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Bacterial Examination in Spoiled Fruits, its Isolation, Identification and Antibacterial Activity of Vinegar

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Abstract: ***Fruits are one of the natural products that are considered to have nutritional and commercial importance. They provide good and healthy diet in daily life of human. Eating foods such as fruits that are lower in calories instead of higher calorie food may be useful in helping to lower calorie intake. One negative impact of the fruits is that they have short shelf life. It may cause due to their contact with microorganisms during their exposure to environment. Fruits provide suitable environment for the survival and growth of many types of microbes especially bacteria. Several bacteria have been isolated and identified based on morphology of colony, staining procedure and also by shape and arrangement of bacteria. The observed bacteria includes E.coli, Staphylococcus sp, Bacillus Sp, Klebsiella sp.Vinegar is an aqueous solution of acetic acid and trace compounds that may include flavourings. Vinegar typically contains 5–8% acetic acid by volume. White vinegar is the most common type of vinegar which offers a sharp taste and harsh smell. Apple cider vinegar is mostly apple juice, but adding yeast turns the sugar in the juice into alcohol. Vinegar can kill microorganisms such as bacteria and viruses and treat yeast infections due to its antimicrobial properties. Zone of inhibitions were formed in well diffusion method of agar for vinegar (Normal Distilled Vinegar and Apple Cider Vinegar) and they showed their antibacterial activity against bacteria.***

Keywords: Fruits, Nutritional value, Spoilage, Natural Distilled Vinegar, Apple Cider Vinegar, Antibacterial Activity

1. Introduction

Fruits are known to have nutritional and commercial importance. They play vital role in human nutrition by supplying with necessary nutrients such as vitamins and essential minerals in human nutrition daily diet that can help to keep a good and normal health. Fruits are daily consumed. One negative impact of the fruits is that they have short shelf life. It may cause due to their contact with microorganisms during their exposure to environment. (Khatri and Sharma, 2018).

Eating a diet high in fruits will provide health benefits and can reduce several human diseases. People who eat more fruits as part of an overall healthy diet are likely to have reduced risk of some chronic diseases such as heart disease, including heart attack, stroke and certain types of cancers. Eating foods such as fruits that are lower in calories instead of higher calorie food may be useful in helping to lower calorie intake.

The microbial contamination has various reasons such as contact of fruits with soil, dust, water and also due to handling methods during harvest and post harvest time, marketing condition, during storage and also condition after purchased by the consumers. (Mohapatra and *et al*)

Fruits are vital sources of nutrients to human beings. They give supplement the body with necessary nutrients such as vitamins, fat, minerals and oils in proper proportion for growth and development.Fruits provide suitable environment for the survival and growth of many types of microbes especially bacteria.(Hasan and Zulkahar, 2020).

The different types of microorganisms can be seen including plant and human pathogens. They will cause fruit spoilage. The term spoilage refers to the change in the condition of food in which the food becomes undesirable for human consumption. (Chaudary and Dhaka, 2016). The spoilage in fruits first shows the softening of tissues. The unpleasant odour and flavours develop. Hence spoilage of fruits will make them unpalatable and toxic for human consumption.

Fresh fruits contain natural micro flora coming from soil, water, air and other sources. The presence of air, high humidity and high temperature as extrinsic factors and oxidation, respiration, enzymatic activity as intrinsic factors during storage of fruits increase the chance of microbial growth and spoilage. Previously, several studies have reported the occurrence of bacteria in spoiled fruits which are as follows
Pseudomonas, Klebsiella, Erwinia, Xanthomonas, Bacillus, Clostridium, E.coli, Staphylococcus, Lactobacillus, Enterobacter, etc.

Vinegar
Vinegar is an aqueous solution of acetic acid and trace compounds that may include flavourings. Vinegar typically contains 5–8% acetic acid by volume. Usually, the acetic acid is produced by a double fermentation, converting simple sugars to ethanol using yeast, and ethanol to acetic acid by acetic acid bacteria.

Distilled White Vinegar
White vinegar is the most common type of vinegar which offers a sharp taste and harsh smell, making it one of the most distinct vinegar types on this list. That's because white vinegar is distilled from grain which results in a crisp and clear product. It may be too pungent for most recipes

Apple Cider Vinegar
Apple cider vinegar is mostly apple juice, but adding yeast turns the sugar in the juice into alcohol. Bacteria turn the alcohol into acetic acid. That's what gives vinegar its sour taste and strong smell. It is one of the most common types of vinegar which is used to both flavour and preserve food. It is made by adding bacteria and yeast to the liquid of crushed and strained apples to create a fermentation process. Sugar is then added to the mixture, making the liquid alcoholic. It is this alcoholic juice that is fermented once more and converted into vinegar.

Antibacterial activity can be defined as the collective term for all active principles (agents that inhibit the growth of bacteria, prevent the growth of microbial colonies and may destroy microorganisms.

Microbiological analysis is the valuable way of evaluating the emerging risk that concern both the monitoring authorities and food consumers as well. Disinfectant wash is essential to reduce fresh fruits and vegetables microbial loads. There are several strategies such as physical and chemical treatments , which have been studied to decontaminate fresh cut fruits and vegetables. (Rahman M *et al*, 2021).

2. Materials and Methods

Sample collection:
Five types of unwashed and unprocessed spoiled fruits comprising of banana, orange, apple, grapes and pomegranate were collected in plastic bag from fruit shop of Krishnapuram, Tirunelveli.

Figure 1: Collection of Spoiled fruits

Apple:
Apple (*Musa domestica*) is an edible fruit that offers multiple health benefits. They are rich in fibres and antioxidants. They are used to make fresh juices and milled to produce cider vinegar. If a piece of apple is left out long, it may spoil. Soft spots, bruising, wrinkled skin, wholes and brown blemishes, liquid oozing from its skin, a mushy texture and bland or grainy taste are the conditions for spoiled apple.

Banana:
Banana (*Musa paradisiaca*) is one of the most important commercial fruit and vegetable crop found all over the world. It is the world's oldest cultivated crop. It is known as 'Dollar earning crop'. It provides Vitamin B6 which is needed in daily diet. It helps in metabolism and to maintain immune system health. The spoiled banana will be grown with mould. The flesh will become brown colour and very mushy.

Grapes:
Grapes (*Vitis vinifera*) is a good source of potassium, a mineral that helps to balance fluids in our body. It is perishable fruit and its market life depends on the time and the temperature exposure. The deterioration takes place if it is directly exposed to the high temperature. The unpleasant flavour and odour will be produced on deterioration and its appearance will be changed.

Orange:
Orange (*Citrus sinensis*) provides vitamin C. It absorbs iron to fight against anaemia. It is consumed in the form of jam, syrup, juice etc. It is used to produce peel oil, citric acid and cosmetics which have international market value. It helps to even out skin tone and texture by supporting the production of collagen. Acid is the major factor in spoilage. The taste will become sour and smell sour and mouldy.

Pomegranate:
Pomegranate (*Punica granatum*) is one of the oldest edible fruit. Its juice is one of the most powerful antioxidants which guard our body against free radicals. It is rich in vitamin C, Riboflavin, iron, phosphorous and protein. The spoiled pomegranate will have seeds with brown colour and appear soft and mushy. In early stage of spoilage pith is only brown around the edges but seeds will be red which can be consumed but later seeds will also become brownish appearance.

Preparation of Agar Medium
A gelatinous substance obtained from certain seaweeds and used in biological culture media and as a thickener in food. Nutrient agar and EMB agar have choosen for bacterial culture.

Nutrient Agar Medium Preparation : Take 2.8grams of Nutrient agar and dissolve it in 100 ml of distilled water and sterilize it in autoclave at 15lbs pressure (121^{o}C temperature) for 15 minutes.

EMB Agar Medium Preparation: Take 3.596 grams of EMB agar and dissolve it in 100 ml of distilled water and sterilize it in autoclave at 15lbs pressure (121^{o}C temperature) for 15 minutes.

Isolation of Bacteria
Serial Dilution is done at different concentrations
It is a stepwise dilution of a substance in a solution. The dilution factor at a each step is constant resulting in a geometric progression of the concentration in a logarithmic fashion.

Culture method
The sterilization of laminar air flow chamber must be done using UV light prior to start of work. The petriplates, prepared agar media, prepared fruits samples and other

required materials were sterilized in autoclave and they were taken to the laminar air flow chamber. Ethanol is used for further maintenance of sterilization condition. Allow the agar media to get cooled but molten for pour plating. A 100µL of selected dilutions of each fruit suspension was poured over labelled nutrient agar (NAM) plates from each selected dilution (Pour plate method or streak plate method).

Pour Plate: It refers to a plate prepared by mixing the inoculum with cooled but still molten medium before pouring the latter into the Petri dish. It is the method of choice for counting the number of colony-forming bacteria present in a liquid specimen.

Streak Plate: It is a rapid qualitative isolation method. The techniques commonly used for isolation of discrete colonies initially require that the number of organisms in the inoculums be reduced. It is essentially a dilution technique that involves spreading a loopful of culture over the surface of an agar plate.

Incubation
The inoculated petriplates were incubated at 37^{O}C for 24 hours to allow bacterial growth. Consequently, bacterial colonies were sub cultured, maintained and stored on NAM slants at 40^{O}C for further use.

Colony counting
After 24 and 48 hours of incubation, the bacterial growth were observed. They were counted and tabulated. Further the Morphological analysis was conducted to determine different bacterial colonies and to observe morphological variation of bacterial growth.

Identification of bacteria
Morphological analysis was observed to determine type of bacteria based on colonies and morphological variation of bacterial growth. Gram staining was conducted to study the cellular morphology of isolated bacteria.

Simple staining: The simple stain can be used as a quick and easy way to determine the cell shape, size, and arrangements of bacteria. It is a very simple staining procedure involving a single solution of stain. Any basic dye such as methylene blue, safranin or crystal violet can be used to colour the bacterial cells.

Gram staining: The Gram staining is a differential method of staining used to assign bacteria to one of two groups (gram-positive and gram-negative) based on the properties of their cell walls.

Antibacterial Activity: Antibacterial activity of vinegar (i.e for Natural Distilled and Apple Cider Vinegar) were performed by Well diffusion method and the zone of inhibition were recorded**.**

Well Diffusion Method
Autoclaved agar medium is poured into the petridishes (20ml each) under the laminar airflow chamber and allow them to solidify. Innoculate the bacterial sample using streak method. After that, well was made using the cork borer and pour them with each samples (100µl) with different concentrations which have to been tested for antibacterial property. Keep the petridishes for incubation for 24 hours.

Zone of inhibition will be formed and their measurements were noted in millimetres and the results are interpreted.

Zone of inhibition: This is an area of media where bacteria are unable to grow due to presence of drugs that impedes their growth. (ZOI).

3. Results and Discussion

In this study, Various bacteria were isolated and identified from 5 different spoiled fruits such as apple, grapes, banana, orange and pomegranate which were collected from Krishnapuram, Tirunelveli. (Fig 1). The isolation of bacteria have been done by pour and streak plate methods in two types of agars such as Nutrient agar and EMB agar using 5 different samples of spoiled fruits at different concentrations.

The culture of bacteria in various spoiled fruit samples were observed after 24 and 48 hours and the total bacterial count is done using Colony counter. The different concentration showed varied bacterial growth. As numerous bacterial growth were observed in 10^{-2} concentration which is not feasible for counting. (Table 1, 2 and 3)

Table 1: Total Bacterial Count in Nutrient Agar

Serial Number	Type of fruit	Concen-tration	Total number of colonies (CFUs/ml)	
			24 hours	48 hours
1	Apple	10^{-2}	Too numerous	Too numerous
2	Apple	10^{-3}	3.32 x 10^{5}	4.8 x 10^{5}
3	Apple	10^{-5}	2.20 x 10^{7}	4.04 x 10^{7}
4	Banana	10^{-2}	Too numerous	Too numerous
5	Banana	10^{-3}	6 x 10^{5}	7.08 x 10^{5}
6	Banana	10^{-5}	4.8 x 10^{7}	6.24 x 10^{7}
7	Pomegranate	10^{-2}	Too numerous	Too numerous
8	Pomegranate	10^{-3}	4.12 x 10^{5}	5 x 10^{5}
9	Pomegranate	10^{-5}	3 x 10^{7}	3.96 x 10^{7}
10	Orange	10^{-2}	Too numerous	Too numerous
11	Orange	10^{-3}	4.4 x 10^{5}	6.64 x 10^{5}
12	Orange	10^{-5}	2.4 x 10^{7}	3.52x 10^{7}
13	Grapes	10^{-2}	Too numerous	Too numerous
14	Grapes	10^{-3}	3.44 x10^{5}	3.92x10^{5}
15	Grapes	10^{-5}	1.88 x 10^{7}	2.76 x 10^{7}

Total Bacterial Growth in EMB Agar

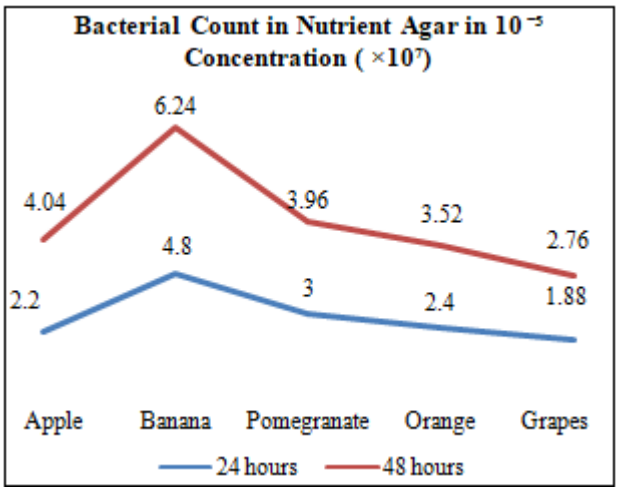
Table 2: 24 Hours

Serial Number	Type of fruit	Concentration	Total number of colonies (CFUs/ml)
1	Apple	10^{-5}	No growth
2	Banana	10^{-5}	No growth
3	Pomegranate	10^{-5}	6 x 10^{6}
4	Orange	10^{-5}	2.4 x 10^{7}
5	Grapes	10^{-5}	4x 10^{7}

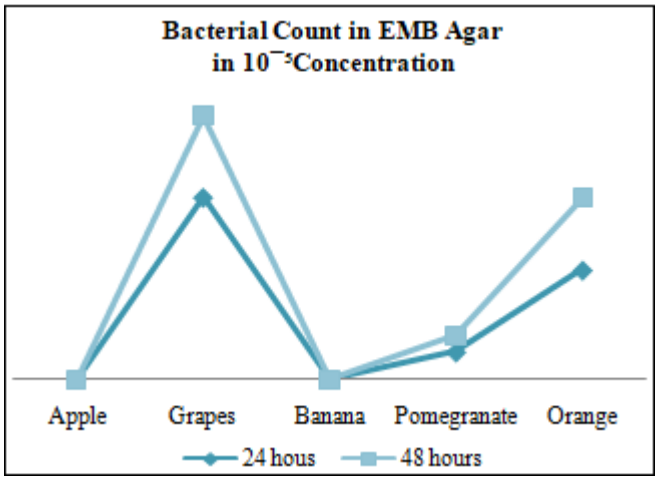
Table 3: 48 hours

Serial Number	Type of fruit	Concentration	Total number of colonies (CFUs/ml)
1	Apple	10^{-5}	No growth
2	Banana	10^{-5}	No growth
3	Pomegranate	10^{-5}	9.6×10^{6}
4	Orange	10^{-5}	3.96×10^{7}
5	Grapes	10^{-5}	5.76×10^{7}

The graph 1 (Plotted for Bacterial growth in 10^{-5} concentration of Nutrient Agar) shows that the highest bacterial count was observed in Banana both in 24 and 48 hours in view of Nutrient Agar. The graph 2 shows that in EMB Agar (Concentration 10^{-5}), apple and banana showed no growth at all. While comparing other fruits, Grapes showed the prominently high bacterial growth.



Graph 1



Graph 2

According to classical bacteriology, most species of bacterial isolate can be differentiated based on Simple Gram Staining Technique. Gram Positive Bacteria were found in Banana, Pomegranate and Orange and Gram Negative Bacteria have been found in Grapes, Pomegranate and apple. Gram stain reaction is based on the difference in the chemical essence of bacterial cell walls. Gram positive cells have thick peptidogly can layer whereas in Gram negative cells it is much thinner and contains outer lipid layers. In result, Gram positive bacteria appear purple because iodine and crystal violet precipitate in the thickened cell wall and they are not eluted by the alcohol. But in gram negative bacteria the crystal violet will be eluted from the bacteria and it will appear pink colour. (Fig 2 & 3)

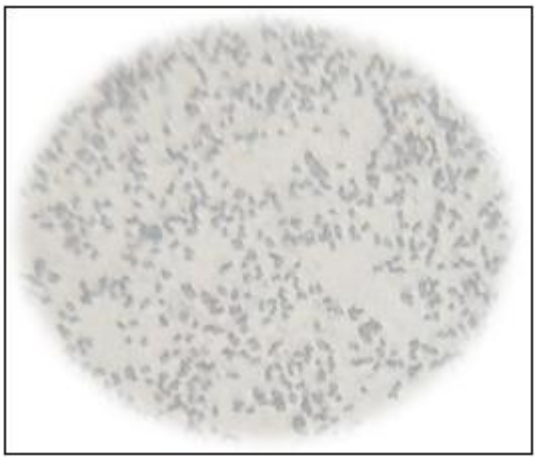


Figure 2: Gram Postitive Bacteria

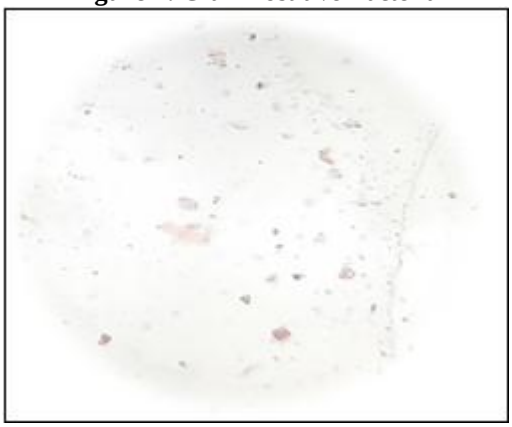


Figure 2: Gram Negative Bacteria

Based on the morphology such as shape, size, texture, arrangement and colour of bacterial isolates they have been grouped due to their similarity. In Table 4, the morphology of bacterial colony have been observed and were noted. In Table 5, the shape and arrangement of bacteria were observed and they were recorded.

Table 4: Morphology of Bacterial Colony

Serial Number	Type of fruit	Morphology of colony
1	Apple	Whitish, flat, large , Smooth , Opaque and glistering Moist
2	Banana	Golden Yellow, Circular large, Opaque, Smooth and shinny White and dry
3	Pomegranate	Whitish, Flat large, Smooth, Opaque and glistering White waxy and abundant growth
4	Orange	Golden yellow, large and circular, Smooth and shinny
5	Grapes	White translucent and raised growth

Table 5: Identification of bacteria

Serial Number	Type of fruit	Gram Type	Shape and Arrangement	Bacteria Predicted
1	Apple	Negative	Short rods	*E. coli*
2	Banana	Positive	Cocci in bunches Long rods with round edges in scattered arrangement	*Staphylococcus species, Bacillus species*
3	Pomegranate	Negative & Positive	Short rods Rods in chain	*E. coli, Bacillus cereus*
4	Orange	Positive	Cocci in bunches	*Staphylococcus species*
5	Grapes	Negative	Short rods in scattered arrangement	*Klebsiella species*

From all these data (Stain type, Colony Morphology and Size and arrangement), The bacteria have been predicted. In Apple sample, bacteria observed was *E.coli.* The banana sample was observed with *Staphylococcus sp and Bacillus sp.* *E.coli* and *Bacillus ceres* have been found in Pomegranate. The bacteria observed in Orange sample was *Staphylococcus sp* and the grapes sample was found to be observed with *Klebsiella sp.* (Table 5).

The *E.coli* bacteria has been isolated from the Apple sample and pure cultured and using this culture as sample the antibacterial activity for vinegar (Normal Distilled vinegar and Apple cider Vinegar) have been performed by Well Difussion method and the zone of inhibition is noted and the result is interpreted

Table 6 shows that the zone of inhibition of Normal Distilled Vinegar at various concentrations. It showed no zone of inhibition in lower concentrations (in 2.5, 5, 10, 20)% as it resembled same as control (Distilled water) so that we have tested for zone of inhibition in higher concentrations (in 50, 100)% and the result were observed and it showed the zone of inhibition. It was observed that the maximum zone of inhibition in concentration 100% (10.33mm) while it is compared with the standard (Erythromycin), it showed (13.33mm).

Table 7 shows that the zone of inhibition of Apple Cider Vinegar at various concentrations. It showed no zone of inhibition in lower concentrations (in 2.5, 5, 10, 20)% as it resembled same as control (Distilled water) so that we have tested for zone of inhibition in higher concentrations (in 50, 100)% and the result were observed and it showed the zone of inhibition. It was observed that the maximum zone of inhibition in concentration 100 (11.33mm) while it is compared with the standard (Erythromycin), it showed (13.33mm).

Table 6: Antibacterial activity of natural distilled vinegar against bacteria isolated from apple

Concentration (%)	Replication 1	Replication 2	Replication 3	Mean
Control (Distilled water)	0	0	0	0
Standard (Erythromycin)	14	12	14	13.33
2.5	0	0	0	0
5	0	0	0	0
10	0	0	0	0
20	0	0	0	0
50	9	9	8	8.67
100	10	11	10	10.33

Table 7: Antibacterial Activity of Apple Cider Vinegar Against Bacteria Isolated from Apple

Concentration (%)	Replication 1	Replication 2	Replication 3	Mean
Control (Distilled water)	0	0	0	0
Standard (Erythromycin)	14	12	14	13.33
2.5	0	0	0	0
5	0	0	0	0
10	0	0	0	0
20	0	0	0	0
50	10	9	9	9.33
100	11	12	11	11.33

While comparing Natural Distilled Vinegar and Apple Cider Vinegar, Apple Cider Vinegar showed the more zone of inhibition as it has the more antibacterial activity against *E.coli.*

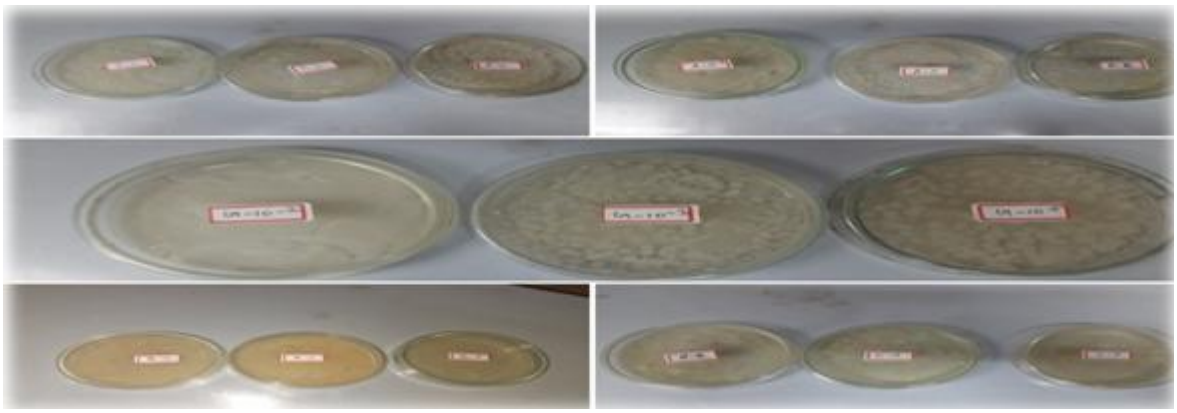


Figure 3: Bacterial Growth in Nutrient Agar

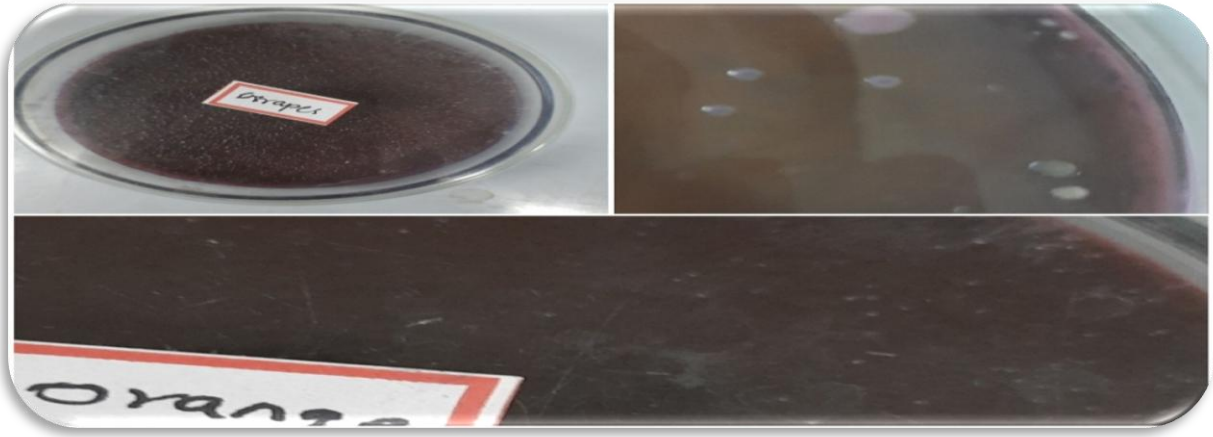


Figure 4: Bacterial Growth in EMB Agar

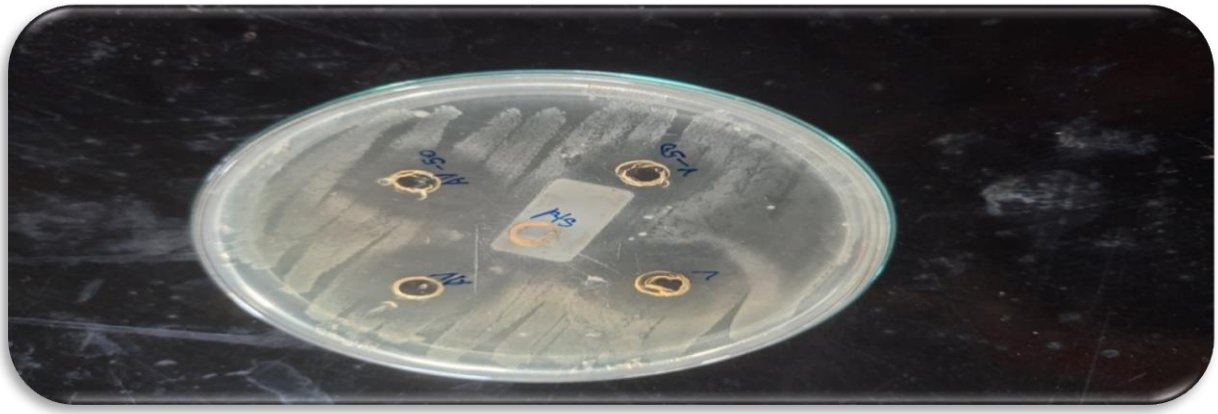


Figure 5: Antibacterial Activity of Vinegar (Zone of Inhibition Observed)

4. Conclusion

The presence of pathogens in fruits may imply possible risk of transmission of pathogens from fruits to human and causes diseases. To prevent this, control methods for pathogens and proper handling methods of fruits should be done. Proper sanitary conditions should be followed from the harvest itself. Hygienic conditions should be maintained in the fruits stalls. The fruit vendors should be educated with the proper maintenance of the fruits and proper discarding methods of the spoiled fruits because the spoiled fruits may transmit various pathogens here and there in environment through various factors. After buying fruits, they should be checked for spoilage before consumption.

Moreover, they should be washed with water to free from dirts and other contaminants and they are advised to disinfect with naturally available disinfections such as salt solution and vinegar. They may remove the pathogens such as bacteria due to their antibacterial activity. So that, we can consume disease free and pathogen free fruits which will provide proper nutritional value of the fruits. Even though shelf life of fruits are important, it is not advisable to use chemical disinfectants to extend the longevity of the fruits.

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