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Shelf-life and pattern of deterioration in firm Tofu

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ABSTRACT

The firm tofu production process in Nigeria was evaluated for contaminants and biodeteriogens involved in the degradation of the food material under stated conditions with the view of determining its biodeterioration pattern and shelf-life. Aspergillus niger, Cladosporium sp., Mucor sp., Schizosaccharomyces, Saccharomyces spp., Bacillus sp., B. subtilis, Micrococcus luteus, Streptococcus sp. and Pseudomonas sp. were implicated as processing contaminants. Successional studies at 8h intervals for 72h yielded a total of 29 isolates consisting of 9 bacteria and 5 fungi with B. cereus and A. niger being the most frequently occurring bacteria and fungi, respectively. The activities of the deteriogens brought about a progressive reduction in pH (5.77–4.89), protein (24.98–0.81%) and lipids (22.00–10.10%) but a constant increase in ash content (4.20–9.60ppm). Available moisture content increased and decreased based on prevailing activities in the microenvironment. Bacillus strains were better producers of amylase, lipase and protease, than other bacteria while the late fungal colonizers showed a higher lipase and protease activity. The shelf-life without refrigeration was 24h.

Keywords: *Tofu; biodeterioration; deteriogens; succession; enzyme; shelf-life*

INTRODUCTION

Tofu, a soybean curd, is a bland, cheese-like food of Chinese origin, made by coagulating fresh hot soymilk and then pressing the resulting curd into blocks (Wang, 1984). Shurtleff & Aoyegi (2000) categorized *tofu* on the basis of their texture after production as silken, soft and firm or regular. This highly nutritious plant-protein based food can be used in soups, salads, pastries, sandwiches, etc, as it is easy to digest and is substituted for meats, cheeses and certain dairy products in diets for dairy-sensitive individuals, vegans and the elderly. Based on the coagulant type, *tofu* can be a good source of calcium added to its inherent B-vitamins, isoflavones, minerals, fibre and unsaturated fat content (Rollins & Joseph, 2000). It has very low levels of saturated fat and no cholesterol.In Nigeria, *tofu* consumption is gradually increasing as the need for a solid plant-based protein substitute for the much needed but very expensive protein diets, such as meat, fish and egg, is rising. *Tofu* production is very easy and relatively cheap as it is made in several homes, hawked in streets and markets, and served in several boarding homes. The production process involves sorting of beans, steeping, dehulling, grinding, filtration, boiling of milk, coagulant addition and curd formation, pressing, dicing and frying in the case of firm *tofu* (Shurtleff & Aoyegi, 2000).

A large number of bacteria and moulds have been implicated as contributing spoilers of *tofu* while there has been no available report on their successive spoilage patterns and the contaminants associated in the production process. The bacterial colonizers include *Pseudomonas* sp., *Streptococcus* sp. (Fouad & Hegeman, 1993); *Bacillus* sp. (S08), *B. megaterium* (S10), *B. cereus* (S17, S27, S28, S32), *Enterobacter sakazakii* (No *et al.*, 2002); *Listeria monocytogenes* (Dong *et al.*, 2003) and *Enterococcus faecium* (LiLiTe *et al.*, 2003) while moulds are *Aspergillus niger* and *Penicillium* sp. (Takeshi, 1985).

No involvement of yeasts has been reported. These deteriogens no doubt have had their colonizing and degrading potential enhanced by the possession and action of various extracellular enzymes (Fapohunda & Olajuyigbe, 2006) although there is a paucity of information as regarding the screening of *tofu* deteriogens for extracellular enzymes. The quality of any food and its shelf-life are totally dependent on the production processes and conditions as well as its chemical (nutritional) composition and associated microflora (Dainty, 1971; Frazier & Westhoff, 1988). Also the successful colonization of a food material by an initial microbial population or community, subsequent development of seral and climax communities that leads to the ultimate alteration of food quality is determined by several intrinsic and extrinsic factors including food type, nutritional composition, pH, water activity, storage temperature, possession of suitable enzymes, etc (Hueck, 1968; Forsythe, 2002; Tucker, 2007). Among these, temperature is considered the most important factor (Dotsom *et al.*, 1977). The aim of this study was therefore to evaluate the firm *tofu* production in Nigeria (from raw material to finished product) for contaminants and biodeteriogens under stated conditions with the view of determining its biodeterioration pattern and shelf-life.

MATERIALS AND METHODS

Sample preparation

Two kilograms of soybean grains were purchased from markets in Ogun and Osun states of Southwestern Nigeria and divided into 2 batches of 1kg each. Batches were labeled as sample A & B. This study was conducted between January 2003 and April 2007. The production steps of Shurtleff & Aoyegi (2000) with slight modifications in the steeping conditions were employed in the processing of the beans into *tofu*. Sample A was steeped in 2.5L sterile hot tap water for 10mins in a 5L sterile covered thermostable glass jar after sorting of beans but before wet-milling in a Kenwood blender while sample B was steeped overnight (12h) in 2.5L sterile cold tap water. Calcium sulphate (alum) was used as coagulant while vegetable oil was used in frying the diced curd.

Biodeterioration studies

Freshly prepared, well fried *tofu* pieces made from the Sample B grains were counted into ten sterile plastic bowls. Each bowl represented a batch and contained triplicate firm *tofu* samples. From each batch, isolation of the biodeteriogens was made at 8-hour intervals to reflect the successional trend, ranging from T_0 to $T_{72.}$ T_0 represents the batch from which isolation was done immediately after obtaining the sample; T_8, after 8 hours; T_{16}, after 16 hours; T_{24}, after 24 hours, and so on. Sample bowls were well sealed with their lids and kept at ambient temperature (28ºC-30ºC) till a climax community was observed. The kjeldahl, soxhlet, dry ashing and oven-drying to constant weight methods of Nielsen (2002) were employed in the proximate analyses determination of crude protein, crude fat (lipid), ash and moisture contents, respectively, of the samples at the above specified intervals. The organoleptic property viewed as hardness and taste changes were evaluated by 5 untrained panelists at each interval period. Hardness was determined by the firmness or slimy texture of the *tofu* samples without consumption while taste was categorized as very good, good, poor and inconsumable upon consumption. Flavor determination in the samples was also noted as either good, palatable, offensive or repulsive (Dotsom *et al.*, 1977). Changes in pH throughout the experimental period were recorded. These parameters served as indices for the deterioration pattern of *tofu* at the stated intervals.

Isolation, characterization and Identification

For the enumeration of microbial populations during *tofu* production and at intervals as described above, randomly selected unsteeped and steeped grains as well as wet-milled paste (1g), cooked curd (1g) and 10g of each firm *tofu* sample was collected and carefully chopped into tiny pieces, mashed in a laboratory mortar to reduce particle size and then immersed in 90ml sterile 0.1% peptone water with vigorous shaking for 2mins to obtain stock solution. The homogenate was then serially diluted and aliquots of 1ml of each 10^{-7} dilution factor were aseptically poured into sterile Petri plates of molten nutrient agar (NA). The plates were immediately swirled gently for about 10 seconds before setting. The same procedure was repeated for yeast extract glucose agar (YGA), fortified with 50mg/L chloramphenicol (Difco Laboratories, Detroit, MI) to isolate molds and yeasts. Duplicate inoculated plates of NA and YGA were incubated at 30ºC for 48h and 5days, respectively. Each isolate was purified by repeated streaking (3 times) on NA (for bacteria), acidified potato dextrose agar (PDA) (Lab M, UK)

(for molds) and YAG (for yeasts) as pure forms were maintained on corresponding slants at 4ºC. Characterization of bacteria was by means of microscopy and conventional biochemical and physiological tests as described by MacFaddin (2000), Forbres *et al.* (2002) and Leboffe & Pierce (2002). Identification was facilitated by the use of the Bergey's Manual of Systematic Bacteriology (Sneath *et al*., 1986). Identification of fungal species was by macroscopic and microscopic observations and comparison with illustrations of Bulmer (1978), Domsch *et al.* (1980) and Brown (2005).

Qualitative Enzyme Assay

The rapid screening method of Hankin & Anagnostakis (1975) with slight modifications was employed for screening of extracellular amylase and lipase of bacterial and fungal origin. Lipase activity was determined using the modified tributyrin agar with a base consisting of 0.3% beef extract, 0.5% peptone and 1.5% agar (Fapohunda, 2006). Vegetable oil served as the treatment fat (sole carbon source) at 0.2% v/v. Amylase activity, evidenced by the zone of clearance, was demonstrated by flooding the incubated starch agar plates with freshly prepared iodine solution. Sterile Skim Milk Agar (SMA) plates containing skim milk powder (100g/L) and agar No. 2 (15g/L) (Lab M, UK) was used in determining extracellular protease (casease) production (Brown, 2005). For the fungal enzyme analyses, 5mm cork borer discs of isolates were used and plates incubated for 5 days at 30ºC while bacteria isolates were incubated at 25ºC for 48h. Zones of clearance produced by the isolates at the end of incubation period served as indices for enzyme production. The triplicate data from each test was analyzed by the SAS (version 9.1, SAS Institute Inc., Cary, NC). Mean values with standard deviations were separated according to the Fischer's protected least significant difference (LSD) at $P < 0.05$.

RESULTS AND DISCUSSION

Characterization of *tofu* processing contaminants

The moulds isolated from the grains before and after steeping in both hot and cold water and from the wet-milled paste were *A. niger* (Hs5 and Cs2), *Cladosporium* sp. (Hs3) and *Mucor* sp. (Hs2 and Cs5) while yeasts included *Schizosaccharomyces* (Cs1 and Wp2) and *Saccharomyces* spp. (Cs6, Wp1 and Wp3) although at various insignificant proportions. The bacteria isolated from grains were *B. subtilis* (Hs1 and Cs2), *Bacillus* sp. (Cs4), *Micrococcus luteus* (Cs6), *Streptococcus* sp. (Cs3 and Cs7) and *Pseudomonas* sp. (Hs4) while the paste had the same bacteria excluding *M. luteus* and *Streptococcus* sp. Hot water treatment during steeping and reduction in steeping time had no significant influence on the microbial contaminants present but could have had an influence on the numbers, although this was not investigated in this study. There was no visible microbial growth on inoculated plates of the cooked curd. The presence of these isolates at the various stages of soybean grain preparation into *tofu* regardless of the varying steeping conditions show that they have high nutrient affinity and colonizing potentials on this grain, and no doubt would go a long way in the deterioration of the resulting food product. The lack of growth in the culture of the cooked curd could have been due to the immediate heat treatment to which the curd was subjected in the final stage of the preparation. This probably led to protein denaturation, nucleic acid disorientation and microbial cell inactivation and/or death.

Microbial succession on *tofu*

Microbial analysis of samples at 8h intervals yielded a total of 29 isolates, made up of 9 bacteria and 5 fungi, all interacting in successive patterns (Table 1). The occurrence of *Bacillus* sp., *B. cereus*, *B. megaterium*, *L. monocytogenes*, *Streptococcus*, *A. niger* and *Penicillum* on firm *tofu* samples correspond with earlier reports of Takeshi (1985), Fouad & Hegeman (1993), No *et al.* (2002) and Dong *et al.* (2003) who stated that these microorganisms could be involved in the spoilage of *tofu*. *B. cereus*, according to No *et al.* (2002), seemed to be the highest bacterial colonizer of *tofu* sample. Our results are in agreement with this observation, and in addition, *A. niger* was the overall highest colonizer based on the frequency of occurrence. Shede *et al.* (2008) reported the involvement of *Bacillus*, especially *B. cereus*, in the successive degradation of raw buffalo hide. This bacterium exhibits the capacity to biosynthesize a wide range of extracellular enzymes that strengthen its colonizing power. It can therefore be deduced that members of the genus *Bacillus* are good deteriogens of a wide range of materials including *tofu*. However, there has been no report of the occurrence of *A. flavus* and *Fusarium* spp. on *tofu* although we found these as culprits during the succession study. This could be owing to their very late colonizing power of this food material.

Organoleptic property and pH

The activities of the isolated deteriogens brought about a gradual reduction in organoleptic properties (texture, taste and aroma) of *tofu* over time (data not shown). Well fried edible firm *tofu* gradually turned into non consumable, soft-textured (slimy) product with very strong repulsive aroma due to the release of volatile amines from protein degradation. These changes were accompanied by a progressive reduction in pH (Fig. 1) from 5.77 to 4.89, within the experimental period. Deceleration in pH as seen in this microenvironment was due to the release of various metabolites, which are rich in hydrogen ion concentration, at each stage of colonization starting from the initial colonizers through the seral stages to the final interactions of the climax community. Thus, the higher pH value favored more bacteria growth and the lower, for more fungal growth (Adegoke, 2004).

Proximate composition of *tofu* during deterioration

Biochemical analysis of the samples at each spoilage interval (Fig. 2) showed that firm tofu upon degradation lost appreciable amounts of its protein and lipids to enzymatic activities while ash contents progressively increased throughout the experimental period. Consequently, moisture content increased from the initial time up to 24h and gradually decreased. Specifically, the protein content reduced from 24.98–0.81%, lipids from 22.00–10.10% while ash content rose from 4.20–9.60ppm. Therefore within 72h of colonization of this food material, the protein content was rapidly depleted as nitrogen source for deteriogens while the lipid was utilized rather more slowly. The above data correlate with the reports of Fapohunda & Olajuyigbe (2006) who stated that the activity of *Alternaria tenuissima*, a deteriogen of stored cereals, was responsible for great nutrient loss in the cereals.

Dainty (1971) reported the presence and activity of *Bacillus sp.* on food materials rich in carbohydrate. Also, it can be noted that proteins and lipids serve as additional nutrients favorable for microbial growth. Therefore as the initial colonizers attack and invade this food material, they liberate extracellular enzymes that degrade the nutrients so that metabolic water is being released. The high increase in metabolic water recorded at T_{24} followed by the subsequent sharp fall at $T_{32,}$ and rise and fall at T_{40} and T_{48}, respectively, could be explained in terms of microenvironment modification by colonizers to favor their growth. At points of moisture level decrease, it was noted that there was an emergence of new fungal components in the microenvironment. This emergence could have led to competition for available resources and metabiosis, the reconditioning of the environment to suit growth, as moulds usually have lower critical water activity levels than bacteria (Forsythe, 2002; Tucker, 2007).

Amylase, lipase and protease from selected *tofu* biodeteriogens

The rapid screening method for qualitative determination of extracellular amylase, lipases and protease (casease) of bacteria (Table 2) showed that all the isolated species of *Bacillus*, *Listeria* and *C. pilosum* exhibited starch hydrolysis (amylase activity). This is evident in the increase in moisture level seen from the proximate analysis of the samples at specific intervals where these species in combinations occur. *M. kristianae* and all *Bacillus* produced lipases thereby aiding the complete oxidation of all lipids to free fatty acids. *Bacillus* sp., *B. megaterium* and *Listeria* demonstrated proteolytic activity by secreting casease while other *Corynebacterium* sp., *Micrococcus* sp. and *Streptococcus* sp. did not secrete any of these enzymes. The roles of these non enzyme liberating isolates in the microenvironment at points of emergence may not be appropriately explained since at those points, higher moisture content of deteriorating food material was recorded.

For the fungal enzyme analysis, the zones of clearance (mm) after 5days of incubation depicted enzyme activity (Table 3). *A. niger* (T_{48}C) showed the highest significant ($P < 0.05$) capacity for extracellular amylase activity with 30.10±0.02 mean zone of clearance of starch agar but very low insignificant ($P < 0.05$) lipase activity (5.05±0.03) as compared to the high significant lipase activity of *Fusarium* (T_{72}B) at 14.20±0.01mm, *A. niger* (T_{64}B) at 13.20±0.01mm and *Penicillium* (T_{56}A) at 10.05±0.01mm. Casease activity was significantly higher ($P < 0.05$) (23.00±0.01) in *A. niger* (T_{64}B) culture than in *A. flavus* (T_{32}B) at 11.25±0.02mm. Based on the successive patterns, it is seen that fungal components of the microenvironment emerged and gained stability as from T_{32} and the fungi tested for enzyme liberation reflected that the late colonizers showed higher lipase and protease activities than the early ones (Table 3). Thus the presence of *A. niger* from T_{32} to T_{72} confirms the report of Takeshi (1985) who stated the possible association of this fungus with *tofu*. This further shows the ability of this unique fungus to exhibit oligotrophism since it survived, alongside *Fusarium*, on low nutrient environment.

Table 1: Successive pattern and frequencies of occurrence of *tofu* deteriogens

Isolates	Codes	Frequency (f/n)
Bacillus sp.	T_0A, $T_{48}A$	$^2/_{29}$
B. cereus	T_0B, $T_{14}C$, $T_{24}A$	$^3/_{29}$
Schizosaccharomyces sp.	T_8	$^1/_{29}$
M. kristianae	T_0C, $T_{16}D$	$^2/_{29}$
Corynebacterium sp.	$T_{16}A$, $T_{40}A$	$^2/_{29}$
L. monocytogenes	$T_{16}B$, $T_{24}B$	$^2/_{29}$
Micrococcus sp.	$T_{24}C$	$^1/_{29}$
B. megaterium	$T_{32}A$	$^1/_{29}$
Streptococcus sp.	$T_{24}D$	$^1/_{29}$
A. flavus	$T_{32}B$, $T_{56}C$	$^2/_{29}$
A. niger	$T_{32}C$, $T_{48}C$, $T_{56}B$, $T_{64}B$, $T_{72}A$	$^5/_{29}$
C. pilosum	$T_{40}B$	$^1/_{29}$
Penicillum	$T_{32}D$, $T_{40}C$, $T_{48}B$, $T_{56}A$	$^4/_{29}$
Fusarium sp.	$T_{64}A$, $T_{72}B$	$^2/_{29}$

T_0 = pioneer community, T_8-T_{64} = seral community, T_{72} = climax community

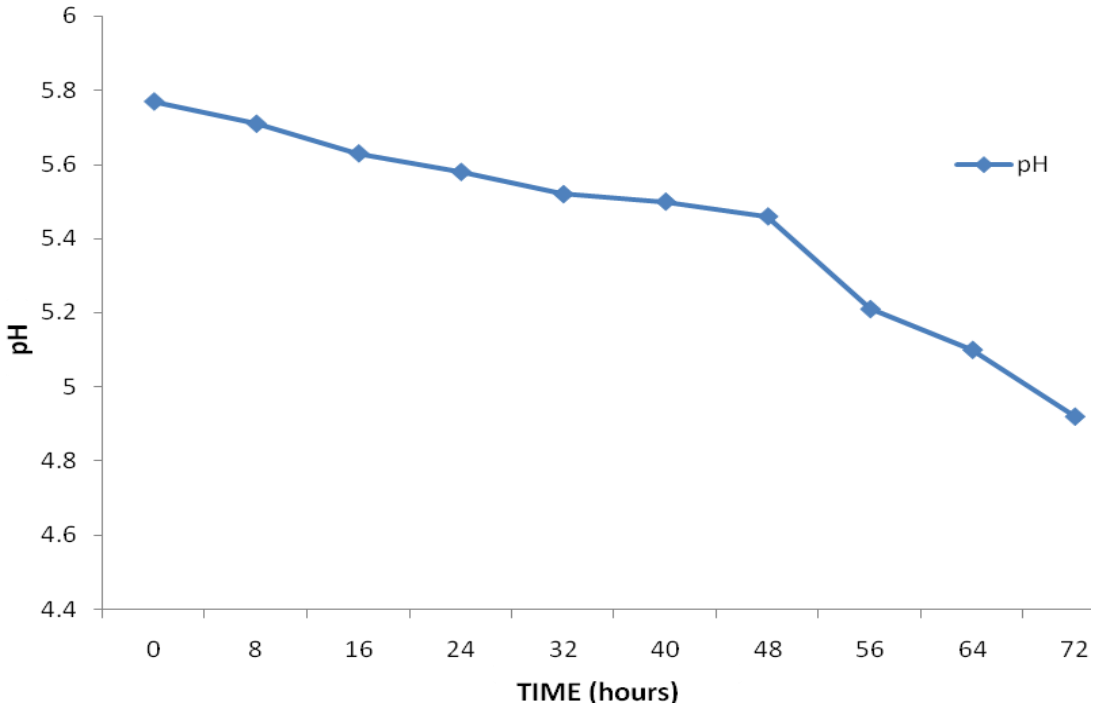

Fig. 1: pH changes of firm *tofu* during biodeterioration

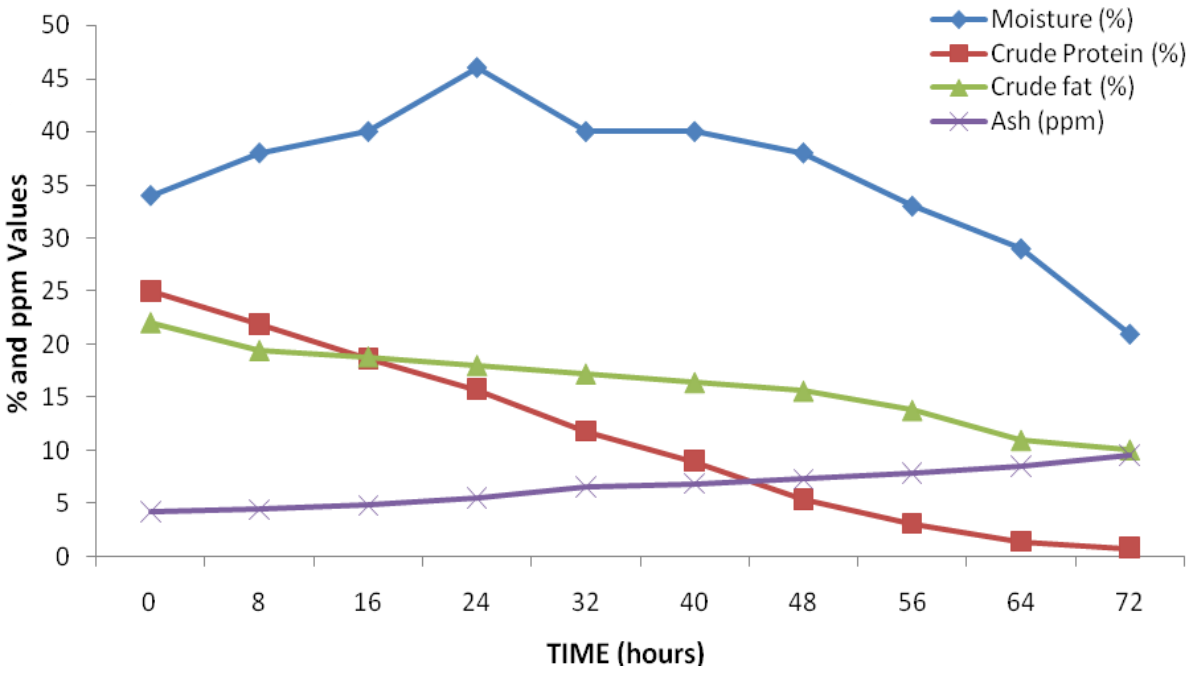

Fig. 2: Biochemical analysis of firm *tofu* samples in percentage (%) and parts per million (ppm) during biodeterioration

Table 2: Enzyme analysis for bacterial deteriogens of *tofu*

Bacteria isolates	Codes	Amylase	Lipase	Casease
Bacillus sp.	T_0A, $T_{48}A$	+	+	+
B. cereus	T_0B, $T_{14}C$, $T_{24}A$	+	+	*NZ
M. kristianae	T_0C, $T_{16}D$	*NZ	+	*NZ
Corynebacterium sp.	$T_{16}A$, $T_{40}A$	*NZ	*NZ	*NZ
L. monocytogenes	$T_{16}B$, $T_{24}B$	+	*NZ	+
Micrococcus sp.	$T_{24}C$	*NZ	*NZ	*NZ
B. megaterium	$T_{32}A$	+	+	+
Streptococcus sp.	$T_{24}D$	*NZ	*NZ	*NZ
C. pilosum	$T_{40}B$	+	*NZ	*NZ

+ = Zone of clearance (enzyme activity) *NZ = No zone of Clearance

Table 3: Enzyme activity (mm) for selected mycodeteriogens of *tofu*

Tested fungal isolates	Amylase	Lipase	Casease
A. flavus $T_{32}B$	5.00±0.01 ^{c}	5.00±0.00 ^{b}	11.25±0.02 ^{b}
A. niger $T_{48}C$	30.10±0.02 ^{a}	5.05±0.03 ^{b}	15.15±0.10 ^{a}
Penicillum $T_{56}A$	21.40±0.06 ^{b}	10.05±0.01 ^{a}	16.10±0.00 ^{a}
A. niger $T_{64}B$	27.50±0.01 ^{a}	13.20±0.01 ^{a}	23.00±0.01 ^{a}
Fusarium $T_{72}B$	*NZ	14.20±0.01 ^{a}	19.50±0.02 ^{a}

*NZ = No zone of Clearance

Results are means and standard deviations of triplicates Means with same superscript alphabets in same column are not significantly different ($P < 0.05$)

Conclusion

This research therefore suggests that based on organoleptic changes brought about by the deteriogens, well fried firm *tofu* should be consumed within 24h for safety purposes. In addition, it has been observed that *Bacillus* sp. are good deteriogens of food materials from the onset, while *A. niger* are responsible for the latter stages. This then forms a basis for investigating possible preservatives for firm *tofu* that could be bacteriostatic or bacteriocidal to initial colonizers (T_0) under ambient temperature since *tofu* consumption is increasing rapidly. Further investigation will focus on the possible metabolites (antimicrobials) elaborated by each isolate especially the late colonizers by which they exclude the early population.

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