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Research Article

Qualitative Determination of Amylase, Lipase, and Protease Production from Microorganisms Causing Deterioration in Raw and Processed Beef

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Abstract

Meat deterioration is a global problem and one of the leading causes of food wastage and substantial economic loss. Although several factors can accelerate this process, microorganisms are perhaps its main promoters due to intracellular enzymes released from lysed microbial cells during metabolism. The present study investigates the microorganisms isolated from meat samples that are responsible for the deterioration of the sample. The assay was carried out in triplicate. Pure cultures of bacteria and fungi used for amylase, lipase, and protease production were previously isolated from raw and processed meat samples. Sterile Bushnell-Hass medium containing 1% starch, olive oil, and skim milk in separate Petri dishes was used and thereafter the basal medium after incubation were flooded with Iodine, Sudan III and biuret solution respectively according to the enzyme assayed. The zones of clearing which were measured and analyzed using one-way ANOVA indicates amylase, lipase or protease secretion. Among the 20 isolates used for the assay, *Klebsiella pneumoniae* was the only bacteria isolate that showed clear zones (mm) for the amylase (4.67 mm), lipase (2.33 mm), and protease (6.33 mm). Other bacterial isolates showed clear zones only for either one or two of the enzymes. There was statistical significant different on the clear hydrolytic zones recorded for the bacterial isolates. The fungal isolates, *Fusarium oxysporum*, *Mucor pusillus*, and *Alternaria alternata* showed hydrolytic clear zones (mm) in all the three enzymes assayed signifying that these organisms were a contributory factor to the deterioration of the beef sample.

Keywords: Deterioration; Microorganism; Amylase; Lipase; Protease; Zones of Clearing

Abbreviations

SPSS: Statistical Package for Social Sciences; mm: Millimeter

Introduction

Meat has been and remains the first choice of animal protein. In developing nations, such as Nigeria, fresh meat constitutes a considerable share of meat consumption. Many countries have different meat consumption patterns and livestock production systems which impact on the products delivered to the market [1]. The authors also reported that "meat industry development is an integral part of the strategy for the advancement of the entire livestock value chain development with a strong degree of integration of the producers and consumers". Meat is an expensive food commodity which contains major growth nutrients like protein, fat, carbohydrate and mineral elements like Na, K, Ca, Mg, P, Fe, Cu, Zn, S, Cl. It also contains a lot of water (which is easily available to microbial deteriogens) and vitamins.

Meat is a nutrient-dense, protein-rich food that is extremely perishable and has a short shelf life if not preserved. The shelf life and maintenance of meat quality are affected by a number of interrelated elements, including keeping temperature, which can result in degraded meat quality characteristics. The most

significant factor affecting the keeping quality of meat is spoilage due to microbial development. The storage of foods as well as meat and meat products is limited by non-enzymatic, enzymatic, or microbial reactions that alter the edible quality of foods, including deterioration, appearance, texture, aroma, flavor, nutrition, and safety and functional properties [2]. Meat and meat products provide excellent growth media for a variety of microflora (bacteria, yeasts, and filamentous fungi) some of which are pathogens [3]. The storage conditions affect the type of microorganisms found in meat and meat products. The favorable pH for the growth of spoilage bacteria in meat is in the range of 5.5-7.0. Slime formation, structural component degradation, off odors, and appearance change were found in meat because of microbial growth within this pH range [4]. Lipid hydrolysis can take place enzymatically or non-enzymatically in meat. In muscle cells of slaughtered animals, enzymatic actions are taken place naturally and they act as catalysts for chemical reactions that finally end up in meat self-deterioration [5]. Elbashir., *et al*. [6] reported that "it becomes highly susceptible to spoilage through a series of chemical reactions, under the action of microorganisms and enzymes due to its high water content and high pH". Microorganisms produce intracellular enzymes to utilize low molecular weight food compounds by transporting them into cells that cause detectable food decay. These enzymes hydrolyze large nutrient molecules of food to metabolize small molecules. Food decays very rapidly with large numbers of microorganisms; an increase in microorganisms causes an increase in the number or amounts of enzymes released by lysed cells resulting in food deterioration [2]. The present study aims to investigate the microorganisms isolated from raw and processed beef samples that are responsible for the deterioration of the samples. Hence, the knowledge will enable one to understand and gain control over the decay process and thus enhancing the shelf life of meat and meat products.

Materials and Methods

Microorganisms

Pure cultures of bacteria and fungi used were previously isolated from raw and processed meat samples in Jos metropolis as reported by Eze., *et al*. [7].

Enzyme production and assay

Screening for amylase, lipase and protease production using plate assay: Preliminary Qualitative screening was done using the modified method of Eggins and Pugh [8] by inoculating 5 mm mycelia discs from the edge of an actively growing 1 and 4-day old bacterial and fungal isolates respectively on sterile Bushnell-Hass medium containing 1% (w/v) starch (HiMedia), olive oil or skim milk separate Petri dishes which served as the carbon sources. The medium was supplemented with I ml fluconazole (200 mg/ml) and 0.5 ml gentamycin (40 mg/ml) to suppress fungal and bacterial growth respectively. The agar culture plates containing various inducible substrates were incubated at 37°C and 25°C for 24 hours and 4 days respectively for bacteria and fungi. After incubation, the mycelium/hyphae were scrapped after the growth of the organisms leaving the basal medium, which was flooded with Iodine or Sudan III or biuret solution according to the enzyme assayed. They were allowed to stand for fifteen minutes, then the excess solutions were discarded and incubated at room temperature for ten minutes and the zones of the clearing were determined which were indications of amylase, lipase, or protease secretion. Control experiment was also set up using basal salt agar plates without the inducing substrate (starch). The plates were observed for a clear zone of hydrolyzed starch against a blue background of unhydrolyzed starch. The experiment was replicated thrice.

Statistical evaluation

The various results that were obtained were subjected to statistical analyses with the aid of one-way ANOVA using SPSS version 23.

Results and Discussion

The organisms isolated were subjected to a different enzymatic test to know their ability to degrade starch, lipid, and protein. The details of the results are presented in tables 1 and 3.

Among the bacteria isolates, *Klebsiella pneumoniae* was the only organism that showed clear hydrolytic zones amylase (4.67 mm), lipase (2.33 mm), and protease (6.33 mm). The highest clear zone of 7.00 mm was recorded for *Proteus mirabilis* for amylase. This was followed by *Staphylococcus simulans* with clear zone of 6.67 mm. It was observed that *B. lentus, Escherichia coli, S. aureus,*

S. intermedius and *S. simulans* recorded no zone of clearance (0.00 mm) for lipase. There was statistical significant different on the clear hydrolytic zones recorded for the bacterial isolates. *Proteus mirabilis*, *Klebsiella pneumoniae*, and *Staphylococcus simulans* were significantly higher than other isolates in their amylase production. *Enterobacter cloacae* differed significantly from others in their lipase production while *K. pneumoniae* and *S. simulans* are significantly higher than other bacterial isolates in their protease activity.

Fusarium oxysporum, *Mucor pusilus* and *Alternaria alternata* showed clear zones (mm) in all the three enzymes tested. It was observed that *M. pusillus* recorded the highest zone of clearance of 9.67 mm. This was followed by that of *Aspergillus ochraceus*, 8.00 mm and *A terreus,* 6.33 mm respectively on protease. The details of the results are presented in tables 2 and 4.

Among the fungal species, *Aspergillus flavus* and *Alternaria alternata* were significantly higher than other isolates in their amylase activity. *Aspergillus ochraceus* differed significantly from others in their lipase activity while *Mucor pusillus* showed the highest activity in the protease test but was significantly similar to *A. ochraceus, Aspergillus fumigatus,* and *Aspergillus terreus*. Enzymes have the property of causing and regulating specific chemical reactions inside or outside living cells [9]. Enzymes are among the most important products obtained for human needs through microbial sources. The enzyme activities assayed showed that *K. pneumoniae* among the bacteria species was the only organism that had hydrolytic clear zones in the three enzymes tested. *K. pneumoniae* was only found in the decayed meat samples as previously reported by Eze., *et al*. [7] implying that the production of the enzymes contributed to the decay process of the samples. According to Erkmen, Osman and Bozoglu [2], microorganisms produce intracellular enzymes to utilize low-molecular-weight food compounds by transporting them into cells that cause detectable food decay. In the amylase assay, *Proteus mirabilis, S. simulans. Bacillus cereus* and *K. pneumoniae* were statistically similar with the highest clear zone shown in *P. mirabilis*. Reports by Oseni and Ekperigin [10] showed that *P. mirabilis* is amylolytic. Though *E. cloacae* did not show any clearing zone the report of Jadhav., *et al*. [11] in their quantitative assay indicated that Enterobacter sp. is an amylolytic producer. *E. cloacae* showed the highest lipolytic activity among the tested organisms. Researchers like Mitra, Khare and Singh [12] reported alkaline lipase production in *E. aerogenes*. These organisms must have produced enzymes that aid in the deterioration since most of them were not found in the non-decayed meat samples as previously reported by Eze., *et al*. [7]. *K. pneumoniae* and *S. simulans* showed the highest protease activity and Bunn., *et al*. [13] in their study showed that *S. simulans* is a protease producer. In the fungal isolates, *A. flavus, Alternaria alternata,* and *A. oryzae* are statistically similar with *A. flavus* and *A. alternata* showing the highest amylolytic activity [14]. Verma and Verma [15] found *A. flavus* and *A. alternata* respectively as amylolytic-producing organisms. *A. ochraceus, Fusarium oxysporum, A. alternata,* and *Mucor pusillus* showed lipolytic activity with the highest activity seen in *A. ochraceus.* In the findings of Yadav., *et al*. [16], *A. ochraceus* was reported to be lipolytic among the lipase producers of *Aspergillus* and *Penicillium spp* that were screened. In addition, Ozturkoglu-Budak, Wiebenga, Bron, and de Vries [17] in their findings reported *Mucor racemosus* as a lipase producer. *Mucor pusillus, A. ochraceus, A. fumigatus,* and *A. terreus* are statistically similar in their protease production with *Mucor pusillus* showing the highest protease activity. Alves, de Campos-Takaki, Okada, Pessoa, and Milanez [18] identified protease-producing enzymes in 12 species of the Genus *Mucor*. There are variations in the literature as regards enzyme assay as in the findings of Geoffry and Achur [19] whose reports showed that agar plate methods vary greatly due to the nature of the substrate employed.

Bacterial species	**Enzymes Production**			**Total**
	AMYLASE	**LIPASE**	**PROTEASE**	
Bacillus cereus	+	_	+	2
B. lentus	_	_	_	0
Enterobacter cloacae	_	+	+	2
Escherichia coli	_	_	+	1
Klebsiella pneumoniae	+	+	+	3
Proteus mirabilis	+	_	+	2
Staphylococcus aureus	_	_	_	0
S. intermedius	_	_	_	0
S. simulans	+	_	+	2
TOTAL	4	2	6	12

Table 1: Enzymes production by the test bacterial isolates.

Bacterial species	Depth of clearing (mm)		
	AMYLASE	LIPASE	PROTEASE
Bacillus cereus	4.00 ± 1.73^{ab}	0.00 ± 0.00^{c}	4.33 ± 0.58^{ab}
B. lentus	0.00 ± 0.00^{b}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
Enterobacter cloacae	0.00 ± 0.00^{b}	4.00 ± 1.00^{a}	5.00 ± 0.00^{ab}
Escherichia coli	0.00 ± 0.00^{b}	0.00 ± 0.00^{c}	3.00 ± 1.00^{b}
Klebsiella pneumoniae	4.67 ± 2.08^{a}	2.33 ± 0.58^{b}	6.33 ± 3.21^{a}
Proteus mirabilis	7.00 ± 5.57^{a}	0.00 ± 0.00^{c}	5.33 ± 0.58^{ab}
Staphylococcus aureus	0.00 ± 0.00^{b}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
S. intermedius	0.00 ± 0.00^{b}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
S. simulans	6.67 ± 3.21^{a}	0.00 ± 0.00^{c}	6.33 ± 1.53^{a}

Table 2: Mean depth of clearing bushnell haaz agar by the bacterial isolates.

Values expressed as mean ± Standard deviation at P < 0.05.

^{a,b,c} Means within a column with different superscripts are significantly different (P < 0.05).

Fungal species	Enzyme Production			Total
	AMYLASE	LIPASE	PROTEASE	
Alternaria alternate	+	+	+	3
Aspergillus flavus	+	_	+	2
A. fumigatus	+	_	+	2
A. niger	+	_	+	2
A. ochraceus	_	+	+	2
A. oryzae	+	_	+	2
A. terreus	_	_	+	1
Fusarium oxysporum	+	+	+	3
Mucor pusillus	+	+	+	3
Penicillium chrysogenum	_	_	+	1
Rhizopus microspores	_	_	_	0
Total	7	4	10	21

Table 3: Enzymes production by the test fungal isolates.

Fungal species	Depth of clearing (mm)		
	AMYLASE	LIPASE	PROTEASE
Alternaria alternate	2.67 ± 0.58^{a}	1.00 ± 0.00^{b}	1.33 ± 0.58^{cd}
Aspergillus flavus	2.67 ± 0.58^{a}	0.00 ± 0.00^{c}	3.33 ± 1.53^{bcd}
A. fumigatus	1.00 ± 0.00^{c}	0.00± 0.00^{c}	5.33 ± 5.13^{abcd}
A. niger	1.67 ± 0.58^{bc}	0.00± 0.00^{c}	1.67 ± 0.58^{cd}
A. ochraceus	0.00± 0.00^{d}	5.00 ± 1.00^{a}	8.00 ± 6.93^{ab}
A. oryzae	2.33 ± 1.15^{ab}	0.00 ± 0.00^{c}	2.33 ± 0.58^{bcd}
A. terreus	0.00± 0.00^{d}	0.00± 0.00^{c}	6.33 ± 1.15^{abc}
Fusarium oxysporum	1.67 ± 0.58^{bc}	1.00 ± 0.00^{b}	2.33 ± 0.58^{bcd}
Mucor pusillus	1.33 ± 0.58^{c}	1.00 ± 0.00^{b}	9.67 ± 5.86^{a}
Penicillium chrysogenum	0.00± 0.00^{d}	0.00± 0.00^{c}	1.67 ± 0.58^{cd}
Rhizopus microspores	0.00± 0.00^{d}	0.00± 0.00^{c}	0.00± 0.00^{d}

Table 4: Mean depth of clearing of bushnell haaz agar by the Fungal Isolates.

Values expressed as mean ± Standard deviation at P < 0.05.

^{a,b,c,d} Means within a column with different superscripts are significantly different (P < 0.05).

Plate 1: Qualitative enzyme assay: A = Protease, B = Amylase, C = Lipase.

Conclusion

Klebsiella pneumoniae, Fusarium oxysporum, Mucor pusillus, and *Alternaria alternata* were the only organisms that showed hydrolytic zones (mm) in the amylase, lipase, and protease enzymes assayed signifying that these organisms were a contributory factor to the deterioration of the beef sample.

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