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Exploring Biodiversity: Sampling, Analysis, and Identification of Microbial Treasures in Diverse Soil Ecosystems

Venci Candida X[1*], Reneefa P[2], Pison S[3], Prakash Shoba S[4], Arokya Glory P T[5], Shenkani K[6]

[1,2,3,4,5]Department of Zoology, Holy Cross College (Autonomous), Nagercoil, Tamilnadu, India
[6]Department of Zoology, JKKN College of Arts and Science, Komarapalayam, Erode, Tamilnadu, India.

**Corresponding author's E-mail: venciaugustine@gmail.com*

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Abstract

In the present study, bacterial colonies in Cashew Oil Industry Soil (COS), Fertilizer-applied Field Soil (FFS) and Control samples were enumerated and characterized across varying dilution factors. The results revealed significant variations in bacterial colony formation across dilution factors, indicating diverse growth rates and population densities. The investigation aimed to discern the microbial populations and characteristics in these contrasting samples. COS and FFS exhibited dilution-dependent colony counts, showcasing distinctions in bacterial populations compared to Control samples. Morphological analysis revealed diverse colony sizes, shapes, and colours, ranging from small to medium, with hues spanning yellow to orange. Margins, elevations, and opacities varied, and Gram staining indicated both positive and negative strains and Gram-positive cocci and diplococci, were identified illustrating taxonomic diversity. Biochemical tests unveiled diverse metabolic traits, identifying Cellobiosococcus sp. and Staphylococcus sp. at the genus level in COS and Micrococcus sp. in FFS. Given that these three bacterial strains were isolated from contaminated soil and considering the prior use of Micrococcus sp. and Staphylococcus sp. in remediation efforts, our upcoming research will specifically concentrate on exploring the bioremediation potential of pollutants using Micrococcus sp., Cellobiosococcus sp., and Staphylococcus sp., with particular emphasis on the unique capabilities of Cellobiosococcus sp as there are nostudies related to Cellobiosococcus sp. involved in bioremediation.

Keywords: *Bacterial characterization, Cashewnut oil industry soil (COS), Fertilizer applied field soil (FFS), Biochemical test, Gram staining.*

1. Introduction

The intricate world of soil serves as a critical component of terrestrial ecosystems, hosting a myriad of microorganisms that play pivotal roles in nutrient cycling, soil health, and environmental sustainability. This hidden ecosystem, teeming with bacteria, is of paramount importance as humanity faces the challenges posed by rapid industrialization and its environmental consequences. Bacteria, with their simplicity and remarkable adaptability, constitute a diverse kingdom of life, participating in essential processes such as nutrient cycling, nitrogen fixation, disease pathogenesis, and various biotechnological applications. Symbiotic and non-symbiotic bacteria, including *Rhizobium*, *Azotobacter*, *Azospirillum*, *Bacillus*, and *Klebsiella* species, are utilized globally to enhance plant productivity (Cocking 2003).

Industrial soils, shaped by human activities, harbour bacterial communities specialized in pollutant degradation. These bacteria exhibit unique metabolic pathways for breaking down industrial pollutants, making them valuable for bioremediation. The genetic adaptations of bacteria in industrial soils allow them to thrive in the presence of contaminants, emphasizing the need to understand how bacterial communities adapt to environmental transformations (Zaidi *et al*. 2008). Factors such as soil acidity, vegetation types, texture, and nutrient availability influence microbial communities. Physicochemical factors, including fertilizers and industrial waste disposal, impact soil fertility and microbial growth. The continuous use of chemicals may reduce bacterial richness, but the integration of organic fertilizers can regulate microbial growth, promoting the sustainability of crop production (Prashanthi *et al*., 2021).

Pesticide application in agriculture may impact ecosystem health, and microbial degradation is a primary mechanism for pesticide breakdown in soils. Phosphorus, a vital macronutrient, influences microbial activity in different soil layers. Bacteria's ability to survive in various conditions, from dry to flooded, highlights their adaptability and ecological importance (Rani, S. *et al*., 2007). Soil bacteria, though often challenging to cultivate, are crucial for nutrient cycling, organic matter decomposition, and soil structure improvement. The shift from traditional cultivation techniques to molecular methods has revealed the existence of uncultured microbes with diverse functions. However, challenges persist in cultivating these bacteria due to a lack of knowledge about their growth requirements (Van *et. al*., 2016).

The microbial activities in soil are influenced by various factors, including soil properties, chemical conditions, and interactions with other organisms. The soil, a dynamic and vital natural resource, requires preservation and improvement to maintain its equilibrium and productive capacity. Soil quality measurements, including biological and biochemical properties, provide valuable insights into soil health and respond sensitively to changes.

Our research was employed on isolating bacterial strains from control, fertilizer applied field soil and cashew oil industrial soils, employing precise sampling and cultivation methods to reveal microbial diversity. Through comprehensive characterization, we aim to depict the bacterial landscape in both ecological niches, highlighting their adaptive capabilities. This project explores soil microbiomes near a pristine stream (control), cashew oil industrial soils and in fertilizer applied field soil, isolating and characterizing these microbes with meticulous biochemical testing. Our goal is to unravel nature's unique microbial tapestry and envision a future where these resilient microorganisms play a key role in eco-friendly pesticide bioremediation, contributing to sustainable agricultural practices.

2. Materials And Methods

Collection of soil samples

Soil samples were gathered from a stream, the cashew nut oil industy and a field treated with fertilizers in Kumara Puram. Control samples were obtained from a fertilizer-free and uncontaminated canal. These samples were carefully placed in sterile plastic bottles and transported to the microbiology laboratory for analysis.

Isolation of bacteria

The Colle and Miles method was employed for bacterial isolation. Stock solutions of the sample were created by measuring one gram in 100ml of sterile water. A ten-fold (10^{-1} to 10^{-10}) serial dilution was performed to reduce bacterial populations. Subsequently, 0.1ml from the 10^{-5}, 10^{-6}, and 10^{-7} dilutions were pipetted onto the surfaces of Petri dishes containing 20ml of sterile Nutrient Agar. After incubating the plates for 24 hours at 37°C, microbial colonies were counted, and the number of cells per ml of sample (CFU/ml) was determined using the formula: Number of colonies counted / volume plated × Dilution factor (Colle and Miles method).

Morphological identification and characterization of bacteria

Morphological identification and characterization of bacteria involved assessing shape, size, and arrangement, as well as distinguishing between gram-negative and gram-positive bacteria. The Gram staining method was employed: a bacterial suspension smear on a glass slide was heat-fixed, stained with crystal violet, iodine mordant, decolorized with ethanol, and counter-stained. The resulting slide was examined under a microscope at 100X magnification (Kannan *et al*., 2018).

Biochemical Identification:

Various biochemical tests were conducted for bacterial identification. The Indole Test involved inoculating peptone broth, incubating, and adding Kovac's reagent to observe cherry red colour formation. The Methyl Red (MR) Test required inoculating glucose phosphate broth, with red colour indicating a positive result. Voges Proskauer (VP) Test involved adding alpha-naphthol and KOH to glucose phosphate broth, with red colour indicating a positive reaction. Nitrate Reduction, Citrate Utilization, Urease Utilization, Catalase, Triple Sugar Iron, Motility, Casein Hydrolysis, Gelatine Hydrolysis, and Starch Hydrolysis tests were also performed with specific procedures and observations for bacterial characterization (Ahirwar *et al*., 2016; Mohite *et al*., 2001; Masi *et al*., 2021; Akter & Huq, 2018).

Gram Staining

The Gram staining protocol (Coico, 2005) was employed for bacterial morphology identification. A bacterial suspension drop was heat-fixed on a glass slide, stained with crystal violet, iodine mordant, decolourized with ethanol, and counter-stained. Examination under a microscope (100X magnification) was followed.

Bacterial Enumeration

For bacterial enumeration, Colle and Miles' method (1989) involved serial dilution, spreading onto Petri dishes with sterile Nutrient Agar, and incubation at 37°C. Colonies were counted, and colony forming units per gram (CFU/g) were computed. Isolated colonies were further identified through biochemical reactions, including gram staining, coagulase test, catalase test, and microscopy (Eleyowo *et al*., 2016).

3. Results and Discussion

Bacterial colony enumeration was performed in three samples: COS (cashew nut office soil), FFS (Fertilizer Field Sample) and Control, employing varying dilution factors. Figure 1 shows the formation of bacterial colonies in nutrient agar. Table 1 shows the enumeration of bacterial colonies in COS, FFS and control. The number of colonies formed in control, COS and FFS are displayed in Graph 1, Graph 2 and Graph 3 respectively.

Figure 1: Formation of bacterial colonies in nutrient agar

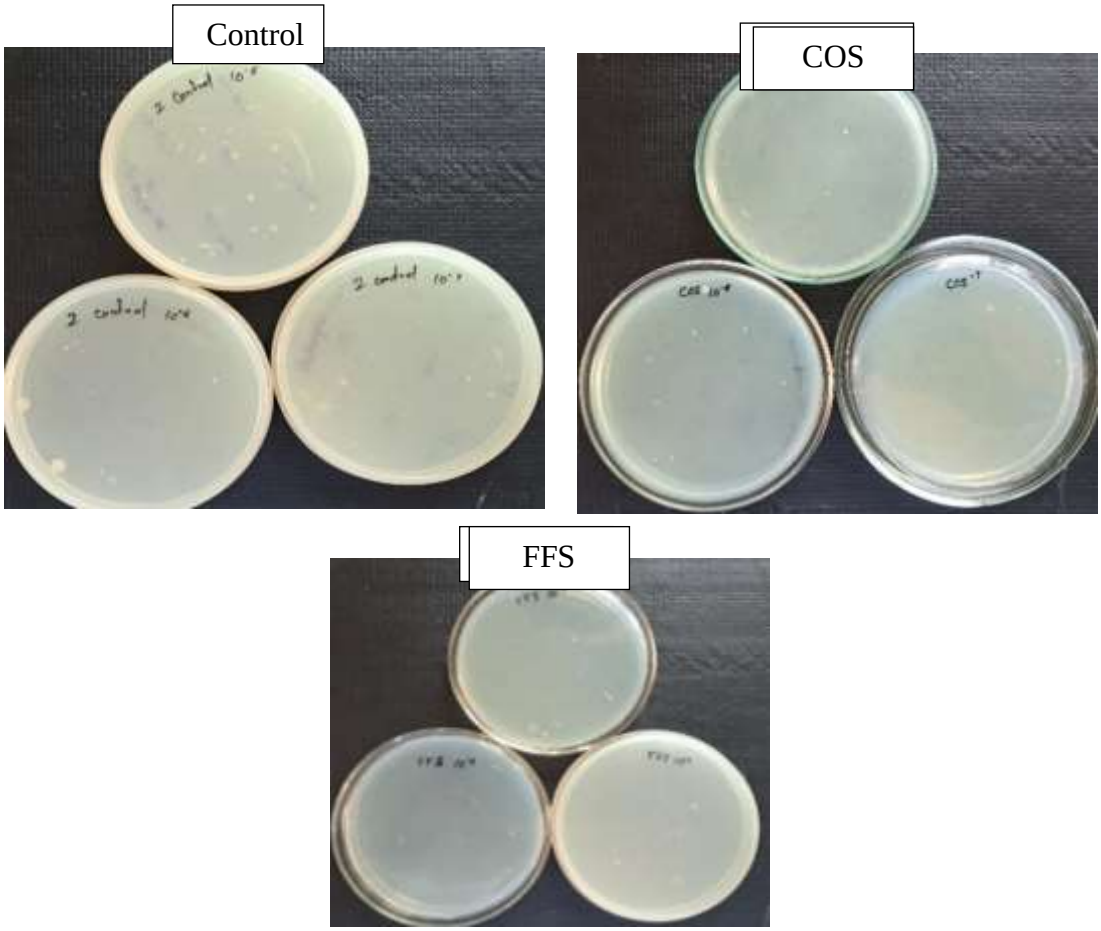
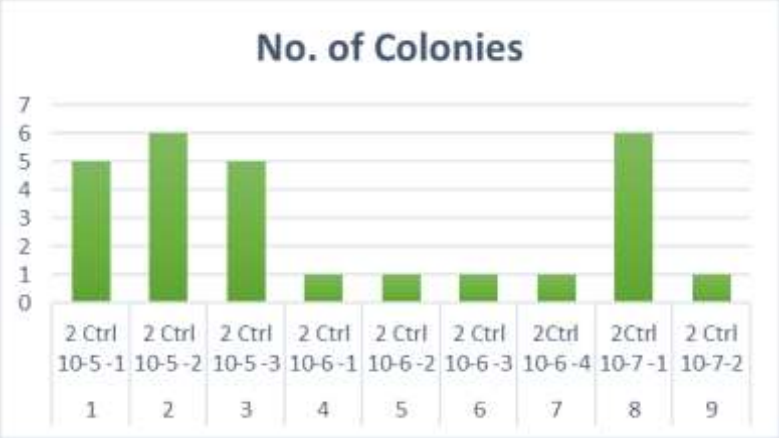


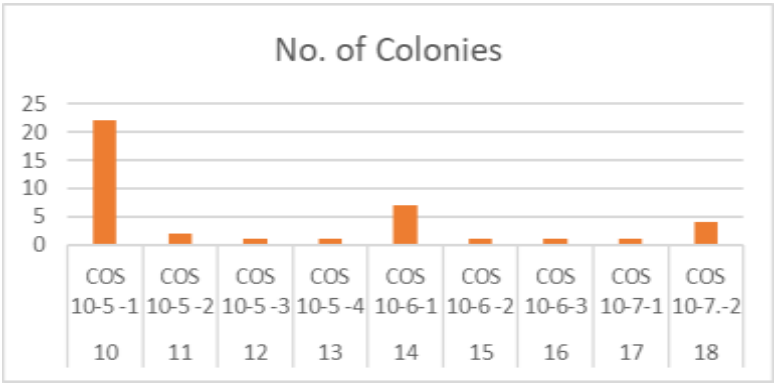
Table 1: Enumeration of Bacterial Colonies in Control, COS and FFS

Sl. No.	Sample code	Dilution factor	No. of colonies/CFU
1	Control	10^{-5}	12 x 10^{-6}
		10^{-6}	10 x 10^{-7}
		10^{-7}	7 x 10^{-8}
2.	COS	10^{-5}	48 x 10^{-6}
		10^{-6}	13 x 10^{-7}
		10^{-7}	10 x 10^{-8}
3.	FFS (Fertilizer Field Sample)	10^{-5}	55 x 10^{-6}
		10^{-6}	26 x 10^{-7}
		10^{-7}	15 x 10^{-8}

Graph 1: Number of colonies formed in Control



Graph 2: Number of colonies formed in COS



Graph 3: Number of colonies formed in FFS

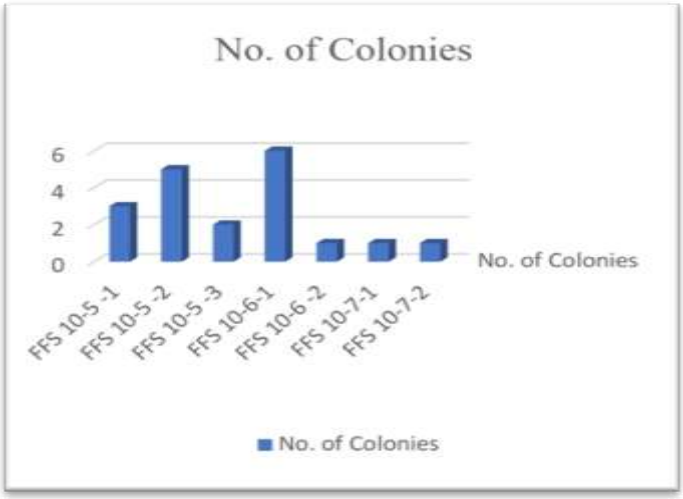


Table 2: Morphological Characteristics of Selected Microorganisms from Control, COS and FFS Sample

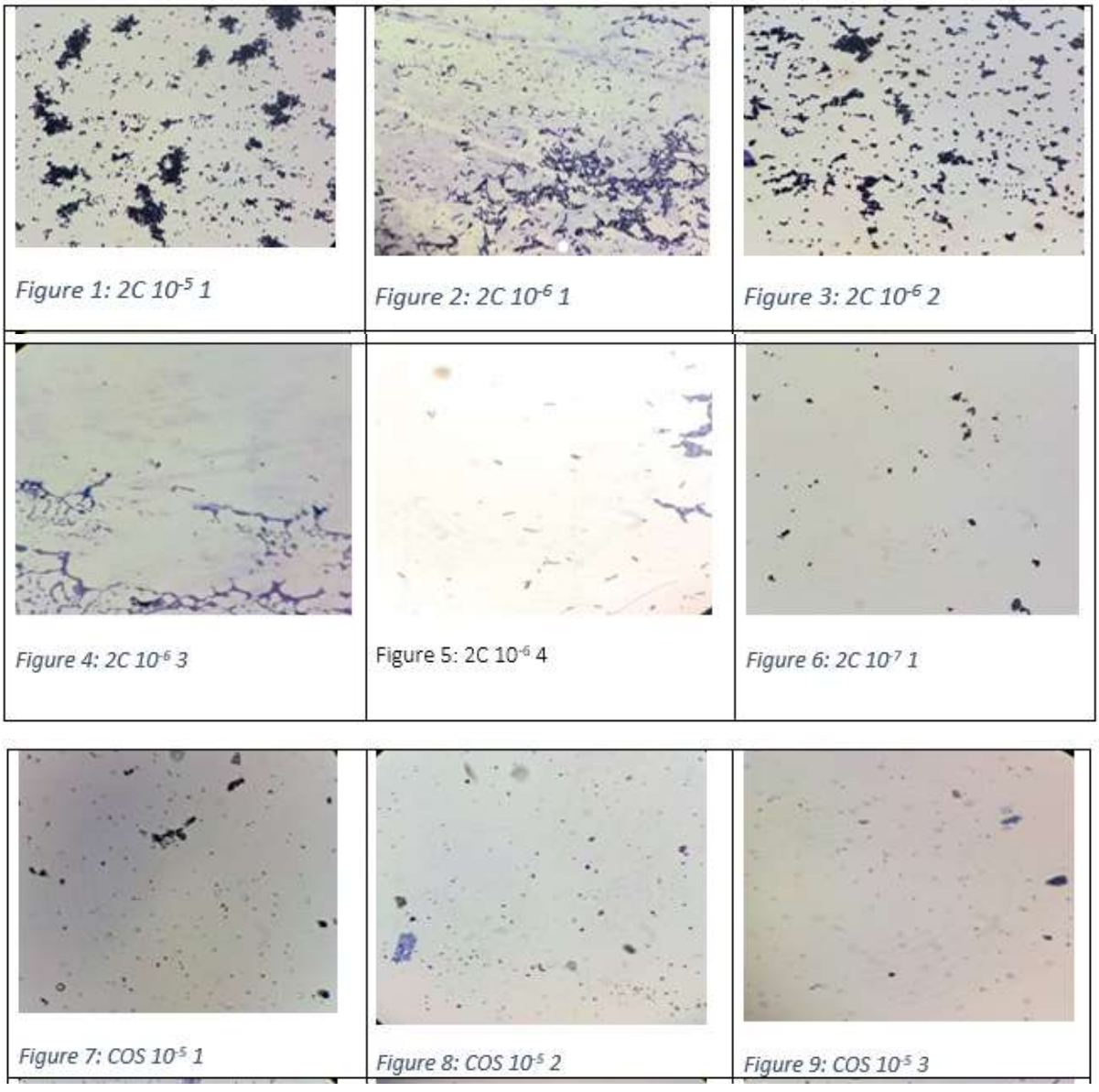
Sl.NO	Name	Size	Shape	Colour	Margin	Elevation	Opacity	Appearance	No. of Colonies	Gram Staining
1	Ctrl 10^{-5} -1	Small	Round	Yellow	Entire	Convex	Opaque	Mucoid	5	-
2	Ctrl 10^{-5} -2	Medium	Round	White	Entire	Growth into Media (Flat)	Semi - Transparent	Watery	6	-
3	Ctrl 10^{-5} -3	Medium	Irregular	Antique White	Entire	Growth into	Opaque	Watery	5	$+^{ve}$ Cocci Bunch

						Media (Flat)				
4	Ctrl 10^{-6} -1	Large	Round	Antique White	Entire	Umbonate	Opaque	Mucoid	1	$+^{ve}$ Rod Chain
5	Ctrl 10^{-6} -2	Small	Round	Yellow	Entire	Convex	Opaque	Mucoid	1	$+^{ve}$ Cocci Bunch
6	Ctrl 10^{-6} -3	Small	Round	Pinkish white	Entire	Convex	Opaque	Shiny	1	$+^{ve}$ Cocci Bunch
7	Ctrl 10^{-6} -4	Large	Curled	Antique White	Undulate	Flat	Opaque	Mucoid	1	$+^{ve}$ Rod Chain
8	Ctrl 10^{-7} -1	Small	Round	Yellow	Entire	Convex	Opaque	Mucoid	6	$+^{ve}$ Cocci Bunch
9	Ctrl 10^{-7}-2	Medium	Round	White	Entire	Umbonate	Opaque	Mucoid	1	-
10	COS 10^{-5} -1	Small	Round	Yellow	Entire	Convex	Opaque	Mucoid	22	$+^{ve}$ Cocci Bunch
11	COS 10^{-5} -2	Small	Round	Yellowish Orange	Entire	Raised	Opaque	Mucoid	2	$+^{ve}$ Diplococci,
12	COS 10^{-5} -3	Small	Round	Orange	Undulate	Flat	Opaque	Mucoid	1	$+^{ve}$ Coma shape
13	COS 10^{-5} -4	Small	Round	white	Entire	Growth into media (Convex)	Opaque	Powdery	1	$+^{ve}$ Diplococci,
14	COS 10^{-6}-1	Small	Round	Yellow	Entire	Convex	Opaque	Mucoid	7	$+^{ve}$ Cocci Bunch
15	COS 10^{-6} -2	Medium	Round	Antique White	Entire	Umbonate	Opaque	Rough	1	$+^{ve}$ Rod Chain
16	COS 10^{-6}-3	Pinhead	Round	white	Entire	Convex	Opaque	Rough	1	$+^{ve}$ DiploCocci
17	COS 10^{-7}-1	Medium	Round	Light Brown	Entire	Umbonate	Opaque	Shiny	1	$+^{ve}$ DiploCocci
18	COS 10^{-7}-2	Medium	Round	Antique White	Entire	Umbonate	Semi - Transparent	Rough	4	$+^{ve}$ DiploCocci
19	FFS 10^{-5} -1	Small	Irregular	Light Orange	Undulate	Umbonate	Opaque	Rough	3	$+^{ve}$ Cocci, Chain
20	FFS 10^{-5} -2	Small	Round	Yellow	Entire	Convex	Opaque	Mucoid	5	$+^{ve}$ Cocci Bunch
21	FFS 10^{-5} -3	Small	Round	Pinkish white	Entire	Convex	Opaque	Shiny	2	$+^{ve}$ Cocci Bunch
22	FFS 10^{-6}-1	Small	Round	Yellow	Entire	Convex	Opaque	Mucoid	6	$+^{ve}$ Cocci Bunch
23	FFS 10^{-6} -2	Small	Round	Pinkish white	Entire	Convex	Transparent	Shiny	1	$+^{ve}$ Cocci Bunch

24	FFS 10^{-7}-1	Small	Round	Pinkish white	Entire	Convex	Opaque	Shiny	1	-
25	FFS 10^{-7}-2	Medium	Round	Antique White	Entire	Growth into Media (Flat)	Opaque	Rough	1	$+^{ve}$ DiploCocci

Table 2 shows the morphological identification of morphological characteristics of Selected Microorganisms from Control, COS and FFS Sample. Cocci bunch and rod chain were seen in control, Cocci bunch, rod chain, coma shaped and diplococci were seen in COS. Cocci chain, cocci bunch and diplococci were seen in FFS. The gram staining images were recorded for control COS and FFS in Graph 2. After morphological identification the bacterial colony which was different from others were selected from both COS and FFS and subjected to biochemical characterization. COS 10^{-5} and 10^{-7}, FFS 10^{-5} and 10^{-6} were selected for biochemical characterization. Figure 2 shows the gram staining images of bacteria isolated from control, COS and FFS. Figure 3 and Figure 4 shows the purification of isolated microorganisms from COS 10^{-5} 4 and COS 10^{-7} 1**,** FFS 10^{-5} 1 and FFS 10^{-6} 2 (Quadrant Streak) respectively. Table 3 shows the biochemical characterization of bacterial isolates form soil. Figure 5 and figure 6 shows the biochemical tests done for COS 10^{-5} 4 and COS 10^{-7} 1**,** FFS 10^{-5} 1 and FFS 10^{-6} 2 respectively.

Figure: 2 Gram Staining Images



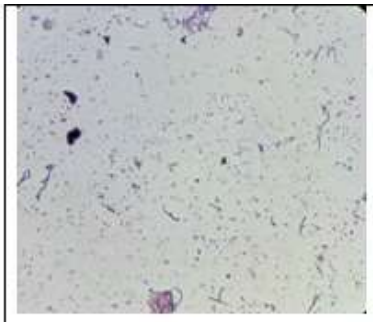


Figure 10: COS 10^{-5} 4

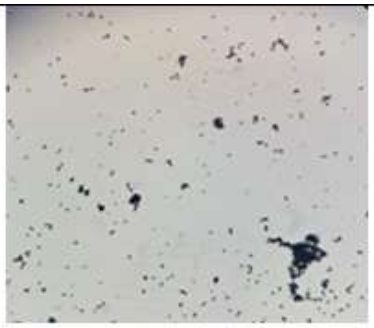


Figure 11: COS 10^{-6} 1

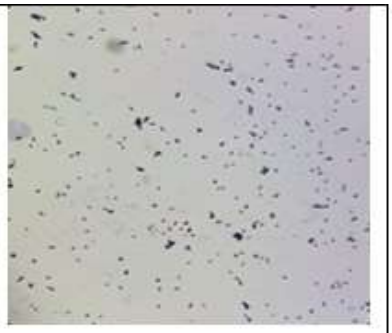


Figure 12: COS 10^{-6} 2

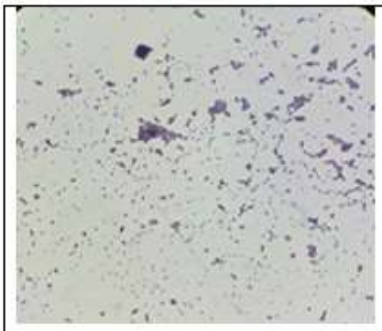


Figure 13: COS 10^{-6} 3

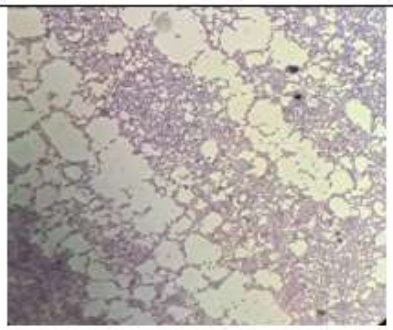


Figure 14: COS 10^{-7} 1

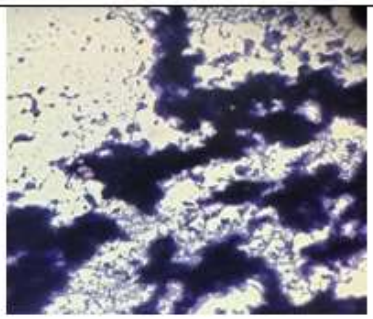


Figure 15: COS 10^{-7} 2

Figure 10: 1C 10-7 3

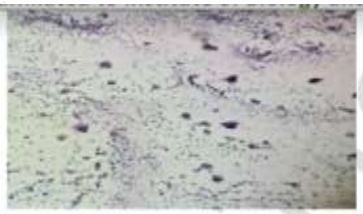


Figure 11: FFS 10-5 1

Figure 12: FFS 10-5 2

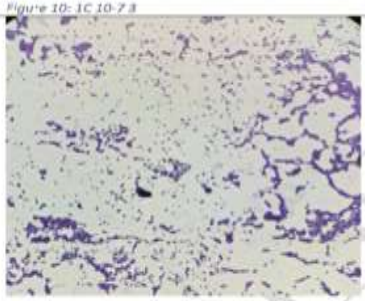


Figure 13: FFS 10-5 3

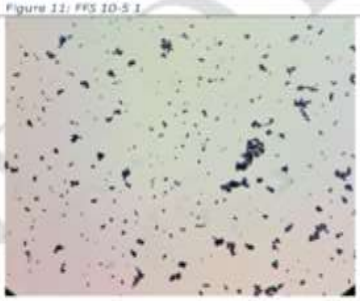


Figure 14: FFS 10-6 1

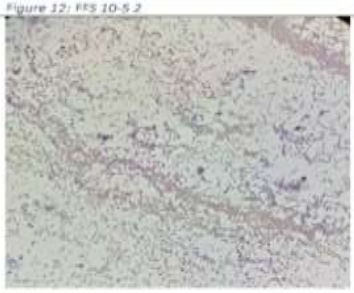


Figure 15: FFS 10-6 2

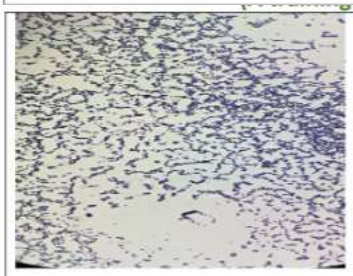
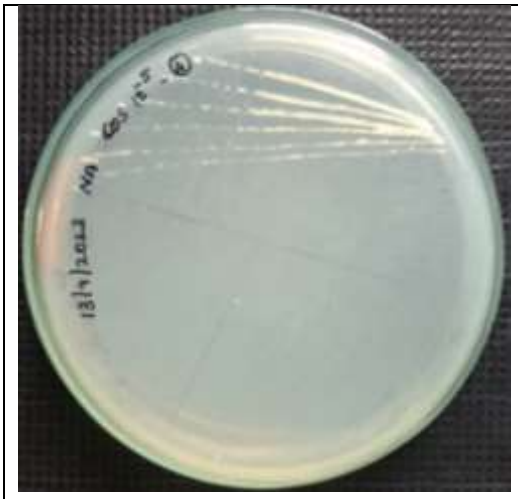


Figure 16: FFS 10-7 2

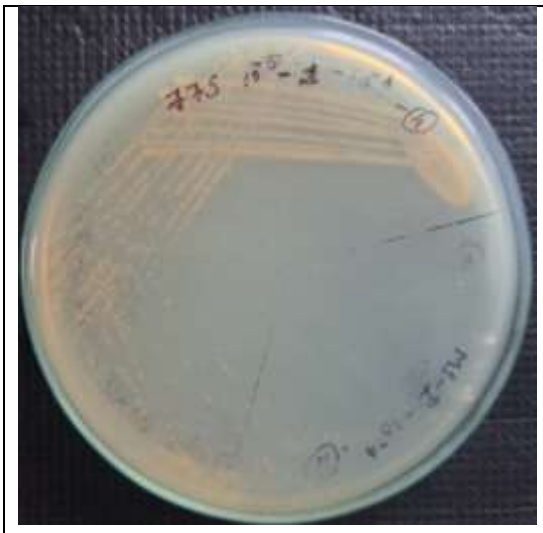
Fig. 3: Purification of isolated microorganisms COS 10^{-5} 4 and COS 10^{-7} 1 (Quadrant Streak)



COS 10^{-5} 4

COS 10^{-7} 1

Fig. 4: Purification of isolated microorganisms FFS 10^{-5} 1 and FFS 10^{-6} 2. (Quadrant Streak)



FFS 10^{-5} 1

FFS 10^{-6} 2

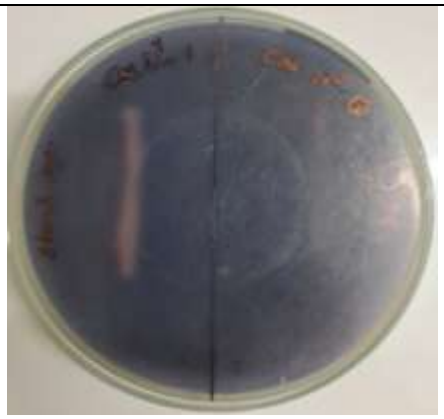
Table 3: Biochemical characterization of bacterial isolates form soil.

S. No.	Biochemical test	FFS 10^{-5} 1	FFS 10^{-6} 2	COS 10^{-5} 4	COS 10^{-7} 1
1	Indole	-	-	-	-
2	Methyl Red (MR)	+	+	+	+
3	Voges Proskauer(VP)	-	-	-	-

4	Nitrate Reduction	+	+	+	+
5	Triple sugar Iron	+	-	-	-
6	Citrate	+	-	-	-
7	Catalase	+	+	+	+
8	Urease	-	-	-	+
19	Starch Hydrolysis	-	+	+	+
10	Caesin Hydrolysis	-	-	-	-
11	Gelatin Hydrolysis	-	-	-	+
12	Motility	Motile	Non-Motile	Motile	Motile

Figure 5: Biochemical tests for isolates from COS 10^{-5} 4 and COS 10^{-7} 1

Gelatin Hydrolysis



Starch hydrolysis

Caesin Hydrolysis

Catalase test

Figure 6: Biochemical tests for isolates from FFS 10^{-5} 1 and FFS 10^{-6} 2

Gelatin Hydrolysis

Starch hydrolysis

Caesin Hydrolysis

Catalase test

Identification at the genus level, based on the macroscopic, microscopic, and biochemical characteristics of isolated microorganisms, revealed that COS 10^{-5}4 and COS 10^{-7}1 were identified as *Cellobiosococcus* sp. and *Staphylococcus* sp., respectively. The species identified in both FFS 10^{-5} 1and 10^{-6} 2 were *Micrococcus Sp*. This knowledge is valuable for bioremediation initiatives, as certain microorganisms possess the capability to degrade pollutants, playing a crucial role in cleaning up contaminated sites and enhancing environmental quality. The presence of microorganisms like *Staphylococcus sp*. is particularly significant, given that certain strains can be pathogenic and pose potential health risks to humans. Recognizing their existence in environmental samples is a crucial step in assessing potential health hazards.

Based on biochemical and molecular characterization done by Tariq M *et al*., 2019, the identified bacterium falls within the *Staphylococcus* genus, specifically as sub-species *aureus* strain K1. It demonstrated the ability to thrive in metal concentrations of up to 22 mM of Cr^{6+} and exhibited a remarkable capacity to eliminate 99% of chromium (1 mM $Cr^{6+)}$ within 24 hours under optimal growth conditions. The findings of Akerele and Akinyemi, 2022 indicated that bacteria have the capability to degrade and utilize plastics as part of their metabolic activity. They recommended to employ plastic-degrading microorganisms as a strategy to mitigate the rate of plastic pollution using strains of microorganisms—*Staphylococcus aureus, Pseudomonas sp, Bacillus sp, Streptococcus sp, Micrococcus sp, Aspergillus flavus, Aspergillus niger*, and *Trichoderma viridae* in bioremediation efforts.

Ten dominant bacterial isolates belonging to the genera *Pseudomonas, Staphylococcus, Micrococcus, Salmonella, Cellobiococcus,* and *Pneumonia* were identified through a combination of morphology assessment, biochemical tests, and the PIB tool by Badrunnisa. S *et al*., 2011. To characterize the optimal conditions for their growth, the isolates were exposed to four different types of media, various pH levels, and a range of temperatures. Furthermore, the ten isolates displayed optimal growth at varying temperatures, spanning from 20°C to 90°C, and at different pH levels, ranging from acidic to alkaline. This comprehensive characterization provides valuable insights into the diverse preferences and adaptability of these bacterial isolates under different environmental conditions.

4. Conclusion

Based on the results of the present study and the insights gathered from the discussions among various scientists, it is evident that the identified species *Micrococcus sp., Cellobiosococcus sp. and Staphylococcus sp*. hold potential for application in bioremediation efforts and has been isolated from various soil types, including fields treated with fertilizers. Given its association with fertilizer-applied fields ad cashew oil industry soil, consequently, our upcoming research will be centered on exploring the bioremediation capabilities of pollutants using *Micrococcus sp., Cellobiosococcus sp. and Staphylococcus sp*.

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