

ANTIMICROBIAL ACTIVITIES OF *Citrus aurantiifolia* PEELS ON MICROORGANISMS ISOLATED FROM SPOILT ONIONS SOLD IN AWKA, ANAMBRA STATE, NIGERIA

ABSTRACT

One of the most significant monocotyledon crops is the onion, which also contains antioxidants. This research aims to isolate microorganisms that cause spoilage in onion and to study the anti-microbial activities of lime peel extract on the isolates. Spoilt Onion samples were collected from three different markets in Awka Metropolis (Eke Awka, Nnamdi Azikiwe temporary site (Temp. site) and Amenyi). They were then transported to Alpha research laboratory Awka, for analysis. The spoilt onions samples were sterilized and cultured using the standard procedure. The culture media used for the study were Nutrient Agar and Sabroud Dextrose Agar, both were placed into a conical flask and subjected to an autoclave at a rate of 121°C at 15psi for 20 minutes and the plates were incubated at room temperature. In the study, various microorganisms that can caused spoilage were identified; the bacteria include *Klebsiella* sp, *E-coli*, *Staphylococcus spp* and *pseudomonas spp*. The fungal isolates are *Aspergillus sp*, *Penicillium sp*, *Mucor sp*, *Rhizopus sp*. They were isolated and identified both morphologically and microscopically. Samples from Amenyi had the highest fungal counts ($4.50 \times 10^4 \pm 0.00a$ cfu/g) while samples from Eke-Awka had the lowest fungal count ($2.70 \times 10^4 \pm 0.200c$ cfu/g) on SDA. Also, samples from the Temp. site had the lowest bacterial counts ($4.71 \times 10^4 \pm 0.100c$ cfu/ml) while samples from Eke-Awka had the highest fungal count ($6.10 \times 10^4 \pm 0.03a$ cfu/ml). The result showed that ethanol extract from lime peel has good inhibitory effects of the organisms. *Rhizopus* sp. had the highest inhibition at 100% of lime peel extract while *Aspergillus* sp. has no inhibition at 100%, 50%, and 25% of lime peel extract. Also, *Escherichia coli* had the highest inhibition of (43.00 ± 0.30) at 100% of lime peel extract while *Pseudomonas* had no inhibition at 100%, 50%, and 25% of lime peel extract. As a result, the study recommended that farmers utilize lime peel extract, which is a much safer and more effective technique of controlling onion spoilage.

Keywords: Onion; Microorganism; Lime; media

INTRODUCTION

The family Alliaceae, which also includes leeks, shallots, and garlic, includes the onion (*Allium cepa*). An estimated 88.510 tons of onion bulbs are produced worldwide, with majority produced in Canada (Buchanan and Gibbons, 2014). The prevalence of bulb rot disease continues to be a major cause of post-harvest loss in the food and agricultural industries. It has been estimated that in Nova Scotia, the disease bulb rot alone causes the loss of up to 20% of stored onion bulbs (Charles, 2012). Several pre- and post-harvest factors, including genotypic traits, soil characteristics, climatic factors, and management techniques, can be blamed for the bacterial infection of onion bulbs and the resulting loss of onion bulbs (Gllis *et al.*, 2007). The grey mold fungus, the black mold fungi, the blue mold fungi, and the bacterial rot are onion diseases (Barth *et al.*, 2009).

Phosphorus, calcium, and carbohydrates are all abundant in onions. The volatile oil known as ally-propyl disulphide is what gives onions their pungent flavor. With a global production of roughly 25 million tonnes, onions are a significant crop on all continents (Frazier and Westhoff, 2015).

Bulb rot in onions is brought on by bacterial infections, and it can appear at any point between pre-harvest and storage. The onion's neck softens when pressed, the bulb tissue softens and soaks up water, and the color changes from yellow to brown. The majority of these illnesses or germs affect onions. For the germs to enter and spread, water is necessary. Through wounds and dead lower leaves, bacteria invade the bulb.(Asagbara and Oyewole, 2011; Berger *et al.*, 2007). The soil-borne viruses might disperse by irrigation water or rainwater splashes. Warm temperatures (above 85°F) and moist environments are favorable to the majority of these microorganisms that induce onion spoilage (Dodona *et al.*, 2005).

This research intends to isolate and identify the microbes responsible for onion deterioration in several locations in Anambra state, Nigeria. Also, to determine the antimicrobial activities of lime peel extract on these isolates.

MATERIALS AND METHODS

2.1 Sample Collection

Three (3) markets in Anambra state (Eke Awka, Nnamdi Azikiwe temporary site (Temp. site), and Amenyi) were where the samples of spoiled onions were purchased. Alpha Research Laboratory in Awka, Anambra state provided the lab and other facilities needed for the practical work.

2.2 Fungal Isolation

2.2.1 Culture Media: In this research, Sabouraud Dextrose Agar (SDA), a commercially available media, were used.

2.2.2 SDA media preparation: One liter of distilled water was added to a suspension of around 65g of the medium, which was thoroughly mixed before being heated to boiling and then dissolved. It was heated for one minute to dissolve the solution, and then sterilized for 15 minutes at 118 to 121⁰ C in an autoclave. After that, while the solution was still molten, 500 milligrams of the antibiotic streptomycin was added. If any of the solution was left over at that point and wasn't immediately needed, it was kept in the refrigerator between 8 and 15⁰ C until then.

2.2.3 Isolation of Fungi

To enhance with the dirt removal, distilled water was used to clean spoiled onion surfaces. Using a sterilized scalpel, a little piece of the spoiled fruit was cut off and put onto freshly made PDA and SDA agar, where it was cultured for three (3) days at room temperature.

2.2.4 Sub-culturing Techniques

This was achieved using the technique described by Chuku *et al.*, 2017. When more than one colony of fungi was seen in the petri dishes, the resulting colonies were subsequently subcultured onto Potato dextrose agar (PDA). This process was continued until pure cultures were obtained.

2.2.5 Identification of isolated Fungi

All of the different fungal species that were isolated were identified, and both macroscopic and microscopic aspects as well as their varied properties were examined, including color, texture, hyphae and conidial forms, conidiophore presence, and conidial head shapes. The correct taxonomic keys assisted in the microscopic identification.

2.2.6 Determination of Fungal Frequency (%)

The prevalence of fungi will be assessed location-wise, in terms of cultural media, and later, in connection to the percentage of diseases calculated based on symptoms. The following formula will be used for fungal frequency percentage determination:

$$\text{Fungal Frequency (\%)} = \frac{\text{Number of particular fungus colonies observed in plates} \times 100}{\text{Total number of colonies of all fungi}}$$

2.3 Isolation of bacteria

Using nutritional agar and EMB agar, the procedure was utilized to separate the bacteria from the tomato seeds. The seeds (weighing one gram) were aseptically collected before being serially diluted in saline. The agar was inoculated with a mixture of nutrients agar and EMB, and they were incubated at 35°C for 24 hours in order to count all of the aerobic heterotrophic bacteria and fecal coliforms in the sample.

2.3.1 Total plate count of bacteria (CFU/ml)

The microbial load in agar plate samples was calculated using a formula.

$$\text{Cfu/ml} = \{(\text{No. of colonies} \times \text{dilution factor}) / \text{volume of inoculum}\}$$

2.3.2 Purification of isolates

The selected colonies were then subcultured for 24 hours on nutrient agar plates. They were then subjected to microscopic identification and biochemical analyses.

2.3.3 Identification of microorganisms

Morphological identification: The isolated bacteria were identified based on motility and Gram-staining.

2.4 Pathogenicity Test

Pure culture of the fungi was isolated using inoculation loop of length 5cm and sterilized using 100% ethanol. These fungal cultures were subsequently isolated in pure forms by sub-culturing and incubated for 24hrs and used for microscopic characterization and biochemical analysis.

Statistical Analysis

Analytical Statistics Sigma plot version 12 statistical software was used to conduct a two-way analysis of variance (ANOVA) on the collected data in order to determine the level of significance of the LSD 0.05% treatment.

RESULTS

Table 1 showed the colony count of bacteria from the different markets. Eke Awka market had the highest colony count, while the Temp site had the lowest colony count.

Table 1: Total bacterial count of the Onion Samples

Sample site	Total bacterial count (cfu/ml)
Eke awka market	$6.10 \times 10^4 \pm 0.03a$
Temp site	$4.71 \times 10^4 \pm 0.100c$
Amenyi market	$5.64 \times 10^4 \pm 1.101b$

Table 2 showed the fungal count from the three markets. Amenyi had the highest fungal count while Eke awka had the lowest.

Table 2: Mean Fungal count of Onion Samples

Sample site	Mean total fungi count (CFU/g)
Eke Awka	$2.70 \times 10^4 \pm 0.200c$
Temp site	$3.15 \times 10^4 \pm 0.100b$
Amenyi market	$4.50 \times 10^4 \pm 0.00a$

Table 3 showed that *Escherichia coli* had the highest inhibition of (43.00 ± 0.30) at 100% of lime peel extract while *Pseudomonas* had no inhibition at 100%, 50%, and 25% of lime peel extract.

Table 3: In-vitro antimicrobial activity of lime peel extract

ISOLATES	Lime peel extract 100%	Lime peel extract 50%	Lime peel extract 25%	Std antibiotics 30µg/ml
<i>Staphylococcus sp.</i>	27.00±0.110	14.90±3.01	6.00±0.50	34.83±1.11
<i>Escherichia coli</i>	43.00±0.30	36.00±1.110	30.00±1.00	34.83±0.30
<i>Klebsiella sp.</i>	18.00±1.00	15.70±2.00	11.00±2.00	19.16±1.00
<i>Pseudomonas spp</i>	0.000±0.00	0.000±0.00	0.000±0.00	24.30±0.20

Table 4 showed that *Rhizopus* sp. had highest inhibition at 100% of lime peel extract while *Aspergillus* sp. had no inhibition at 100%, 50%, and 25% of lime peel extract.

Table 4: In-vitro antifungal activity of lime peel extract

ISOLATES	Lime peel extract at 100%	Lime peel extract at 50%	Lime peel extract at 25%	Std antibiotics 30µg/ml
<i>Aspergillus</i> sp.	0.000 ± 0.00	0.000 ± 0.00	0.000 ± 0.00	29.60 ± 1.00
<i>Penicillium</i> sp.	15.00 ± 1.00	12.00 ± 2.00	7.00 ± 1.00	39.00 ± 1.00
<i>Mucor spp</i>	35.00 ± 0.11	23.70.3.01	21.30 ± 3.01	30.00 ± 1.00
<i>Rhizopus</i> sp.	41.00 ± 0.01	29.00 ± 3.01	22.00 ± 0.01	38.00 ± 3.01

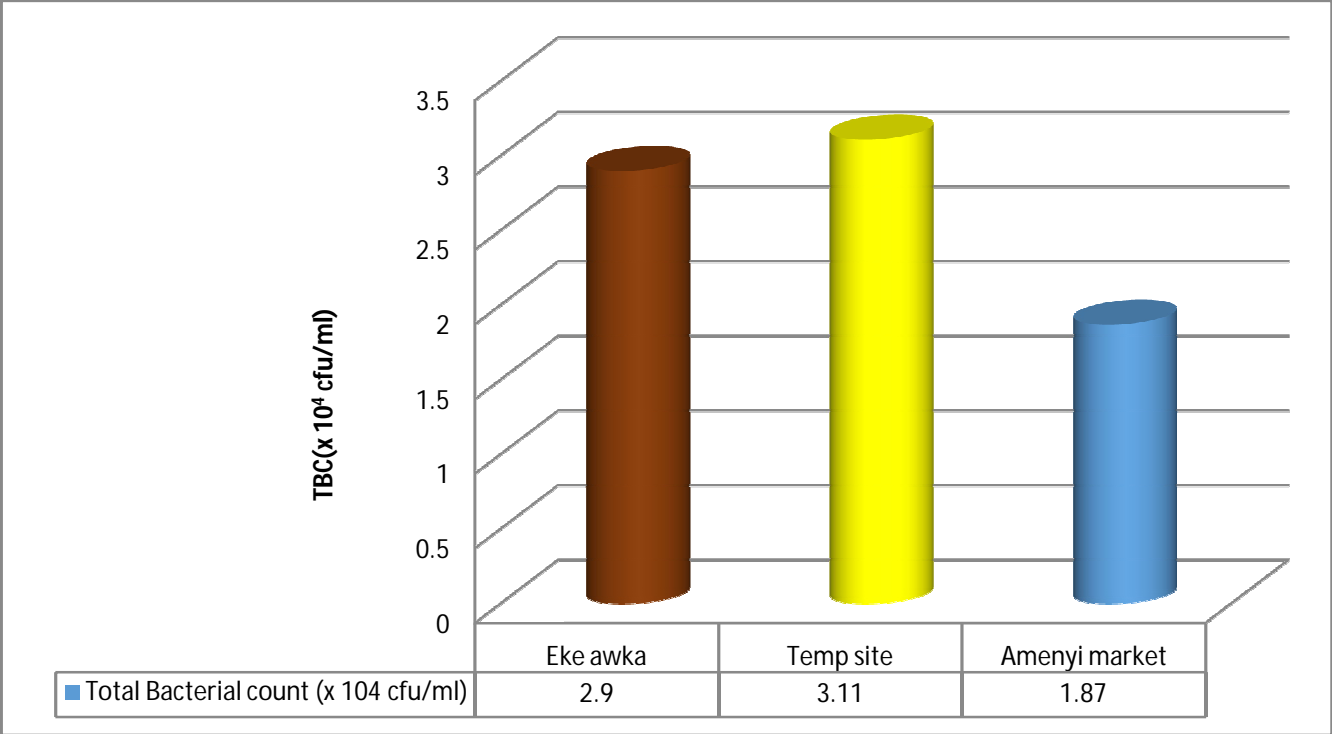


Fig. 1: Total Bacterial count of onion samples

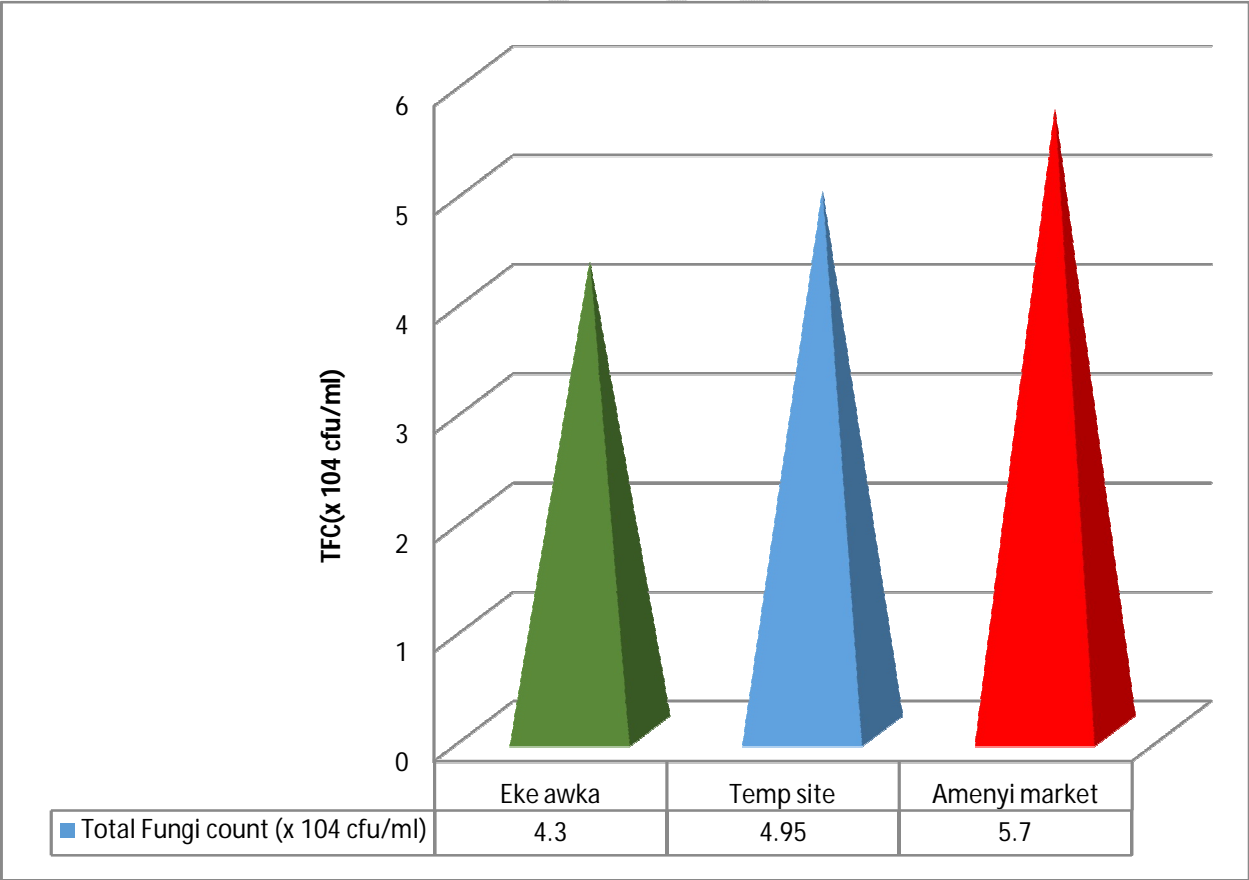


Fig. 2: Total Fungi count of onion samples

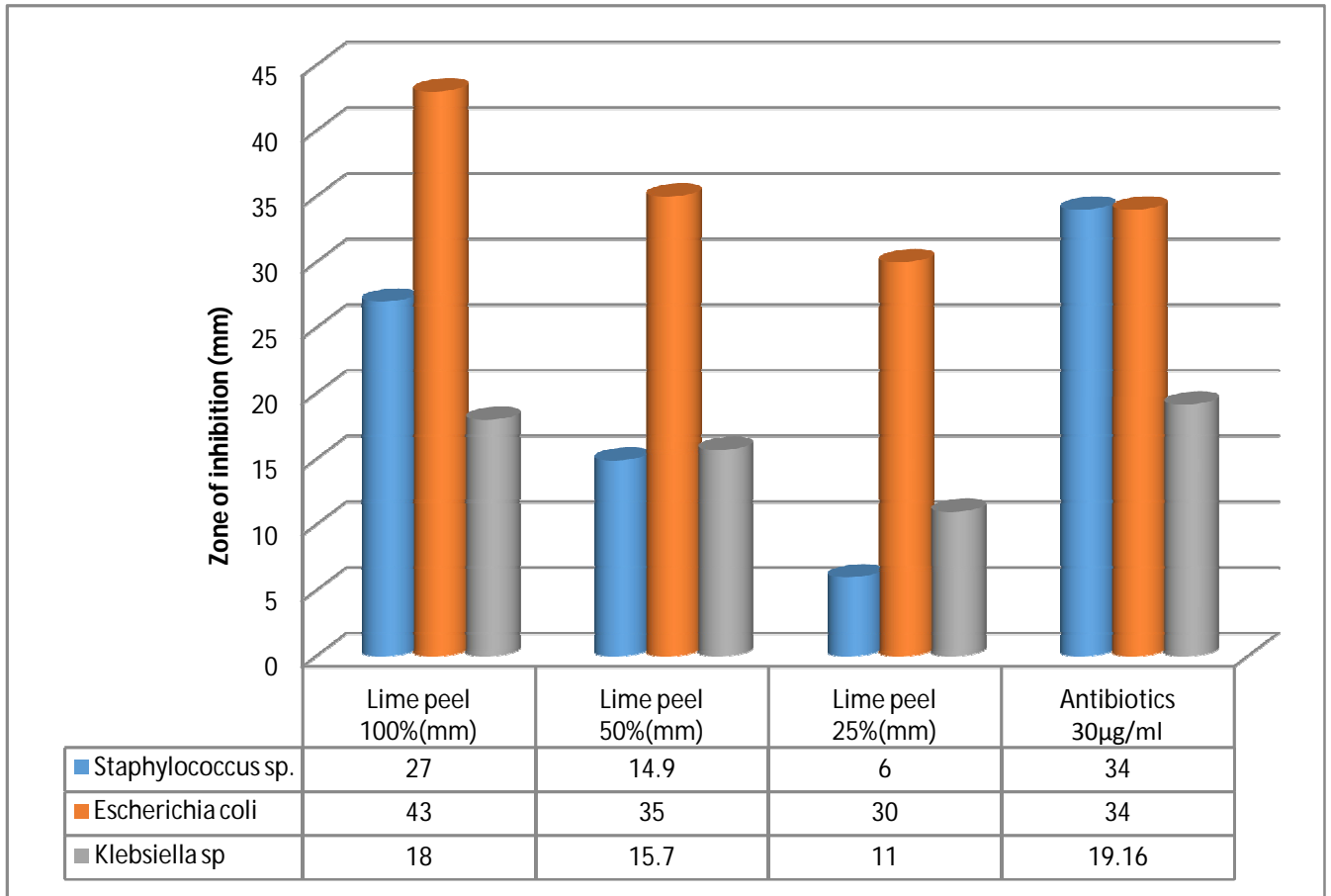


Fig. 3: In vitro antibacterial activity of lime peel extract

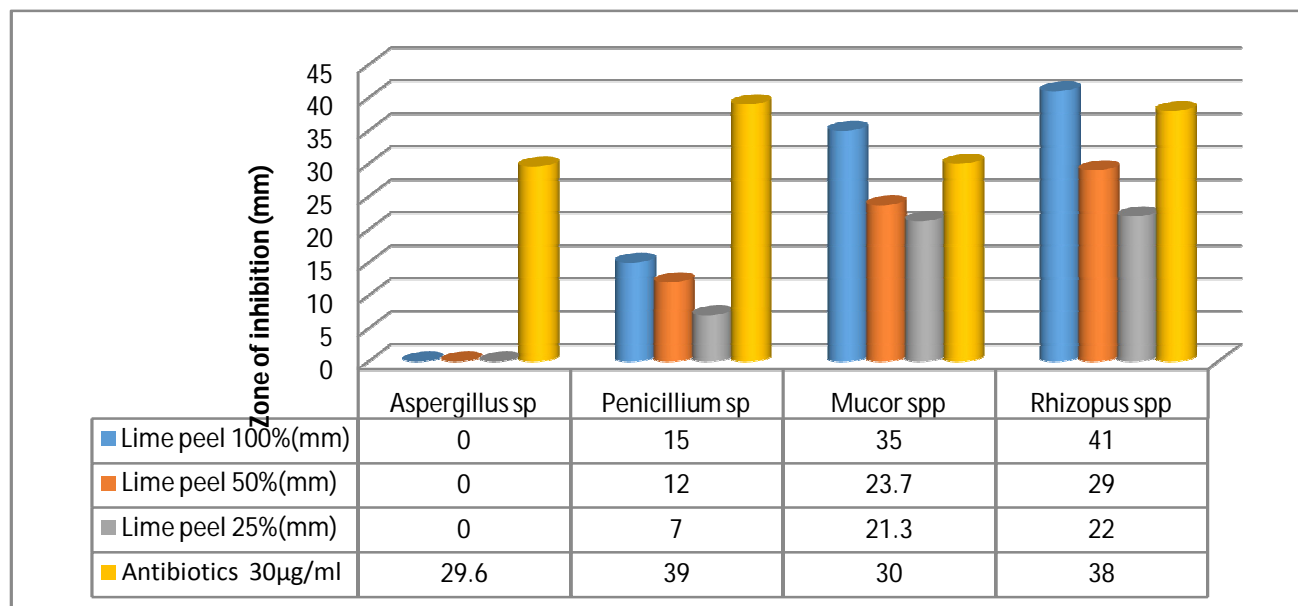


Fig. 4: In vitro antifungal activity of lime peel extract

UNDER PEER REVIEW



Plate 1: Pure culture of *klebsiella* sp

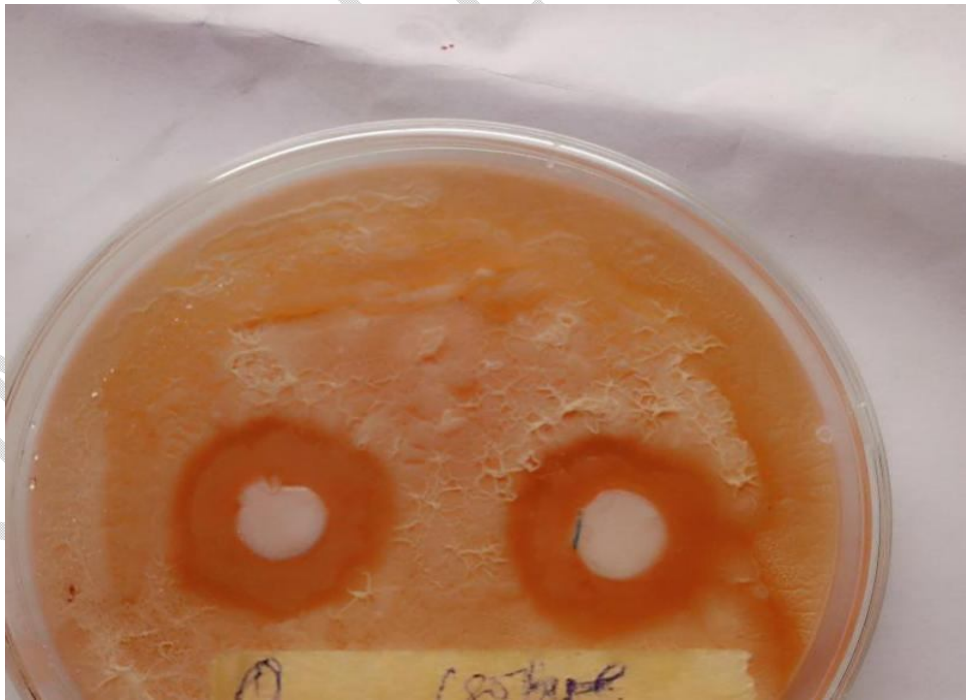


Plate 2: Pure culture of *E- coli*

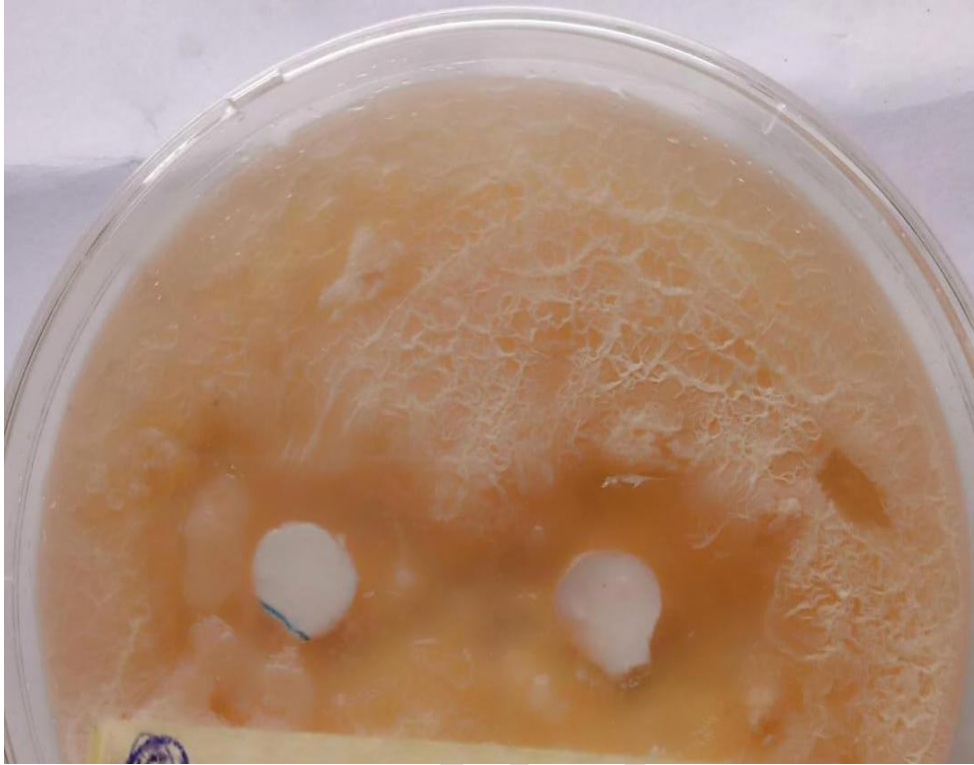


Plate 3: Pure culture of *staphylococcus spp*

UNDER PEER

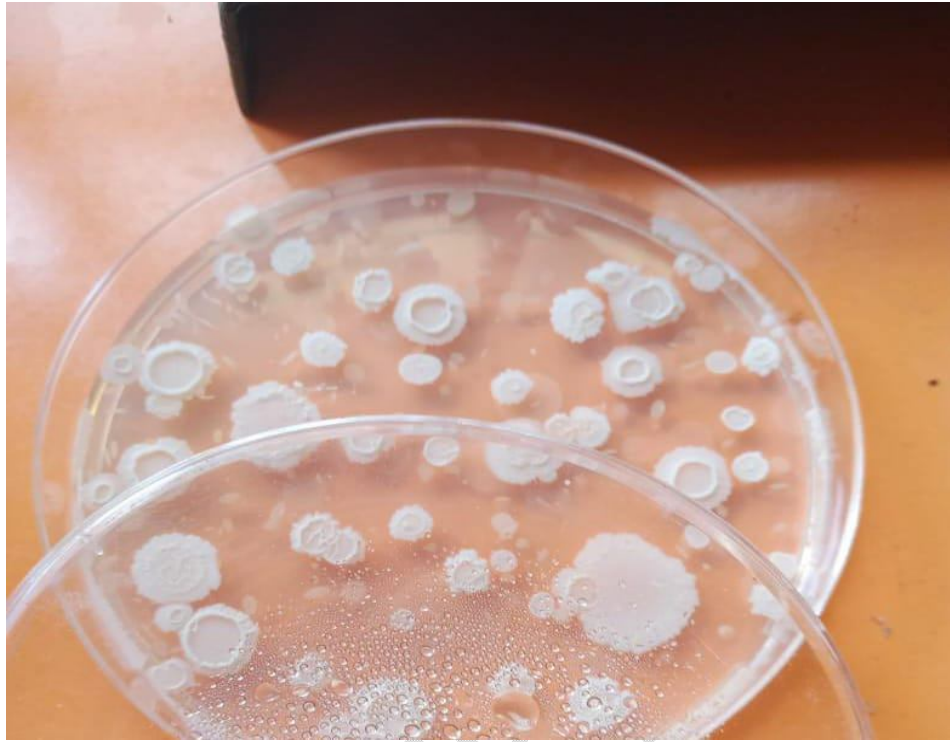


Plate 4: Pure culture of *Penicillium* sp.

UNDER PEER

DISCUSSION

4.1 Discussion

Studies on the microbes that cause onion deterioration in several locations of Anambra State have revealed the existence of a host of microbes. The fungal counts ranged from (2.70, 3.15, and 4.50) $\times 10^4$ cfu/g) while the bacteria ranged from (4.71, 5.64, and 6.10) $\times 10^4$ cfu/ml. *Klebsiella* sp, *E- coli*, *Staphylococcus* sp and *Pseudomonas* sp were the bacteria isolated from the onion samples from the different locations while *Aspergillus* sp, *Penicillium* sp, *Mucor* sp, *Rhizopus* sp. were the fungal isolates.

The study revealed that there was relatively high incidence of deterioration in the different locations and this can be attributed to pre-harvest handling and storage conditions. This supports the findings of Ghaffor *et al.* (2003) who hypothesized that damage to product caused during harvest and inadequate storage is a major contributor to infection because the majority of spoilage bacteria enter the produce through such damaged tissues. In addition, unsanitary behaviors including cross-contamination and contact infections during product transit might make infections more common (Nasir *et al.*, 2007).

Antioxidants and anti-inflammatory substances are found in onions. Their strong anti-inflammatory capabilities could perhaps lower blood pressure as well (Muhammad *et al.*, 2011). Several pre- and post-harvest factors, including soil characteristics, climatic factors, and management practices, can be blamed for onion bulb infection by microorganisms and the resulting loss of onion bulbs (Negash *et al.*, 2009).

4.2 Conclusion

Onion (*Allium cepa* L.) is one of the most important vegetable crops commercially grown in the world. Hence, there is a need to develop and promote the use of antiseptics and antibiotics in the control of these microorganisms in the study area.

Although there are many restrictions on growing onions, one of the main issues with growing onions and increasing productivity in the studied area is farmers' inappropriate agronomic

methods. Farmers are also encouraged to make use of the liquid substance of lime peel to control microbial growth of onion, either by spraying the onions with lime peel extracts and also take much care during Onion harvest and storage to reduce physical damage which encourage the growth of these bacteria to cause onion spoilage.

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APPENDIX

Table 1: MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF ISOLATES

Parameters	Isolate 1	Isolate 2	Isolate 3	Isolate 4
Colony characterization	Milky circular with flat elevation	Whitish irregular shape with flat elevation	Yellowish irregular shape with flat elevation	Milky irregular shape with flat elevation
Cell characterization	Rod in singles	Rods in clusters	Cocci in clusters	Cocci in clusters
Gram's Test	Positive	Negative	Positive	Negative
Motility Test	Negative	Positive	Positive	Positive
Catalase	Positive	Negative	Positive	Positive
Coagulase	Negative	Negative	Positive	Positive
Citrate	Negative	Positive	Negative	Positive
Indole	Positive	Negative	Negative	Positive
Oxidase	Negative	Positive	Negative	Positive

Urease	Positive	Positive	Negative	Positive
Probable organism	<i>Klebsiella sp</i>	<i>E- coli</i>	<i>Staphylococcus spp</i>	<i>Pseudomonas spp</i>

Table 2. List of isolates and their Probable identity

Isolate	Description	Probable identity
Isolate 1	They are Gram-negative bacteria. They are rod-shaped. Identification of <i>klebsiella</i> are pink	<i>Klebsiella spp</i>
Isolate 2	They are rough or a smooth form. Colonies are rough flat and irregular. <i>E- coli</i> are pink in color	<i>Escherichia coli</i>
Isolate 3	They are Gram-positive cocci. They are non-motile, anaerobic, and catalase-positive, and in pus they form clusters like bunches of grapes	<i>Staphylococcus spp</i>
Isolate 4	They are Grams-negative, rod shaped. They are non-sporing bacterium	<i>Pseudomonas spp</i>

Table 3. Extraction from different bacteria

EXTRACT	Lime peel extract of 100%	Lime peel extract of 50%	Lime peel extract of 25%	Std antibiotics 30
<i>Staphylococcus</i> sp.	27.00	14.90	6.00	34.83
	27.00	14.50	6.00	34.80
	28.00	14.50	6.00	34.86
<i>Escherichia coli</i>	43.00	35.00	30.00	34.83
	45.00	35.00	28.00	34.00
	40.00	35.00	23.00	34.00
<i>Klebsiella coli</i>	18.00	15.70	11.00	19.16
	18.00	15.80	11.00	20.00
	18.00	15.10	11.00	19.00
<i>Pseudomonas</i> spp	0.00	0.00	0.00	24.30
	0.00	0.00	0.00	24.00
	0.00	0.00	0.00	24.60

Table 4. Total Bacteria count ($\times 10^4$ CFU/ml) from different sites

SAMPLE SITE	Total Bacteria count ($\times 10^4$ CFU/ml)
Eke awka	6.10 6.3 6.0
Temp site	4.70 4.74 4.71
Amenyi market	5.60 5.68 5.65

Table 5: Prevalence of Isolates

Collection site	Bacterial isolates
Eke awka	<i>Klebsiella</i> sp. <i>Staphylococcus</i> spp <i>Escherichia coli</i> <i>Pseudomonas</i> spp
Temp site	<i>Staphylococcus</i> spp <i>Escherichia coli</i>
Amenyi market	<i>Bacillus</i> sp. <i>Staphylococcus</i> spp <i>Pseudomonas</i> spp

Table 5 shows that *Staphylococcus* spp has the highest occurrence in the three markets while *Bacillus* sp. has the lowest occurrence

Table 6: occurrence of Fungal isolate

Collection site	Fungal isolates
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Eke awka	<i>Aspergillus spp</i> <i>Mucor spp</i> <i>Rhizopus sp.</i>
Temp site	<i>Aspergillus spp</i> <i>Mucor spp</i> <i>Penicillium</i>
Amenyi market	<i>Aspergillus spp</i> <i>Mucor spp</i> <i>Rhizopus sp.</i> <i>Penicillium spp</i>

Table 6 shows that *Aspergillus spp* and *Mucor spp* has the highest percentage occurrence while *Rhizopus sp.* and *Penicillium* has the lowest percentage occurrence.