WOLKITE UNIVERSITY

COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCE

DEPARTMENT OF BIOTECHNOLOGY

SCREENING AND IDENTIFICATION OF PECTINOLYTIC BACTERIAL ISOLATES FROM FRUIT AND VEGETABLE WASTE DUMP SOIL

A Senior Project Submitted to Department of Biotechnology For Partial Fulfillment of Bachelor of Science Degree in Biotechnology

By;

NAME	ID/NO
MEKIYA MEHDI	NCSR/ 275/11
HIKMA MOHAMMED	NCSR/ 200/11
FIREZER ABEBAW	NCSR/148/11
BOSENA SHEBABAW	NCSR 085/11
MULUKEN TEREFE	NCSR 338/10

Advisor:Mulugeta Belay (Msc)

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Wolkite, Ethiopia

Wolkite University

College of Natural and Computational Science

Department of Biotechnology

Senior Project Submission Form

This is to certify that senior project entitled as "screening and identification of pectinolytic bacterial isolates from fruit and vegetable waste dump soil", submitted in partial fulfillments for the Bachelor of Science in Biotechnology by below listed students, complies with the regulations of the university and meets the accepted standard with respect to originality and quality.

Name of the students	ID/NO
1. Mekiya Mehdi	NCSR/ 275/ 11
2. Hikma Mohammed	NCSR/ 200/ 11
3. Firezer Abebaw	NCSR/ 148/11
4. Bosena Shebabaw	NCSR/ 085/11
5. MulukenTerefe	NCSR/ 338/ 10

Signed by Examining committee:

Examiner: _____ Signature _____ Date_____

Examiner: _____ Signature _____ Date_____

Advisor: _____ Signature _____ Date_____

Chairperson: _____ Signature_____ Date_____

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Chairperson :Signature:_____Date:_____

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LIST OF ABBREVAITION

Hz:Hertz

KB:Kirby Bauer

KOH:Potassium Hydro oxide

LSR:Liquid Solid Ratio

MAE:Microwave Assisted Extraction

MS:Mimimum salt

MR-VP:Methyl red and Voges Proskauer

PAE :pectin acetyl esterase

PG:Polygalacturonases

PL:Pectate Lyases

PME:Pectin Methyl Esterases

PNL:Pectin Lyases

W:Watt

ABSTRACT

Naturally occurring polysaccharide pectin, is very important in both scientific and commercial world due to its biodegradability. A large group of pectinase enzymes causing breakdown of pectin polysaccharides of plants and fruit are used in industrial sector to increase the yield and clarity of fruit juices. In this study bacterial strains were isolated using serial dilutions of 10^{-7} of rotten oranges. Organisms were isolate and identified based on staining and biochemical tests. The pectinolytic activity was determined by using pectin containing minimal essential medium. The methodology applied was KB agar well diffusion method at the temperature is 35 ± 20^{0}C. Based on Gram staining, and biochemical tests, bacterial strains were isolated and identified strains showed different pectinolytic zones depending on the concentration of inoculum and the largest pectinolytic zones of 3cm was observed by PB4. These strains were efficient and have potential to be used commercially to increase the clarity and quality of fruit juices.

Keywords:Fruit Waste, Pectin, pectinase activity, polygalactutonic acid, Pectinolytic bacteria,

1. INTRODUCTION

1.1 Background of the study

Citrus fruits are the world's most abundant crop-producing over 115 million tons annually of which around 45-60% weight of fruits including peels and seeds are disposed of as a by-product having a negative impact on the environment (Azad ***et al.,*** 2014; Putnik ***et al.,*** 2017). The disposal of these biodegradable wastes causes serious environmental problems. Pectin is an essential hydrocolloid is a complex polysaccharide found in the higher plant's cell wall and middle lamella.

Pectin is the methyl-esterified polygalacturonic acid that consists of 300–1000 galacturonic acid units. It is used as a food additive in the food, pharmaceutical, and cosmetic industry due to its gelling, thickening, and stabilizing property (Thakur ***et al.,*** 1997*;* Quoc ***et al.,*** 2015; Guo ***et al****.,* 2017). The amount of carboxylic acid contained in total carboxylic acid units is named as the degree of esterification (DE) that influences the physical property of pectin .

The widely recognized raw materials for commercial pectin production are orange, sugar beet pulp, and apple pomace. Recently, researchers have pointed some new sources for pectin production such as banana, grapefruit rind, cacao pod husk, jackfruit, mango peel, melon rind, pumpkin, carrot, pomace, papaya and passion fruit (Pasandide ***et al.,*** 2017). Numerous techniques have been applied for the extraction of pectin such as conventional heating with acid, ultrasonic extraction, sub critical water, enzyme extraction, ultra-high pressure, and microwave-assisted extraction (MAE). The most common method of heating with acidified water is time-consuming and causes loss of energy (Putnik ***et al.,*** 2017).

1.2 Statement of the problem

The raise in production of fruit products, such as marmalade, low-caloric foods, juice, frozen foods, jellies and jams, has led to the generation of large volume of fruit wastes as a by-product. These agro-industrial wastes cause serious environmental pollution, and

hence, there is an urgent need for their recycling and appropriate utilization via extraction and production of biologically and chemically functional ingredients (i.e. pectin). The disposal of these biodegradable wastes causes serious environmental problems. Fruit wastes have gotten a reputation for one of the prime sources of municipal wastes, which have been an undeniably extreme enviromental issue. The conventional scouring process in textile industry involves the use of strong chemicals and these chemicals cause serious environmental problems.

1.3 Significance of the study

The significance of this study is isolation of pectinase producing bacterial strain for different advantages for industrial and agricultural purpose and also very important to generate base information for further study on economic importance of pectinase.

1.4 Objective of the study

1.4.1 General objective

- To Extract of pectin and isolates of pectinolytic bacteria from fruits and vegetable waste dump soil

1.4.2 Specific objectives

- To Extract of pectin from orange peel
- To Isolate of pectinolytic bacteria from fruits and vegetable waste dump soil
- To Characterize of pectinase producing bacterial isolates
- To Determine of pectinolytic activity of pectinase producing bacterial isolates

2. REVIEW OF LITERATURE

2.1 Pectin

Pectic substance is the generic name used for the compounds that are acted upon by the pectinolytic enzymes. They are high molecular weight, negatively charged, acidic, complex glycosidic macro-molecules (polysaccharides) that are present in the plant kingdom. They are present as the major components of middle lamella between the cells in the form of calcium pectate and magnesium pectate (Jayani ***et al.***, 2005).

2.2 Sources of pectin

Pectin is abundantly present in citrus fruits such as oranges, lemons and grapefruits. Fruits like apples, guavas, quince, plums and gooseberries contain high levels of pectin, while soft fruits like cherries, grapes and strawberries contain lower amounts . Pectic substances occur in the middle lamella and the primary cell wall of higher plants and prominent in parenchymatous tissue. These are abundantly present in apple, lemon, orange, mango, tomato, beet, and carrots (Lal ***et al***, 1998).

2.3 Pectinases

Enzymes hydrolysing pectic substances are called as pectinolytic enzymes or pectinases (Saad ***et al***., 2007). Food processing enzymes including pectinases account for 45% of enzyme usage. Pectinases are phytopathogenic substances (Yadav ***et al.,*** 2005). Microbially derived pectinases find more use due to their advantage over plant and animal derived pectinases. The reasons being cheap production, easier gene manipulations, faster product recovery, further microbial enzymes are usually free of harmful substances.

Pectinases are naturally produced by many organisms, including bacteria, fungi, yeasts, insects, nematodes, protozoa and plants. A quarter of the global food enzymes sale is met with microbial pectinases (Jayani ***et al.***, 2005).

2.4 Microbial Pectinases

Pectinolytic enzymes are naturally produced by many organisms like bacteria, fungi, yeasts, insects, nematodes, protozoan and plants. Microbial pectinases are important in the phytopathologic process, in plant-microbe symbiosis and in the decomposition of dead plant material, contributing to the natural carbon cycle. Pectinases are abundantly produced by saprophytic fungi, and decaying plant tissue represents the most common substrate for pectinase producing microorganism (Gummadi ***et al.***, 2003). Plant attack by pathogenic microorganisms. Usually starts by pectinolytic enzymes attack since pectic substances are more accessible than other fibers in plant tissue (Rombouts ***et al.***, 1980)

2.5 Classification of Pectinase

The group of enzymes which are involved in the degradation of ‘‘smooth region’’ (homogalacturonan) include deesterifying enzymes i.e. pectin methyl esterases (PME) and pectin acetyl esterase (PAE). Pectinases, the pectolytic enzymes include one esterase, six polygalacturonases and four Lyases, which removes methoxyl and acetyl residues of pectin producing polygalacturonic acid (Whittaker, 1991). The other subclass of homogalacturonan degrading group are broadly termed as depolymerases which break the α-1, 4-linkages either by hydrolysis i.e. polygalacturonases (PG) or via transelimination mechanism namely pectate lyases (PL)and pectin lyases (PNL). The main role of these enzymes (pectinases) is basically to degrade pectin. However, there are other accessory enzymes involved in the degradation of side chains of pectins which include α-arabinofuranosidase , β-galactosidase , endogalactanase and feruloyl and p-coumaroyl esterase (Jayani ***et al.***, 2005).

2.6 Applications

Over the years, pectinases have been used in several conventional industrial processes, such as plant fiber processing, tea, coffee, oil extraction, treatment of industrial waste containing pectinacious material etc. They have also been reported to work on purification of viruses (salazar and Jayasingle,1999) , in paper making (Reid and Richard, 2004),fruit juice manufacturing and clarity, paper and pulp industry, animal feed, Waste water tretment, Textile processing and biosecuring of cotton fiber, tea fermentation and improvment of chromaticity and stability of wine.

2.6.1 Textile Processing

Pectinase, in combination with other enzymes such as amylase, lipase, cellulase, and hemicellulase, has been used in the textile industry to remove sizing agents from cotton, substituting the usage of harsh chemicals(Rehman***,et al.,*** 2021). Different combinations of enzymes, such as cellulose with pectinase and protease, have been utilized for the bioscouring of cotton to achieve effective whiteness and absorbency of the textile fabric(Anand***, et al.,*** 2020). The use of enzymes such as pectinases in conjunction with amylases, lipases, cellulases and other hemcellulolytic enzymes to remove sizing agents has decreased the use of harsh chemicals in the textile industry, resulting in a lower discharge of waste chemicals to the environment, improving both the safety of working conditions for textile workers and the quality of the fabric (Hoondal***,et al.,*** 2002).

2.6.2 Fruits and Vegetable Processing

Pulp treatment, fruit juice extraction, and clarity are all factors in the use of microbial pectinases in the fruit and vegetable industry. Pectinases contribute to the reduction of viscosity, the clarity of juice, and the maceration of vegetables, as well as the reduction of fermentation time(Anand ***et al.,*** 2020).

2.6.3 Wine Processing

Pectinolytic enzymes' primary roles in the wine making process are to aid in extraction, maximize juice yield, facilitate filtration, and intensify flavor and color. The use of

pectinases in wine making accelerates maceration, enhances juice extraction yield, speeds up filtration, and improves flavor and color. This technique improves the wine's quality. The addition of pectic enzymes to the crushing of fruits during the wine making process enhances the volume of free flow juice and reduces pressing time. It also aids in the filtration and clarity of juice as well as also improves the chromaticity and stability of red wines (Lavanya ***et al.,*** 2021).

2.6.4 Coffee and Tea Fermentation

In the process of tea fermentation, instant tea powders have a great role to make drinking tea. This instant tea powder has a huge concentration of pectin because it is made from leaves. The preparation of tea by using this powder leads appearance of foam formation on tea due to the high concentration of pectin. Pectinase such as Polygalacturonase is used in the tea process to destroy the foam-forming property of instant tea powders by destroying pectins, increasing the quality of tea, color changes and highly valuable in the market . Pectinase is also used in the coffee fermentation process. The coffee bean has had covers that surrounded its internal structures these hardcovers of coffee beans are called mucilage. The mucilage also has viscous and gelatinous properties that are not comfortable to make drinkable coffee. During the process of coffee fermentation the alkaline pectinase is used to remove mucilage coat from the coffee bean before using the coffee bean. Coffee is fermented with pectinolytic microbes to remove the mucilage coat from the beans and to improve tea fermentation and froth-producing properties (Sharma***,et al.***, 2012).

2.6.5 Oil Extraction

Pectinase and other cell wall degrading enzymes have been widely explored for oil extraction from various sources such as flax seed, olives, dates,and so on . Citrus oils, such as lemon oil, can be extracted with pectinases because these enzymes disrupt

pectin’s emulsifying properties, which prevent oils from being removed from citrus peel extracts (Oumer***,et al.,*** 2017).

2.6.6 Paper and pulp industry

During paper making, pectinase can depolymerize and subsequently lower the cationic demand of pectin solutions and the filtrate from peroxide bleaching (Reid and Richard, 2004).

3 . METHODOLOGY

3.1.Description Of Study Area

The study was conducted at the Department of Biotechnology Molecular laboratory, Wolkite University. Wolkite is located 156 km away from south west direction Addis Ababa at latitude and longitude 80 37’N370,47/8.283N37.7835 E with an altitude between 1910 and 1935Meter above sea level.

3.2. Method Of Sample Collection

For the isolation of pectinase producing bacterial isolates partially decayed fruit and vegetable wastes from Wolkite University cafeteria were taken in sterile polythene bags. The samples were then stored at 4℃ until further processing.

Figure 1 Rotten fruit and Soil Samples

3.3 Method Of Substrate Preparation

Fresh orange peels were collected in plastic bags from juice houses located in Gubrei town and taken to the lab for pectin extraction. The peels were washed with tap water and sliced thinly to remove the white peel (*i.e.,albedo*). The cut pieces were then dried overnight at 50℃ in a hot air oven, and grinded into fine powder using mortar and pestle to pass through a 40-mesh sieve. After that, the powdered peel was packed in poly-ethylene bags and stored in a dry place until needed for the extraction processes.

Figure 2 Stage of substrate preparation. A. Orange peel, B. Cut pieces of a peel, C. Pieces of peel in hot air oven, D. Grinding of the dried peel, and E. Peel powder

3.4 Microwave-assisted Extraction of Pectin

Pectin extraction from powdered orange peels were performed with the help of microwave extractors, at working frequency input 50Hz and maximum power input 3300 W, as described by (Hosseini ***et al.,*** 2016) . An aqueous solution of citric acid was mixed with powdered peels at liquid-solid ratio (LSR) of 30 (v/w) with constant stirring until the pH reached 1.5. The solution was then placed over a rotating disc in the middle of the microwave oven, and extracted with a power of 660 W and 10 minutes of irradiation time.After the microwave treatment, the extract was cooled to room temperature, filtered through a nylon cloth. A double volume of ethanol (96%) (v/v) was added to the supernatant and allowed to sit for 2hrs (at 4℃) to allow precipitation of the extracted pectin (Leong ***et al.,*** 2016). To remove the monosaccharides and disaccharides, the coagulated pectin mass was washed three times with 96% ethanol. The extracted wet pectin was dried in the hot air oven at 40℃ to achieve constant weight. The dried pectin was packed in glass jar. Pectin yield (PY) was calculated according to(Li*et al*.2012): PY = $m/m_0 \times 100$; where m (g) is weight of dried pectin and m_0 (g) is peel powder weight.

Figure 3 Micro-wave assisted extraction of pectin. A. Dissolve the powder and boiled, B. Filtered via nylon cloth, C. Addition of ethanol, D. Filtered via filter paper, E. Oven dried, and F. Pectin powder

3.5 Isolation of Pectinolytic Bacteria

Pectin degrading bacteria were isolated from fruit and vegetable dump waste soil. One gram of soil sample was mixed in 100 ml of sterile distilled water and kept in shaker for one hour, which was then serially diluted to 7 dilutions. A volume of 0.1mL from 10^{-7} dilutions was inoculated in modified Minimum salt(MS) medium containing pectin (1.0%), ammonium chlosride (0.2%), di-sodium hydrogen phosphate (0.6%), sodium chloride (0.5%), potassium dihydrogen phosphate (0.3%), magnesium sulfate heptahydrate (0.01%), and agar (2.0%) adjusted at pH 7 and incubated at 37℃ for 48

hours. After incubation the isolates which are able to utilize pectin as a source of carbon develop colony on MS media and were selected as positive cultures. Isolates were further purified by sub-culturing on same medium under the same conditions and maintained in nutrient agar slants for further characterization (Gerhardt ***et al.,***1994).

3.6 Identification of Bacterial Isolates

Identification of the selected bacterial isolates were done on the basis of colony characteristics (size, shape and color of colonies), Gram staining and a series of biochemical tests (catalase, citrate, TSI (triple sugar iron), indole, MR-VP, motility and starch hydrolysis were employed) (Roy ***et al*** 2018).

3.7 Pectinase Activity Assay

The isolates were screened for pectinase activity by Kirby Bauer (KB) disc diffusion method on MS medium supplemented with 1% pectin as sole source of carbon. A total of 30µl of each isolate was aseptically poured in bored wells of MS plates. Plate were then incubated at 35°C (pH 7.0) for 48 hours (Karabi ***et al.,*** 2018).

After incubation, plates were flooded with iodine-potassium iodide solution (1.0 g iodine, 5.0 g potassium iodide, and 330 ml H_2O) and left undisturbed for 5-10 minutes. Then the clear zone diameter was measured in order to select isolates with highest pectinase activity. The largest clear zone of hydrolysis is assumed having the highest activity (Beg ***et al.***, 2000).

4. RESULTS AND DISCUSSIONS

4.1 Pectin Production

The present study successfully attempted to extract pectin from orange peels using microwave-assisted technology. The pectin yield obtained from orange peel was 20%(w/w) with a power of 660 W and irradiation time of 10 min. The findings showed a higher yield compared to the result obtained by (Zakaria ***et al.*** 2021) that obtained

2.08% yield from pineapple peel using microwave technology. The results obtained by (Mahmud **et al.** 2021) showed the highest amount of pectin (24%) from citrus fruit waste.

4.2 Isolation of Pectinase Producing Bacteria

Serial dilution, spread plating, and streaking methods were used to isolate potential pectinase producing bacteria from fruit and vegetable waste dump soil. A total of four morphologically distinct colonies, which were able to grow on MS media containing pectin as the only carbon source, were selected as potential pectin degrading bacteria. The four pure isolates were obtained after streaking on nutrient agar plates. To make it easier to distinguish between them, the pure isolates were labeled (i.e., PB1, PB2, PB3 and PB4) and preserved in nutrient agar slant for further phenotypic characterization.

Figure 4 Isolated pectinolytic bacterial strain

4.3 Pectinase Activity Assay

The pectinase activities of the selected isolates were evaluated using Kirby Bauer (KB) disc diffusion method by measuring the clear zone in MS plates. There was a clear hydrolysis zone among all four bacterial isolates, indicating the existence of pectinase activities. The pectinase producing bacterial isolates were classified based on (Soares ***et al***. 1999) as very good pectinase producer (clear zone ≥ 15 mm), good pectinase producer(clear zone ≥10 mm), weak pectinase producer (clear zone ≥ 5 mm) and poor pectinase (clear zone < 5 mm). Similar results of hydrolysis zone (20-25mm) were shown by previous studies (Varghese ***et al.,*** 2013; Hitha and Girija, 2014; Setegne Haile ***et al.,*** 2021).

Table 1 clear zone of the isolated bacteria

Isolates	PB1	PB2	PB3	PB4
Clear zone diameter (mm)	25	25	15	30

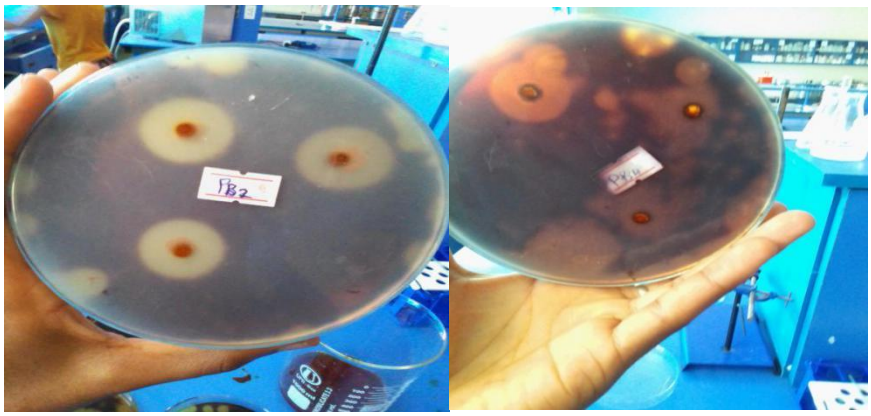

Figure 5 Assessment of pectinase activity of a bacterial isolate PB2 and PB4

4.4 Phenotypic Characterization of Bacterial Isolates

Microscopically, bacterial isolates PB1, PB3 and PB4 was found to be Gram-negative rods, while isolate PB2 was found to be Gram-positive cocci. Furthermore, isolates were subjected to different type of biochemical tests (Catalase test, MR-VP test, citrate utilization test, sugar fermentation test etc.) for identification. Based on the morphological and biochemical characteristics the isolates were tentatively described as *Pseudomonas* sp., and *Bacillus* sp. [Table 2] using Bergey's Manual of Determinative Bacteriology (Breed **et al.**,2020). Biologists have used a series of biochemical tests to distinguish closely related bacteria in the detection of bacteria; Bergey's Manual of Determinative Bacteriology were used to identify pectinolytic bacteria based on their morphological and biochemical characteristics.(Setegn Haile **et al.,** 2021) reported

pectinase producing *Bacillus sp* from avocado peel waste. Other investigations indicated that the highest amount of pectinase produced by *Enterobacter sp. (*Obafemi **et al.,** 2019), *Chryseobacterium* sp, *Bacillus*, *Pseudomonas* and *Micrococcus*(Karabi ***et al.***, 2018).

Table 2 Phenotypic characteristics of bacterial isolates

Characteristics	**Bacterial Strain**			
	PB1	**PB2**	**PB3**	**PB4**
Morphology: Colony and Cellular				
Color	Gray, opaque	Whitish, translucent	Grey. Opaque	Grey, opaque
Shape	Spherical	Spherical	Spherical	Spherical
Gram staining	Negative, rod	Positive, rod	Negative, rod	Negative, rod
Biochemical Tests				
Citrate	_	+	+	+

Catalase	_	_	_	_
Triple sugar Iron	+/+/+	+/+/+	+/+/+	+/+/+
Methyl Red	_	+	_	_
Voges-Proskauer	_S	+	+	**-**
Starch Hydrolysis	+	+	+	+
Indole	_	_	_	_
H_2S	_	_	_	_
Tentative ID	*Pseudomonas*	*Bacillus*	*Pseudomonas*	*Pseudomonas*

5 . CONCLUSIONS

Orange peel has the potential to become one of the sources for pectin production due to the high content of pectin, which is then can be used as food thickener, emulsifier, stabilizer and gelling agent in food industry. Thus, microwave assisted extraction of pectin from orange peel has shown high potential to accelerate the extraction process and produce higher yield .Pectin is an important hydrocolloid widely used in the food, pharmaceutical, and cosmetic industry. In the present study, a cost-effective and efficient method of microwave-assisted extraction (MAE) has been successfully applied using organic citric acid to extract pectin from orange peels.

Bacillus sp. And *Pseudomonas* sp. were isolated from the rotten fruit and were found as efficient pectinase producing bacterial strains that can be used for various industrial applications including extraction and clarification of fruit juices, processing of cotton fabric in textile industries, bleaching of paper, removal of pectic waste waters and maceration of tea leaves.

Among the various pectinase, bacterial extracellular pectinase are the most significant, compared with animals, plants, viruses and fungal extracellular pectinase. Extracellular pectinase produced by Bacillus and Pseudomonase species are of main interest from a biotechnology perspective, and are not only in scientific fields of protein chemistry and

proteins engineering but also in applied fields such as foods, pharmaceutical and paper industries. These pectinases account for 10% of the total worldwide production of enzyme. The genus Bacillus and Pseudomonase contains a number of industrially simportant species and approximately half of the present commercial production of bulk enzymes derives from the single class of enzymes which play an important part in the metabolism of almost all organisms.

6. RECOMMENDATION

Based on the result of this study, it is good to recommend the following recommendation

- Microwave assisted extraction of pectin from orange peel is better to extract pectin with high yieid and quality.
- Bacillus and Pseudomonas bacterial strains were good source for pectinase, so it is better used at industrial scale.
- This research should be conducted in depth in large scale for further identification.

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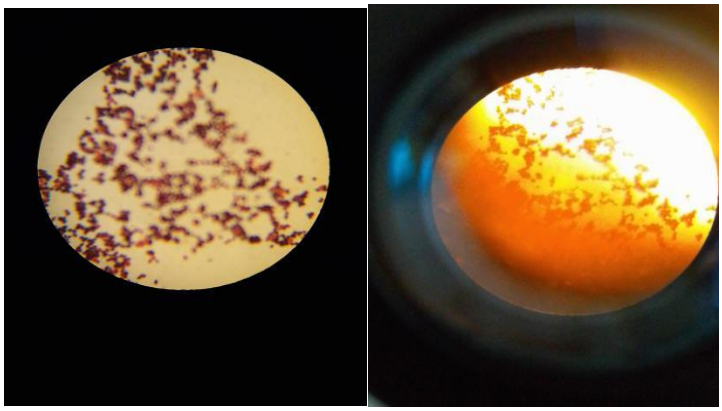
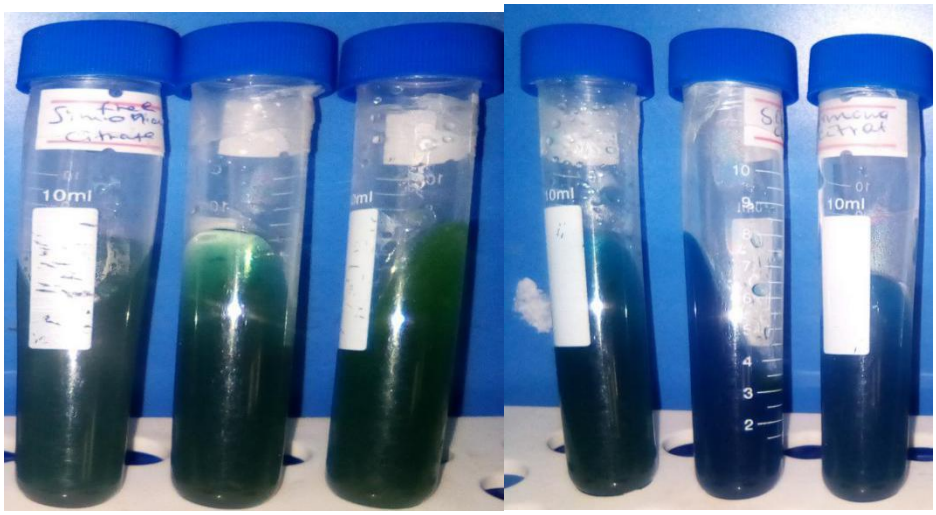
8.APPENDICES

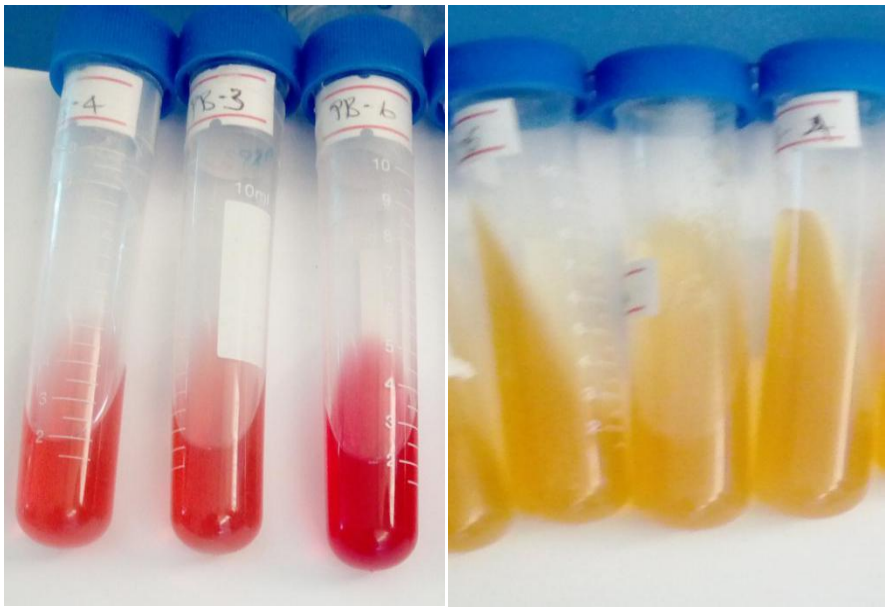


Appendices 1 shape and color under microscopy



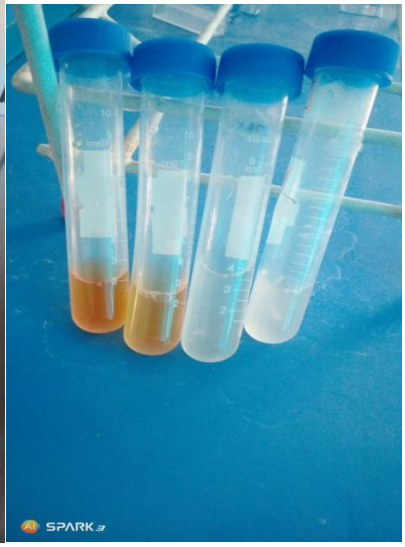
A.Before incubation B.After incubation

Appendices 2 Simon citrate test



A Before incubation B.After incubation

Appendices 3 Triple sugar agar test



A. Before incubation

B.After incubation and addition of KOH and alpha naphtol

Appendices 4 VP test