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IDENTIFICATION OF BACTERIA IN CONTAMINATED FRUIT WITH BIOCHEMICAL TESTS AND MALDI-TOF TO IMPLEMENT ANTIMICROBIAL MEASURES AND PREVENT POTENTIAL HARM TO CONSUMERS

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ABSTRACT

With a rise in actions to reduce food wastage, it is imperative to study the bacterial causes of this degradation. This not only allows one to understand the types of bacteria commonly present in spoiled foods but also enables the administration of correct measures to curb their growth, thus elongating shelf life. Thus, this paper explores biochemical tests and MALDI-TOF methods of identifying bacteria present in a spoilt fruit sample.

***KEY-WORDS**: bacteria, bacterial contamination, biochemical tests, food spoilage*

INTRODUCTION

Bacteria are unicellular prokaryotes, lacking membrane bound organelles. They are largely classified into gram positive and gram negative depending on the composition of their cell walls. Other methods of identification can be based on shape(cocci, spirillum, bacillus), the organization of cells (lone cells, clusters, chains) and size of cell. Abundantly present in the environment, bacteria play a vital role in human bodies, many food industries and bacterial infections(Murray, et al. 2021).

Food spoilage is the process by which food becomes impermissible for human consumption and is brought about by the metabolic process of several microorganisms. While spoiled food may not always be harmful for the consumer, it is considered inedible due to changes in 'texture, smell, taste, or appearance'(Rawat 2015). This is mainly caused

by bacteria, fungi, viruses and parasites. This spoilage usually occurs during production(for example on farms), while packaging or transportation or by the user. In either of the cases, once the bacterial colony comes in contact with the fruit or vegetable,the ability to proliferate depends on environmental factors such as nutrients present in the medium, levels of moisture, pH and temperature. Some bacteria that commonly cause spoilage in fruits are lactic acid bacteria., *Enterobacter aerogenes* and *Penicillium spp.*(Steele 2004).

Food spoilage is a major cause of food wastage, with about half of fruits and vegetables produced discarded due to contamination either by fungi or bacteria(Pitt and Hocking 2009). Given the implication of food wastage, identification of bacterial contaminants is necessary to discern the appropriate methods of food preservation required for unique species. An extension of this identification allows one to understand which, if any, bacterial infection could be caused through consumption of the spoilt sample, thus enabling more effective administration of medication.

AIM: TO IDENTIFY BACTERIAL CONTAMINANTS IN SPOILT FRUIT SAMPLES.

METHODOLOGY

To isolate the initial mixed cultures, first the medium was prepared. The media chosen were Hicrome Universal Agar, Plate Count Agar and Sabouraud Dextrose Agar. The Hicrome Universal Agar is a chromogenic agar which consists of chromogens that act like substrates for enzymes present in microorganisms. The presence of a particular enzyme in the bacteria would hydrolyse the substrate to release a specific colour, thus enabling identification of certain bacterial families (Perry and Freydière 2007). Media was prepared as per the instructions on the label. As per the requirement, 300ml of the medium was prepared and sterilized by autoclaving at 15lbs pressure (121°C) for 15 minutes to ensure sterility.

SERIAL DILUTIONS:

10g of the spoilt fruit sample (papaya) was taken and added to the saline. After mixing to make sure the sample was evenly distributed, serial dilutions were carried out. The initial sample is taken to be 10^{-1} colonies. After serial dilutions were done, each dilution of the colonies became 10^{-2} and 10^{-3}. Once these suspensions were prepared, 100 microlitre of each dilution was plated out on the surface of the agar. The plates were incubated as Plate Count Agar and HiCome Universal Agar at 35- 37̆°C for 24 hours for bacterial cultures. Sabouraud Dextrose Agar at 20-25°C for 48-72 hours for fungal cultures

After incubation , four typical bacterial isolates with different characteristics were observed. They were yellow, white translucent, white opaque and white small.

These 4 isolates were then isolated by streaking on 4 individual plates with agar. To do so, a sterile disposable loop was used for isolation and the four quadrant streak method was followed. This included spreading the bacterial sample in the zone of inoculation. The plate was then turned, allowing the streaking to take place in 4 quadrants. These plates were then incubated for 24 hours at 37°C.

BIOCHEMICAL TESTS:

In order to identify the bacteria, biochemical tests had to be performed. The following tests were conducted on each colony:

1. Gram Staining
2. Oxidase test
3. Catalase test
4. Bile Esculin test
5. Sugar Utilisation test
6. Motility test

For ease of testing, 2 sets of biochemical identification strips were also used that were developed by HiMedia.

Test 1: Gram Staining

While the exact reason for gram staining is not identified, there are multiple theories involving the composition of the cell wall of bacteria or its permeability that causes either a purple or pink colour. Some have proposed differences in lipoprotein, nucleic acids or carbohydrates in different bacteria cell walls, leading to the retention of a particular stain(SALTON 1963). Four solutions are used- Gram's crystal violet, Gram's iodine, Gram's decolorizer and Safranin. The crystal violet originally stains the cells and the iodine strengthens its binding to the cell wall. The decolouriser helps remove the stain from cell walls where the binding is weaker and these cells take up the stain of Safranin. A gram positive bacteria will show a purple colour while a gram negative bacteria will show a pink colour(HiMedia Laboratories Pvt. Limited 2019).

To conduct the test, first the bacterial suspension was made and a few drops were taken using a loop and placed on a glass slide to make a smear. This was then heat fixed by moving the glass slide across a bunsen burner until all the water had evaporated. Then, crystal violet was added for 1 minute and washed off. Following this was Gram's iodine, also placed for 1 minute and then washed off. Gram's decolorizer was added next for 30 seconds and

washed. Finally, Safranin was flooded for 2 minutes and then washed. The slides were air dried and observed under a microscope using oil immersion.

RESULTS OF GRAM STAINING:

All bacterial cultures appeared to be gram positive and had a cocci shape. The yellow and white small cultures appeared in clusters.

Test 2: Oxidase Test

The oxidase test is an indication of the use of the enzyme cytochrome oxidase in bacterial cells. While carrying out cell respiration, this enzyme allows for the rapid oxidation of cytochrome c, which is a part of the electron transport chain and helps convert oxygen to water. Thus to conduct this test, discs containing tetra-methyl-p-phenylenediamine dihydrochloride are used. This compound is oxidised by cytochrome c using cytochrome oxidase, releasing a blue pigment indophenol blue(Cathcart and Shields 2010).

To conduct this test, isolated colonies were taken from the plates using a sterile disposable loop. They were then added onto a disk and the colour change was recorded. The positive control used was Pseudomonas aeruginosa and the negative control was Escherichia coli.

RESULTS OF THE OXIDASE TEST:

Bacterial Isolate	Positive/negative reaction
Yellow	Negative
White translucent	Positive
White opaque	Positive
White small	Negative
Pseudomonas aeruginosa	Positive
Escherichia coli	Negative

Test 3: Catalase Test

To protect themselves from hydrogen peroxide, some bacteria have the enzyme catalase. This helps to catalyse the breakdown of hydrogen peroxide into water and oxygen. Thus, a positive reaction would be the presence of effervescence, indicating the production of oxygen gas. The equation for this reaction is as follows:

$2H_2O_2 +$ catalase $\rightarrow 2H_2O + O_2$ (Reiner 2010)

To conduct the test, a micropipette was used to add one drop of 3% hydrogen peroxide on a glass slide. A loop was used to take a small sample of the pure colonies and was placed in the hydrogen peroxide. The presence of effervescence was noted. The positive control was taken as *Staphylococcus aureus.*

RESULTS OF THE CATALASE TEST:

Bacterial Isolate	**Positive/negative reaction**
Yellow	Positive
White translucent	Positive
White opaque	Positive
White small	Positive
Staphylococcus aureus	Positive
Enterococcus faecalis	Negative

Test 4: Bile Esculin Test

This test showcases whether a bacterial cell is able to hydrolyse esculin in the presence of bile. Those that can hydrolyse this compound would produce esculetin from esculin. This esculetin reacts with iron ions (Fe^{3+}) in ferric citrate to produce a dark colour(Aryal 2019).

To conduct this test, two methods were used. The first used a bile esculin disk. Each colony was spread on individual plates with Bile Esculin Agar Base. The disks were then added to the center of the plates and were incubated for 24 hours at 37°C. A positive test would show blackening around the disks.

The second method of conducting this test was using a KB016 strip, manufactured by Himedia. A suspension of each sample had to be prepared in saline. Then 500 microliter was pipetted into the respective wells.Each strip was then incubated for 24 hours at 37°C. A positive test would show a dark brown/black colour while a negative test would show no colour change.

RESULTS OF THE BILE ESCULIN TEST:

Bacterial Isolate	**Positive/negative reaction**
Yellow	Positive
White translucent	Positive
White opaque	Positive
White small	Positive
Enterococcus faecalis	Positive
Escherichia coli	Negative

Test 5: Sugar Utilisation Test

This test is based on the bacteria's ability to ferment a carbohydrate(sugar). On doing so, it produces various compounds like 'lactic acid, formic acid, acetic acid, butyric acid, butyl alcohol, acetone, ethyl alcohol, carbon dioxide, and hydrogen'. These reduce the pH of the medium they are incubated in. Common indicators used are phenol red, which changes from red to yellow if pH reduces. A Durham's tube can also be added to check for the presence of gases. If gases are present, one will be able to see air bubbles at the top of the tube. If there is a color change and gas formation, the bacteria is considered to be a fermenter with acid and gas production. One with only

colour change is a fermenter with acid production and one with no colour change or gas production is a non-fermenter (Tankeshwar 2021).

To conduct the test, Carbohydrate Fermentation Media, Phenol Red Broth base was prepared as per the directions. Different carbohydrates (dextrose, lactose, mannitol, xylose and arabinose) at concentrations of 0.5% were added . 10 ml of the medium was distributed in the test tubes and inverted Durham's tubes were added to the tubes. The tubes were sterilized by autoclaving at 15 lbs pressure at 121°C for 15 minutes. After sterilization the isolate suspension was added and tubes were incubated at 35-37°C for 24 hours. The colour change and gas in the tube were then noted.

RESULTS OF SUGAR UTILISATION TEST:

Carbohydrate fermentation	**Yellow isolate**	**White Opaque isolate**	**White Translucent isolate**	**White Small isolate**
Dextrose	Fermentation and no gas	Fermentation and gas	Fermentation and gas	Fermentation and gas
Lactose	No fermentation and no gas	Fermentation and gas	No fermentation and no gas	No fermentation and no gas
Mannitol	Fermentation and no gas	No fermentation and no gas	Fermentation and gas	Fermentation and gas
Xylose	Fermentation and no gas	Fermentation and gas	Fermentation and gas	Fermentation and no gas
Arabinose	Fermentation and no gas	Fermentation and gas	Fermentation and gas	No fermentation and no gas

Test 6: Motility Test

This test determines whether the bacteria is able to move through the presence of a flagella. Organisms that are motile will be able to spread through the agar while those that are non-motile will only grow in straight lines down the agar(Tankeshwar 2021).

To conduct the test, semi-solid agar is added to test tubes and they are inoculated with the bacteria. These tubes are then incubated for 24 hours at 37°C and the pattern of growth is observed.

RESULTS FOR MOTILITY TEST:

Bacterial Culture	Motile/ Immotile
Yellow	Negative
White translucent	Negative
white opaque	Negative
white small	Negative

KB016 TESTING STRIPS:

Given the intensive and time-consuming nature of many biochemicals, HiMedia has created its own testing strips. Each strip has numbered wells, filled with mediums that are suitable for their respective biochemical test. From a suspension of the bacteria, 500 microliter is added to each well using a micropipette. The strips are then incubated for 24 hours. The strip used had the following tests:

	Strip 1		Strip 2
1	ONPG	1	Esculin Hydrolysis
2	Lysine Utilisation	2	Sucrose fermentation

3	Ornithine Utilisation	3	Sorbitol fermentation
4	Urease	4	Trehalose fermentation
5	Phenylalanine	5	Glucose fermentation
6	Voges Proskauer's Test (VP)	6	Cellobiose fermentation
7	Methyl Red (MR)	7	Melibiose fermentation
8	Indole	8	Salicin fermentation
9	PYR	9	Mannose fermentation
10	$\beta - glucuronidase$	10	Maltose fermentation
11	$\alpha - galactosidase$	11	Raffinose fermentation
12	$\beta - xylosidase$	12	Lactose fermentation

Certain tests also required reagents to be added to obtain results. The following table provides a the tests and their respective reagents:

Test	Reagent
Phenylalanine	TDA
Voges Proskauer's Test	Barritt Reagent A and B
Methyl Red	Methyl Red Reagent
Indole	Kovac's Reagent
PYR	PYR Reagent

Along with the strips, Hi Media provides a result interpretation chart with possible colour changes. These were used for interpretation of results.

Strip I	Result Interpretation chart					
No.	Test	Reagents to be added after incubation	Principle	Original colour of the medium	Positive reaction	Negative reaction
1	ONPG	—	Detects β – galactosidase activity	Colourless	Yellow	Colourless
2	Lysine utilization	—	Detects Lysine decarboxylation	Olive green to Light Purple	Purple / Dark Purple	Yellow
3	Ornithine utilization	—	Detects Ornithine decarboxylation	Olive green to Light Purple	Purple / Dark Purple	Yellow
4	Urease	—	Detects Urease activity	Orangish yellow	Pink	Orangish yellow
5	Phenylalanine Deamination	2-3 drops of TDA reagent	Detects Phenylalanine deamination activity	Colourless	Green	Colourless
6	Voges Proskauer's	2-3 drops of Baritt reagent A and 1 drop of Baritt reagent B	Detects acetoin production	Colourless / Light Yellow	Pinkish red	Colourless/ slight copper
7	Methyl red	1-2 drops of Methyl red reagent	Detects acid production	Colourless	Red	Yellowish- orange
8	Indole	1-2 drops of Kovac's red reagent	Detects deamination of tryptophan	Colourless	Pinkish Red	Colourless
9	PYR	1-2 drops of PYR reagent	Detects PYR enzyme activity	Cream	Cherry Red	Cream
10	β - Glucuronidase	—	For Enzymatic hydrdysis of Glucuronidase	Colourless / Light Yellow	Bluish Green	Light Yellow
11	α- Galactosidase	—	For Enzymatic hydrdysis of Galactosidase	Colourless / Light Yellow	Pink	Colourless / Light Yellow
12	β-Xylosidase	—	For Enzymatic hydrdysis of Xylosidase	Colourless / Light Yellow	Purple	Colourless Light Yellow

Strip II	Result Interpretation chart				
No.	Test	Principle	Original colour of the medium	Positive reaction	Negative reaction
13	Esculin hydrolysis	Esculin hydrolysis	Cream	Black	Cream
14	Sucrose	Sucrose utilization	Pinkish Red / Red	Yellow	Red / Pink
15	Sorbitol	Sorbitol utilization	Pinkish Red / Red	Yellow	Red / Pink
16	Trehalose	Trehalose utilization	Pinkish Red / Red	Yellow	Red / Pink
17	Glucose	Glucose utilization	Pinkish Red / Red	Yellow	Red / Pink
18	Cellobiose	Cellobiose utilization	Pinkish Red / Red	Yellow	Red / Pink
19	Melibiose	Melibiose utilization	Pinkish Red / Red	Yellow	Red / Pink
20	Salicin	Salicin utilization	Pinkish Red / Red	Yellow	Red / Pink
21	Mannose	Mannose utilization	Pinkish Red / Red	Yellow	Red / Pink
22	Maltose	Maltose utilization	Pinkish Red / Red	Yellow	Red / Pink
23	Raffinose	Raffinose utilization	Pinkish Red / Red	Yellow	Red / Pink
24	Lactose	Lactose utilization	Pinkish Red / Red	Yellow	Red / Pink

Image 9: HiMedia Interpretation Chart

RESULTS FOR KB STRIP:

Test	Yellow	White Small	White Opaque	White Translucent
ONPG	Positive	Negative	Positive	Positive
Lysine Utilisation	Negative	Negative	Negative	Negative
Orthinine	Negative	Negative	Negative	Negative
Urease	Negative	Negative	Positive	Negative

Phenylalanine Deaminase	Negative	Negative	Negative	Negative
VP	Negative	Negative	Negative	Negative
MR	Positive	Positive	Positive	Positive
Indole	Negative	Negative	Negative	Negative
PYR	Negative	Negative	Negative	Negative
$\beta - Glucuronidase$	Negative	Negative	Negative	Negative
$\alpha - galactosidase$	Positive	Negative	Positive	Positive
$\beta - xylosidase$	Negative	Negative	Negative	Negative
Esculin Hydrolysis	Positive	Positive	Positive	Positive
Sucrose	Positive	Negative	Negative	Positive
Sorbitol	Positive	Negative	Negative	Positive
Trehalose	Negative	Negative	Positive	Negative
Glucose	Positive	Positive	Positive	Negative
Cellobiose	Positive	Negative	Positive	Positive
Melibiose	Positive	Negative	Positive	Positive
Salicin	Positive	Negative	Positive	Positive

Mannose	Negative	Negative	Positive	Negative
Maltose	Positive	Negative	Positive	Positive
Raffinose	Positive	Negative	Negative	Positive
Lactose	Positive	Negative	Positive	Positive

MALDI-TOF:

While these biochemical tests give a strong indication of which bacterial family these samples belong to, all strains might not provide the same response due to mutations caused by the environment, thus making identification more complex. For a more certain identification, HiMedia Laboratories also uses MALDI-TOF for analysis. For each bacterial sample, this software generates a peptide mass fingerprint based on the proteins in the cells, which it then compares to those stored in the database. It also detects ribosomal proteins and, less commonly, certain biomarkers to give an accurate identification(Singhal, et al. 2015). To prepare each bacterial colony for this software, first a loop is used to add the culture to the plate provided by the machine. Then 70% formalin is added to each sample. This is air dried and re-inserted into the machine for testing.

RESULTS FOR MALDI-TOF:

Bacterial Isolate	**Organism(best match)**
White Small	Staphylococcus sciuri
White Transparent	Enterobacter cloacae
Yellow	No Organism Identification Possible
White Opaque	Klebsiella Pneumoniae

CONCLUSION

Initial isolation of the spoilage microorganisms on microbiological media yielded four bacterial isolates. Preliminary biochemicals were carried out for the isolates. The MALDI-TOF results revealed the possible spoilage causing microorganisms to be *Staphylococcus sciuri,Enterobacter cloacae* and *Klebsiella pneumoniae.* The yellow isolate is unidentified, possibly due to the limitations of the database of the MALDI software. Despite this, the main bacterial species causing food spoilage in a papaya plant were identified to the maximum capacity. Using these results, one must implement the correct precautionary measures to prevent their growth, due to their pathogenicity and potential harm for the consumer.

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