.7H20. It was maintained at 300C for 24hours in shaking incubator (G 24 Environmental incubator shaker, New Brunswick Scientific Co. INC, Edison, N.J. U.S.A) 150rpm. At the end of fermentation period, the whole fermentation broth was centrifuged at 10,000rpm at 40C for 10mins. This was followed by decantation of supernatant; the clear supernatant was then used as crude enzyme preparation.

F. DETERMINATION OF PROTEASE ACTIVITY

Protease activity was measured using Casein as substrate generally in 0.1M citrate phosphate buffer (pH 7.0). 1% (w/v) Casein solution was prepared in 0.1M citrate phosphate buffer solution and was heat – denatured at 1000C for 15mins in a water bath and

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allowed to cool after which casein was kept at 100C in a refrigerator. 10% trichloroacetic acid was prepared and kept refrigerated. Protease activity (assay) was determined by adding 1ml of supernatant into 1ml of casein solution and incubates for 1hour at 350C. The reaction was stopped by addition of 3ml of trichloroacetic acid solution. The test solutions were kept in the refrigerator at 40C for 1 hour and centrifuged using high speed refrigerated centrifuge (HITACHI CR 21GII) at 10,000 rpm for 5mins at 40C. The absorbance was read at 280nm using spectrophotometer (Jenway).

G. CHARACTERIZATION OF PROTEASE

I. EFFECT OF INCUBATION TEMPERATURE OF PROTEASE

This experiment was performed by incubating crude protease and substrate (casein) at different temperatures (350C, 370C, 400C and 550C respectively). This was carried out by adding 1ml of supernatant to 1ml of casein solution follow by incubation at 350C, 370C, 400C and 550C for 1hour. The reaction was stopped through addition of 3ml of Trichloroacetic acid solution. The test solutions were kept in the refrigerator at 40C for 1 hour and centrifuged at 10,000rpm for 5 minutes at 40C. The absorbance was read at 280nm using Spectrophotometer (Jenway).

II. EFFECT OF VARYING PH VALUES ON PROTEASE ACTIVITY

This experiment was performed to investigate the effect of varying pH values of citrate phosphate buffers on crude protease activity. 1ml of crude protease was added to 1ml of casein solution prepared by adjusting citrate phosphate buffers to different pH of 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5. This was followed by incubation at 350C for 1hour. The reaction was stopped through addition of 3ml of Trichloroacetic acid solution. The test solutions were kept in the refrigerator at 40C for 1hour and centrifuged at 10,000rpm for 5mins at 4oC.The absorbance was read at 280nm using spectrophotometer (Jenway).

III. EFFECT OF INCUBATION PERIOD ON PROTEASE ACTIVITY

The effect of different incubation time's viz: 1, 6, 12,18, and 24hrs at 35oC was observed on protease activity. 1ml of supernatant was added to 1ml of casein solution. This was followed by incubation at different periods (time) of 1, 6, 12, 18, and 24hrs. The reactions were stopped by addition of 3ml trichloroacetic acid. The test solutions were kept in the refrigerator at 4oC for 1hour and centrifuged at 10,000rpm for 5mins at 40C. The absorbance was read at 280nm using spectrophotometer (Jenway).

III. RESULT AND DISSCUSSION

A. BACTERIAL COUNTS OF FRESH AND STALE SAMPLES OF 'TSIRE'

The total bacterial count of fresh and stale 'tsire' samples on different culture media are shown in 1. The microbial load ranged from 1.1x105 to 2.2 x 106 for fresh 'tsire' sample while it ranged from 1.96 x 106 to 2.4 x 106 for stale sample Table 1. The highest bacterial count was observed in nutrient agar while the least was in Tryptone soy agar fresh sample Table 1. However, for stale sample, highest bacterial count was recorded in nutrient agar while the least was observed in Mannitol salt agar but there was no observable rnicrobiai load in Eosin Methylene blue agar for both fresh and stale tsire sample.

The different in the result recorded for bacterial counts may be linked to the fact that the fresh samples might contained limited or few amount of contaminated/ spoilage bacteria because of heat involved during the final processing the pre contamination by bacteria as a result of the packaging materials used, contamination by flies, method of handling and transportation and the air flora of the environment will not escape or survival final heat processing involved. The results obtained for stale tsire samples is highest and this can be attributed to post processing contamination as a result of packaging materials, spices and period of storage. The results recorded here for fresh tsire were within the acceptable standard specified by International Commission on Microbiological Specification (i.e. < 1.0 x 106 cfu/g) (ICMS, 1986) whereas the contrary results was recorded for stale tsire samples.

Tsire samples examined in this work are within the acceptable and satisfactory range under the Public Health Laboratory Service guidelines for the bacteriological quality of ready-to-eat foods samples at the point of sale (PHLS, 2000) since no pathogenic organism was isolated from all the tsire samples.

B. FREQUENCY OF OCCURRENCE OF BACTERIAL ISOLATES OBTAINED FROM 'TSIRE' SAMPLE

The frequency of occurrence of each isolate was shown in Table 2 with isolate F1t having the highest frequency of occurrence of 25% while W4M, F2n, W4n, F3t, W4t and W2t had lowest frequency of occurrence of 12,5% (Table 2).

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C. SCREENING EXPERIMENTS OF ISOLATES FOR PROTEOLYTIC ACTIVITY

A total number of 19 isolates were obtained for both fresh and stale tsire samples Plate 1. The isolates were screened for proteolytic activity, 8 out of the 19 isolates showed inhibition on skim milk agar plate, an indication of protease producer Plate 1. The clearance zones observed for respective isolates shows the ability of the isolates to utilize skim milk agar (as source of nitrogen), an indication that they are capable of producing protease enzyme. Isolates F1t, W4M, and F2n had higher diameter of zones (38, 30.5 and 27.5mm respectively. The least diameter zones (15, 17.5 and 18mm) were recorded for isolates W4n, W1n and F3t, while isolates W4t and W2t had diameters zones 20.5 and 24 mm respectively Figure 1.Three isolates (F1t, W4M, and F2n) with higher diameter of zones, a depiction of high presence of protease activity through high hydrolysis of skim milk, were considered for cultural, morphological, sugar fermentation, biochemical and further experimental studies.

Table 1: Total bacterial count of fresh and stale "tsire" samples on different culture media (cfu/ml)

Culture Media	Microbial loads of Fresh 'tsire' (cfu/ml)	Microbial loads of Stale 'tsire' (cfu/ml)
Nutrients Agar	2.9×10^5	2.4×10^6
Tryptone Soy agar	1.1×10^5	2.2×10^6
Mannitol Salt agar	1.3×10^5	1.96×10^6
Eosin Methylene blue agar	NG	NG

Key: NG= No Growth

Table 2: Frequency of Occurrence of Isolates obtained from UI Tsire Samples

Isolates code	Number of Occurrence	Percentage of Occurrence (%)
F_1t	2	25
F_2n	1	12.5
F_3t	1	12.5
W_4n	1	12.5
W_2t	1	12.5
W_4t	1	12.5
W_4m	1	12.5
Total	8	100

Note: UI=University of Ibadan

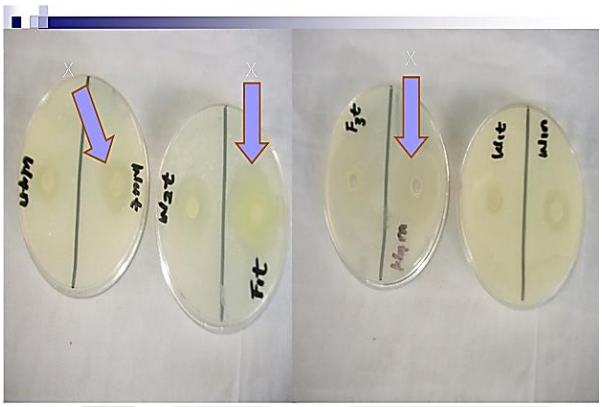

Plate1: Proteolytic activity of Isolates as depicted by clearance Zones (x) during screening experiment on skim milk agar.

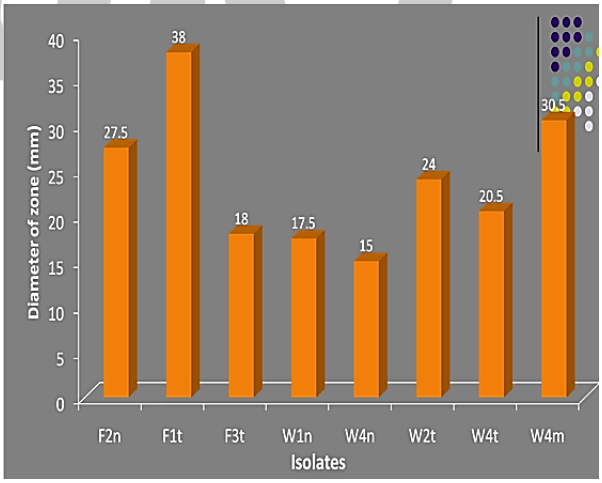

Figure 1: Proteolytic activity of the Isolates as depicted by diameter of inhibition zones during screening experiments on skim milk agar

D.IDENTIFICATION OF THE EFFICIENT PROTEASE PRODUCING ORGANISMS

Cultural, Morphological, Sugar fermentation and biochemical characterization were performed in accordance was Bergey's manual of determinative bacteriology (Cappuccino and Sherman, 2002) and by comparing their morphological and biochemical

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characteristics with standard reference organisms (Cheeshrough, 2009). 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 3). All the bacterial isolates are gram positive, rod shape, spore formers and motile (Table 4). It was identified that strain F1t belonged to Bacillus licheniformis, strain F2n belonged to Bacillus stearothermophilus and strain W4m belonged to Bacillus subtilis. The results were showed in (Table: 3, 4, 5 & 6). Studies have showed that high yielding strains are the major producers of Microbial proteases. This species includes bacillus sp., among these, Bacillus subtilis and Bacillus licheniformis are the most important group of bacteria that are involved in the enzyme industry and they are effective protease producers (Boominadhan et al., 2009).

Table 3: 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 4: Morphological characterization of bacterial isolates from UI "tsire" samples

Isolates Code	**Gram staining**	**Shape**	**Spore formation**	**Motility**
F_1t	+	rod	+	+
F_2n	+	rod	+	+
W_{4m}	+	Cocobacillus	+	+

Table 5: Carbohydrate fermentation of bacterial isolates from UI "tsire" samples

Isolates Code	**Glucose**	**Lactose**	**Maltose**
F1t	+	+	-
F2n	-	-	+
W4m	+	+	+

Isolates Code	**Mannitol**	**Sucrose**	**Arabinose**	**Xylose**
F_1t	+	+	+	-
F_2n	-	+	+	-
W_{4m}	+	+	+	+

Table 6: Biochemical characterization of bacterial isolates from UI "tsire" samples

Isolates Code	Cat	Oxi	S.H	V.P	M.R	N.R
F_1t	+	-	+	-	-	+
F_2n	+	+	-	-	+	+
W_{4m}	+	+	+	-	-	+

Isolates Code	Citrate	In	Co	Ur	Citrate	N.R
F1t	-	-	-	+	Bacillus licheniformis	+
F2n	-	-	+	+	Bacillus	+
W4m	-	-	+	-	Stearothermophilus Bacillus subtilis	+

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

E. EFFECT OF TEMPERATURE ON PROTEASE ACTIVITY OF BACILLUS STEAROTHERMOPHILUS (F2N), BACILLUS LICHENIFORMIS (FIT) AND BACILLUS SUBTILIS (W4M)

For the three Bacillus species, as temperature increases, then the protease activity increased. However, the Bacillus licheniformis had the highest activity when the temperature reaches us 55C, this was followed by Bacillus subtilis while the Bacillus stearothermophilus (F2n) had the least activity (Figure 2). In Figure 2, B. stearothermophilus (F2n) had an increase in growth within temperature range of 400C to 550C, though the optimum growth was at 550C, this was followed by a decrease in growth at 600C. Bacillus licheniformis (Fit) had its optimum growth at 45oC, however there is a gradual decrease in growth within temperature range of 400C to 500C, its optimum growth was at 50oC, and however a decrease in growth was observed within temperature range of 55oC to 60oC.

The protease activity were analyzed at different temperatures ranging from 35-550C at a constant pH7. Protease activity increased with temperature within the range of 35-550C (Figure 2). However, Bacillus licheniformis (Fit) had the highest activity as temperature increases, this was followed by Bacillus Subtilis (W4m) and Bacillus stearothermophilus (F2n). The results of this study agree with the report by Ammar et al. (2003) who observed that the optimum temperature for thermostable protease activity was 550C; Dhandapani and Vijayaragavan (1994) also observed that a maximum temperature of 550C was recorded for an alkaline protease activity form Bacillus stearothermophilus AP-4;, A similar type of result was observed by Horikoshi (1990), Banerjee et al (1999), Fujiwara and Yamamoto (1987) they all reported to be 600C. A related result was also observed by Zyidzia, and Zvauya, (2007), Adinarayana et al, (2003). In literature, optima temperature have been reported between 30-70oC for Bacillus sp. protease in Sevine et al.(2011).Optimum temperature for protease of B. lichenformis UV-9 was found to be 60oC by Muhammad et al. (2013). In similar view, Abebe Bizuye et al. (2014) reported optimum temperature for proteolytic activity of protease producing bacteria was 37oC- 50oC.

However, this was in contrast to the results recorded for protease activity by Bacillus subtilis BS1 which exhibited its maximum protease activity at 500C (Shaheen et al., 2008). The results of the present study was also in discordant with work of Sen and Saytyanarayana (1993) who reported 500C as optimum temperature for protease activity of Bacillus licheniformis S-40

F.EFFECT OF PH ON PROTEASE ACTIVITY OF BACILLUS STEAROTHERMOPHILUS (F2N), BACILLUS LICHENIFORMIS (FIT) AND BACILLUS SUBTILIS (W4M)

Studies of the influence of pH on the protease activity have also been carried out for three Bacillus species (Bacillus stearothermophilus (F2n), Bacillus licheniformis (Fit) and Bacillus Subtilis (W4m)) (Figure 3). The activity of the protease was very low at pH values between 5.5 and 6.5 for three Bacillus species and then increased sharply beyond pH of 6.5. Bacillus stearothermophilus (F2n) has its highest activity at pH values of 8.5, 7.0 and 7.5 respectively (Figure 3). The results of this study agree with the report of Sookkheo et al. (2000) who observed three proteases S, N and B from thermophilic Bacillus stearothermophilus TLS33 with optimum pH values of 8.5, 7.5 and 7.0 respectively. In related view, Sevine et al. (2011) showed that the enzyme also gave high activity in the alkaline pH range 6.0-9.0. Similarly, Abebe Bizuye et al. (2014) reported optimum pH for Protease producer at pH 8-10.

Bacillus subtilis (W4m) exihibited its maximum protease activity at pH ranges between 7.0 and 8.5 (Figure 3). Nascimento and Martins (1996) and Sookkheo et al (2000) were earlier reported that the optimum pH for protease activity was 7.0 to 8.5. According to Borris (1987) alkaline protease activity is found to be maximum at pH 9-13. This was also in complete accordance with the findings of many workers. The optimum pH for maximum protease activity was 7.5 by Bacillus subtilis (Remeikaite, 1997), 7.2 by Bacillus subtilis K11 and Bacillus licheniformis (Massuco, 1980; Schindler, 1981), and 7 Bacillus spp. Tku004 (Wang et al. (2006). Optimal activity was found at pH 8.5 for protease produced by Bacillus subtilis (Figure 3). The enzyme studied by Malath, and Dhar (1987) and Manachini et al. (1988) had a pH optimum range of 9.0 – 9.5. This indicates that the enzyme studied by them was alkaline in nature. The present results show it to be slightly less alkaline even when compared with the protease produced by Bacileus No. 221 (Horikoshi, 1971) and Bacillus licheniformus MIR 29 Ferrero et al. (1996) with an optimum pH of 11.5, 12.0 and 13

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respectively. In similar view, maximum activity at alkaline pH 9 was reported by Odu et al. (2012). In literature it was shown that the enzyme also gave high activity in the alkaline pH range 6.0-9.0(Sevine et al., 2011). Abebe Bizuye et al (2014) reported optimum pH for Protease producer was pH 8-10

Bacillus licheniformis (Fit) had its maximum protease activity at pH range beween 7.0 to 8.0 studies (Figure 3). The highest protease activity was found at pH 7.0. The results of this study agree with the report of Abdul Rouf (1990) who reported that the optimum PH for protease activity in their reaction mixture was found to be 7.2. Massucco, (1980) and Schindler (1981) also reported pH of 7.2 for optimum activity of protease produced by Bacillus licheniformis. Dutta and Banerjee (2006) reported that 7 are required for the maximum protease activity in Pseudomonas. A related result was also observed by Nascimento et al. (2004) who reported optimum activity of pH 6.9 for protease produced by Bacillus sp. SMIA -2.

G. EFFECT OF PH ON PROTEASE ACTIVITY OF BACILLUS STEAROTHERMOPHILUS (F2N), BACILLUS LICHENIFORMIS (FIT) AND BACILLUS SUBTILIS (W4M)

Effect of different incubation period on protease activity showed that the three Bacillus species had their highest activity after incubation period of 1 hour (Figure 4). Subsequent incubation periods (6, 12, 18 and 24 hours) showed gradual decline in protease activity. However, this was contrary with the work reported by Kunamneni et al. (2003) who found that the highest activity is after 48 hours of incubation with Bacillus subtilis PE – 11.

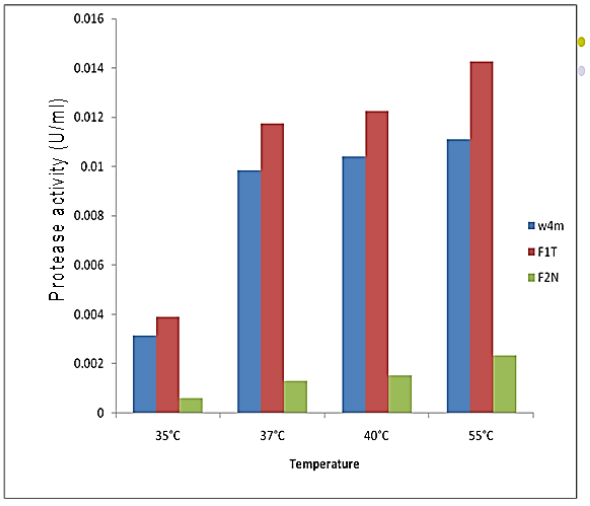

Figure 2: Effect of different temperatures on protease activity of Bacillus licheniformis (F1t), Bacillus subtilis (W4m) and Bacillus stearotherm ophilius (F2n) From UI 'tsire' samples

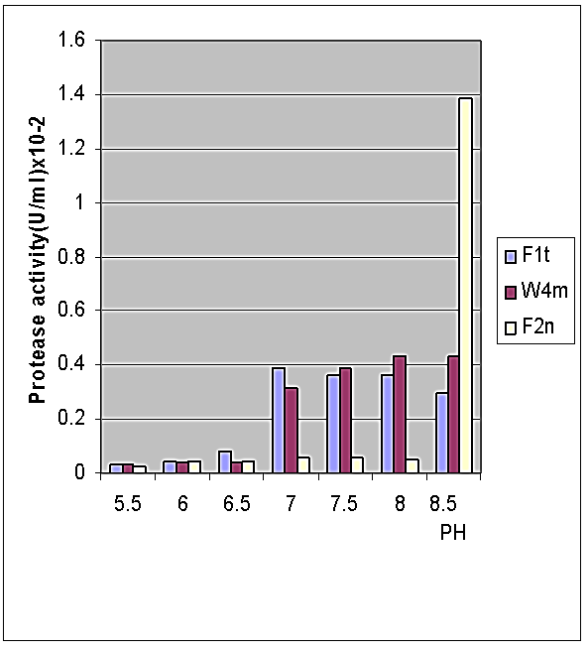

Figure 3: Effect of varying pH on protease activity of Bacillus licheniformis (F1t) Bacillus stearothermophilus (F2n) and Bacillus subtilis (W4m) From UI 'tsire' samples

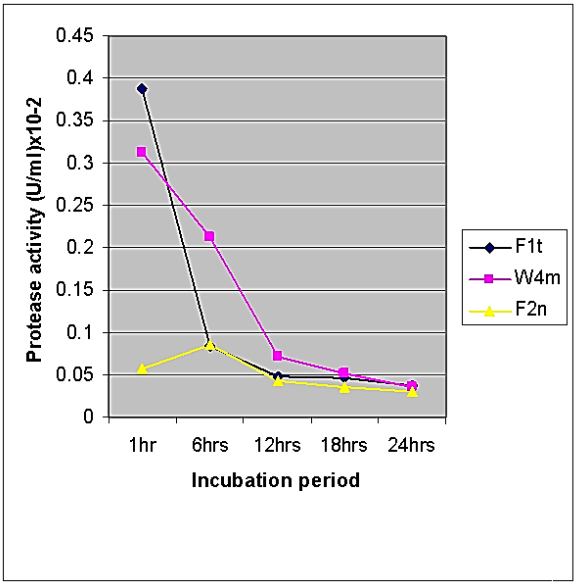

Figure 4: Effect of different Incubation periods on Protease activity of Bacillus licheniformis (F1t), Bacillus subtilis (W4m) and Bacillus stearothermophilus (F2n) From UI 'tsire' samples.

IV. CONCLUSION

The presence of these bacteria (Bacillus licheniformis (F1t), Bacillus subtilis (W4m) and Bacillus stearothermophilius (F2n)) in the tsire-suya meat is hazardous to public health. These organisms are producers of potent enterotoxins and the ingestion of food containing these toxins can cause a sudden onset

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of illness, with nausea, vomiting and diarrhea as the major symptoms. The presence of these organisms in the tsire samples is attributable of the filthy environment, poor personal hygiene of the processors and retailers, the use of contaminated utensils during processing, use of contaminated materials for packaging, activities of flies as well as the addition of spices and seasonings after processing. Bacillis spp are abundant in the nose and throat as well as the skin of humans. They are found in the air and even in the spices and the spores are heat resistant. This may have accounted for its occurrence in the tsire samples. The findings from this study showed that the tsire-suya was bacteriologically unsafe and constitutes a food safety risk; to its numerous consumers. There may be outbreak of food poisoning and or food borne infections due to the consumption of contaminated tsire meat if appropriate quality control measures not put in place. There is need to enlighten the producers, retailers and consumers of tsire-suya meat on the importance of good environmental and personal hygiene.

The alkaline protease isolated from Bacillus stearothermophilus (F2n) is a thermostable protease. It is stable at alkaline pH and high temperatures. The optimal pH and optimal temperature of the protease were at pH 8.5 and 550C. The protease isolated from Bacillus subtilis (W4m) is a thermostable protease. The optimal pH and optimal temperature of the protease were at pH 8.0 and 550C. The thermophilic neutral protease from Bacillus licheniformis (Fit) is a thermostable protease. It is stable at neutral pH, at high temperatures. The optimal pH and optimal temperature of the protease were at pH 7.0 and 550C. Bacillus species isolated from tsire are thermotolerant, thermopholic these are responsible for their survival during tsire production and ability to produce thermostable alkaline and neutral proteases that are advantageous in some industrial applications i.e. faster reaction rates as well as increase in solubility of non-gaseous reactants and products.

ACKNOWLEDGMENT

Special gratitude to God for successful completion of this work, our appreciation also goes to members of our family for support and understanding during the course of this research.

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