“Isolation, Biochemical Characterization and Identification of Microorganisms from Spoilt Brinjals Obtained from Local Markets of Dhaka City, Bangladesh”

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN MICROBIOLOGY

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Declaration

I hereby declare that the thesis project titled "**Isolation, Biochemical Characterization and Identification of Microorganisms from Spoilt Brinjals Obtained from Local Markets of Dhaka City, Bangladesh**" has been submitted by me, Shorna Sheikh and has been carried out under the supervision of Nazneen Jahan, Lecturer, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka.

It is further declared that this thesis has been composed solely by me and it has not been submitted, in whole or in part, in any previous institution for a degree or diploma. All explanations that have been adopted literally or analogously are marked as such.

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DEDICATED

TO

MY

BELOVED MOTHER

Acknowledgement

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Shorna Sheikh

September, 2018

Abstract

Vegetables are the most common food item in our everyday life. And brinjal is the most popular one. Starting from tradditionl curries to various Italian, French, Arabian cuisines brinjal is quite wellknown. It has vast nutritional value also. This study was done to isolate, identify, to see the temperature tolerance and enzymatic activity of the microorganisms responsible for the spoilage of brinjals. Six spoilt brinjal samples were collected from from various vegetable markets of Dhaka city and cultured using various selective and differential agar media using spread plate technique. A total of 53 bacterial isolates were identified where *Eshcherichia coli* and *Shigella*spp. showed the highest prevalence 9 (16.98%), followed by *Vibrio* spp. and *Bacillus* spp. 8 (15.09%), *Salmonella* spp. 7(13.21%),*Klebsiella* spp. 6(11.32%), *Staphylococcus* spp. 4(7.55%) and *Pseudomonas* spp. 2(3.77%)by using preliminary tests, plating on selective media and biochemical tests. Again in the cellulase degradation test done by Congo-Red Agar or CMC agar (Carbooxy-methyl-cellulose agar) only *Klebsiella spp., Shigella spp. and Salmonella* spp. showed positive results. These microbes were grown in three temperature ranges to observe their ability of growth. After incubation, it was seen that none of 53 isolates were able to grow in 4 and 60 whereas all of the isolates 53 (100%) could grow in the room temperature 25. This result indicates that all the isolates were strictly mesophiles and no thermophiles or sacrophiles were present in the suspected bacterial isolates.

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List of Abbreviations:

MSA	Mannitol Salt Agar
MR	Methyl Red
VP	Voges-proskauer
TSI	Triple Sugar Iron
CMC	Carboxymethylcellulose Agar
MIU	Motility Indole Urease
spp.	Species
µl	Microliter

Chapter 1: Introduction

1.1 Introduction

Eggplant is widely cultivated as vegetable in both temperate and tropical areas, especially in Asia. Eggplant is a major fruit vegetable with world production exceeding 32 million tons (Mt).

Some of these species are important for their medicinal qualities (Argoreo and Obanor, 2014). These nutritional and medicinal benefits have helped enhance their wide-spread cultivation across continents and cultural boundaries. (Grubben, 2004).Vegetables are related to outbreaks of food borne illness in several countries and also the organisms concerned embrace microorganism, viruses and parasites. These outbreaks vary in size from some persons affected to several thousands. Contamination of vegetables could happen in the least bit stages throughout pre and post-harvest techniques (De Roever, 1999). Cultivation and operation or preparation of the vegetables area unit is to blame for this contamination (Sumner and Peter, 1997). Unsafe water used for rinse the vegetables and sprinkling to stay them contemporary is additionally a supply of contamination (Mensah et al., 2002). Different potential sources of microorganisms embrace soil, ordure (human and animal origin), water (irrigation, cleaning), ice, animals (including insects and birds), handling of the merchandise, gather and process instrumentation and transport (Johannessen et al., 2002).

1.2 Factors of spoilage

Spoilage microorganisms can be introduced to the crop on the seed itself, during crop growth in the field, during harvesting and postharvest handling, or during storage and distribution. Some of the factors of spoilage are given below-

- growth and activities of micro-organisms, principally bacteria, yeasts and moulds
- insects, parasites and rodents;
- temperature, both heat and cold;
- moisture and dryness;
- air and in particular oxygen;
- light;

1.3 Common spoilage flora

After harvest, vegetables are often spoiled by a wide variety of microorganisms including many bacterial and fungal species.The bacteria were identified as Serratia marcescens, Escherichia coli and Corynebacteriumbovis while the fungi was identified on the basis of their colonial and microscopic characteristics as Penicilliumdigitatum, Rhizopusstolonifer, Aspergillusniger and Alternariaalternata. Escherichia coli was predominantly isolated among the bacterial isolates (50%) while Aspergillusniger occurred most frequently than the other fungal species (40%).

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1.4: Literarture review

The bacterial isolates were identified as *Staphylococcus aureus, Pseudomonaaeruginosa, Eshcherichia coli, Vibrio cholera* and *Salmonella typhi*by using preliminary tests,plating on selective media and biochemical tests. (3P. Saranraj, P.Sivasakthivelan, S. Sivasakthi and S. KaviKarunya 2016).

Mahmud et al., (2013) identified bacteria responsible for spoilage of some vegetables like carrot, tomatoes, cucumber, pepper, potatoes from Kaduna central market and Kawo market of Northern Nigeria. The presence of both gram positive and gram negatives were seen. Such as *Staphylococcus* (80%), *Streptococcus*(10%)straisns, *Eshcherichiacoli*(30%), *Citrobacter*(30%), *Klebsiella (*30%)along with a least occurrence of *Enterobacter*from Kaduna central market. Similar results were found from the other market with absence of *Eshcherichia coli* and the presence of *Edwardsiella*spp., *Staphylococcus aureus*and *Klebsiella*spp. were found with the highest percentage at Kawo market.

Zacharia and Philip (2016) and Gambari et al. (2013), isolated *SolanaceaeAlternariasolani, Fusariumsolani, Colletotrichumgloeosporioides, Botrytis cinerea, Penicillium sp.,Rhizopusnigricans, Curvularialunata, Botryodiplodiatheobromae, Mucor sp., Rhizoctoniasolani*and *Aspergillusniger*from rotten fruits of eggplant. *Alternariasolani, A. alternata, A. flavus, M. hiemalis*and *R. stolonifer*were identified and responsible for spoilage of eggplant.

1.5: Aims and objectives:

The objectives of this research work were to demonstrate the prevalence of bacteriain spoilt brinjals.On the basis of above context, the objectives of the present study are:

- Determine the microorganisms responsible for spoilage of brinjals.
- Determine the frequency of occurrence of isolated bacteria
- Biochemical characterization of spoilage causing bacterial isolates
- Determine the presence of spoilage related enzymes in isolated microbes
- Ability to grow in different temperatures

Chapter 2: Materials & Methods

2. Materials and methods:

2.1 Study area and duration:

The laboratory processing, analysis of data and the overall experimental work were done in the Microbiology Research Laboratory of the Department of Mathematics and Natural Sciences of BRAC University. The study was conducted during the period January- April, 2018.

2.2 Sample size:

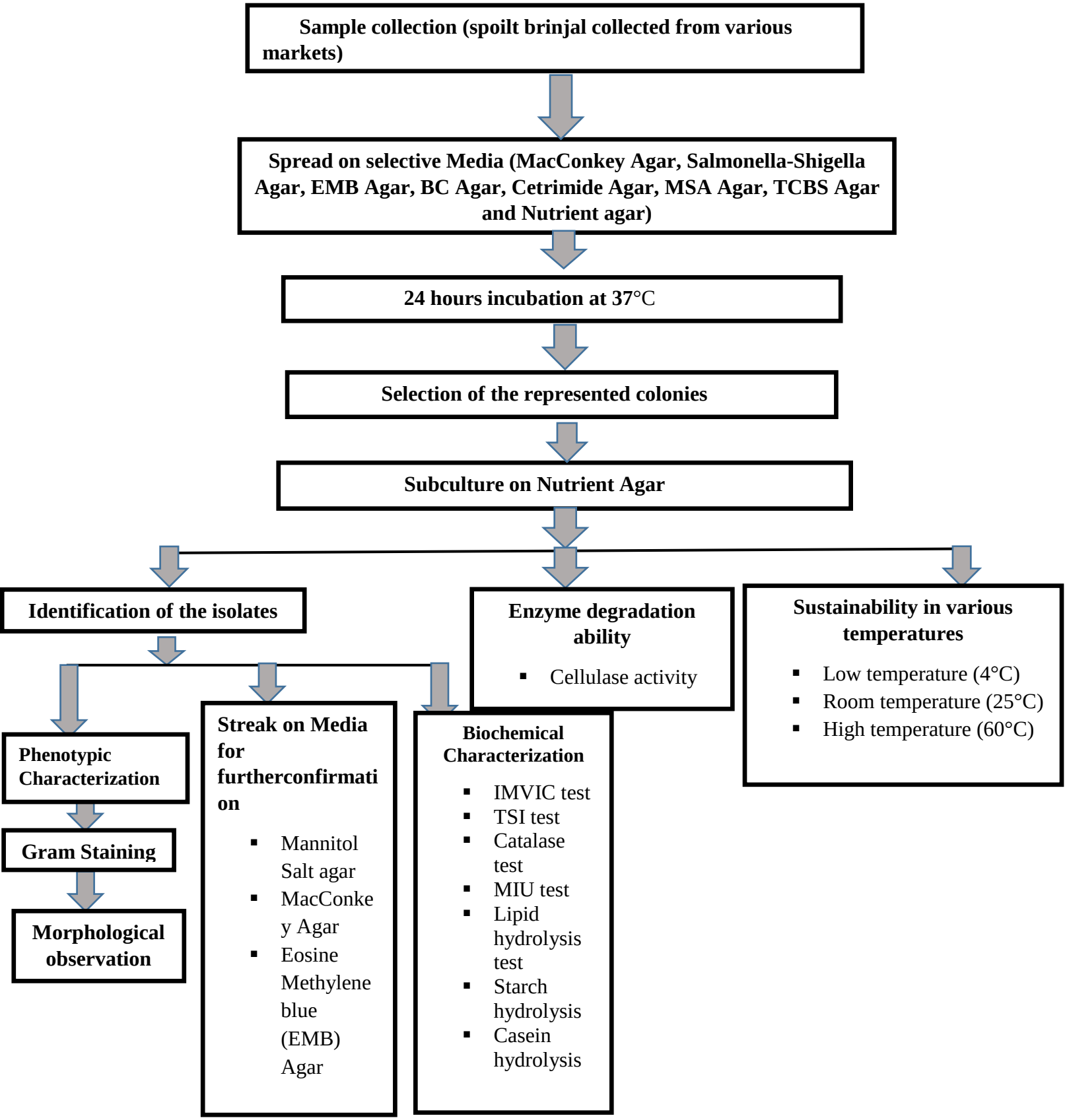
A total of about 6 brinjal samples were collected from various vegetable markets of Dhaka city.

2.3 Sample collection and processing:

A total of 6 spoilt brinjal samples were collected from different markets such as Kawran Bazar, Abdullapur Bazar, Kusul market, Mohammedpur market, Banani market, MohakhaliKhacha Bazar located inside Dhaka city.

Once the spoilt brinjal were collected, it was kept in a zip lock bag and brought to the laboratory for further experimental procedures. The spoilt portion was cut using knife and then grinded using mortar-pestel to make a paste and dissolved into 5ml saline.

2.4 Flow Diagram of the Study Design:



2.6 Isolation,purification and storage of sample:

Sources of 6 spoilt brinjal samples collected from different local markets inside Dhaka city along with their collection date, number of isolates and isolate IDs are given below-

Table: Sample collection: Source, Number of isolates found and their given name in the study

Sample No.	Source	Date	Time	Number of isolates	Isolates ID
01	Kawran Bazar	23.01.2018	7:00 am	9	B_1a, B_1b, B_1c, B_1d, B_1e, B_1f, B_1g, B_1h, B_1i
02	Abdullapur Bazar	04.02.2018	7:00 am	9	B_2a, B_2b, B_2c, B_2d, B_2e, B_2f, B_2g, B_2h, B_2i
03	Kusul market	18.02.2018	7:00 am	10	B_3a, B_3b, B_3c, B_3d, B_3e, B_3f, B_3g, B_3h, B_3i, B_3j
04	Mohammedpur market	04.03.2018	7:00 am	8	B_4a, B_4b, B_4c, B_4d, B_4e, B_4f, B_4g, B_4h
05	Banani market	18.03.2018	7:00 am	7	B_5a, B_5b, B_5c, B_5d, B_5e, B_5f, B_5g
06	MohakhaliKhacha Bazar	08.04.2018	7:00 am	8	B_6a, B_6b, B_6c, B_6d, B_6e, B_6f, B_6g, B_6h

After sample collection, samples were spreaded on Nutrient Agar and some selective media plates such as Mannitol Salt Agar, Eosine Methylene Blue Agar, Bacillus cereus Agar, Salmonella Shigella Agar, TCBS Agar, Cetrimide Agar from the saline. Next the plates were incubated for 24hours at 37^0C. After that, isolates from the selective media were streaked on nutrient agar to continue further confirmation using biochemical procedures and later on stored as stocks.

Long term preservation:

T_1N_1 media was prepared in a sterile vial. For long term preservation, bacteria was taken from the culture plate with a sterile inoculating needle and stabbed 3-5 times into the T_1N_1 media and incubated for 24hours at 37^0C. After incubation 200µl of paraffin oil was added on the culture and the vial was wrapped with parafilm and stored at room temperature.

2.8 Biochemical identification:

Biochemical identification of the isolates was done using methods from Bergey's Manual of Systematic Bacteriology.

2.8.1 Indole test:

Indole production test was done to determine the ability of microorganisms to degrade the amino acidtryptophan by the enzyme tryptophanase.

- For indole test each indole broth containing 6ml of peptone, sodium chloride was taken.
- Using sterile technique, small amount of the experimental bacteria from fresh culture was inoculated into the tubes by means of loop inoculation method with an inoculating loop
- The tubes were then incubated for 24 hours at37ºC.
- In order to detect the indole production, 10 drops of Kovacs reagent was added to all the tubes.
- If red reagent layer develops then it indicates indole positive and absence of red color indicates that the substrate tryptophan was not hydrolyzed and it indicates indole negative reaction. (Cappuccino &Sherman, 2005)

2.8.2 Methyl red (MR) test:

Methyl red test was done to determine the ability of the bacteria to oxidize glucose with the production and stabilization of high concentration of acid end products.

- For methyl red test each MR broth containing 5 ml of dipeptone, dextrose and potassium phosphate was taken.
- Using sterile technique, each tube was inoculated by fresh culture of experimental bacteria by means of loop inoculation method.

- The tubes were then incubated for 48 hours at37ºC.
- After 48 hours, 5 drops of methyl red indicator was added to each tube and the colour of the tubes was observed.
- If red colour develops then it indicates that the organism was capable of fermenting glucose with the production of high concentration of acid.
- If orange or yellow colour develops then it indicates methyl red negative result (Cappuccino &Sherman, 2005).

2.8.3 Voges-Proskauer (VP) test:

The Voges-Proskauer (VP) test was done to determine if an organism produces acetyl methylcarbinol from glucose fermentation.

- For Voges-Proskauer test each VP broth containing dipeptone, dextrose and potassium phosphate was taken.
- Using sterile technique, each tube was inoculated by fresh culture of experimental bacteria by means of loop inoculation method.
- The tubes were then incubated for 48 hours at37ºC.
- After 48 hours, 10 drops of Barritt's reagent A was added to each tube and the tubes were shaken. Then immediately 10 drops of Barritt's reagent B was added and the tubes were shaken.
- The colour was observed after 15-30 minutes of the reagent addition.
- If red colour developed then it indicates that the organism was capable of fermenting glucose with ultimate production of acetyl methyl carbinol and it indicates positive result.
- If no colour developed then it indicates voges- proskauer negative result. (Cappuccino &Sherman, 2005)

2.8.4 Citrate utilization test:

Citrate utilization test was done to differentiate among enteric organisms on the basis of their ability to ferment citrate as a sole source of carbon by the enzyme citrase.

- For citrate utilization test each vial containing 2.5 ml of Simmons citrate agar was taken.

- Using sterile technique, small amount of the experimental bacteria from 24-hours fresh culture wasinoculated into the vials by means of a streak inoculation method with an inoculating loop.
- The vials were then incubated at 37ºC for 24-48 hours.
- After 48 hours incubation, if the Prussian blue colour developed then it indicates the citrate positive result which means the organism was capable of fermenting citrate as a sole source of carbon.
- If there was no colour change then it indicates citrate negative result.

2.8.5 Catalase test

The differentiation of bacteria that produce the enzyme catalase from non-catalase producers is achieved using this test. Catalase acts as a catalyst in the breaking down of hydrogen peroxide to Oxygen and water, two to three ml of 3% hydrogen peroxide solution was poured into a test tube. A 24 hour culture of the test organism from the nutrient agar was emulsified in the hydrogen peroxide solution. The release of bubbles immediately indicated a positive test while it was negative when no bubble was formed.

2.8.7 Triple sugar-iron (TSI) agar test:

Triple sugar iron agar test was done to differentiate between Gram negative enteric bacilli based on their ability to ferment carbohydrate and reduce hydrogen sulfide.

- For TSI test each tube containing TSI agar was taken.
- Using sterile technique, small amount of the experimental bacteria from fresh culture was inoculated into the tubes by means of stab inoculation method with an inoculating needle.
- The tubes were then incubated at 37˚C for 24-48 hours.
- After 24-48 hours the color of both the butt and slant of agar slant cultures were observed.

The results were recorded based on the following interpretation-

Table Interpretation of Triple sugar-iron (TSI) test result

Result	**Interpretation**	**Symbol**
Yellow slant/ yellow butt	Glucose and lactose and/or sucrose fermentation with acid accumulation in slant and butt.	A/A
Red slant/yellow butt	Glucose fermentation with acid production. Proteins catabolized aerobically (in the slant) with the alkaline products (reversion).	K/A
Red slant/red butt	No fermentation. Peptone catabolized aerobically with alkaline products. Not from *Enterobacteriaceae.*	K/K
Red slant/no change in butt	No fermentation. Peptone catabolized aerobically with alkaline products. Not from *Enterobacteriaceae.*	K/NC
No change in slant/no change in butt	Organism is growing slowly or not at all. Not from *Enterobacteriaceae.*	NC/NC
Black precipitate in the agar	Sulfur reduction. (an acid condition, from fermentation of glucose or lactose and/or sucrose, exists in the butt even if the yellow color is obscured by the black precipitate).	H_2S
Cracks or lifting of agar	Gas production.	G

2.8.8 MIU (Motility-indole-urease) test:

MIU test was done for determining the motility of bacteria, indole production and urea degradation by means of the enzyme urease.

- Using sterile technique, smallamount of the experimental bacteria from fresh culture was inoculated into the tubesby means of stab inoculation method with an inoculating needle
- The tubes were thenincubated for 24 hours at 37ºC.
- The growth of the organism would spread throughout the test tube from downward to the upward of the test tube, if the organism is motile.
- The colour of the media will turn to deeppink if the organism is positive for urease test. If yellow colour develops then it indicates urease negative result.
- To confirm the indole test, five drops of Kovac's reagent was added following overnightincubation. Then the colour of the media was examined and the results were recorded.

- Formation of a rose red ring at the top indicates a positive result. A negative result canhave a yellow or brown layer (Cappuccino &Sherman, 2005).

2.8.9 Starch hydrolysis test:

Starch hydrolysis test was done to observe if the microbes can usestarch, acomplex carbohydrate made from glucose, as a source of carbon and energy for growth. Use ofstarchis accomplished by an enzyme calledalpha-amylase.

- Soluble starch media was dissolved in a small amount of water and was heated slowly with constant stirring. Then all the ingredients were added to it and was transferred into a conical flask and sterilized by autoclaving at 121.5°C.
- The sterilized agar medium was poured into the sterilized Petri plates and was allowed to solidify.
- Each plate was inoculated at the center with the bacterial inoculum.
- Plates were incubated at 37°C for 24–48 hrs.
- To test the hydrolysis of starch, each plate was flooded with iodine.
- An appearance of clear zone around the growth is considered as positive result.(Cappuccino &Sherman, 2005)

2.8.10 Casein hydrolysis test:

This test was done to determine the ability of microorganisms to excrete hydrolytic extracellular enzymes capable of degrading the protein casein.

- Using sterile technique, skim milk agar plates were inoculated with the test organism by using a sterile inoculating loop.
- Then the plates were incubated for 24 hours at 37°C.
- If the organisms secrete proteases, it will exhibit a zone of proteolysis which is demonstrated by a clear area surrounding the bacterial growth. It represents a positive result. In the absence of protease activity, the medium surrounding growth of the organism remains opaque which is a negative result.

2.8.11Lipid Hydrolysis

This media tests for the ability of an organism to break down and use a vegetable lipid (tributyrin) present in the agar plates. If an organism is able to secrete lipase the lipid can be hydrolyzed. The media usually contains spirit blue or methylene blue as an indicator. Use of the lipid can be observed as a zone of clearing around areas of growth. The zone has to be transparent for the test to be considered positive; color changes are not considered to be positive.

Chapter 3: Results

3.1: Bacterial Isolation and Identification:

3.1.1 Cultural and morphological characteristics of the bacterial isolates:

In the Table 3.1 the color, shape of the bacterial colonies on selective, differential and enriched media and the morphology of the bacterial colonies on nutrient agar are explained.

Table 3.2: Biochemical characteristics of the bacteria isolated from spoilt brinjals

Isolates ID	Gram staining		Indole Test	Methyl red Test	VP Test	Citrate test	Triple Sugar Iron Test						MIU Test			Catalase Test	Lipid Hydrolysis	Starch Hydrolysis	Casein hydrolysis	Suspected Organism
	Gram reaction	Shape					Slant/Butt	Glucose	Lactose	Sucrose	H_2S	Gas	Motility	Indole	Urease					
B_1a	-	Rod	+	+	-	-	Y/Y	+	+	+	-	+	+	+	-	+	-	-	-	***E.coli*spp.**
B_1b	-	Rod	-	+	+	+	Y/Y	+	+	+	-	+	-	-	+	+	+	-	-	***Klebsiella*spp.**
B_1c	-	Rod	-	-	-	+	R/R	+	-	-	-	-	+	+	+	+	+	+	+	***Pseudomonas* spp.**
B_1d	+	cocci	-	+	+	+	Y/Y	+	+	+	-	+	-	-	+	+	+	-	-	***Staphylococcus* spp.**
B_1e	+	cocci	-	+	+	+	Y/Y	+	+	+	-	+	-	-	+	+	+	-	-	***Staphylococcus* spp.**
B_1f	+	cocci	-	+	-	-	Y/Y	+	+	+	-	-	-	-	+	+	+	-	+	***Staphylococcus* spp.**
B_1g	+	cocci	-	+	-	-	Y/Y	+	+	+	-	-	-	-	+	+	+	-	+	***Staphylococcus*spp.**
B_1h	-	Rod	+	+	-	-	Y/Y	+	+	+	-	+	+	+	-	+	-	-	-	***E.coli*spp.**
B_1i	-	Rod	-	+	+	+	Y/Y	+	+	+	-	+	-	-	+	+	+	-	+	***Klebsiella*spp.**

Table 3.2: Biochemical characteristics of the bacteria isolated from spoilt brinjals

Isolates ID	Gram staining		Indole Test	Methyl red Test	VP Test	Citrate test	Triple Sugar Iron Test						MIU Test			Catalase Test	Lipid hydrolysis	Starch Hydrolysis	Casein hydrolysis	Suspected Organism
	Gram reaction	Shape					Slant/Butt	Glucose	Lactose	Sucrose	H_2S	Gas	Motility	Indole	Urease					
B_2a	+	rod	-	-	-	-	Y/Y	+	+	+	-	-	-	-	-	+	+	+	+	***Bacillus spp.***
B_2b	-	rod	-	+	-	+	Y/Y	+	+	+	+	-	+	-	-	+	-	-	-	***Salmonella spp.***
B_2c	-	rod	-	+	-	+	Y/Y	+	+	+	+	-	+	-	-	+	-	-	-	***Salmonella* spp.**
B_2d	-	rod	-	-	+	+	Y/Y	+	+	+	-	+	-	-	+	+	+	-	-	***Klebsiellaspp***

Table 3.3: Biochemical characteristics of the bacteria isolated from spoilt brinjals.

Isolates ID	Gram staining		Indole Test	Methyl red Test	VP Test	Citrate test	Triple Sugar Iron Test						MIU Test			Catalase Test	Lipid Hydrolysis	Starch Hydrolysis	Casein hydrolysis	Suspected Organism
	Gram reaction	Shape					Slant/Butt	Glucose	Lactose	Sucrose	H_2S	Gas	Motility	Indole	Urease					
B_2e	-	rod	-	+	+	+	Y/Y	+	+	+	-	+	-	-	+	+	+	-	-	***Klebsiella*spp.**
B_2f	-	rod	+	+	-	-	Y/Y	+	+	+	-	+	+	+	-	+	-	-	-	***E.coli*spp.**
B_2g	-	rod	-	+	-	+	Y/Y	+	+	+	+	-	+	-	-	+	-	-	-	***Salmonella* spp.**
B_2h	-	rod	-	+	+	+	Y/Y	+	+	+	-	+	-	-	+	+	+	-	-	***Klebsiella* spp.**
B_2i	-	rod	-	+	+	+	Y/Y	+	+	+	-	+	-	-	+	+	+	-	-	***Klebsiella*spp.**
B_3a	+	cocci	-	+	-	-	Y/Y	+	+	+	-	-	-	-	+	+	+	-	-	***Staphylococcus* spp.**
B_3b	+	cocci	-	+	-	-	Y/Y	+	+	+	-	-	-	-	+	+	+	-	-	***Staphylococcus*spp.**
B_3c	+	cocci	-	+	-	-	Y/Y	+	+	+	-	-	-	-	+	+	+	-	-	***Staphylococcus* spp.**
B_3d	-	rod	-	+	-	+	Y/Y	+	+	+	+	-	+	-	-	+	-	-	-	***Salmonella* spp.**

Table 3.3: Biochemical characteristics of the bacteria isolated from spoilt brinjals.

Isolates ID	Gram staining		Indole Test	Methyl red Test	VP Test	Citrate test	Triple Sugar Iron Test						MIU Test			Catalase Test	Lipid Hydrolysis	Starch Hydrolysis	Casein hydrolysis	Suspected Organism
	Gram reaction	Shape					Slant/Butt	Glucose	Lactose	Sucrose	H_2S	Gas	Motility	Indole	Urease					
B_3e	-	rod	+	+	-	-	Y/Y	+	+	+	-	+	+	+	-	+	-	-	-	***E.coli*spp.**
B_3f	-	rod	-	+	+	+	Y/Y	+	+	+	-	+	-	-	+	+	+	-	-	***Klebsiella*spp.**
B_3g	-	rod	-	+	+	+	Y/Y	+	+	+	-	+	-	-	+	+	+	-	-	***Klebsiella*spp.**
B_3h	+	cocci	-	+	+	+	Y/Y	+	+	+	-	+	-	-	+	+	+	-	-	***Staphylococcus* spp.**
B_3i	+	rod	-	-	-	-	Y/Y	+	+	+	-	-	-	-	-	+	+	+	+	***Bacillus* spp.**
B_3j	-	rod	-	+	-	+	Y/Y	+	+	+	+	-	+	-	-	+	-	-	-	***Salmonella*spp.**
B_4a	-	rod	-	-	-	+	R/R	+	-	-	-	-	+	+	-	+	-	-	-	***Pseudomonas* spp.**
B_4b	-	rod	-	-	-	+	R/R	+	-	-	-	-	-	-	+	+	+	-	+	***Pseudomonas* spp.**
B_4c	-	rod	-	-	-	+	R/R	+	-	-	-	-								***Pseudomonas* spp.**

Table 3.3: Biochemical characteristics of the bacteria isolated from spoilt brinjals

Isolates ID	Gram staining	Indole	Methyl	VP Test	Citrate	Triple Sugar Iron Test	MIU Test	Catalas	Lipid Hydrol	Starch Hydrol	Casein hydrolr	Suspected Organism

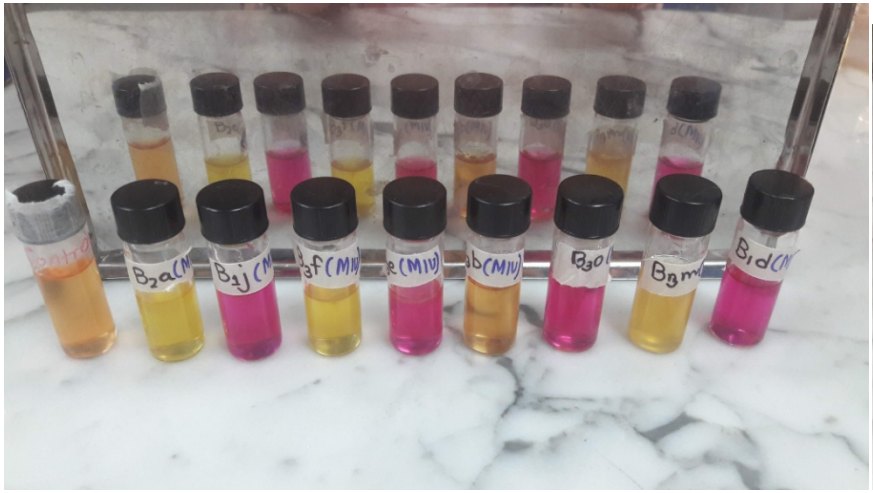
Isolates ID	Gram staining		Indole Test	Methyl red Test	VP Test	Citrate test	Triple Sugar Iron Test						MIU Test			Catalase Test	Lipid Hydrolysis	Starch Hydrolysis	Casein hydrolysis	Suspected Organism
	Gram reaction	Shape					Slant/But t	Glucose	Lactose	Sucrose	H_2S	Gas	Motility	Indole	Urease					
B_4d	-	rod	-	-	-	+	R/R	+	-	-	-	-	+	+	+	+	+	+	+	***Pseudomonas* spp.**
B_4e	-	rod	-	-	-	-	Y/Y	+	+	+	-	-	+	-	-	+	+	-	-	***Vibrio* spp.**
B_4f	-	rod	-	-	-	-	Y/Y	+	+	+	-	-	+	-	-	+	+	-	-	***Vibrio*spp.**
B_4g	-	rod	-	-	-	-	Y/Y	+	+	+	-	-	+	-	-	+	+	-	-	***Vibrio* spp.**
B_4h	-	rod	-	-	-	-	Y/Y	+	+	+	-	-	+	-	-	+	+	-	-	***Vibrio*spp.**
B_5a	-	rod	+	+	-	-	Y/Y	+	+	+	-	+	+	+	-	+	-	-	-	***E.coli*spp.**

	Gram reaction	**Shape**					**Slant/But t**	**Glucose**	**Lactose**	**Sucrose**	**H_2S**	**Gas**	**Motility**	**Indole**	**Urease**					
B_5b	-	rod	-	-	-	+	R/R	+	-	-	-	-	+	+	+	+	+	+	+	***Pseudomonas* spp.**
B_5c	-	rod	-	-	-	-	Y/Y	+	+	+	-	-	+	-	-	+	+	-	-	***Vibrio* spp.**
B_5d	-	rod	-	-	-	-	Y/Y	+	+	+	-	-	+	-	-	+	+	-	-	***Vibrio*spp.**
B_5e	-	rod	-	-	-	-	Y/Y	+	+	+	-	-	+	-	-	+	+	-	-	***Vibrio* spp.**
B_5f	-	rod	-	-	-	-	Y/Y	+	+	+	-	-	+	-	-	+	+	-	-	***Vibrio*spp.**
B_5g	-	rod	+	+	-	-	Y/Y	+	+	+	-	+	+	+	-	+	-	-	-	***E.coli*spp.**

Table 3.3: Biochemical characteristics of the bacteria isolated from spoilt brinjals

Table 3.3: Biochemical characteristics of the bacteria isolated from spoilt brinjals

Isolates ID	Gram staining		Indole Test	Methyl red Test	VP Test	Citrate test	Triple Sugar Iron Test						MIU Test			Catalase Test	Lipid Hydrolysis	Starch Hydrolysis	Casein hydrolysis	Suspected Organism
	Gram reaction	Shape					Slant/But t	Glucose	Lactose	Sucrose	H_2S	Gas	Motility	Indole	Urease					
B_6a	-	rod	-	-	-	+	R/R	+	-	-	-	-	+	+	+	+	+	+	+	***Pseudomonas* spp.**
B_6b	-	rod	-	-	-	-	Y/Y	+	+	+	-	-	+	-	-	+	+	-	-	***Vibrio* spp.**
B_6c	-	rod	-	-	-	-	Y/Y	+	+	+	-	-	+	-	-	+	+	-	-	***Vibrio*spp.**
B_6d	-	rod	-	-	-	-	Y/Y	+	+	+	-	-	+	-	-	+	+	-	-	***Vibrio* spp.**
B_6e	-	rod	-	-	-	-	Y/Y	+	+	+	-	-	+	-	-	+	+	-	-	***Vibrio*spp.**
B_6f	-	rod	+	+	-	-	Y/Y	+	+	+	-	+	+	+	-	+	-	-	-	***E.coli*spp.**
B_6g	-	rod	+	+	-	-	Y/Y	+	+	+	-	+	+	+	-	+	-	-	-	***E.coli*spp.**
B_6h	-	rod	+	+	-	-	Y/Y	+	+	+	-	+	+	+	-	+	-	-	-	***E.coli*spp.**



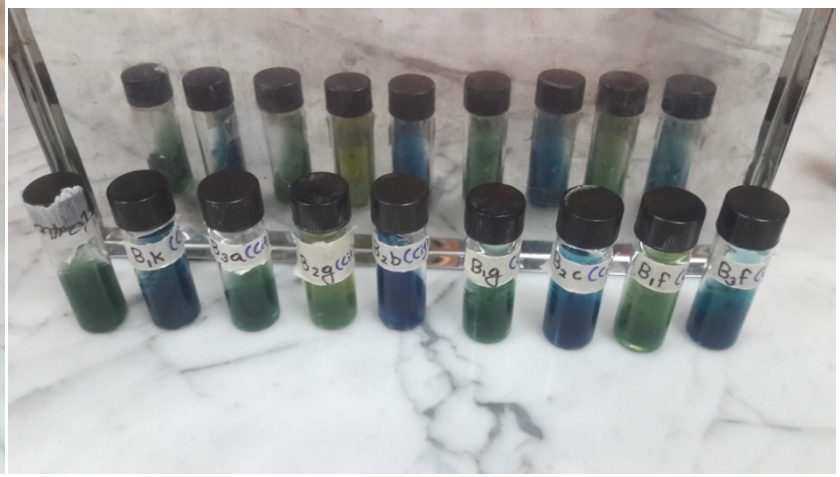


Fig. MIU test showing positive (pink color) and negative (orange) results Fig. citrate test showing positive (prussian blue) and negative (green) result

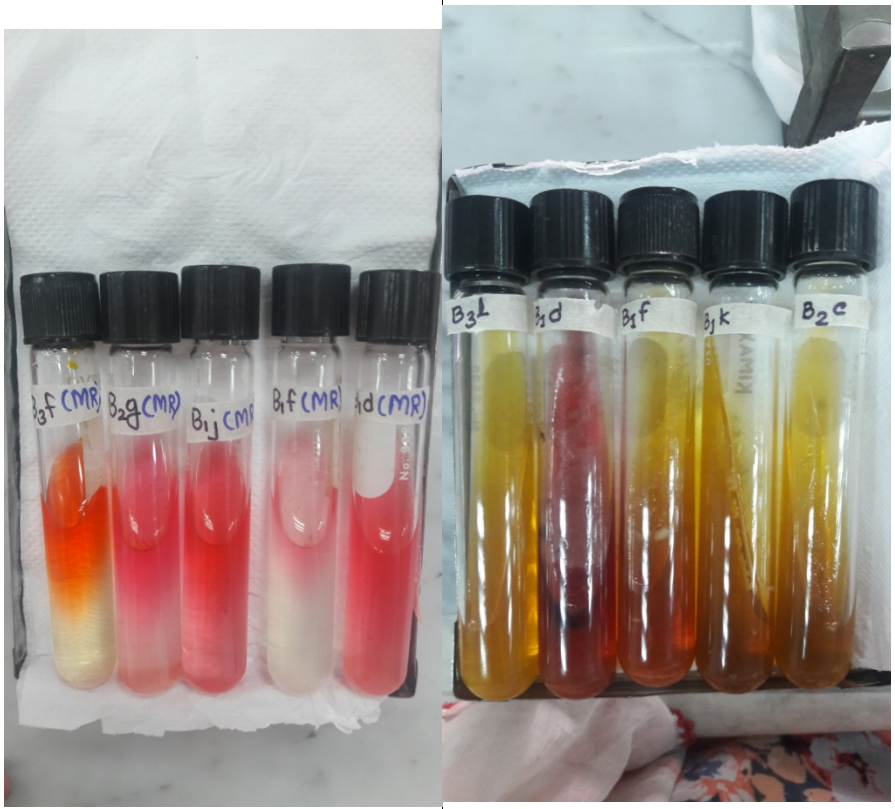


Fig. Triple sugar iron test

Fig. Methyl red test

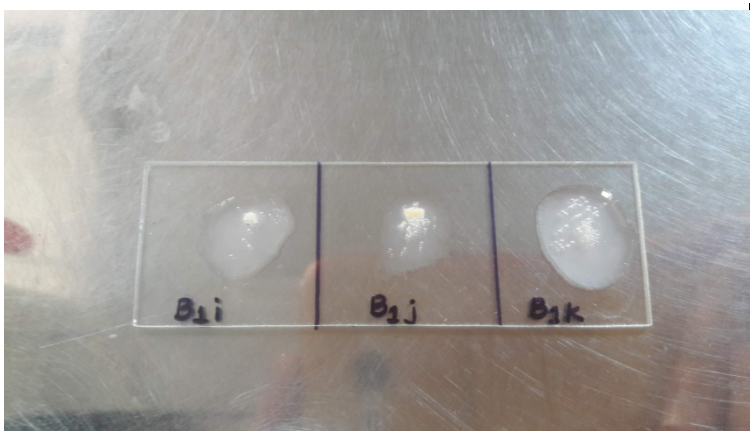


Fig. catalase test showing positive result

Fig. starch hydrolysis test showing positive result

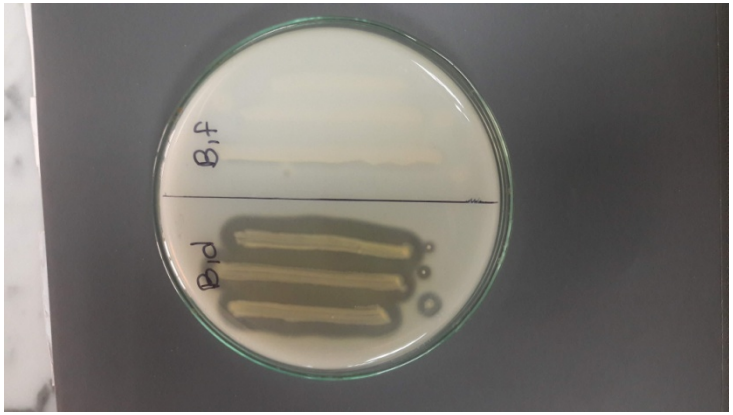


Fig. casein hydrolysis test showing both positive and negative results

After observing the cultural and morphological characteristics of bacterial isolates and performing the biochemical tests, 40 isolates have been identified. The isolates that have been confirmed include *Staphylococcus* sp., *Pseudomonas* spp., *Bacillus* spp., *E.coli*, *Vibrio* spp., *Salmonella* spp.,and*Klebsiella*spp.. The total number and the percentage of the isolates obtained from the samples are shown in table 3.3and figure 3.4

Table 3.4: Prevalence of bacteria species isolated from spoilt brinjals

Bacterial isolates	**Number of the isolates**	**Total bacterial isolates**	**% Prevalence**
Staphylococcus spp.	4	53	7.55
Pseudomonas spp.	2		3.77
Bacillus spp.	8		15.09
Vibrio spp.	8		15.09
Salmonella spp.	7		13.21
*Klebsiella*spp.	6		11.32
E.coli	9		16.98
Shigella spp.	9		16.98

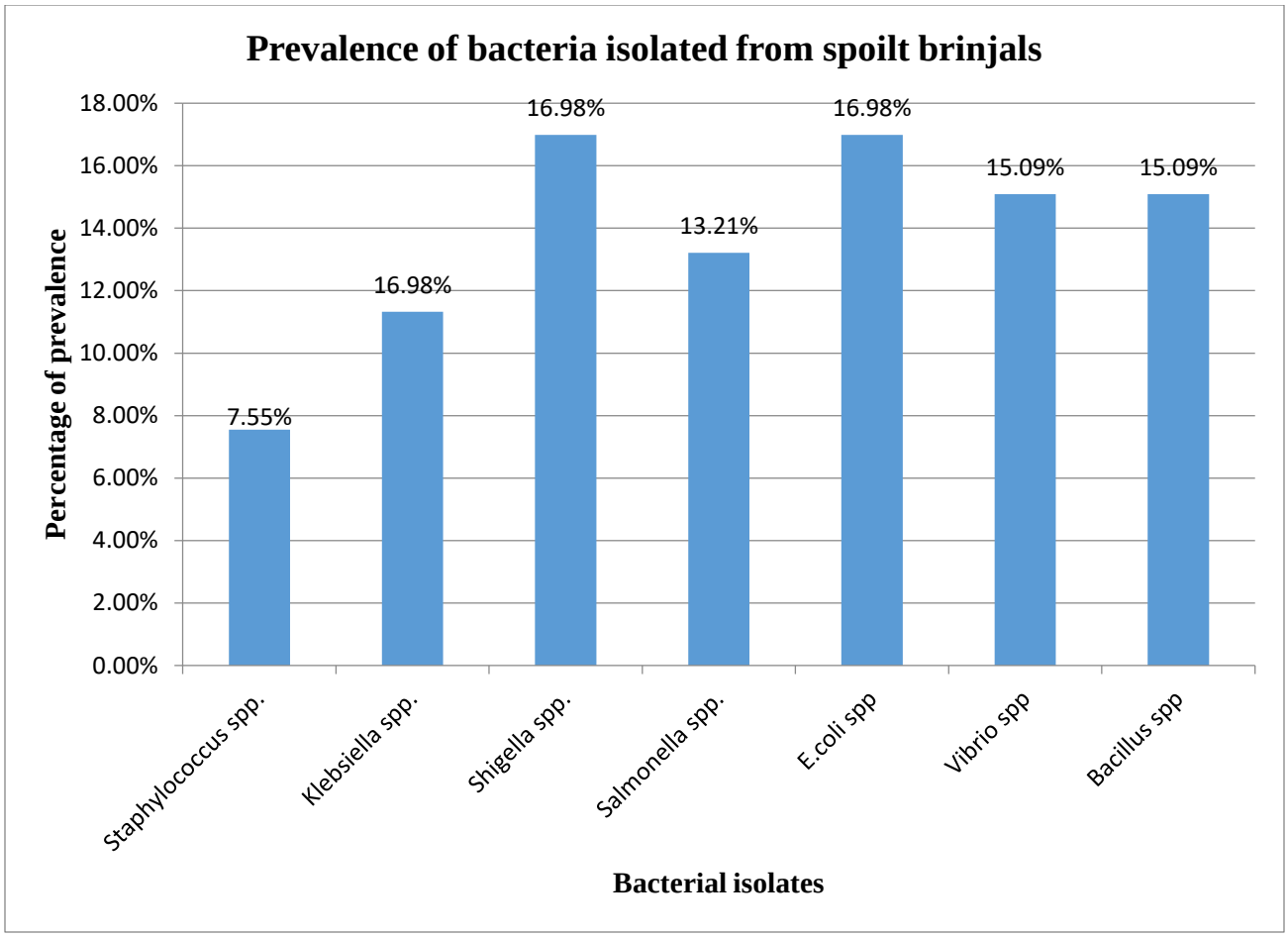


Figure 3.3: Percentage of prevalence of isolated bacteria from spoilt brinjals

Among the identified isolates, both the Gram positive and Gram negative organisms were found. The Gram positive organisms that have been identified include *Staphylococcus* spp and *Bacillus* spp. The Gram negative organisms that have been identified include *E.coli*, *Klebsiella*spp, *Vibrio*spp, *Pseudomonas*spp and *Salmonella* spp. The differentiation, number and the percentage of the identified bacterial isolates based on Gram reaction are shown in Table 3.4 and Figure 3.5

Table 3.5: Distribution of the isolates according to Gram's Reaction

Gram's Reaction	**Number of isolates found**	**Percentage (%)**
Gram positive	10 (out of 37)	27.07%
Gram negative	27 (out of 37)	72.97%

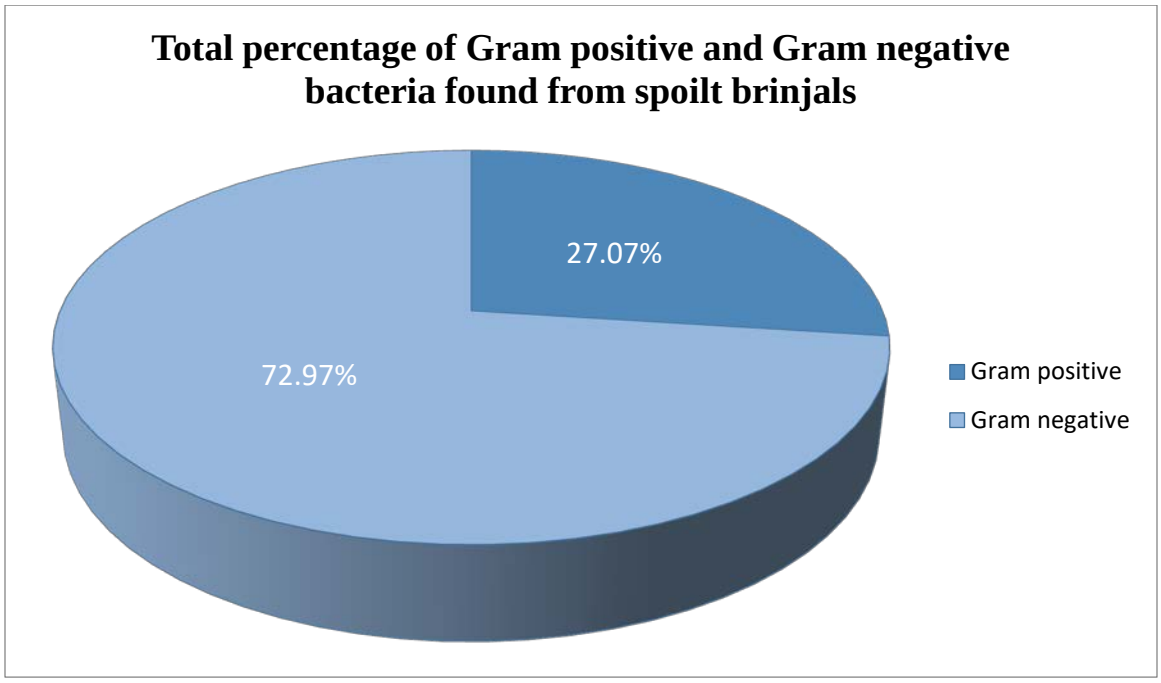


Figure 3.4: Total percentage of Gram positive and Gram negative bacteria identified from spoilt brinjals

3.3 Temperature tolerance of the tested organism:

Temperature tolerance of the organisms was determined by growing the isolates in different temperature like 4°C, 25°C and 60°C. All the isolates showed growth at 25°C but in 4°C and 60°C0% isolates showed viable growth.

Table 3.9: Temperature tolerance of bacterial isolates:

IsolatesID	4°C	25°C	60°C
B_1a	-	+	-
B_1b	-	+	-
B_1c	-	+	-
B_1d	-	+	-
B_1e	-	+	-
B_1f	-	+	-
B_1g	-	+	-
B_1h	-	+	-
B_1i	-	+	-
B_2a	-	+	-
B_2b	-	+	-
B_2c	-	+	-
B_2d	-	+	-
B_2e	-	+	-
B_2f	-	+	-

IsolatesID	4°C	25°C	60°C
B_2g	-	+	-
B_2h	-	+	-
B_2i	-	+	-
B_3a	-	+	-

B_3b	-	+	-
B_3c	-	+	-
B_3d	-	+	-
B_3e	-	+	-
B_3f	-	+	-
B_3g	-	+	-
B_3h	-	+	-
B_3i	-	+	-
B_3j	-	+	-
B_4a	-	+	-
B_4b	-	+	-
B_4c	-	+	-
B_4d	-	+	-
B_4e	-	+	-

IsolatesID	4°C	50°C	60°C
B_4f	-	+	-
B_4g	-	+	-
B_4h	-	+	-
B_5a	-	+	-
B_5b	-	+	-
B_5c	-	+	-

B_5d	-	+	-
B_5e	-	+	-
B_6a	-	+	-
B_6b	-	+	-
B_6c	-	+	-
B_6d	-	+	-
B_6e	-	+	-
B_6f	-	+	-
B_6g	-	+	-
B_6h	-	+	-

(+) = growth (-) = no growth

Table: 3.10Total number of positive bacterial growth:

Total bacterial isolates	**Bacterial growth at 4**°C	**Bacterial growth at 25**°C	**Bacterial growth at 60**°C

53	0 (0%)	53(100%)	0(0%)

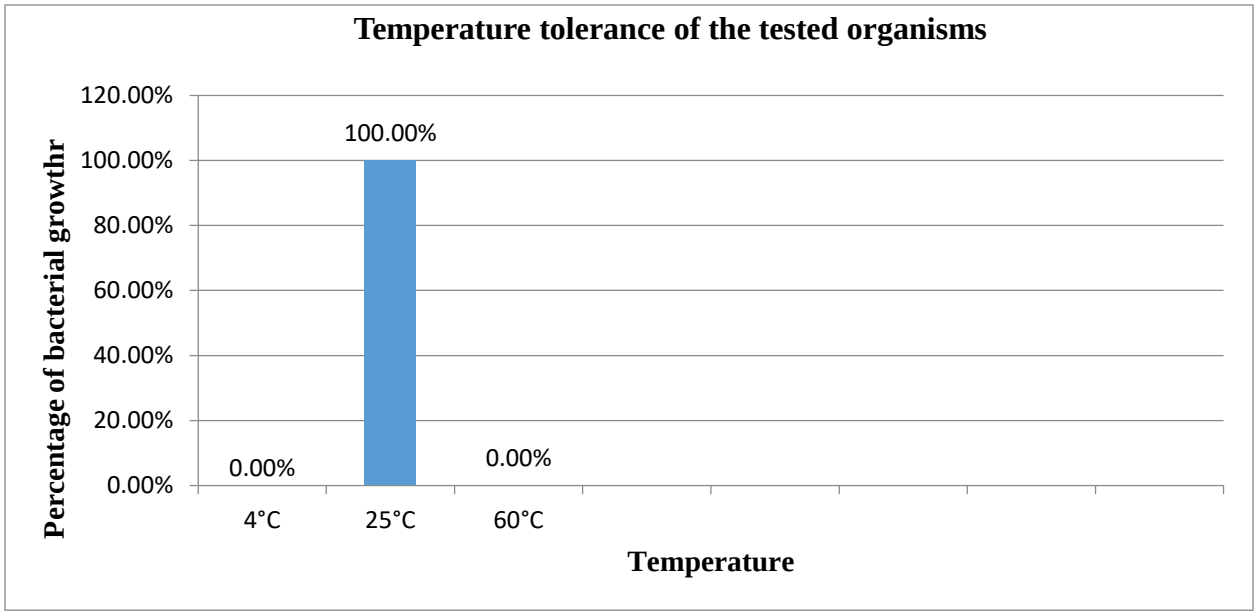


Figure 3.7: Temperature tolerance percentage of the tested organisms.

3.4 Cellulase activity

Cellulase activity of abundant identified organisms is also observed

Table 3.7: Cellulose Hydrolysis test

Identified Organism	Cellulose Hydrolysis
Klesiella spp.	+
E.coli	-
Vibrio spp.	-
Shigella spp.	+
Bacillus spp.	-
Salmonella spp.	+
Shigella spp.	-

Chapter 4: Discussion and Conclusion

Discussion

After conducting the experiment the results include that the spoilage of the brinjals were due to the presence of spoilage causing microoragnisms. Though there are some other factors which can also be equally responsible for the degradation such as poor post-harvest handling practices and unhygienic storage conditions in the local markets.

In this study, a total of 53 isolates had been identified from 6 different samples collected from different local vegetable markets of Dhaka city. The bacterial isolates were suspected by observing the cultural and morphological characteristics shown in different selective and differential media including biochemical test results. Out of 53 isolates, both *E.coli*and *Shigella*spp. showed the highest prevalence 9 (16.98%), followed by *Bacillus* spp. and *Vibrio* spp. 8 (15.09%), *Salmonella* 7 (13.21%), *Klebsiella*6 (11.32%), *Staphylococcus* spp. 4(7.55%) and *Pseudomonas* spp. 2(3.77%). This experiment also shows the degradation capability of cellulase enzyme where *Klebsiella* spp., *Salmonella* spp. and *Shigella spp.* showed positive results.

In this study, bacterial growth was observed at 4°C, 25°C and 60°C. All bacterial isolates showed growth at 25°C whereas; none of them were able to survive at 4°C and 60°C respectively.

So from the above experiment it can be said that in order to avoid spoilage of vegetables it is better to keep them in refrigerator and obviously eat them after proper cooking. As the microbes cannot survive such high and low temperatures ranges. Under no circumstances the vegetables should be kept in room temperature to prevent any spoilage.

Conclusion

The study showed the prevalence of microbes in spoilt brinjals. Quite a number of potential pathogens like *Staphylococcus* spp, *Escherichia coli*, *Salmonella* spp., *Vibrio* spp., *Pseudomonas* spp., *Klebsiella* spp., Bacillus spp., were found. Besides microbes can grow in room temperature but not in low temperature or a higher temperature. This suggests that the vegetables should always be stored in refrigerator to avoid spoilage and further hazards. As brinjal is one of the most consumed vegetable in our country so it should cooked properly to prevent all sorts of microbial contamination.

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Appendices

Appendix- I

Media compositions:

The composition of all media used in the study is given below:

Nutrient Agar

Component	Amount (g/L)
Peptone	5.0
Sodium chloride	5.0
Beef extract	3.0
Agar	15.0
Final pH	7.0

Saline

Component	Amount (g/L)
Sodium Chloride	9.0

Nutrient broth

Component	Amount (g/L)
Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Final pH	7.4±0.2 at 25ºC

Mannitol Salt Agar

Component	Amount (g/L)
Proteose peptone	10.0
Beef extract	1.0
Sodium chloride	75.0
D-mannitol	10.0
Phenol red	0.025
Agar	15.0
Final pH	7.4 ± 0.2 at 25°C

Cetrimide Agar

Component	Amount (g/L)
Pancreatic Digest of Gelatin	20.0
Potassium Sulfate	10.0
Magnesium Chloride	1.4
Cetyltrimethylammonium Bromide	0.3
Glycerin	10.0
Agar	13.6
Final pH	7.2± 0.2 at 25°C

Salmonella Shigella Agar

Component	Amount (g/L)
Peptic digest of animal tissue	15.0
Proteose peptone	5.0
Dextrose	1.0
Lead acetate	0.2
Sodium thiosulphate	0.08
Agar	15.0
Final pH	7.0± 0.2 at 25°C

MacConkey Agar

Component	Amount (g/L)
Peptic digest of animal tissue	1.5
Casein enzymatic hydrolysate	1.5
Pancreatic digest of gelatin	17.0
Lactose	10.0
Bile salt	1.50
Crystal violet	0.001
Neutral red	0.03
Agar	15.0
Final pH	7.1 ± 0.2 at 25°C

TCBS Agar

Component	Amount (g/L)
Proteose peptone	10.0
Yeast extract	5.0
Sodium thiosulphate	10.0
Sodium citrate	10.0
Oxgall	8.0
Sucrose	20.0
Sodium chloride	10.0
Ferric citrate	1.0
Bromothymol blue	0.04
Thymol blue	0.04
Agar	15.0
Final pH	8.6± 0.2 at 25°C

Eosine Methylene Blue Agar (EMB):

Component	Amount (g/L)
Peptone	10.0
Dipotassium Phosphate	2.0
Lactose	5.0
Sucrose	5.0
Eosin yellow	0.14
Methylene Blue	0.065
Agar	13.50
Final pH	7.1 ± 0.2 at 25°C

Simmon's Citrate Agar

Component	Amount (g/L)
Magnesium sulphate	0.2
Ammoniundihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bacto agar	15.0
Bactobromothymol blue	0.08

Methyl Red -VogesProskauer(MR-VP) Media

Component	Amount (g/L)
Peptone	7.0
Dextrose	5.0
Dipotassium hydrogen phosphate	5.0
Final pH	7.0

Triple Sugar Iron Agar (TSI)

Component	Amount (g/L)
Bio-polytone	20.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous ammonium sulphate	0.2
Sodium thiosulphate	0.2
Phenol red	0.0125
Agar	13.0
Final pH	7.3

Motility Indole Urease (MIU) Agar

Component	Amount (g/L)
Tryptone	10
Phenol red	0.1
Agar	2.0
Sodium chloride	5.0
pH (at 25°C)	6.8 ± 0.2 at 25°C

Indole broth

Component	Amount (g/L)
Peptone	10.0
Sodium chloride	5.0

Phenol Red Maltose Broth

Component	Amount (g/L)
Proteose peptone	10.0
Beef extract	1.0
Sodium chloride	5.0
Maltose	5.0
Phenol red	0.018
pH (at 25°C)	7.4 ± 0.2 at 25°C

Starch Agar

Component	Amount (g/L)
Meat extract	3.0
Peptic digest of animal tissue	5.0
Starch, soluble	2.0
Agar	15.0
pH (at 25°C)	7.2 ± 0.1 at 25°C

Skim Milk Agar

Component	Amount (g/L)
Skim milk powder	28.0
Casein enzymichydrolysate	5.0
Yeast extract	2.5
Dextrose	1.0
Agar	15.0
pH (at 25°C)	7.0 ± 0.2 at 25°C

Appendix – II

Reagents and buffers

Gram's iodine (300 ml)

To 300 ml distilled water, 1 g iodine and 2 g potassium iodide was added. The solution was mixed on a magnetic stirrer overnight and transferred to a reagent bottle and stored at room temperature.

Crystal Violet (100 ml)

To 29 ml 95% ethyl alcohol, 2 g crystal violet was dissolved. To 80 ml distilled water, 0.8 g ammonium oxalate was dissolved. The two solutions were mixed to make the stain and stored in a reagent bottle at room temperature.

Safranin (100ml)

To 10 ml 95% ethanol, 2.5 g safranin was dissolved. Distilled water was added to the solution to make a final volume of 100 ml. The final solution was stored in a reagent bottle at room temperature.

Kovac's Reagent (150 ml)

To a reagent bottle, 150 ml of reagent grade isoamyl alcohol, 10 g of p-dimethylaminobenzaldehyde (DMAB) and 50 ml of HCl (concentrated) were added and mixed. The reagent bottle was then covered with an aluminum foil to prevent exposure of reagent to light and stored at 4°C.

Methyl Red (200 ml)

In a reagent bottle, 1 g of methyl red powder was completely dissolved in 300 ml of ethanol (95%). 200 ml of destilled water was added to make 500 ml of a 0.05% (wt/vol) solution in 60% (vol/vol) ethanol and stored at 4°C.

Barrit's Reagent A (100 ml)

5% (wt/vol) a-naphthol was added to 100 ml absolute ethanol and stored in a reagent bottle at 4°C.

Barrit's Reagent B (100 ml)

40% (wt/vol) KOH was added to 100 ml distilled water and stored in a reagent bottle at 4°C.

Catalase Reagent (20 ml 3% hydrogen peroxide)

From a stock solution of 35 % hydrogen peroxide, 583 µl solution was added to 19.417 ml distilled water and stored at 4°C in a reagent bottle.

Urease Reagent (50 ml 40% urea solution)

To 50 ml distilled water, 20 g pure urea powder was added. The solution was filtered through a HEPA filter and collected into a reagent bottle. The solution was stored at room temperature.

Appendix-III

Instruments

Autoclave	Model: WIS 20R Daihan Scientific Co. ltd, Korea
Laminar airflow cabinet	Model-SLF-V, vertical, SAARC group Bangladesh
Incubator	Model-0SI-500D, Digi system Laboratory Instruments Inc. Taiwan
Vortex Mixer	Digi system Taiwan, VM-2000
Electronic Balance	RADWAG WagiELEktroniczne Model: WTB 200
Refrigerator (4°C)	Model: 0636 Samsung