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Isolation and Antimicrobial Screening of Actinomycetes spp. From Processed Mango Waste

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A B S T R A C T

Actinomycetes strains were isolated from mango Mangifera indica L. compost and yard waste. Actinomycetes strains were propagated in Bennett medium and later tested for antimicrobial and antifungal activity using the agar plug method. Strain A and strain C were isolated using the swirling method, and strain B was isolated by the spread plate method. Three of the compounds showed antagonistic activity against Gram-positive and Gram-negative bacteria and fungi. The colour of the substrate mycelium and aerial spore mass was varied. The three strains also showed positive results in biochemical tests such as protein hydrolysis, starch hydrolysis, catalase test, and negative results in nitrate reduction. The strains also showed a positive reaction in glucose fermentation in the triple sugar iron medium. Further purification and extraction of active substances are recommended, including the use of electron microscopy and molecular tools for strain characterization and identification.

Keywords: ***Actinomycetes, anti-microbial, Mangifera indica L.***

Introduction

Identification of drug-resistant microbial pathogens responsible for increasing the demand for efficient antibiotics, antioxidants, anti-tumor, and other natural bioactive compounds is one of the problem areas inhibiting the development of therapeutic agents. In search of new bioactive compounds, the continuation of the traditional approach of screening large numbers of microbial cultures from nature is being followed.

Actinomycetes are ubiquitous; they are a heterogeneous group of filamentous Gram-positive bacteria that are well known for their capability to produce a huge array of chemically diverse and medically important secondary metabolites (Shatoury *et al.*,2006). The majority of Actinomycetes will grow on the standard bacteriological media of the laboratory, such as nutrient agar, trypticase soy agar, blood agar, and even brain heart infusion agar. Actinomycetes used as a biocontrol agent have been reported to play a defense mechanism in protecting the plant host against disease as well as exert effects on the growth and physiology of plants from their metabolic products (Sun Og *et al*.,2008). Actinomycetes are both in terms of quantities as producers of useful antibiotics and enzymes known to be predominant soil population growth (Rivera et al.,2005). Actinomycetes produce best-known products being antibiotics. The morphology of an actinomycete on agar can be a good and rapid indicator of its identity, but examination of isolated colonies can provide little useful information (Brun *et al*.,2000). Various accounts of Actinomycetes that produce antibiotic, from marine, brackish and terrestrial sediments have been reported. It is seen that the terrestrial site is the richest source of antibiotic producing actinomycetes (Parungao *et al.*, 2007). Actinomycetes that live within the host plant tissue are known as endophytic actinobacteria. Actinobacteria are rich sources of novel natural products, which can be utilized for their application in medicine, agriculture and industry. Actinobacteria of the genus streptomyces are especially good quality antibiotic producers. Evidence has accumulated rare Streptomyces species, might is an unusual source of new biologically active compounds. Endophytic actinobacteria were increasingly important as some strains of actinomycetes can synthesize bioactive metabolites that suppress some of the pathogenic fungi and bacteria (Wanbajob,2008). Actinomycetes can degrade complicated molecules or oxidize organic material such as cellulose,lignin and chitin. Actinomycetes are everywhere in nature and can be isolated from soil, water, and on decaying organic matter. Research for new antibiotics has made scientists look for varied habitats for potential Actinomycetes isolates exhibiting antimicrobial activity.

Composting is a microbial process that involves a sequence of mixed microbial population decomposing heterogenous organic material. Composting is one such process which relies heavily on such rich actinomycetes activity (Mc Carthy,1990). Its shielding role of compost microorganism to crops is as significant as they compete with plant pathogens. Compost improves the aeration and drainage of clay soil. Compost can be seen as a divider that "shoulders apart" tightly packed clay particles so water and air can enter. Compost enables sandy soil to hold water and nutrients. Compost absorbs moisture "like a sponge" and releases nutrients slowly.it also encourages earthworm and other natural soil organisms to act, which create a growth-promoting environment for plants. Composting is one way of reducing the size of organic waste and putting them back into the earth to feed crops. Composting can be applied to all types of yard trash such as leaves, flower and vegetable plant matter, straw and small amounts of woody pruning's, grass clippings and weeds. The resins don't allow the material to degrade and extend the amount of time that it will take to compost versus other materials

of plant. Every yard produces waste in the form of pruning, lawn mowing, and other common plant maintenance routines. Yard waste refers to waste from plants during the maintenance and upkeep of gardens, lawns, areas, and landscape. As far as antimicrobial potential of actinomycetes is concerned, researches have been conducted that have demonstrated the successful isolation of actinomycetes from mango waste and can be utilized to waste product. For instance, Signh *et al*.., (2019), mentioned the proof of isolating a strain which was found to exhibit considerable antibacterial activity against pathogens including *Escherchia coli* and *Staphylococcus aureus*. Kumar *et al*., 2021 quotes that waste isolates of actinomycetes demonstrated antifungal activity against *Candida albicans, Aspergillus niger* and established that they can be a natural source of antifungal compound.

Mangoes (*Mangifera indica L.*) are among the most widespread trees cultivated in various regions of the country. A combined system employing mango waste was composed of anaerobic digestion for biogas production and utilized as a digested waste. Mango peels are utilized for the manufacturing of dietary fiber with high antioxidant activity (Borup,1992). Most of the research work in mango peel exploitation is focused on the origin of pectin that has been regarded as a high-grade dietary fiber. Masibo,2008 reveals that bioactive compounds from the mango pulp, peels seed, leaves, flowers and stem bark are rich sources of antimicrobial agent used for the treatment of infectious diseases caused by pathogenic bacteria. Processed mango waste, a byproduct of the mango industry, presents a new and as-yet-untapped niche for isolating actinomycetes. Mango waste contains an abundance of organic matter and nutrients, providing a good substrate for the growth of these microorganisms. Utilization of agricultural waste not only alleviates environmental concerns but also presents a sustainable approach towards the discovery of new antimicrobial agents.

This study aims to isolate Actinomycetes spp. from processed mango waste and determine their antimicrobial activity against certain pathogenic microorganisms. With this new source, we aim to find new strains of actinomycetes that could be used for the development of new antibiotics.

Objectives of the Study

The study generally aimed to determine the possible microbial community in the processed mango waste, a valuable source for abundant actinomycetes using different culture media. The specific objectives of the study are:

- To characterize and identify the isolated Actinomycetes spp. by morphological and biochemical.
- To evaluate the antimicrobial property of the isolated Actinomycetes spp. against several pathogenic microorganisms including bacteria and fungi.
- To contribute to environmental sustainability by the use of processed mango waste as a valuable input for biotechnological R&D.

Materials and Method

Sample collection

Samples of processed mango were obtained from the Industrial Technology Development Institute-Department of Science and Technology Samples were placed in sterile plastic container, taken to the laboratory and subjected to isolation procedure within 96 hours.

Selective isolation procedure and media

The actinomycetes were tested for their viability and propagated in different selective media prior to the antimicrobial and enzymatic tests. (*Qin et al.,2009 & El-Shatoury et al.,2006*)

Method 1. Samples were directly diluted at 10^{-6} with sterile water and spread plated onto the medium. The Petri dishes were incubated at room temperature for 2 weeks. The isolated actinomycetes were propagated in Bennett medium before the antimicrobial and enzymatic tests.

Method 2. Some samples (1.0 g) were diluted 10^{-6} with sterile water, and 100 microliters of diluted samples were carefully incorporated onto the medium by swirling the media. The petri dishes were maintained at room temperature for 2 weeks. The isolated actinomycetes were propagated in Bennet medium before the antimicrobial and enzymatic tests.

Screening of Actinomycetes isolates

Preparation of plates for antibacterial and antifungal activity

Antimicrobial activities of the actinomycete isolate were tested against pathogenic bacteria. *Bacillus subtillis, Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris, Staphylococcus aureus, Candida albicans, Aspergillus niger*, and *Penicillium sp.* by agar plug method. Actinomycetes were streaked on Bennett medium and incubated for 7 days. A loopful of bacteria was inoculated into 5 ml of nutrient broth: one ml was swirled in the nutrient agar, plated, cooled, and allowed to solidify.

Antibacterial and Antifungal Assay

Six mm. agar disc were prepared using sterile cork borer from well-grown culture of *Actinomycetes sp.* and placed on fresh lawn culture of test organisms: *Bacillus subtillis, Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris, Staphylococcus aureus, Candida albicans, Aspergillus niger*, and

Penicillium sp. The filter paper disc (6 cm) was dipped in the streptomycin sulphate for the control of bacterial assay and the clotrimazole for fungal assay, respectively.

Interpretation of Inhibition Zones

The plates were inverted when measurements of the zone of inhibition were done. The diameter of zone of inhibition was measured in the nearest tenth of mm with a ruler. For the purpose of standardization, the following interpretative range of standard zones was adapted.

	Diameter of Zones of Inhibition (mm)
Resistant	10 or less
intermediate	11-15
Susceptible	16 or more

Source: https://www.accessexcellence.org/AE/AEC/CC/chance activity

Characterization of Actinomycetes

The potent actinomycetes selected from screening were characterized morphologically and biochemically.

Classification of actinomycetes

Isolated strains were transferred onto Bennett agar medium, pH 7.2, and incubated for 14 days. The color of aerial mycelium (on the surface of agar), substrate mycelium (underside of plate), and diffusible pigment were observed.

Biochemical method

Different biochemical tests were performed for the identification of the potent actinomycetes.

Protein hydrolysis

Emerson agar with 10% skim milk was inoculated with 7-day-old agar plugs of isolates and was incubated for 24 to 48 hours. The zone of hydrolysis was measured as a diameter in millimeters (mm). The tests have three replicates per trial.

Starch hydrolysis

Emerson agar with 1% soluble starch was cooled to 45 ºC and allowed to solidify before an old agar isolate was inoculated. Incubation was for 24 to 48 hours. Few drops of iodine were used to visualize the zone of hydrolysis around the organism. The zone of hydrolysis was measured as a diameter in millimeters (mm). The test was triplicated per trial.

Catalase test

The 7-day-old agar plugs of local isolates were propagated in tubes. A few drops of hydrogen peroxide were added. Observation of the appearance of oil bubbles or foaming indicates a positive reaction in catalase production and non-bubble means a negative reaction. The test was duplicated per trial.

Nitrate reduction

The 7-day-old agar plugs of isolates were inoculated in a nitrate broth consisting of beef extract (3.0 g/L), peptone (4.0 g/L), potassium nitrate (1.0 g/L), and incubated for 5 days at room temperature. Two reagents were used: 1 ml of sulfanilic acid for reagent A and 1 ml of dimethyl-alpha-naphthylamine for reagent B as an indicator for reduction. The production of a red color occurs in the presence of nitrite and indicates the ability of the organisms to reduce nitrate to nitrite. The positive result does not change its color, and the negative one turns red. Zinc dust was used for the isolates that showed negative results.

Triple sugar iron

Isolates were inoculated into triple sugar iron media by stabbing the butt and streaking the slant and was incubated at 37 ºC for 24 to 72 hours. The yellow butt color and red slant color means glucose only fermented. Both yellow color in butt and slant means glucose, also lactose and or sucrose fermented. Both red color in butt and slant means no action in glucose, lactose or sucrose. H_2O production is an indication of the presence of black precipitates

Results and Discussion

Actinomycetes strains were isolated from mango compost and yard wastes. Strains A and strain C were isolated using a swirling method, and strain B was isolated by the spread plate method. All isolates grew on a range of agar media, showing morphology typical of streptomycetes (William *et al.*, 1989). The color of the substrate mycelium and aerial spore mass was varied. Isolates produced diffusible pigments on dextrose-casein peptone agar media.

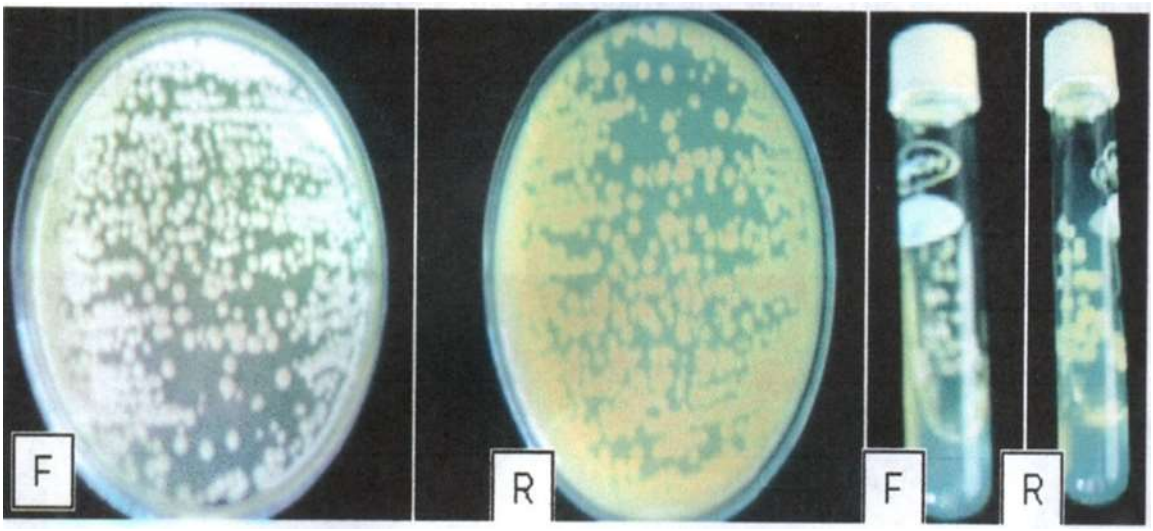

Figure 1. Microscopic appearance of isolate A propagated for 7 days in Bennet medium in plates and in slants (F- front, R- reverse side)

The spore surface of strain A is hairy and warty. Sparse aerial mycelium when produced is white. The reverse is pale yellow to yellowish brown. It must probably belong to Streptomyces with series. (Goodfellow, 1989)

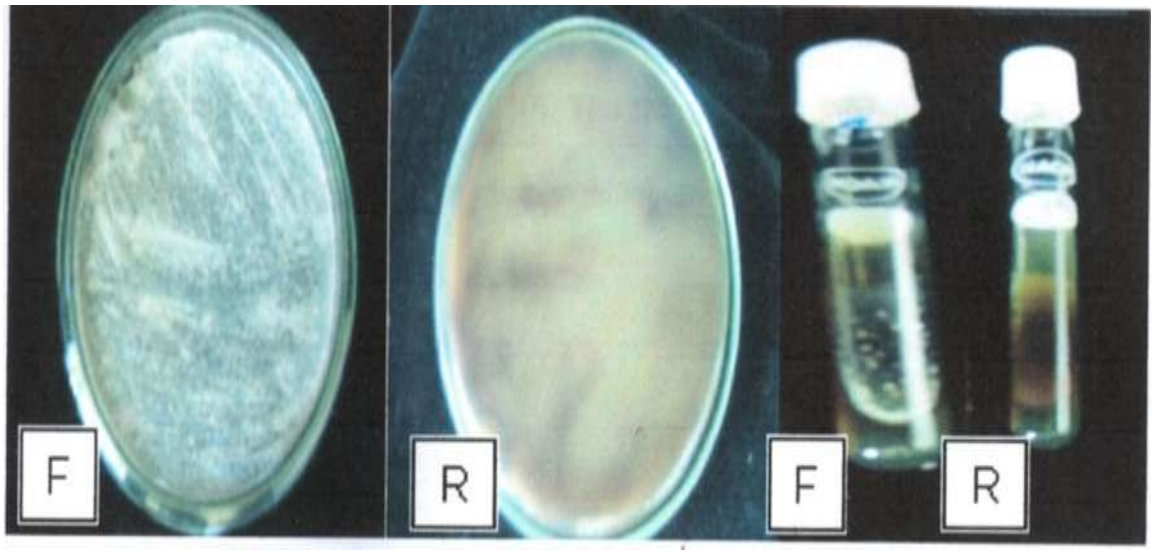

Figure 2. Macroscopic appearance of isolate B propagated for 7 days in Bennett medium in plates and in slants. (F-front, R- R-reverse side)

The spore surface of strain B is spiny and warty. The spore mass is in gray. The reverse is gray to black. It must probably belong to *Streptomyces* grey series (Goodfellow, 1989)

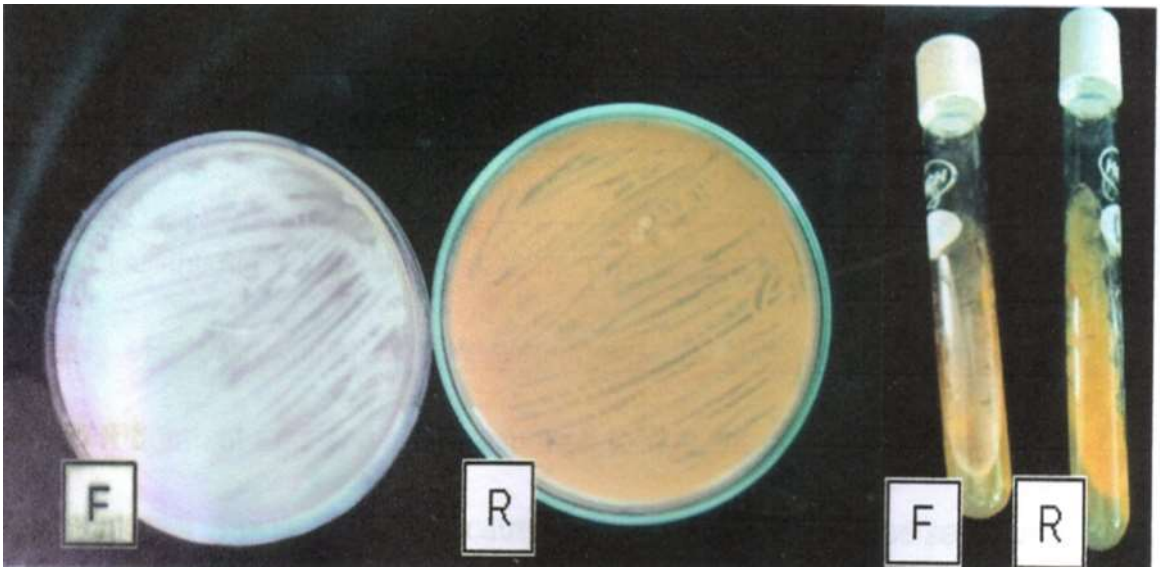

Figure 3. Macroscopic appearance of isolate C propagated from 7 days in Bennet medium in plates and in slants (F- front, R- reverse)

The spore surface of strain C is smooth. The spore mass is in light gray. The reverse is yellow brown. It must probably belong to Streptomyces grey series (Goodfellow, 1989)

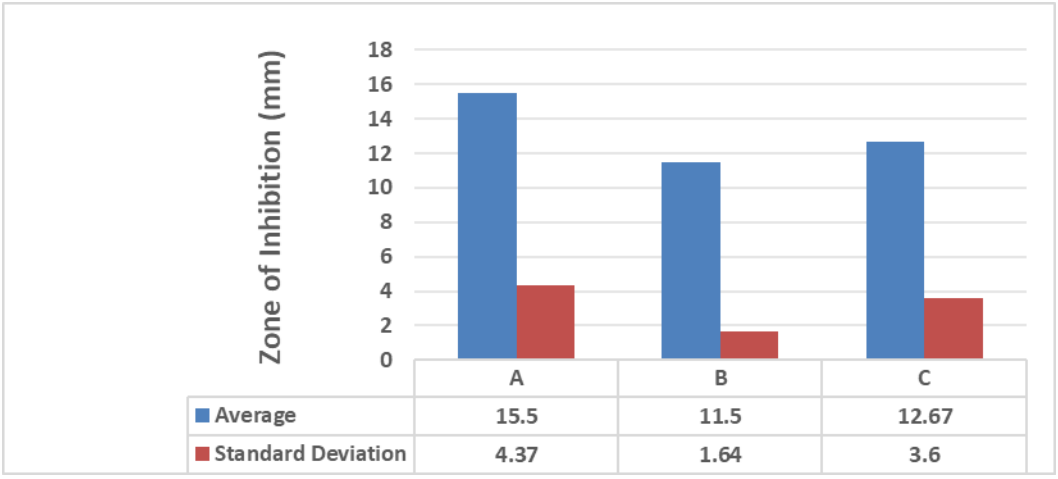

Figure 4. Activity of 7 day- old isolates propagated in Bennet medium against *Bacillus subtillis* (010). *Subtillis.*

Figure 4 shows the activity of 7-day-old isolates propagated in Bennet medium against *Bacillus subtillis* (010). the three strains inhibitions showed high antibacterial activity that can resist against Gram positive B. *Subtillis.*

Bacillus subtilis is a remarkably diverse bacterial species that is capable of growth with many environments. *B. subtilis* can form highly resistant dormant endospores in response to nutrient deprivation and other environmental stresses. It can cause dermal allergic or hypersensitivity reaction in individual (Earl, 2008).

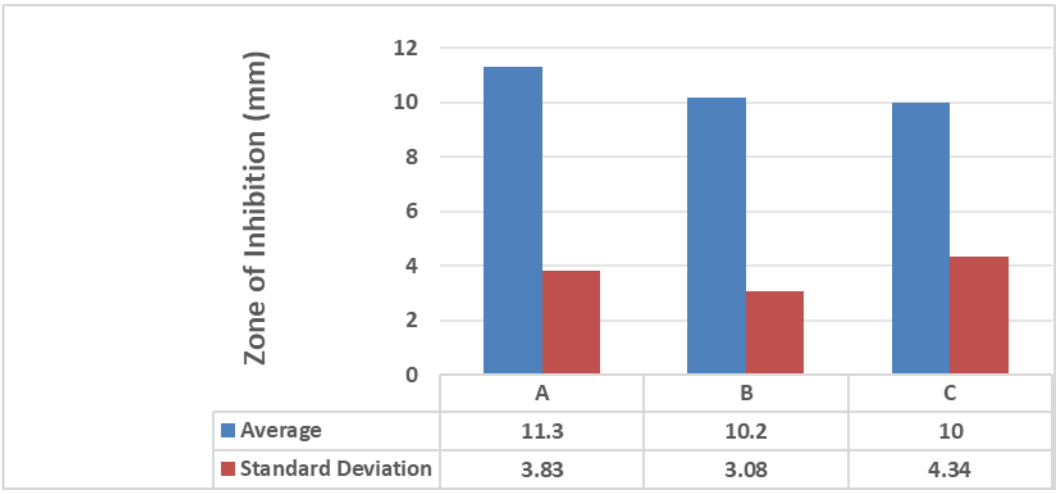

Figure 5. Activity of 7-day-old isolates propagated in Bennett medium against *Escherichia coli (002)*

Figure 5 shows the activity of 7-day-old isolates in Bennett medium against *Escherichia coli* (002). Strain A has high anti-microbial activity against *E. coli*, followed by strain B and strain C.

Escherichia coli is a Gram-negative bacillus; it is a common kind of bacteria that lives in the intestines of animals and humans, and most are harmless. People can spread the disease through contact with one another if they do not wash their hands after using the bathroom. Bloody diarrhea and stomach pains are the most common symptoms for people infected with *E. coli* (Nelson, 2008).

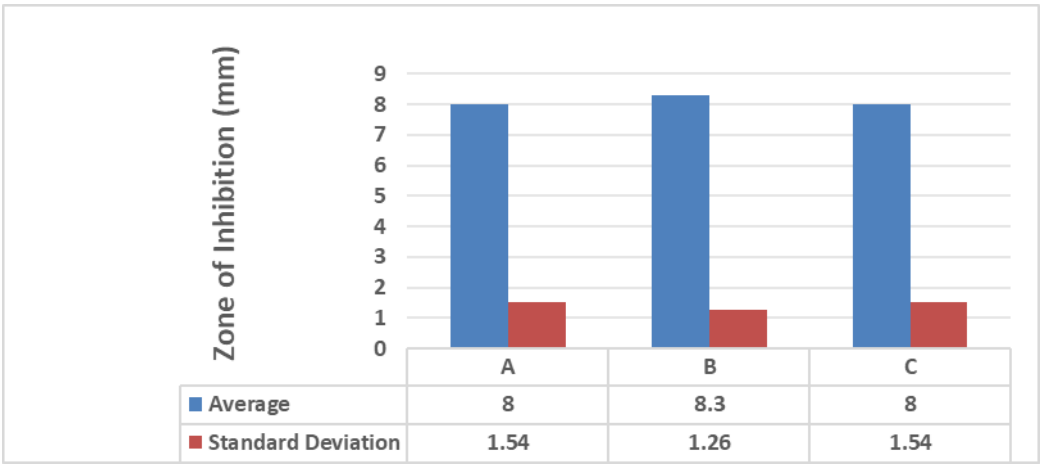

Figure 6. Activity of 7-day-old isolates propagated in Bennett medium against *Pseudomonas aeruginosa* (005)

Figure 6 shows the activity of 7-day-old isolates propagated in Bennett medium against *Pseudomonas aeruginosa* (005). Strains A, B, and C showed moderate activity against gram-negative bacteria.

Pseudomonas aeruginosa is a Gram-negative, motile, rod-shaped bacterium. The bacterium is capable of both aerobic and anaerobic growth. It is abundant in various types of moist environments and can adapt to numerous others. The opportunistic pathogen is commonly associated with hospital-acquired infection, most notably in immunocompromised individuals. *Pseudomonas aeruginosa* accounts for 10% of all hospital-acquired infections (Stojek, 2008).

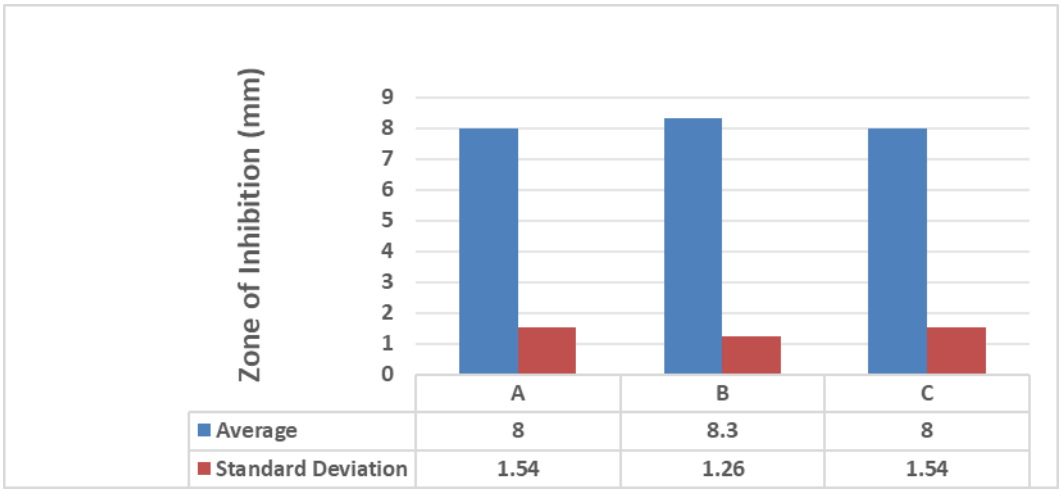

Figure 7. Activity of 7-day-old isolates propagated in Bennett medium against *Proteus vulgaris* (012)

Figure 7 shows the activity of 7-day-old isolates propagated in Bennett medium against *Proteus vulgaris* (012). Strain A has a high inhibitory action against the pathogenicity of *P. Vulgaris*, followed by Strains B and C.

Proteus vulgaris is a Gram-negative bacillus and facultative anaerobe. *P. vulgaris* usually is found to inhabit the intestinal tract of animals, however, it can be found in soil, water, fecal matter, and raw meat. It is considered to be an enteric pathogen; however, it can cause urinary tract and wound infections. It may also cause respiratory tract infections that persist even after antibiotic treatment (Sharma, 2011).

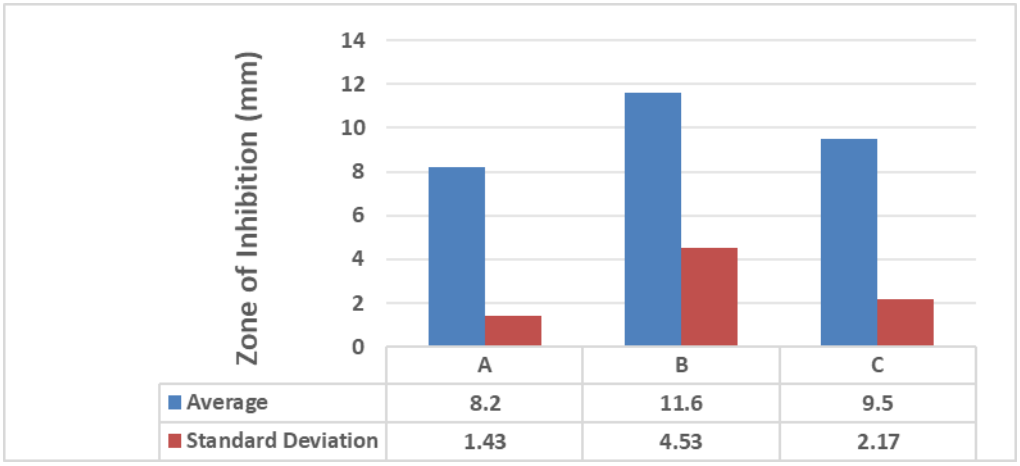

Figure 8. Activity of 7-day-old isolates propagated in Bennett medium against *Staphylococcus aureus* (004)

Figure 8 shows the activity of 7-day-old isolates in Bennett medium against *Staphylococcus aureus* (004). The results indicate that Strain B was highly active against *S. aureus* strains. The zones of inhibition of strains A and C are close to each other.

Staphylococcus aureus (004) is a Gram-positive, non-motile coccus, often found in grape-like clusters. *S. aureus* is transmitted through aerosol or direct contact with fomites, infected animals, or infected people. Approximately 30% of healthy humans carry *S. aureus* in their nasopharynx or on their skin. Any chemical or mechanical sterilant will also serve to remove S. *aureus* from the environment. However, S. *aureus* is resistant to drying and may remain infectious for weeks on dried skin or secretions (River, 2009).

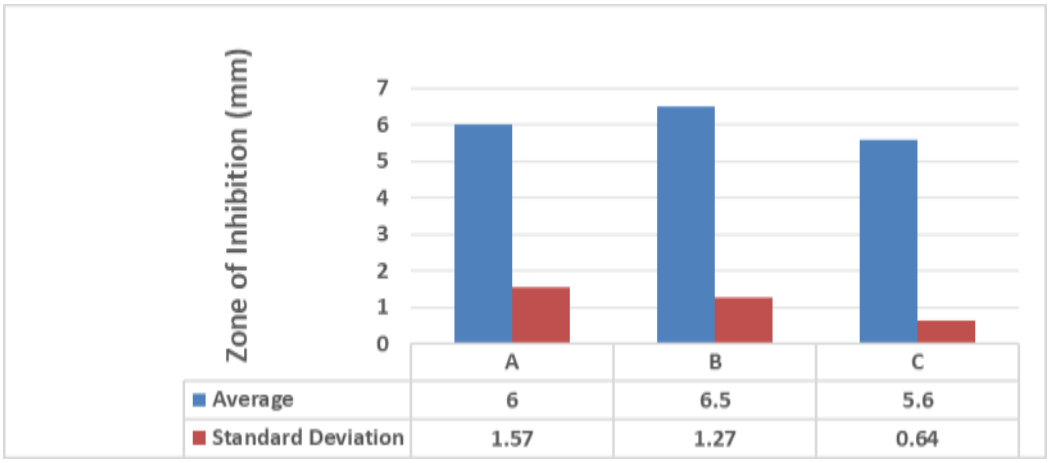

Figure 9. Activity 7-day-old isolates propagated in Bennett medium against *Aspergillus niger*

Figure 9 shows the activity 7-day-old isolates propagated in Bennett medium against *Aspergillus niger*. The strain C has the high inhibitory action of restraining *Aspergillus niger***.**

Aspergillus niger is a filamentous ascomycete fungus that is ubiquitous in the environment and has been implicated in opportunistic infections of humans. A variety of these enzymes from A. niger are important in the biotechnology industry. A. niger is also an important model organism for several important research areas including the study of eukaryotic protein secretion in general, the effects of various environmental factors on suppressing or triggering the export of various biomass degrading enzymes, molecular mechanisms critical to fermentation process development and mechanisms involved in the control of fungal morphology.

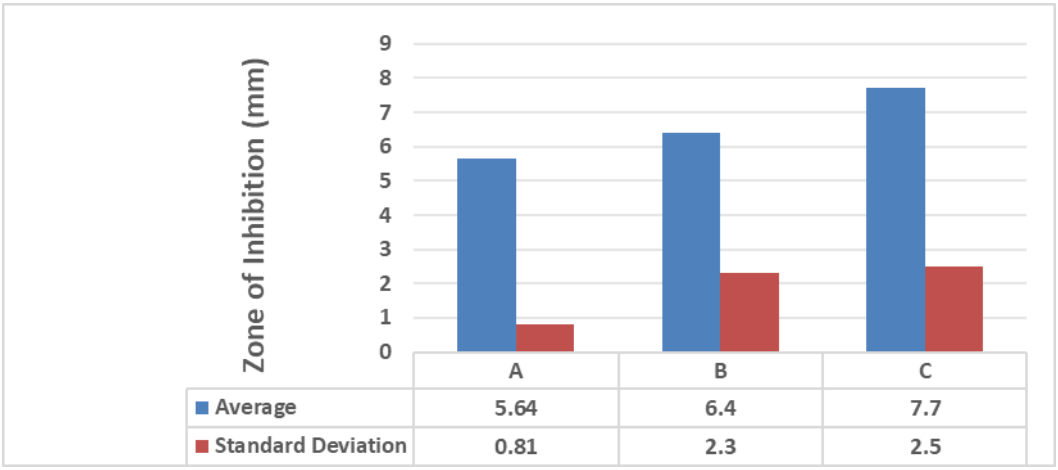

Figure 10. Activity of 7-day-old isolates propagated in Bennett medium against *Candida albicans*.

Figure 10 shows the activity of 7-day-old isolates propagated in Bennett medium against *Candida alibicans*. The three isolates contained antifungal properties and had antagonistic ability by inhibiting the growth of the fungi.

Candida species are normally harmless commensals of the gastrointestinal and genitourinary tract. However, they can also be important pathogens that cause a range of conditions, including painful superficial infections, such as vaginitis in otherwise healthy women, and life-threatening bloodstream infections among vulnerable intensive care patients, especially those undergoing chemotherapy or immunosuppressive therapy following an organ or bone marrow transplant procedure. (Sudbery, 2004)

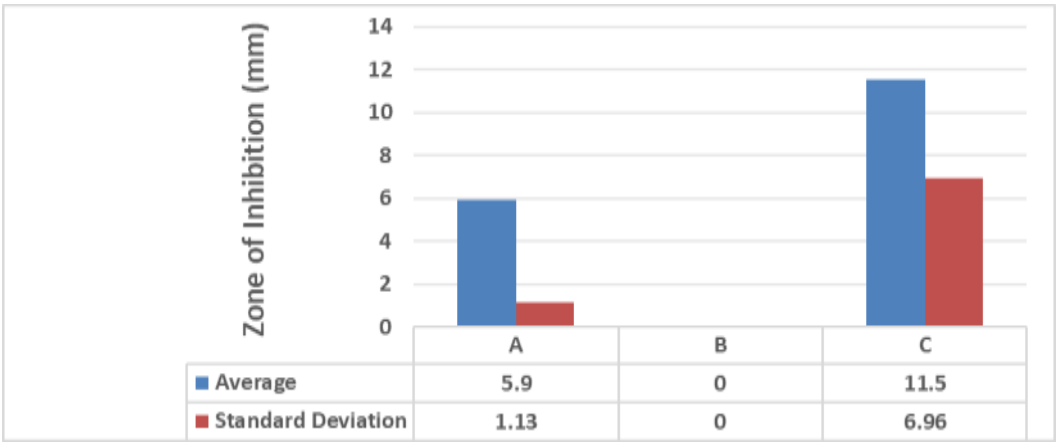

Figure 11. Activity of 7-day old isolates propagated in Bennet medium against *Penicillum sp.*

Figure 11 shows the activity of 7-day old isolates propagated in Bennet medium against *Penicillum sp*. The three strains inhibited the growth of *Penicillum sp*. Strain B has contained high antifungal properties than Strain A and C.

Penicillum sp. is a large anamorphic (asexual state) ascomycetous fungal genus with widespread occurrence in most terrestrial environments. This genus comprises more than 200 described species and many are common soil inhabitants, as well as food borne contaminants or food ingredients used in the preparation of cheese and sausages. *Penicillum* species produce a much-diversified array of active secondary matabolites including antibacterial, antifungal substances, immunosuppressants, cholesterol-lowering agents and also potent mycotoxins (Petit, 2009).

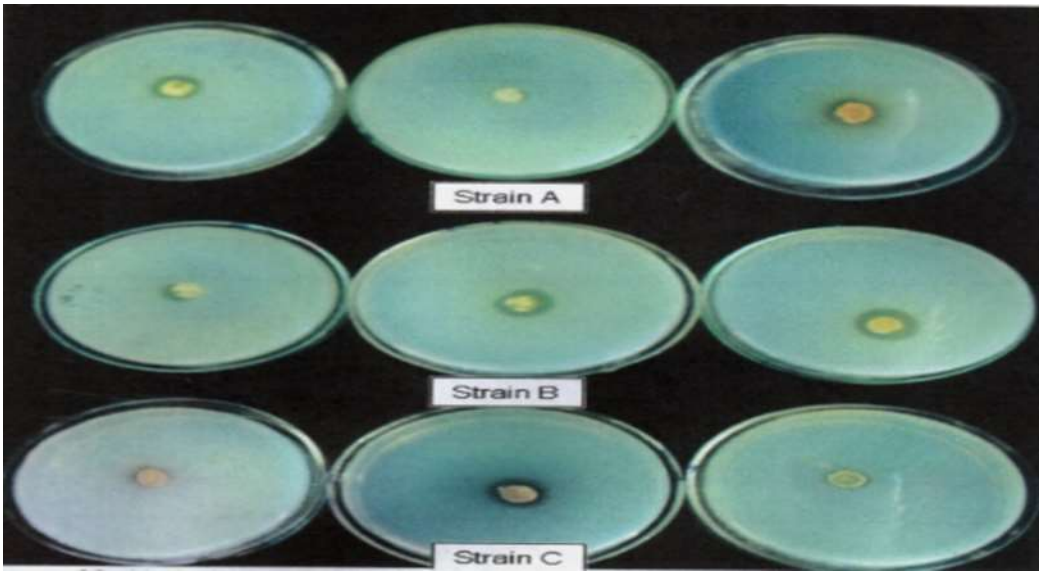

Figure 12. Hydrolysis of protein incorporated in skim milk Emerson medium by 7-day-old agar isolates.

Figure 12 shows that the three strains showed a zone of clearing that indicates protein hydrolysis. Proteins are made up of lots of amino acids joined together by peptide bonds. Hydrolysis of the protein is what happens when the peptide bonds are broken. The protein needs water and an enzyme. The result of hydrolysis is smaller amino acid chains (peptides) and free amino acids. It is well known that proteins are built up of various proportions of different amino acids, and that on hydrolysis they are broken down, giving proteoses, peptones, polypeptides, and finally their constituent amino acids.

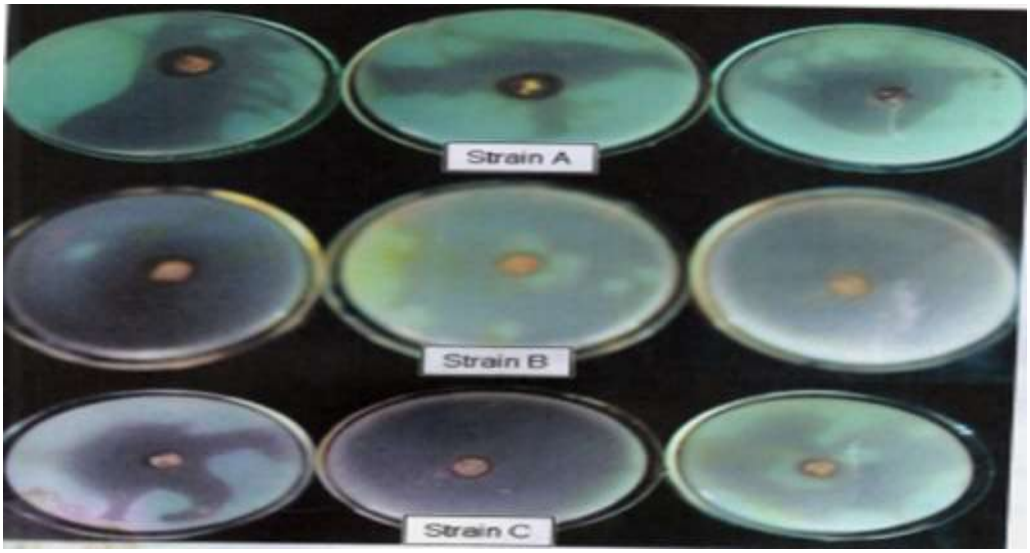

Figure 13. Hydrolysis of starch incorporated in Emerson medium 7-day-old agar isolates.

Figure 13 shows the degradation of glucose that reacts with iodine, forming the zone of hydrolysis. Starch sugar is a differential medium that tests the ability of an organism to produce certain exoenzymes that hydrolyse starch. The attached molecule is too large to enter the bacterial cell, so some bacteria secrete exoenzymes to degrade starch into subunits that can be utilized by the organism. Starch agar is a simple nutritive medium with starch added, no colour change occurs in the medium when the organism hydrolyses starch. Iodine is added to the plate after incubation. Iodine turns blue, black, or purple depending on the concentration of iodine in the presence of starch. A clearing around the bacterial growth indicates that the organism hydrolysed starch.

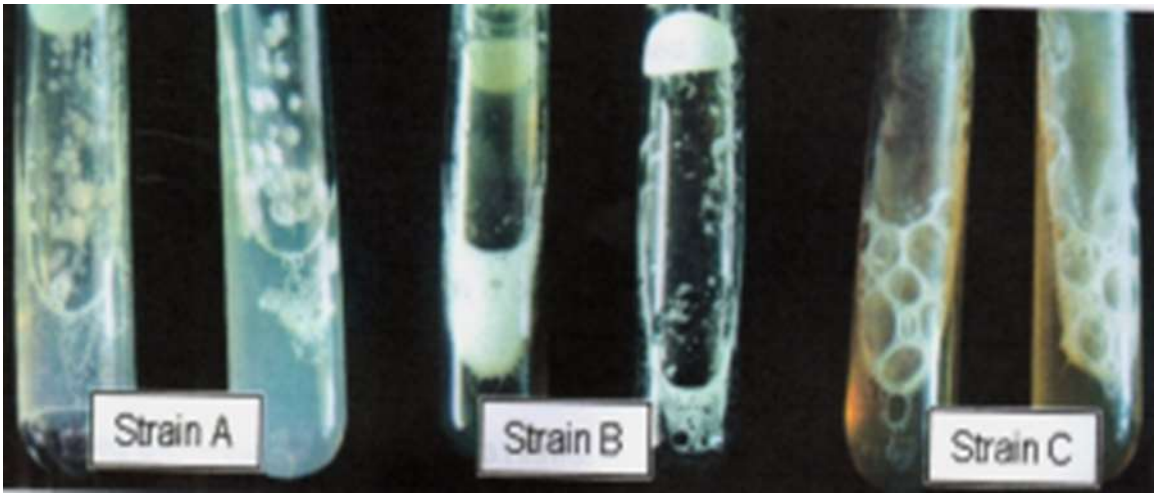

Figure 14. Local isolates' activity in the catalase test, isolates A to C propagated in Bennett medium.

Figure 14 shows the presence of bubbles of the three strains in the catalase test. The catalase test can be used to detect the enzyme catalase. This enzyme is responsible for protecting bacteria from hydrogen peroxide (H_2O_2) accumulation, which can occur during aerobic metabolism. If hydrogen peroxide accumulates, it becomes toxic to the organism. Catalase breaks down H_2O_2 into water and O_2.

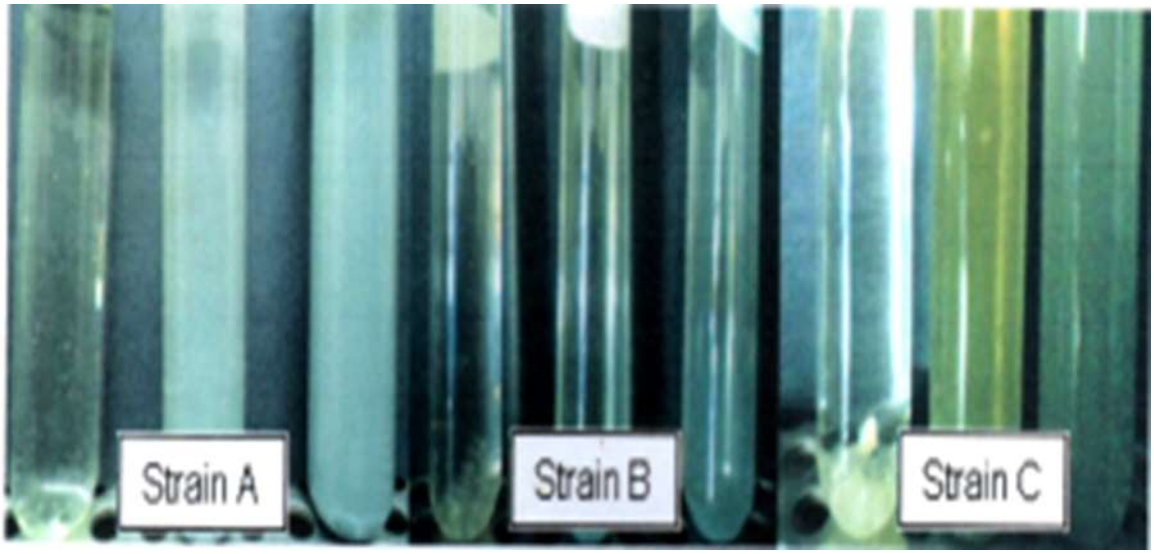

Figure 15 shows the reduction of 7-day-old isolates in nitrate reduction. Isolates A, B, and C were propagated in Bennett medium.

Figure 15 shows that the three strains have a negative result in nitrate reduction. Nitrate, present in the broth, is reduced to nitrite, which may then be reduced to nitric oxide, nitrous oxide, or nitrogen. The nitrate reduction test is based on the detection of nitrite and its ability to form a red compound when it reacts with sulfanilic acid to form a complex (nitrite-sulfanilic acid), which then reacts with a naphthylamine to give a red precipitate (protonsil). Zinc powder catalysis the reduction of nitrate to nitrite.

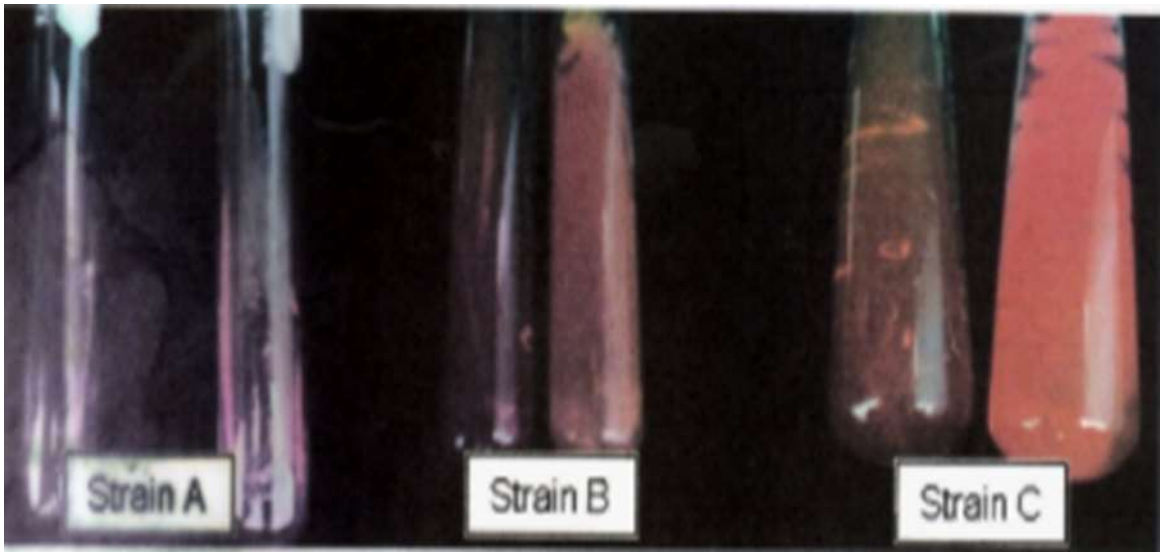

Figure 16. Reaction of 7-day old isolates in triple sugar iron. Isolates A, B and C propagated in Bennet medium.

Figure 16 shows the fermentation of the sugars of the three strains. Triple Sugar Iron (TSI) Agar is a microbiological test used to differentiate bacteria based on their ability to ferment sugars and produce hydrogen sulfide. It contains three sugars—glucose, lactose, and sucrose—along with iron salts and a pH-sensitive dye (phenol red). The test helps identify bacteria by observing color changes in the medium. If glucose is fermented, the butt of the tube turns yellow, while lactose or sucrose fermentation results in both the slant and butt turning yellow. Hydrogen sulfide production is indicated by blackening of the medium.

Conclusion

The three actinomycete strains were obtained from the composted mango waste from ITDI-DOST. They were propagated in Bennett medium for antimicrobial and biochemical testing. The strains show good inhibitory properties in pathogenic bacteria such as *Bacillus subtilis*, *Escherichia coli* and *Psuedomonas aeruginosa*, *Proteus vulgaris* and *Staphylococcus aureus*, and also in pathogenic yeast and fungi such as *Candida albicans*, *Aspergillus niger* and *Penicillium sp.* These three strains also showed positive results in biochemical tests such as protein hydrolysis, starch hydrolysis, catalase test, and negative results in nitrate reduction. The strains also showed a positive reaction in glucose fermentation in the triple sugar iron medium.

Recommendation

Three potential antibiotic and enzyme-producing microorganisms have been isolated from processed mango waste. Purification and extraction of the substances are recommended. The use of a scanning electron microscope is suggested for the characterization of the strains. Identification of Actinomycetes spp. Using molecular tools, which proved to be faster and less tedious, is also recommended.

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Disclosure of Conflict of Interest

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