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GLOBAL DRUG DESIGN & DEVELOPMENT REVIEW

Isolation of Amylase Producing Bacteria From Soil of Peshawar Region and its Biochemical Characterization

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Abstract: *Amylases are a class of biocatalyst enzymes that may break down the amylase biomass that is produced by many different microorganisms, such as fungi and bacteria, and is found in the natural environment. The generation of amylase by an isolated amylase-producing microbe is the focus of the current study. Three soil samples from various locations in the Peshawar region were taken in order to isolate the strain that produces amylase. Using iodine solution and activity zone approaches, all isolated colonies were tested. Only three bacterial colonies out of 16 strains produced amylase in a favourable manner. Tests using morphological and biochemical analysis were then used to characterize these bacterial isolates, which were given the names MB7, MD2, and MD4.*

Key Words: Amylase, Grams Iodine, Amylase-degrading Species, Morphological

Introduction

A vital component of human nutrition, starch is also a prime storage product of many harvests with high commercial value, including potatoes, rice, cereals, maize and other crops(De-Souza and de Oliveira Magalhaes, 2010). With amylose and amylopectin as its two glucose subunits, starch is a complex polysaccharide. Glycosidic linkages α-1,4-glucose join the two subunits of glucose. Starch is converted to simple sugars by a number of important enzyme families, chiefly amylases (Gopinath et al., 2017) Among the most significant industrial enzymes are amylases. Enzymes known as degrading enzymes hydrolyze internal α-1,4-glycosidic connections in starch to break it down into smaller components like glucose, maltose, and maltotriose units(Hasan et al., 2017).

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Exoamylases and endoamylases are the two broad categories into which amylases fall. While endoamylases haphazardly carry out hydrolysis in the interior of starch to produce linear and branched oligosaccharides, exoamylases work on the substrate's non-reducing edges to produce short-end products. β-amylase is an example of an exoamylase, whereas α-amylase is an example of an endoamylase (Pathania *et al*., 2016). These two groups comprise the majority of amylases. Humans and other mammals contain large amounts of α-amylase (Bholay *et al*., 2018).

Amylases are found in a variety of species, including microbes, plants, and animals. Due to benefits including its quick turnaround time, low production space requirements, and affordable mass production capacity, the enzyme derived from microbes is the best substitute source (Alariya *et al.,* 2013; Abel-Nabey and Farag 2016). Nowadays, extracellular enzymatic activity in a variety of microbes is studied using microorganisms as biological sources of commercially significant enzymes (Pathak *et al.,* 2014).

According to Xie *et al.* (2014), amylases have the potential to be used in a wide range of industrial sectors, including the food, fermentation, detergent, leather, textile, paper, pharmaceutical, and sugar industries. The use of α-amylase has expanded to numerous additional domains, including therapeutic, medicinal, and analytical procedures, as a result of recent developments in biotechnological disciplines (Karnwal, 2011). The need for innovative amylases is gradually increasing on a global scale due to the potential applications of amylase in various industrial sectors. The conventional method involves isolating bacteria that generate unique enzymes from different environments and outcompete the existing products. The assessment of these microorganisms' potential economic significance depends critically on their subsequent analysis for the purpose of optimising production parameters (Pathak and Narula, 2013).

In 1894, fungal amylase was the first enzyme from a microbial source to be commercially produced. It is used as a therapy to treat digestive disorders (Singh *et al.,* 2016). About 65% of the global enzyme market is made up of amylase, which is produced in greater quantities than any other enzyme (Abd-Elhalem *et al*., 2015). Microbial amylases have replaced the chemical hydrolysis of starch in the starch hydrolysing industries due to a number of benefits, including uniformity of the produced yields, reaction specificity, reduced energy consumption, and the elimination of neutralisation phases (Pokhrel *et al*., 2013).

There have been several microbes identified as producing amylase; the most often used ones for industrial use are from the genus Bacillus. Due to their diverse nature and exceptional environmental adaptability, Bacillus species exhibit considerable versatility. Their metabolic makeup and the output of enzymes are influenced by a variety of factors (Bozic *et al*., 2011). It is ubiquitous in nature, dependent on the right nutrients for growth, and produces the greatest amount of alpha-amylase (Kalyani and Rajesh, 2018). Large-scale industrial synthesis of extracellular enzymes for a variety of applications is facilitated by the use of Bacillus subtilis, B. stearothermophilus, B. amyloliquefaciens, and B. licheniformis (Singh *et al*., 2015).

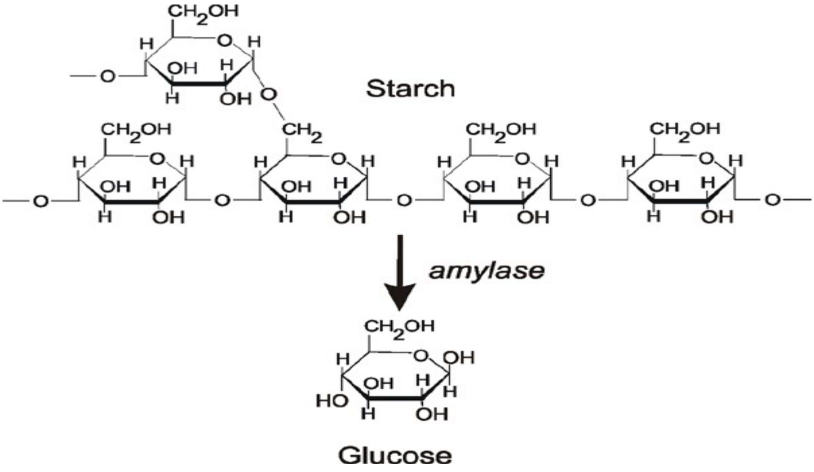
The current study looked at the separation, characterization, and identification of amylolytic bacteria from soil. To get the highest yield, other variables affecting amylase production, such as pH, temperature, sources of carbon and nitrogen, and incubation time, were assessed.

Amylase

The enzymes known as amylases are responsible for breaking down starch, which is a reserve of carbohydrates found in plants, and glycogen, which is a reserve of carbohydrates found in animals, to produce products like maltose, dextrin, and eventually glucose. According to Subedi, B. *et al.* (2023), these are typical in microbial communities, plants, and mammals. As glycoside hydrolases, all amylases are capable of breaking down the α-1,4-glycoside bonds present in a polysaccharide made of starch.

Figure 1.3

Starch Degradation with α-Amylase (Cloete, et al., 2019)



History of Amylase

The first starch-degrading enzyme in wheat was found by Kirchhoff in 1811, which opened the door for the discovery and study of amylase (Tiwari, S. P. *et al.,* 2015). This was preceded by other reports of malt and digestive amylases. Much later, in 1930, Ohlsson proposed classifying the starch-digesting enzymes in malt as α and β amylases, based on the anomeric form of sugars that the enzyme provided (Oluwadamilare *et al.*, 2019).

Types of Amylase

With regard to types, there are two classes of amylases: endo-hydrolase and exo-hydrolase.

Endo-Hydrolase

Endo-hydrolases function within the molecule that they are acting on. α-amylase (E.C.3.2.1.1) is an example of an endo-hydrolase, as it catalyses the hydrolysis of the α-1, 4-glycosidic bonds in starch, resulting in the production of glucose and maltose. α-amylase is also known as a metalloenzyme since it is dependent on the presence of metal ions, such as calcium, for both stability and activity (Sundarram & Murthy, 2014). α-amylase acts faster than β-amylase because it can operate anywhere on the substrate. Its optimal pH range is 6.7–7.0, and it is a key digestive enzyme in mammals (Oluwadamilare *et al.*, 2019). α-amylases are present in many kinds of tissues and organs (Rani *et al.*, 2015).

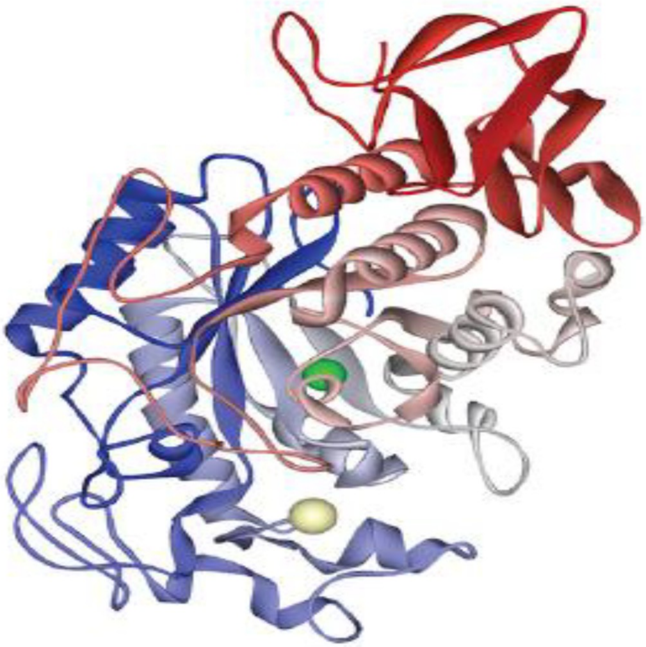
Exo-Hydrolase

Exo-hydrolases function on the substrate's non-reducing edges. β-Amylase (EC 3.2.1.2) is an example of an exo-hydrolase that functions by hydrolyzing α-1, 4-glucan complexes from the nonreducing end of the polysaccharide chain, resulting in the primary end product maltose. During fruit maturation, β-Amylase converts starch to maltose, which gives ripe fruit its sweet flavour. The ideal pH range for β-amylase is 4.0–5.0. Cereals, higher plant seeds, and microorganisms are the main sources of β-Amylase (Rani *et al.,* 2015).

Structure of Amylase

Figure 1.4

Three-dimensional Structure of α-amylase (Gopinath et al., 2017)



An alpha-amylase ribbon diagram with beta-sheets and alpha-helices is depicted in Figure 1.4. The enzyme is made up of 170 water molecules, 496 amino acids, one chloride ion, and one calcium ion. The amino acids aspargine, arginine, aspartate, histidine, and three molecules of water are linked to the calcium ion that is necessary for its function. Furthermore, an arginine, an aspargine and arginine, and a water molecule are all connected to the chloride ion (Sahoo & Roy, 2023)

Starch

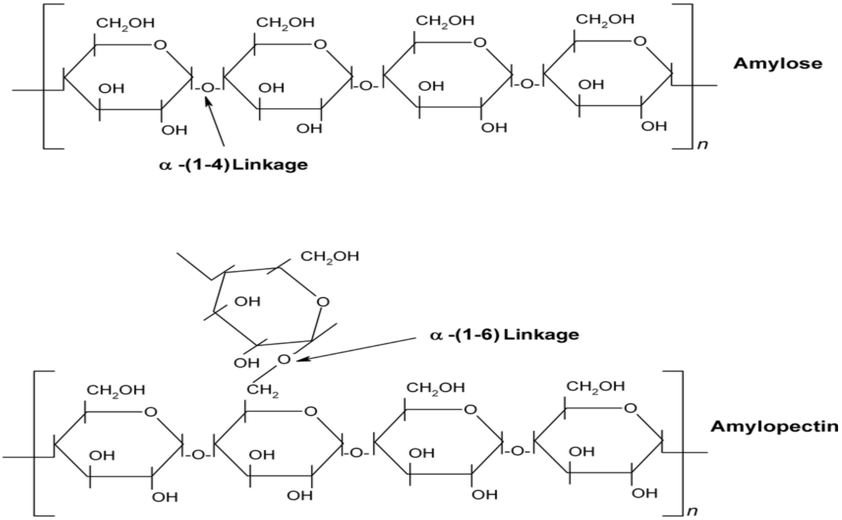
All higher plants use starch, a glucose polymer joined by a glycosidic link through the C1 oxygen, as a significant and plentiful source of food reserves and energy. Plants use photosynthesis to create it as a long-term storage molecule. The primary substrate that the enzyme amylase breaks down the glycosidic bonds that are incorporated into its polymer is this one. According to Pathania *et al.* (2017), it can be found in seeds, fruits, leaves, bulbs, and tubers. Crops based on starch make up a large portion of the human diet. The main industrial sources of starch include tapioca, corn, potatoes, and wheat (El-Fallal *et al.*, 2012), (Fang *et al.*, 2019)

Structure of Starch

A starch molecule is formed by two distinctive glucose polymers, amylose and amylopectin, which involve glucose molecules that are joined by glycoside linkages. The two polymers are featured with different structures and characteristics. A linear chain of amylose polymer has up to 6000 units of glucose bound by α-1-4 glycoside bonds (Gopinath *et al.*, 2027). The relative amylose and amylopectin content varies depending on the starch origin. In most common starches, the average amylose content is 20-30 percent, e.g. in maize, barley and potato (El-Fallal *et al.,* 2012). Amylopectin on the other hand accounts for 75-80% of the starch and is characterized by branched glucose unit chains. The glucose units in the short linear chain of the polymer are linked by α-1, 4-glycosidic linkage while branching takes place every 15-45 glucose units with α-1, 6 glycosidic bonds (Sundarram & Murthy, 2014). In the polymer's short linear chain, α-1, 4-glycosidic linkage connects the glucose units, and the branching occurs every 15–45 glucose units via α-1, 6-glycosidic bonds (Sundarram & Murthy, 2014).

Figure 1.4

Structure of Amylose (left) and Amylopectin (right) (El-Fallal et al., 2012)



Microbial Amylase

It has now been established that microorganisms are a valuable source of enzymes. Scientific and industrial sectors have focused mostly on amylases originating from microorganisms. Microorganisms vary in the amount of amylase they produce, even within the same species. Additionally, the synthesis of amylase varies based on the origin of the microbe; strains isolated from starchy environments produce larger levels of the enzyme. It is now possible to improve microbial strains for optimum enzyme yield thanks to sophisticated genetic engineering techniques (Gopinath *et al.,* 2017).

Surprisingly, fungus-derived amylase was the first microbial enzyme to be produced commercially in 1894. It is used to treat digestive disorders (Singh *et al.*, 2016). Many living things, including humans, plants, and microbes like bacteria, fungi, and yeast, produce the amylases that break down starch. The genus Bacillus contains the majority of the microorganisms used in industrial applications, including bacteria. Due to their diverse nature and exceptional environmental adaptability, Bacillus species exhibit considerable versatility. Their metabolic makeup and the output of enzymes are influenced by a variety of factors (Bozic *et al.*, 2011).

It is ubiquitous in nature, dependent on the right nutrients for growth, and produces the greatest amount of alpha-amylase (Kalyani and Rajesh, 2018). Large-scale industrial synthesis of extracellular enzymes for a variety of applications is facilitated by the use of Bacillus subtilis, B. stearothermophilus, B. amyloliquefaciens, and B. licheniformis (Singh *et al.,* 2015).

Materials and Methods

Sample Collection

The soil sample was collected from different places in Peshawar. The collected soil sample was characterized by their texture, moisture contents and pH. For the collection of soil samples, the top layer of soil was removed followed by sample collection from a depth of approximately 10cm. It was stored in pre-sterilized plastic bags followed by appropriate tags. The samples were further processed in the laboratory of IBS, SUIT.

Preparation and Analysis of Sample

1 gram of each sample was aseptically introduced into 9 ml of sterile distilled water and was serially diluted. Then the spread plate technique was used to transfer bacteria on nutrient agar media and underwent 24 24-hour incubation period at 37°C. The plates showing distinct colonies of bacteria were further sub-cultured(Pokhrel et al., 2013).

Screening and Selection of Potent Amylase-Producing Bacteria Using Starch Hydrolysis Test

Using a sterile wire loop, bacterial colonies were sporadically streaked on newly produced starch agar plates in order to screen for amylolytic strains. After that, the plates were incubated for 24 hours at 37 C. Gram's iodine solution was used for staining in order to detect amylolytic bacteria. The presence of amylase producers was directed by the hydrolysis zone surrounding the colonies. For additional study, isolates with the biggest zone of hydrolysis were chosen (Alariya *et al.*, 2013).

Preservation Of Organism

To preserve the purified organisms, they will be grown in nutrient broth media for a full day before being placed in eppendorf tubes and kept at -20 C with 50% glycerol stock.

Microscopic, Morphological and Biochemical Characterization

The isolates were characterised morphologically and biochemically in addition. The size, form, colour, texture, margin, elevation, surface, and diameter of the morphological features were compared. Gramme staining, catalase, coagulase, indole, triple sugar iron, citrate utilisation, urease, oxidase, Mackonkey, and blood agar tests are among the biochemical identification methods (Singh *et al.*, 2016); (Bergey *et al.*, 1974).

Gram Staining

Refined plates that were 24 hours old were used to get precise results. A little bacterial colony was passed through a sterilised wire loop to create a bacterial stain on a sterile glass slide. It was allowed to air dry and heated by rapidly passing the slide over a Bunsen burner flame. The slide was covered with a crystal violet stain and left for roughly 60 seconds after the culture was heat-fixed. After that, distilled water was used to thoroughly rinse the smear. After rinsing the smear with distilled water once again, it was coated for a minute with Gram's iodine solution. After that, it was decoloured with 95% ethanol by rinsing the slide for about ten seconds and then giving it a quick rinse with distilled water.

Lastly, the smear was cleaned with distilled water after being dyed with safranin for 45 seconds. After completion, the slides were inspected under a 1000X magnification microscope.

Mannitol Salt Agar Test

MSA medium plates were produced under sterile circumstances, and a single colony of the test organism was removed from a fresh culture using a sterilised wire loop. The plates were then injected using the streak plate procedure. The plates were incubated for 24 to 48 hours at 37 C, and then they were inspected (Sharp and Searcy, 2006)

Blood Agar Test

The necessary quantity of nutrient agar powder was first dissolved in distilled water to create a basal medium, from which blood agar media was created. After the mixture was heated to 121 C for

15 minutes while being constantly stirred to dissolve all of the ingredients, it was autoclaved. 5% (vol/vol) of sterile defibrinated sheep blood that had been warmed to room temperature was added to the media after it had cooled to 45–50 C following autoclaving. The solution was gently mixed, then put onto petri dishes and allowed to harden. Plates were streaked with a 24-hour-old pure culture using an inoculating loop in a sterile environment. For twenty-four hours, the culture plates were kept in an incubator at 37 C. Following incubation, the hemolytic phenomena were subsequently noticed.

Catalase Test

The name of the bacterial isolate was written on a dry, clean glass slide. Using a sterile wire loop, a tiny quantity of bacterial colony from an 18–24 hour-old culture was transferred to the other side. Using a dropper, a few drops of 3% hydrogen peroxide (H2O2) were added to the smear. The quick evolution of oxygen into bubbles in less than ten seconds is a sign of a successful outcome.

Coagulase Test

The name of the bacterial isolate was written on a dry, clean glass slide. Using a sterile wire loop, a tiny quantity of bacterial colony from an 18–24 hour-old culture was transferred to the other side. Using a dropper, a few drops of 3% hydrogen peroxide (H2O2) were added to the smear. The quick evolution of oxygen into bubbles in less than ten seconds is a sign of a successful outcome.

Oxidase Test

Isolate from pure culture was used for the test. A newly prepared 1% reagent solution was immersed in a sterile strip of filter paper. Next, new test organism growth was picked up and applied to the damp area using a wooden loop or stick. In less than ten seconds, the tint changed from colourless to a rich purple or blue.

Indole Test

The indole test evaluates a microbe's capacity to convert tryptophan to indole. After being ready, the tryptophan broth medium was autoclaved for 15 minutes at 121. After adding a fresh 24-hour culture to the broth, it was incubated for another 24-hour period at 37 C. Three to four drops of Kovac's reagent were added to confirm indol synthesis. While a yellow or amber-coloured ring indicates negative results, a cherry red ring that appears at the top of the medium indicates a positive indole test.

Citrate Test

Test tubes containing citrate agar slants were made and autoclaved for 15 minutes at 121C. Following sterility testing, test organisms were inoculated onto citrate agar slants using a loop full of new culture, and the inoculation was allowed to sit at 37C for 24 hours. Positive outcomes were shown by the slants' hue changing from green to blue.

Results

Isolation and Selection of Amylase-Producing Bacteria among Isolates

In nutrient agar plates, 16 bacterial colonies were chosen by the colony counting technique after serial dilution. Using a starch hydrolysis assay on a starch agar plate, the amylase production of each isolate was examined. Just three isolates out of the total demonstrated the best amylase hydrolysis, and these were chosen for additional secondary screening. By creating a distinct zone of hydrolysis, the confirmatory Congo red test demonstrated that the strains were capable of producing enzymes and hydrolyzing substrates. The three isolates were given the names MB7, MD2, and MD4.

Screening of Strain Mb7, Md2 and Md4 for Amylase Production

The colony morphology and characteristics of three isolates were discerned from nutrient agar plates after streaking. The growth of the three isolates is shown in Figure 8.1.

Table 4.1

Morphological characteristics of MB7, MD2 AND MD4

S. No	Isolate	Colour	Colony size	Texture	Form	Elevation
1	MB7	Off White	Moderate	Smooth	Round	Flat
2	MD2	Yellowish	Moderate	Dry and rough	Oval + Round	Flat
3	MD4	Off White	Moderate	Smooth	Round	Flat

Table 4.2

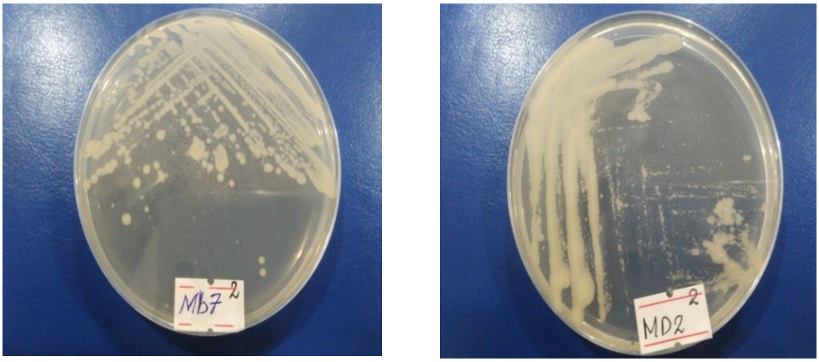
Biochemical Characteristics of the Bacterial Isolates

Sample ID	Citrate test	Eosin methylene blue	Mackonkey	MSA media	Blood agar	Coagulase test	Oxidase test	Urease test	Catalase test	Indole test	Identified Genus
MB 7	Weak positive	Negative	Pink growth	Negative	Greenish growth	Negative	Negative	Negative (yellowish colour)	Positive	Negative	Bacillus
MD 2	Weak positive	Purple growth	Pink growth	Yellow growth	Greenish Growth	Negative	Negative	Medium positive	Positive	Negative	Bacillus
MD 4	Weak positive	Negative	Negative	Yellow growth	Greenish growth	Positive	Negative	Slow positive	Positive	Negative	Bacillus

Appendices

Figure 8.1

Bacterial Growth of MB7, MD2 and MD4 on Nutrient Agar



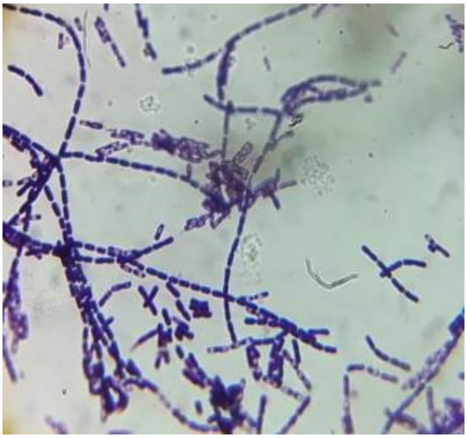
MB7

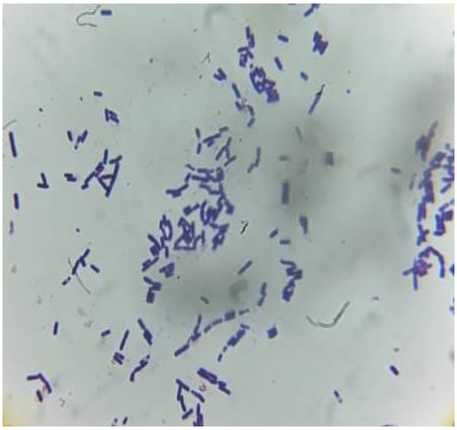
MD2

MD4

Figure 8.2

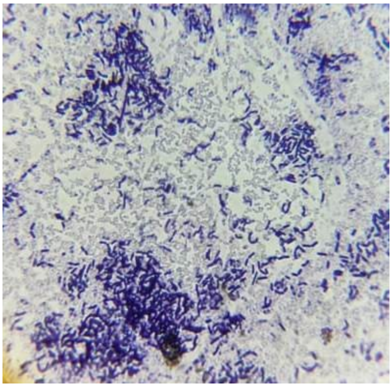
Gram Staining of MB7, MD2 and MD3





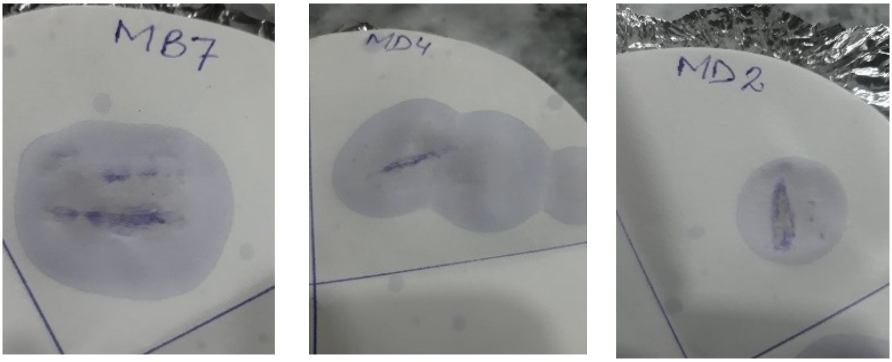
MB7

MD2

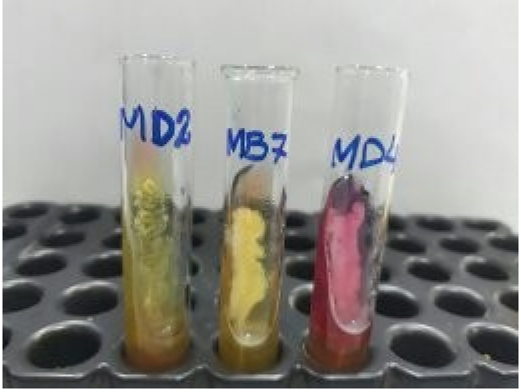


MD4

Figure 8.3

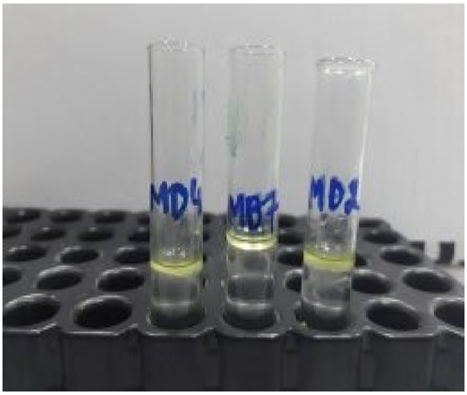


Oxidase test

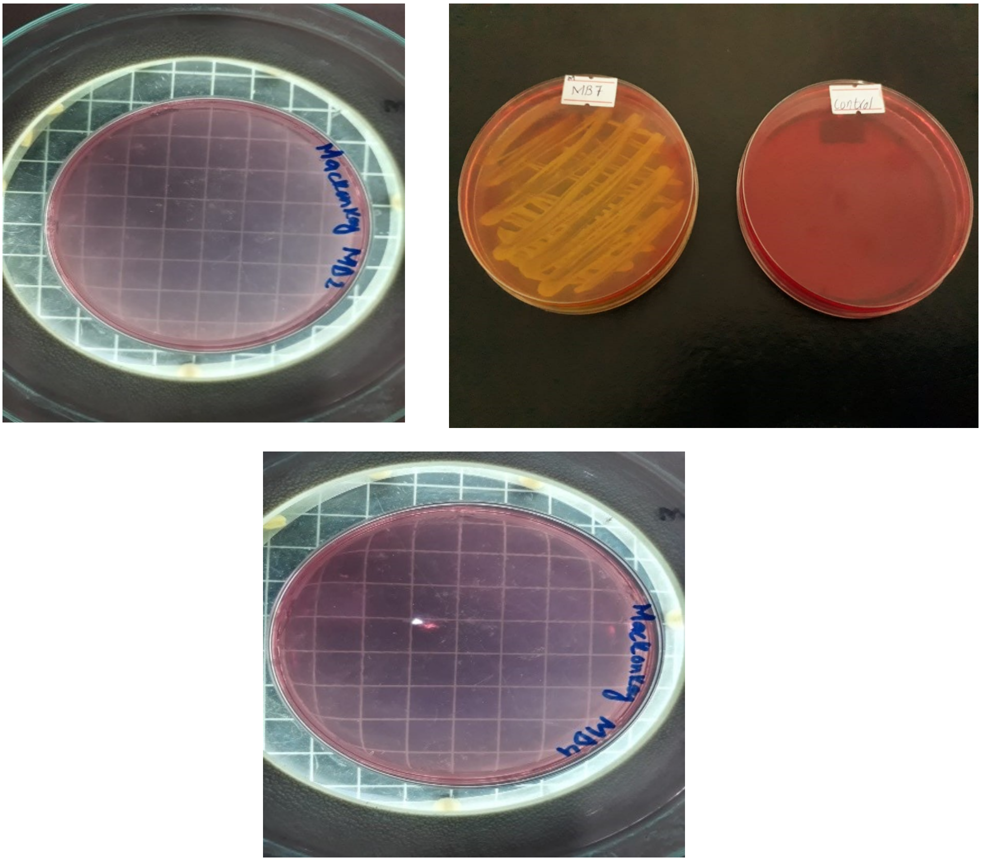


MSA media

Citrate test



Indole test



Mackonkey agar test

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