Original Research Article

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

ABSTRACT

Onion is one of the most important monocotyledons crops and they also contain 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 121ºC at 15psi for 20 minutes and the plates were incubated at room temperature. In the study, various microorganisms that can caused the spoilage were identified; the bacteria include K*lebsiella* sp, *E-coli, Staphylococcus spp* and p*seudomonas spp*. The fungal isolates are *Aspergillus sp*, *Penicillium* sp, *Mucor sp, Rhizopus* sp. They were isolated and identified both morphological and microscopically. Samples from Amenyi had the highest fungal counts ($4.50 \times 10^4 \pm 0.00$a cfu/g) while samples from Eke-Awka had the lowest fungal count ($2.70 \times 10^4 \pm 0.200$c cfu/g) on SDA. Also, samples from Temp. site had the lowest bacterial counts ($4.71 \times 10^4 \pm 0.100$c cfu/mi) while samples from Eke-Awka had the highest fungal count ($6.10 \times 10^4 \pm 0.03$a cfu/ml). The result showed that ethanol extract of lime peel has good inhibitory effects of the organisms. *Rhizopus* sp. had 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. Therefore, the study recommended that farmer should adopt the use of lime peel extract in the control of Onion spoilage as this method of control is much safe and efficient.

Keywords: Onion; Microorganism; Lime; media

INTRODUCTION

Onion (*Allium cepa)* is a member of the family Alliaceae, which include garlic, shallot, and leeks. Global production of onion bulbs is estimated at approximately 88.5×10 tons which are produced in Canada (Buchanan and Gibbons, 2014). Inspire of the importance of onion production to the agriculture and food industry, post harvest loss to bulb rot disease remains high. It has been suggested that as much as 20% of stored onion bulbs are lost to bulb rot disease alone in Nova Scotia (Charles, 2012). Infection of onion bulbs by bacteria and the associated loss of onion bulbs can be attributed to several pre and post-harvest factors such as genotypic characteristic, soil properties, climatic factors, and management practices (Gllis *et al.,* 2007). Onion pathogens are the gray mold fungi, the black mold fungi, the blue mold fungi and bacterial rot (Barth *et al.,* 2009).

Onion is rich in phosphorus, calcium and carbohydrates. The pungency in onion is due to a volatile oil known as ally-propyl disulphide. Onion is an important crop in all continents with world production of about 25 million tonnes (Frazier and Westhoff, 2015).

In onion, bacterial diseases cause bulb rot which develops any time between prehavest and storage. The symptoms include softening and water soaking of the bulb tissue, Yellow to brown discoloration and the neck of the onion becomes soft when pressed. These diseases or bacteria are primarily a problem on onions. Water is essential for entry and spread of the bacterial (Berger *et al.,* 2007). Bacteria enters the bulb through wounds and dying lower leaves (Asagbara and Oyewole, 2011). The pathogens are soil borne and may spread through irrigation water or splashing eater from rain. Most of these pathogens that causes spoilage in onions are favored by warm temperature (over 85°F) and wet conditions (Dodona *et al.,* 2005).

This study aims to isolate and identify the microorganisms associated with the spoilage of onions in a different location in Anambra state, Nigeria. Also, to determine the antimicrobial activities of lime peel extract on these isolates.

MATERIALS AND METHODS

2.1 Sample Collection

The spoilt onion samples were purchased from three (3) markets in Anambra state (Eke Awka, Nnamdi Azikiwe temporary site (Temp. site) and Amenyi). Laboratory and other facilities used in the practical work were obtained from Alpha research laboratory Awka, Anambra state.

2.2 Fungal Isolation

2.2.1 Culture Media: Commercially available media were used in this work, which is Sabouraud Dextrose Agar (SDA).

2.2.2 SDA media preparation: About 65g of the medium was suspended in one litre of distilled water, mixed well, and dissolved by heating to boiling, with frequent agitation. After heating for one minute and dissolving the solution, it was sterilized in an autoclave at 118-121^{0}C for 15 minutes. This was followed by the addition of 500 mg streptomycin antibiotic while the solution was still in a molten state. If all the solution was not used at that moment, the remainder was stored in the refrigerator at 8 – 15^0 C until when needed.

2.2.3 Isolation of Fungi

Spoilt pineapple surfaces were washed with distilled water to enhance the removal of dirt. A small portion of the spoilt area of the fruits was cut in and out using a sterile scalpel and inoculated onto a freshly prepared PDA and SDA agar and incubated at room temperature for three (3) days.

2.2.4 Sub-culturing Techniques

This was done following the method by Chuku et al., 2017. The resulting colonies were then sub-cultured onto Potato dextrose agar (PDA), process was repeated whenever more than a single colony of fungi was observed in the Petri-dishes until pure cultures were obtained.

2.2.5 Identification of isolated Fungi

All the various species of fungi isolated were identified, both macroscopic and microscopic features and their various characteristics studied, (i.e) colour, texture, a form of hyphae, form of conidia, presence of conidiophores, shape of conidial heads. The microscopic identification was aided by appropriate taxonomic keys [Onuorah, **Hemalathao**].

2.2.6 Determination of Fungal Frequency (%)

The fungal frequency will be determined location- wise, as well as cultural media-wise and later its correlation will be observed with the Percent Disease index 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

The method was used to isolate the bacteria from the tomato seeds using nutrient agar and EMB agar. The (One gram) seeds were aseptically collected and then serially diluted in normal saline In order to determine the total number of aerobic heterotrophic bacteria and fecal coliforms in the sample, the agar was inoculated with a combination of nutrients agar and EMB, they were incubated at 350ºC for 24 hours.

2.3.1 Total plate count of bacteria (CFU/ml)

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

Cfu/ml = {(No. of colonies X dilution factor) / volume of inoculums]

2.3.2 Purification of isolates

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

2.3.3 Identification of microorganisms

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

Gram's staining: The samples were stained according to Gram's techniques. A thin smear was prepared on a glass slide and air dried, and then heated to a high temperature. The smear was then covered with iodine for 60 seconds. It was then decolored using 70% ethanol and then washed under tap water. It was then counterstained with safarin for 30 seconds. The slide was then dried using a filter paper. The cells were then examined using the light microscope's oil immersion objective lens. The colors of the organisms were also studied. The purple color of the bacteria is due to their growth rate, while the pink color is due to their shape.

Motility test

The stabbing technique was used to carry out this test. Test tubes containing sterilized Sim Agar were prepared. A sterilized inoculating needle was used to pick up isolates from their pure cultures. Each test tube was stabbed with the needle rubbed with each isolate in the middle. The test tubes were then incubated at 370ºC for 24hours. After 24 hours, the tubes were observed for the motility of the isolates. A motile isolate usually grows away from the point where the medium was stabbed.

Biochemical Identification: The isolated bacterial colonies were confirmed by Biochemical kits (Universal Food pathogen Identification Disc, Hi2TMEnterobacteriaciae Identification Kit, and TSI test) and the results were interpreted as per the interpretation chart and identification index following kit protocol [Chessbourgh].

2.3.4 Biochemical tests

Urease Test

The purpose of this test was to demonstrate the ability of the organisms to produce Urease, which is an enzyme that breaks down urea. A change in the color of the urea-agar after 37^{o}C was confirmed by the test.

Catalase Test

The objective of the test was to determine which of the organisms could produce oxygen from hydrogen peroxide by catalase.

A colony was then placed into a glass slide. After a drop of 3% hydrogen peroxide, the colony produced gas bubbles. The reaction was then confirmed by the presence of catalase.

Methyl Red Test

The purpose of this test was to determine which of the various strains could produce and maintain a stable acid product after glucose fermentation. It is usually used to identify the enterobacteriaceae and to differentiate them from other bacteria. The culture was placed in a warm environment at 37°C for 48 hours. After 5 drops of the methyl red reagent, the red color of the culture immediately changed.

Voges -Proskeur Test (V.P. test)

This test was used to identify the organisms that can produce acetyl methyl carbinol, which is an essential component of carbohydrate metabolism. It is commonly used to differentiate between gram- negative and non-germ-negative organisms. Inoculated glucose broth was prepared with the test organism at a temperature of 370ºC for 3 days. A combination of naphthol and sodium hydroxide solution was added to the mixture and allowed to stand for 1 hour.

Indole Test

The purpose of this test was to determine which of the various strains can split indole from tryptophan in the presence of buffered peptone water. It is commonly used to differentiate Gram-negative and Bacilli. The culture tubes were incubated at 37°C for 48 hours. After 4 drops of Kovac reagent, the positive test was detected by a red color around the upper part of the test tube.

Citrate Utilization Test

The purpose of this test was to identify the various types of bacteria that can utilize citrate as their sole source of carbon for their metabolism. The medium used for this was Simon's citrate. The agar was inoculated with the young cultures of the various species. Inoculated tubes were placed in a Petri dish and subjected to a temperature of 37°C for about 24 hours. The resulting change in color indicated a positive result.

Coagulase Test

The coagulase test is used to identify the presence of a type of Staphylococcus aureus. It can be performed using the presence of a certain protein called coagulase. The method of Barry et al., [5] was employed.

2.4 Procedure

1. A very homogeneous suspension of the inoculated inoculum was mixed with a drop of normal saline. 2. A loopful of rabbit plasma was then added to the suspension and thoroughly mixed for 5 seconds. 3. A control was set up in the same manner without blood plasma. 4. Coagulase positive staphylococci showed clumping or agglutination within 5-15 seconds while negative suspension showed no clumping.

Oxidase Test

This was carried out to identify bacterial species that will produce the cytochrome oxidase enzyme.

A piece of filter paper was placed in a clean Petri dish and 2-3 drops of fresh or nascent oxidase reagent was added. A colony of test organism were collected using a glass rod and smeared on the filter paper and observed. Blue-purple color within few a seconds showed a positive test.

2.5 **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

Statistical Analysis Data collected were subjected to two-way analysis of variance (ANOVA) with the use of Sigma plot version 12 statistical software to ascertain the level of significance of the treatment given to at LSD0.05% .

RESULTS

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

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

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
***Staphylococcus* sp.**	27.00±0.110	14.90±3.01	6.00±0.50	34.83±1.11
Escherichia coli	43.00±0.30	36.00±1.110	30.00±1.00	34.83±0.30
***Klebsiella* sp.**	18.00±1.00	15.70±2.00	11.00±2.00	19.16±1.00
Pseudomonas spp	0.000±0.00	0.000±0.00	0.000±0.00	24.30±0.20

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 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
Aspergillus sp.	0.000 ± 0.00	0.000 ± 0.00	0.000 ± 0.00	29.60 ± 1.00
Penicillium sp.	15.00 ± 1.00	12.00 ± 2.00	7.00 ± 1.00	39.00 ± 1.00
Mucor spp	35.00 ± 0.11	23.70.3.01	21.30 ± 3.01	30.00 ± 1.00
Rhizopus sp.	41.00 ± 0.01	29.00 ± 3.01	22.00 ± 0.01	38.00 ± 3.01

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.

BAR CHART

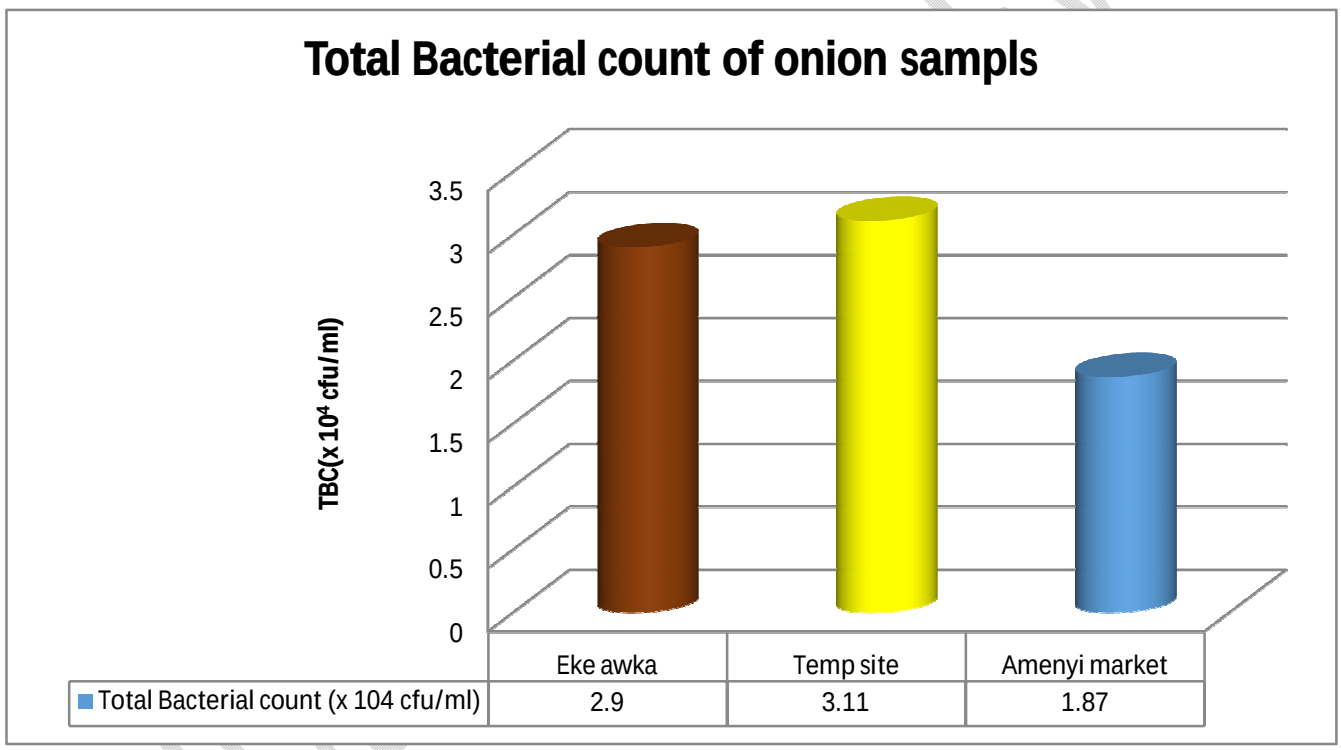


Plate 1: Total Bacterial count of onion sampls

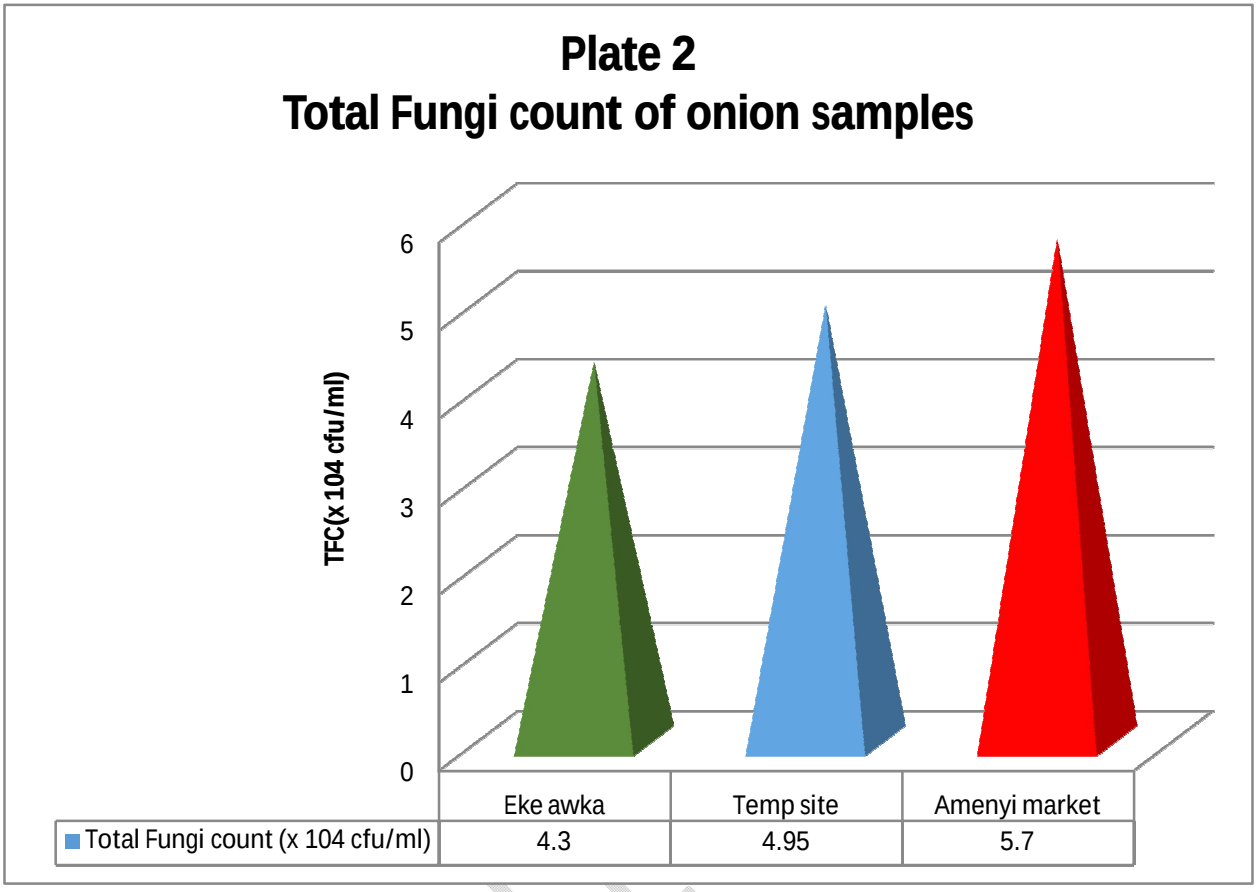
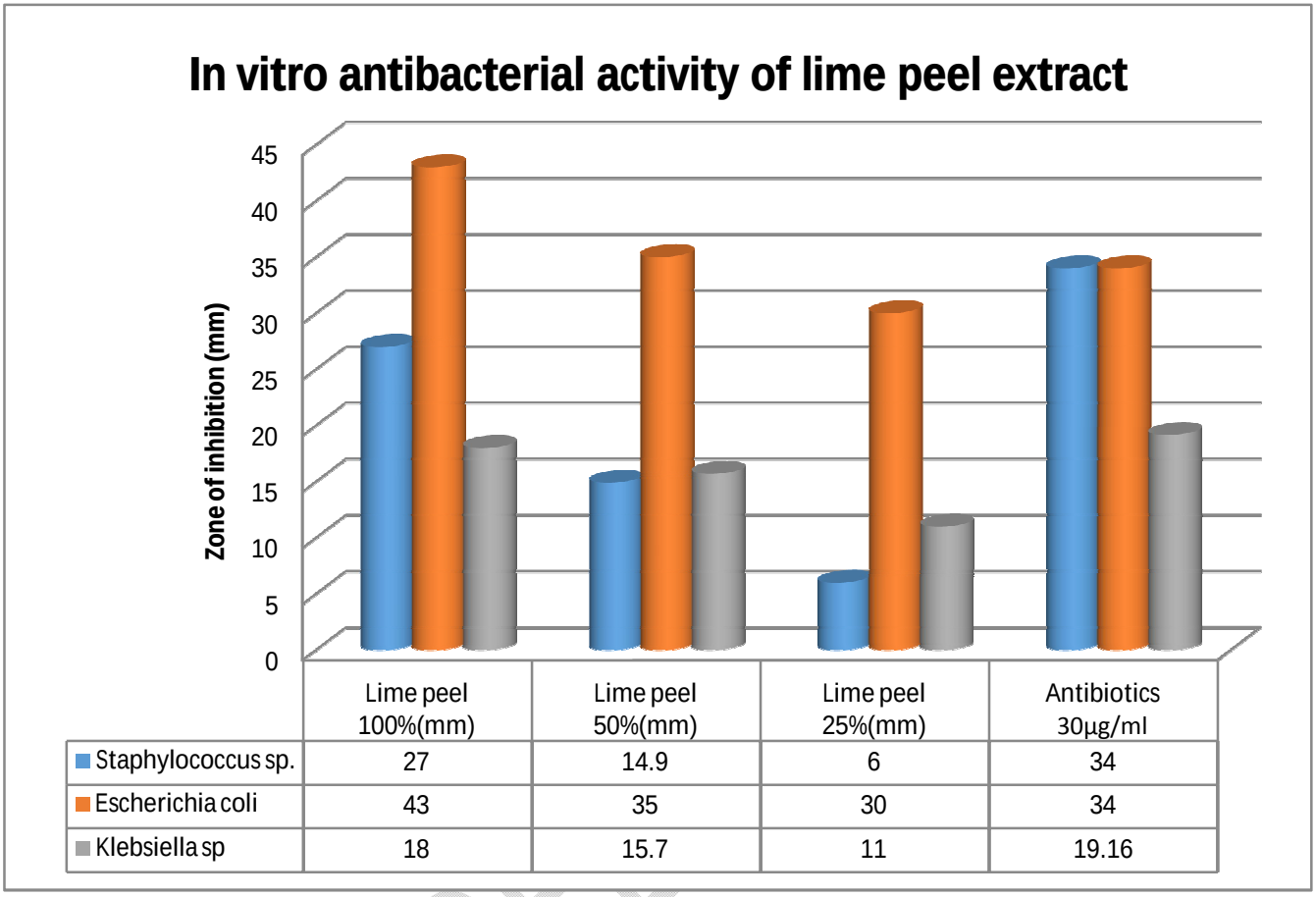
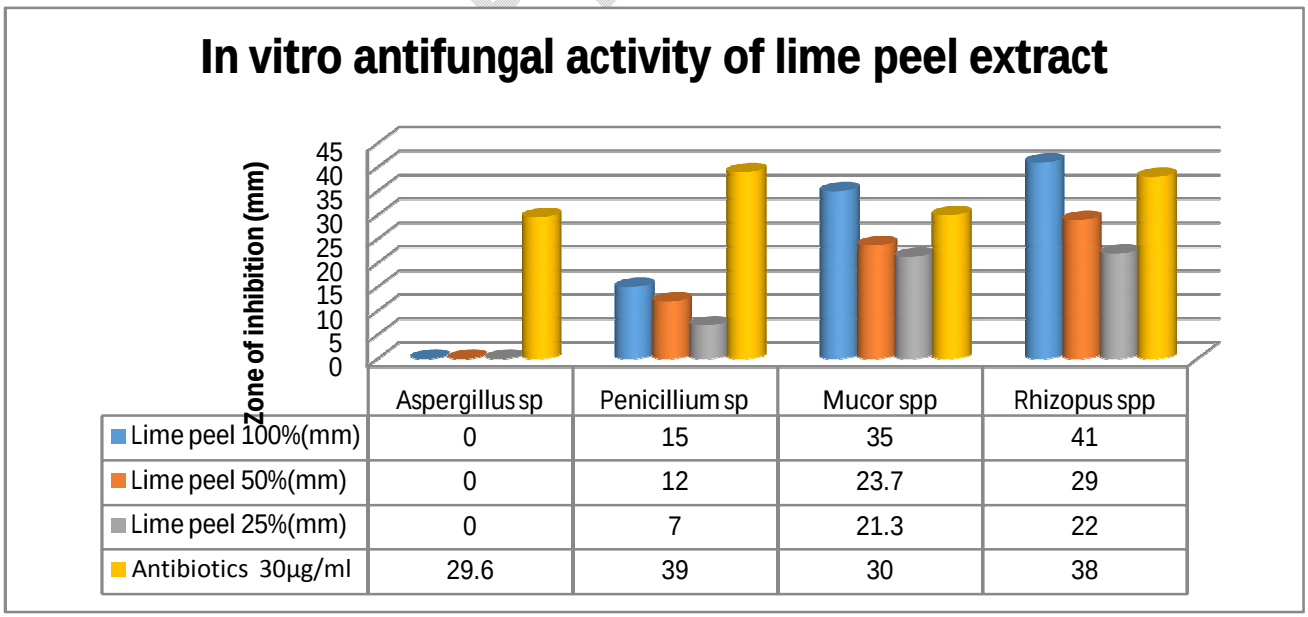


Plate 2



	Lime peel 100%(mm)	Lime peel 50%(mm)	Lime peel 25%(mm)	Antibiotics 30µg/ml
Staphylococcus sp.	27	14.9	6	34
Escherichia coli	43	35	30	34
Klebsiella sp	18	15.7	11	19.16

Plate 3 In vitro antibacterial activity of lime peel extract



	Aspergillus sp	Penicillium sp	Mucor spp	Rhizopus spp
Lime peel 100%(mm)	0	15	35	41
Lime peel 50%(mm)	0	12	23.7	29
Lime peel 25%(mm)	0	7	21.3	22
Antibiotics 30µg/ml	29.6	39	30	38

Plate 4 In vitro antifungal activity of lime peel extract

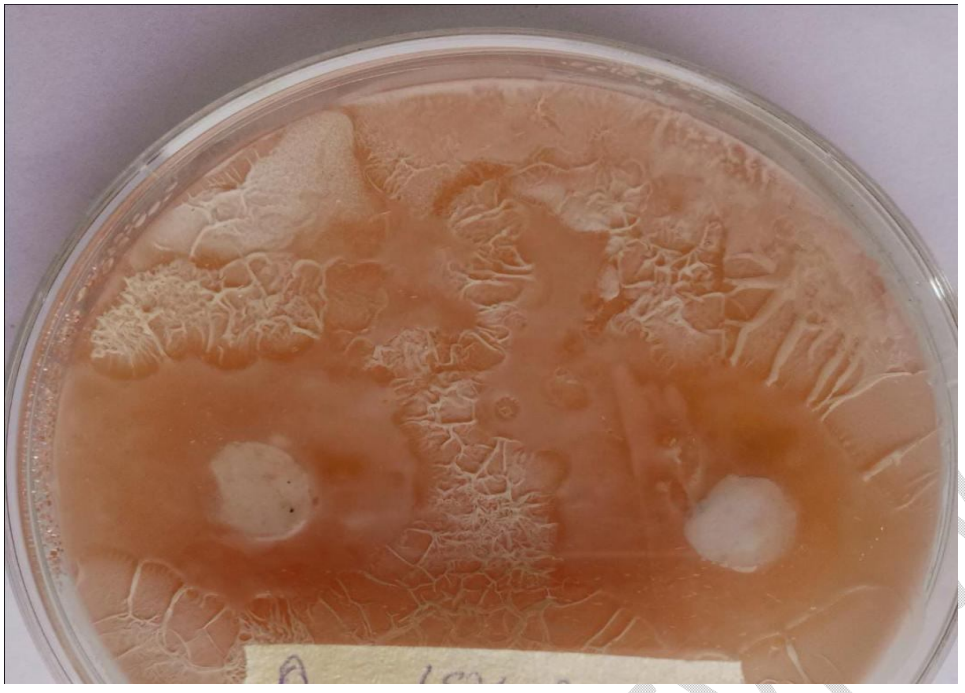


Plate 5: Pure culture of *klebsiella* sp

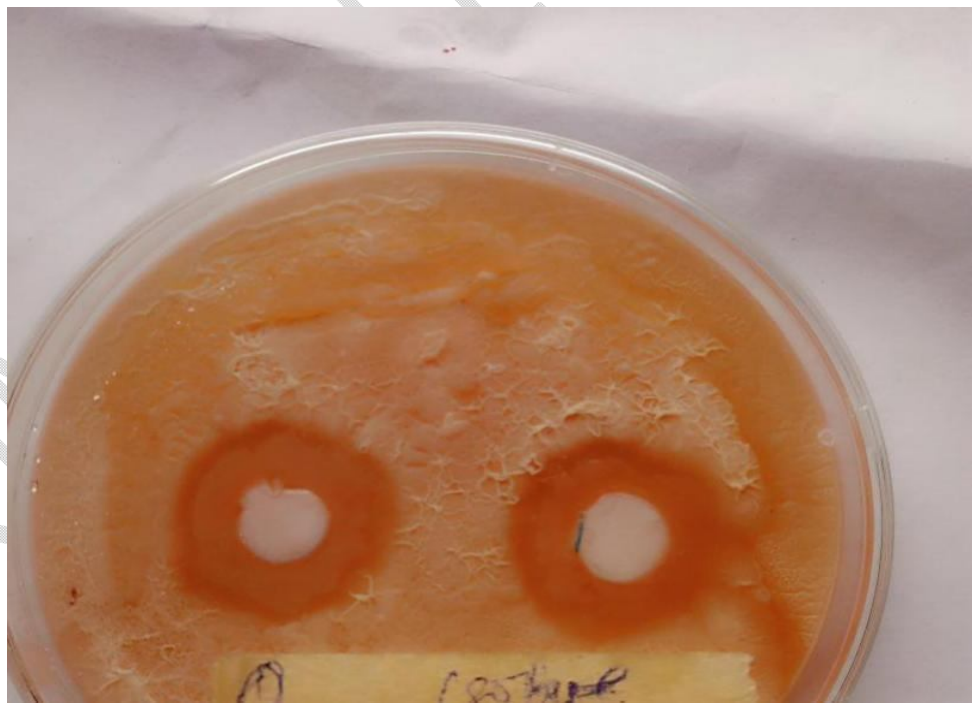


Plate 6: Pure culture of *E- coli*

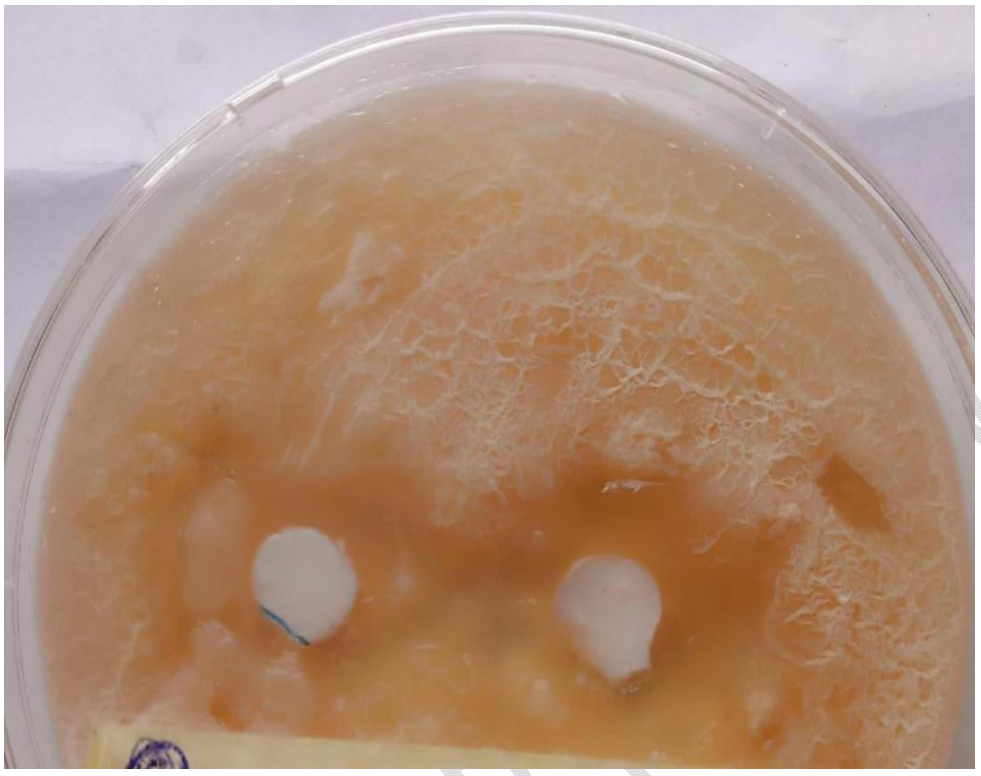


Plate 7: Pure culture of *staphylococcus spp*

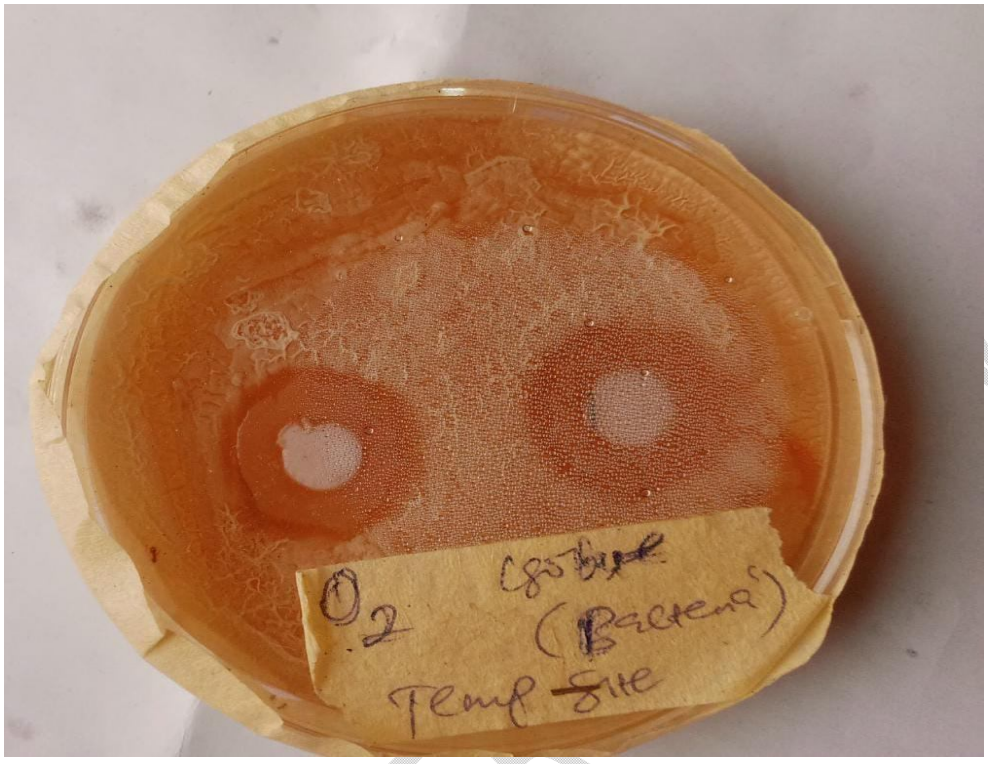


Plate 8: Pure culture of *Pseudomonas*

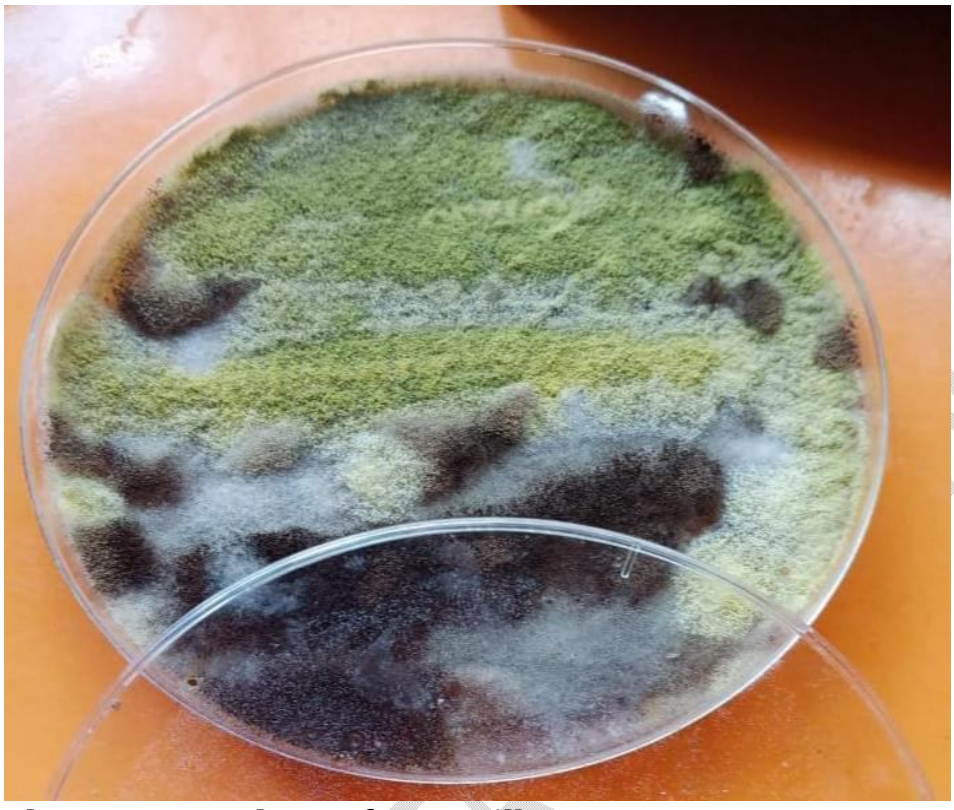


Plate 9: Pure culture of *Aspergillus* sp.

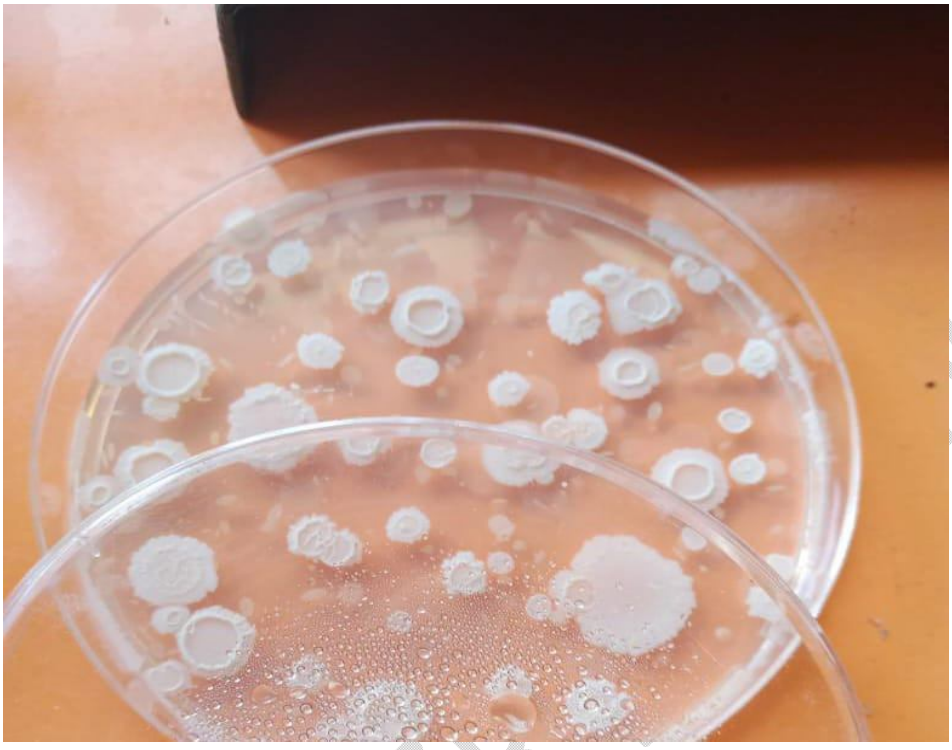


Plate 10: Pure culture of *Penicillium* sp.

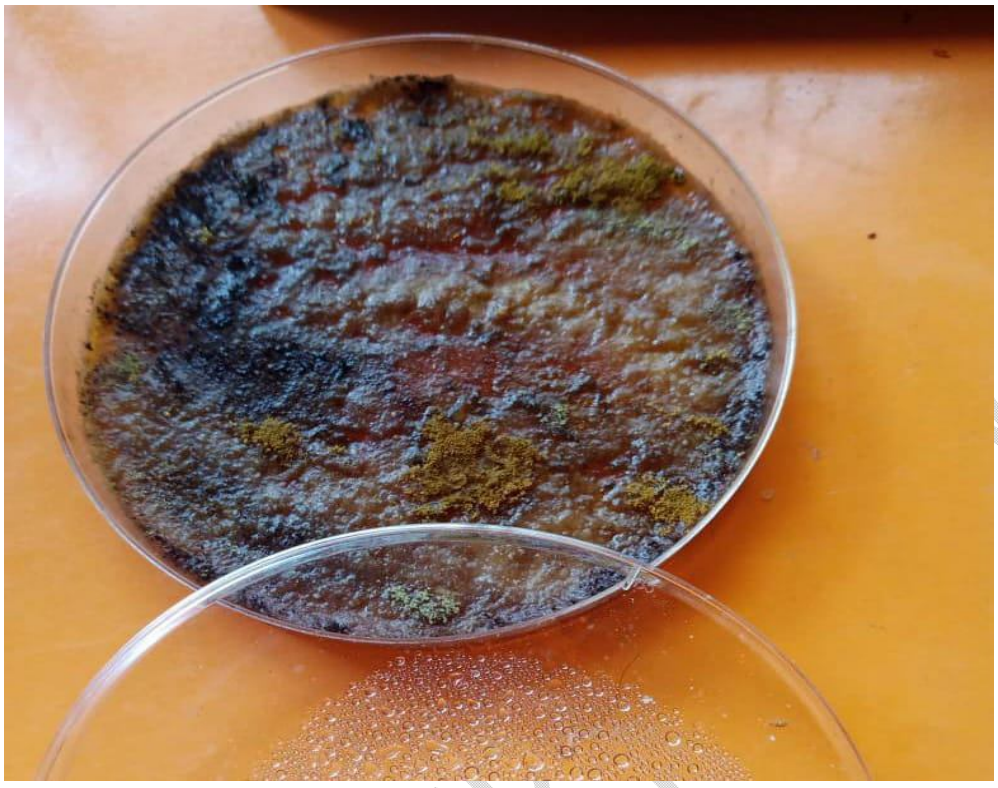


Plate 11: Pure culture of *Mucor spp*

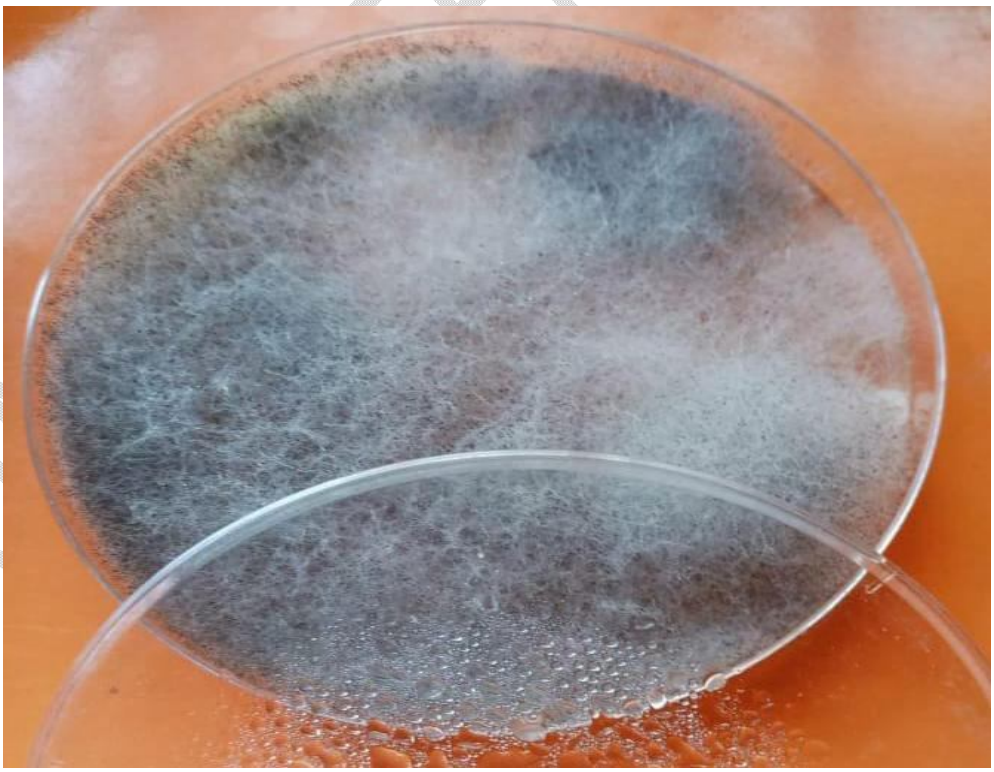


Plate 12: Pure culture of *Rhizopus* sp.

DISCUSSION

4.1 Discussion

Studies on the microorganisms responsible for the spoilage of onion in a different locations in Anambra State showed presence of a teeming population of microbes. The fungal counts ranged from fungal counts ranged from (2.70, 3.15, and 4.50) $x10^4$ cfu/g) while the bacteria ranged from (4.71, 5.64, and 6.10) $x10^4$ 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 of the different locations and this can be attributed to pre-harvest handling and storage conditions. This agrees with Ghaffor *et al.,* 2003 which opined that damage inflicted on produce at the time of harvest and poor storage is a major cause of infection since most of the spoilage microorganisms invade the produce through such damaged tissues. Furthermore, the incidence of infection can be worsened by poor sanitary practices such as cross-contamination, contact infection during the transportation of product (Nasir *et al*., 2007).

Onion contain antioxidants and compounds that fight inflammation. Their potent anti-inflammatory properties may also help reduce high blood pressure (Muhammad *et al.,* 2011). Infection of onion bulbs by microorganism and the associated loss of onion bulbs can be attributed to several pre and post-harvest factor such as soil properties, climatic factors and management practices (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 need to develop and promote the use of antiseptic and antibiotics in the control of these microorganisms in the study area.

Although several constraints are associated with onion production; improper agronomic practice used by farmers are among the major problems to onion production and productivity improvement in the study area. Farmers are also encouraged to make use of 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 bacterial 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	***Klebsiella* sp**	***E- coli***	***Staphylococcus spp***	***Pseudomonas spp***

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 *klebsiella* are pink	*Klebsiella spp*
Isolate 2	They are rough or a smooth form. Colonies are rough flat and irregular. *E- coli* are pink in color	*Escherichia coli*
Isloate 3	They are Gram-postive *cocci.* They are non-motile, anaerobic, and catalase-positive, and in pus they form clusters like bunches of grapes	*Staphylococcus spp*
Isolate 4	They are Grams-negative, rod shaped. They are non-sporing bacterium	*Pseudomonas spp*

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**
Staphylococcus sp.	27.00 27.00 28.00	14.90 14.50 14.50	6.00 6.00 6.00	34.83 34.80 34.86
Escherichia coli	43.00 45.00 40.00	35.00 35.00 35.00	30.00 28.00 23.00	34.83 34.00 34.00
Klebsiella coli	18.00 18.00 18.00	15.70 15.80 15.10	11.00 11.00 11.00	19.16 20.00 19.00
Pseudomonas spp	0.00 0.00 0.00	0.00 0.00 0.00	0.00 0.00 0.00	24.30 24.00 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	*Klebsiella* sp. *Staphylococcus spp* *Escherichia coli* *Pseudomonas spp*
Temp site	*Staphylococcus spp* *Escherichia coli*
Amenyi market	*Bacillus* sp. *Staphylococcus spp* *Pseudomonas 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

Eke awka	*Aspergillus spp* *Mucor spp* *Rhizopus* sp.
Temp site	*Aspergillus spp* *Mucor spp* *Penicillium*
Amenyi market	*Aspergillus spp* *Mucor spp* *Rhizopus* sp. *Penicillium spp*

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