Microbial diversity of indigenous microorganism communities from different agri-ecosystems in Soc Trang province, Vietnam

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ABSTRACT

Indigenous Microorganism (IMO) has great potential for agricultural uses since they have high ability in biodegradation, nitrogen fixation, phosphate solubilization, plant growth hormone production as well as bio-control. This study aimed to determine the presence of some different major groups of microbes in IMO from different Agri-ecosystem habitats like bacteria, fungi, actinomyces, *Salmonella*, *Shigella*, *E. coli*, and *Coliform*. The presence of bacteria, actinomyces, and fungi of IMO samples was identified by 27F/1492R, 243F/1378R and ITS1F/ITS4R primers, respectively. The numbers of bacteria, fungi, and actinomyces were determined by the plate counting method on TSA, PDA and Starch media, respectively. The numbers of *Salmonella* sp. and *Shigella* sp. were determined by the plate counting method on selective *Salmonella* and *Shigella* agar (SS agar) after incubation for 48 hours at 37^{o}C while the density of *Coliforms* sp. and *E.coli* were counted by the Most Probable Number method (MPN). The results of the study showed that 3 major groups of microbes including bacteria, fungi, and actinomyces in 14 collected IMO samples were detected genetically. Moreover, bacterial numbers were dominated and ranged from 10^6 to 10^9cfu/g IMO samples while the density of fungi and actinomyces were lower and varied from 10^5 to 10^7cfu/g IMO sample. Interestingly, all surveyed IMO samples did not contain any human disease pathogens such as *Salmonella*, *Shigella*, *Coliforms* and *E. coli*. These results imply that collected IMO contains a high diversity of major groups of microbes and can be used as safe bio-stimulants for clean vegetable production.

1. Introduction

A concept of Indigenous Microorganism (IMO) was developed by Cho Han Kyu in the 1960s from the Janong Farming Institute, South Korea. IMO cultures contain consortia of beneficial microorganisms, mainly comprising fungi, bacteria, and actinomyces that are collected and cultured from soils to use as bio-fertilizer for the plant (Reddy, 2011). Indigenous microorganism community (IMO) is a group of the innate microbial consortium that inhabits the soil and the surfaces of all living things. It has potential for biodegradation, bioleaching, bio-composting, nitrogen fixation, phosphate solubilization, soil fertility improvement and in the production of plant growth hormones as well as bio-control (Kumar & Gopal, 2015). Application of IMO in agriculture is a friendly environmental method and helps to enhance organic matter decomposition, plant nutrition, soil fertility, crop yields and resistance to plant diseases (Chiemela, Serafin, Ricardo, & Joseph, 2013b). A study by Chiemela, Serafin, Ricardo, and Joseph (2013a) showed that IMO had strong efficacy on the promotion of agricultural and plant residues degradation in a compost heap and produced a large number of soluble micronutrients that are ready to be taken up by plants. In general, it is well-known that IMO brings many benefits to plants and has been applied broadly in agriculture in many countries all over the world. IMO-based technology, a great innovation has been applied widely in many countries in the eastern part of the world. In Vietnam, the application of IMO in organic coffee farming in Daklak showed that IMOs helped to improve the physical, chemical and biological properties of the coffee soils. Moreover, IMOs also reduced 25% chemical fertilizer of the recommended chemical fertilizer formula, but the productivity of this treatment was not significantly different from the recommended chemical fertilizer treatment (Pham & Dok, 2009). However, deep and scientific knowledge about IMO microorganism composition and any harmful bacteria residing in IMO have been still lacked and should be scientifically elucidated. Therefore, this study aimed to determine the presence of some different major groups of microbes in IMO from different agro-ecosystem habitats like bacteria, fungi, actinomyces, *Salmonella*, *Shigella*, *E. coli*, and *Coliform*.

2. Materials and methods

2.1. Materials

Fourteen different IMO were collected from different crop models in Soc Trang province, Vietnam including bamboo, crop rotation (corn, watermelon, courgette), banana, shallot, vegetables, rice, watermelon, grassland, maize, lettuce, oranges, grapefruit, guava, sugarcane by following the method described by Kyu and Koyama (1997) (Figure 1).

Figure 1. The procedure of IMO collection

Sites of sample collection are presented in Table 1. At each sampling site, three plastic baskets with a size (25 x 15 x 8 cm) were used, corresponding to 3 replicates. Each basket was filled with 1 kg of steamed rice and covered on the top of the basket with cloth and waist belt. The baskets were buried underground at each sampling site and covered the top of the baskets with leaf litters for three days. After three days of incubation, all fermented rice colonized by indigenous microorganisms was harvested, put into a glass jar and carried to the laboratory. This source of microorganisms was called IMO1. Collected IMO1 were mixed with brown sugar paste with a ratio of 1:1 (w/w) until the mixed material became gooey. This mixed material was stored in the ceramic pot in a cool area and away from direct sunlight for seven days for another fermentation time. After seven days of fermentation, this source of microorganisms was called IMO2 and the IMO2 was kept in the refrigerator at 4^{o}C for further studies (Le, Nguyen, & Nguyen, 2018).

Table 1

The located of nineteen collected IMO samples in Soc Trang province

Code	Origin of IMO (Farming system)	Located in Soc Trang province
TP-1	Bamboo	Phu Tam commune, Chau Thanh district
MQ-2	Crop rotation	5 Ward, Soc Trang city
CP-3	Banana	7 Ward, Soc Trang city
HV-4	Shallot	Vinh Phuoc commune, Vinh Chau district
RP-5	Lettuce	3 Ward, Soc Trang city
LM-6	Rice	My Xuyen town, My Xuyen district
DL-7	Watermelon	Truong Khanh commune, Long Phu district

Code	Origin of IMO (Farming system)	Located in Soc Trang province
CL-8	Grassland	Truong Khanh commune, Long Phu district
BT-9	Maize	Thanh Tri commune, Thanh Tri district
RM-10	Vegetables	Thanh Quoi commune, My Xuyen district
CK-11	Oranges	Xuan Hoa commune, Ke Sach district
BK-12	Grapefruit	Xuan Hoa commune, Ke Sach district
OK-13	Guava	Xuan Hoa commune, Ke Sach district
MC-14	Sugarcane	Đai An II commune, Cu Lao Dung district

Source: The researcher's data analysis

2.2. Determining the presence of bacteria, fungi, and actinomyces in different IMO2

Firstly, the total DNA of each IMO2 was extracted by MO BIO kit (MOBIO Laboratories, QIAGEN Company, Valencia, CA), then used 27F/1492R primers for PCR reaction to amplify 1500bp sequences of 16S rARN of bacteria (Lane, 1991). The nucleotide sequence of 27F primer includes 5'AGA GTT TGA TCC TGG CTC AG3' and the nucleotide sequence of 1492R contains 5'GGT TAC CTT GTT ACG ACT T3'. All PCR amplifications were carried out in 20µL reactions containing 10µL Green mix (2X), 1µL 27F primer (10µM), 1µL 1492R primer (10µM), 2µL pure DNA and 6.0µL deionized water. All reactions were run on the GeneAmp PCR System 9700. Samples were amplified using the following parameters: 5-min initial denaturation of DNA at 95°C, followed by 30 cycles of 1-minute denaturation at 94°C, 1-minute primer annealing 53^{o}C, and 90-minute extension at 72°C. Amplification was completed by a final extension step at 72°C for 7 minutes. In addition, ITS1/ITS4 primers were used for PCR reaction to amplify 675bp sequences of the ITS gene region of fungi. Fungal DNA amplification began with an initial denaturation at 95°C for 5 min, 30 cycles of 96°C for 1min, 63°C for 1min, 72°C for 1min and followed by the step of 72°C for 7min (Tao, Liu, Hyde, Lui, & Yu, 2008). The nucleotide sequence of ITS1 and ITS4 primers contains 5'-CTTGGTCATTTAGAGGAAGTAA-3' and 5'-TCCTCCGCTTATTGATATGC-3', respectively. Finally, 243F/1378R primers were used for PCR reaction to amplify 1175bp sequences of 16S rARN actinomyces (Heuer, Krsek, Baker, Smalla, & Wellington, 1997). The actinomyces DNA started with an initial denaturation step of 95°C for 5min before 30 cycles of 94°C for 1min, 63°C for 1min, 72°C for 2min and then a final step of 72°C for 10min for the last cycle. To visualize the PCR products, 5µL of the reactions were loaded into 1.5% of agarose gel, 5µL of 100bp DNA ladder was also loaded into a gel as a molecular weight biomarker. Gels were run for 30 minutes at 150 volts and 500 milliamps and then visualized and photographed by UV light from Gel Logic 1500 (Kodak) to find target sequences with the size of 1465bp for bacteria, 675bp for fungi and 1175bp for actinomyces, respectively.

2.3. Cell counting of bacteria, fungi, and actinomyces in collected IMO2

An aliquot of 10 grams of each IMO2 was put into a 250mL glass bottle containing 90mL sterilized distilled water on a shaker at a speed of 150 rpm for an hour and left stand for 5 minutes after shaking. A series dilution with a factor of 10 was prepared. An aliquot of 50µL of each dilution was spread on PDA, TSA, and Starch agar plates as media for bacteria, fungi, and actinomyces, respectively. Each dilution of each medium was repeated triply. The composition of PDA medium (g/L) contained 200g potato infusion, 20g dextrose, 20g agar for 1 L distilled water. The composition of TSA medium (g/L) comprised 17g tryptone, 3g soya peptone, 5g sodium chloride, 2.5g dipotassium hydrogen phosphate, 2.5g dextrose. The composition of 1liter Starch medium containing 20 g Starch, 1.15g K2HPO4, 1.5gMgSO4, 1g NaCl, 2g (NH4)2SO4, CaCO3 and 20g agar. In addition to (500 IU/L) streptomycin antimicrobial compound, TSA supplemented with 500 IU/L nystatin antifungal compound for bacteria and Starch medium supplemented with K2Cr2O7 0.4%for actinomyces. Samples were placed in incubators at 30^{o}C for 3-7 days. Finally, the number of microbes developed on each agar medium was counted to calculate the numbers of colony-forming units.

2.4. Determination of the presence of Coliforms, E. coli, Salmonella, Shigella spp. in collected IMO2

Salmonella and *Shigella* numbers were determined by counting their colonies on selective media for only *Salmonella* and *Shigella* (SS agar) after incubation for 48 hours at 37^{o}C (Taylor & Harris, 1965). An aliquot of 100µL IMO2 solution (prepared in section 2.3) was transferred and spread onto the SS medium agar. Three replications were repeated for each dilution. All agar plates were placed in an incubator at 37°C and the presence of *Salmonella* and *Shigella* colonies after 48 hours of incubation was examined.

Coliform numbers were determined by transferring 0.4mL of each dilution concentration (in 2.3) into a 20mL test tube containing 3.6mL of Lauryl Sulphate Broth medium (LSB) and an upside-down Durham tube (5 replications for each diluted concentration). The composition of Lauryl Sulphate Broth medium (g/L) included 20g Tryptose, 5g lactose, 5g NaCl, 2.75g KH2PO4, 2.75g K2HPO4, and 0.1g Sodium lauryl sulfate. The tubes were incubated at 34.5^{o}C, for 48 hours and then observed and recorded the presence of air bubbles in the sample. The sample was identified as negative for *Coliform* when air bubbles did not exist in the liquid medium of the test tubes and the samples were considered positive for *Coliform* when air bubbles appeared in the liquid medium of the test tubes. To quantify the numbers of *E.coli* in the sample, transferred 0.4mL liquid media of test tubes showing positive *Coliform* into a new test tube containing 3.6mL of Escherichia coli broth (EC broth) and upside-down Durham tube with no air bubbles. The composition of EC broth medium containing 20g tryptose, 1.5g of Bile salts No.3, 5g lactose, 1.5g KH2PO4, 4g K2HPO4, 5g NaCl, pH 6.8 in 1 litter. The test tubes were incubated in an incubator for 24-hour at 44.5°C. The sample was identified as negative for *E.coli* when air bubbles did not exist in the liquid medium of the test tubes and the samples were considered positive for *E.coli* when air bubbles appeared in the liquid medium of the test tubes. The density of *Coliforms* sp. and E.coli were counted by the Most Probable Number method (MPN).

2.5. Data analysis

The data were analyzed by ANOVA and compared by the DUNCAN test with MINITAB version 16 software.

3. Results and discussion

3.1. The presence of bacteria, fungi, and actinomyces in collected IMO2

The results of a polymerase chain reaction for testing the presence of three main groups of microorganisms including bacteria, fungi, and actinomyces are shown in Figure 2 indicating that all three main groups of microorganisms were detected in all collected IMO2 samples. For bacteria, the 27F/1492R primers amplified successfully a fragmented DNA with a 1475bp length of bacteria's 16S rARN gene from DNA of all collected IMO2 (Figure 2A). This result reveals that there was a presence of bacterial community in all collected IMO2 samples from the different farming ecosystems, the presence of bacteria and the clear visual band with different intensities of each well in agarose gel electrophoresis of Figure 2 indicated for the different population of the bacterial community of IMO2. Similarly, all collected IMO2 were recorded to have the size of target sequences (650bp) when running PCR by ITS1F/ITS4R primers for fungi (Figure 2B). Therefore, the fungi community was also presented in all 14 collected IMO2 samples. Moreover, special primers for actinomyces, 243F/1378R also successfully amplified target sequences (1150bp) of the 16S rARN gene for actinomyces in IMO2 (Figure 2C). This proved that in all IMO2 samples collected there was the presence of actinomyces, although it is hard to know which species and genera group of actinomyces. In general, the results of this study suggest that all IMO2 collected from different ecosystem habitats have a very high microbial diversity including bacteria, fungi, and actinomyces as three main groups of microbes in IMO2 samples.

This result was consistent with the study of Kyu and Koyama (1997), Kalsom and Sariah (2006) and Reddy (2011). These studies indicated that indigenous microorganisms composition containing bacteria, fungi, actinomyces, nematodes, protozoa, ... and the activities of these microbial groups would help to increase biodegradation, nitrogen fixation, phosphorus dissolution and synthesis of plant growth promotion, soil fertility recovery and improvement and organic matter decomposition in the soil. Consequently, crop yields will be improved, reducing pathogenic microorganisms and increasing plant defense.

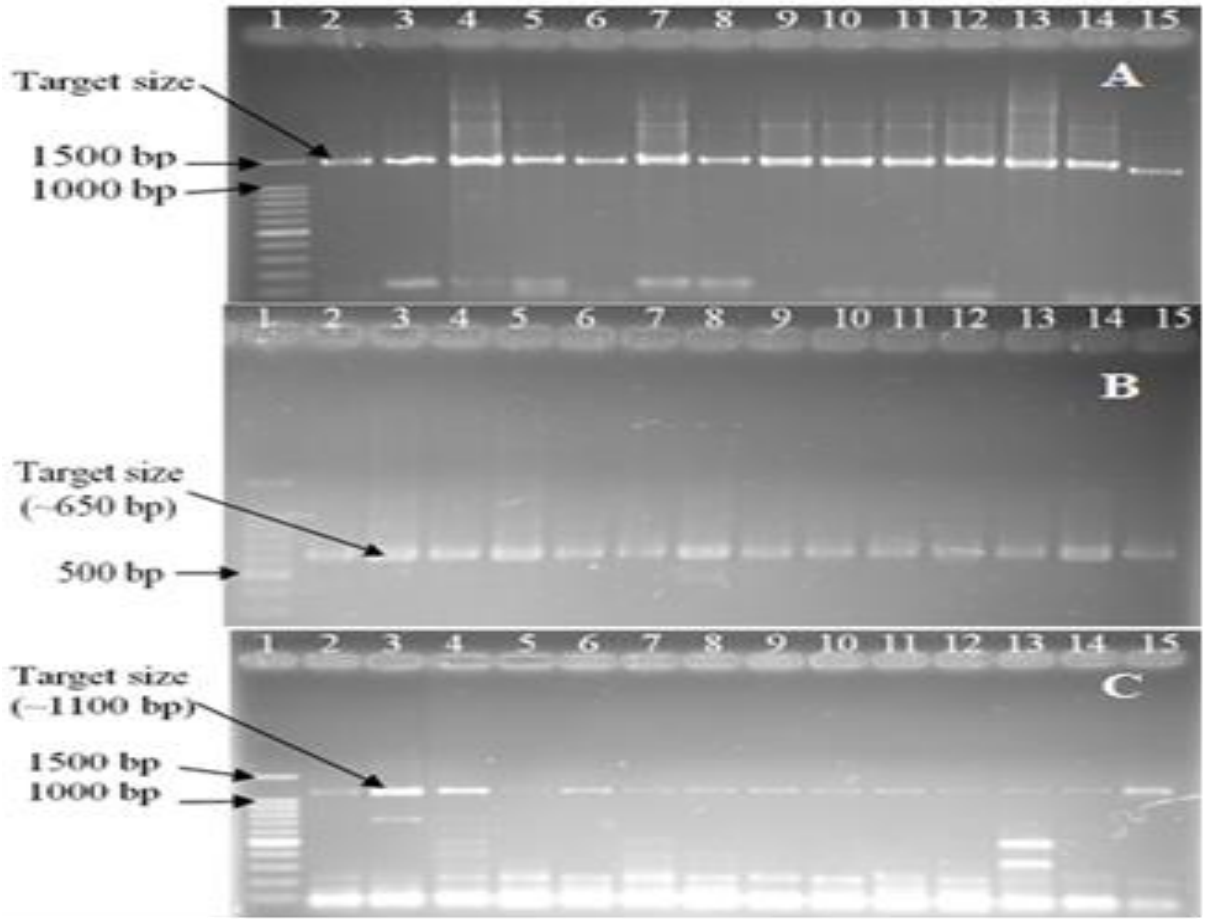

Figure 2. PCR products of specific sequences for bacteria (A), fungi (B) and actinomyces (C) by special primer amplification for 14 collected IMO

*Note: Lane 1: 100bp standard ladder; lane 2: IMO from bamboo; lane 3: IMO from crop rotation; lane 4: IMO from banana; lane 5: IMO from shallot; lane 6: IMO from vegetables; lane 7: IMO from rice; lane 8: IMO from watermelon; lane 9: IMO from grassland; lane 10: IMO from maize; lane 11: IMO from lettuce; lane 12: IMO from oranges; lane 13: IMO from grapefruit; lane 14: IMO from guava; lane 15: IMO from sugarcane

3.2. The numbers of bacteria, fungi, and actinomyces in collected IMO2

The numbers of bacteria, fungi, and actinomyces in fourteen different IMO2 are presented in Table 2. The numbers of 3 main groups of microorganisms within each indigenous microorganism were different and between collected IMO from different soil ecosystems were also different. In general, in each IMO, the bacterial population was significantly higher than the other two groups, and the different microbial composition between IMO was accumulated from different agro-ecosystems.

3.2.1. Bacteria

It can be seen from Table 2 that the number of bacteria in IMO2 obtained from different habitats varied significantly when compared with each other. Bacterial numbers in the IMO ranged from 1.36×10^7 to 2.13×10^9cfu/g IMO in which IMO collected from guava soil has the highest numbers of bacteria (2.13×10^9cfu/g IMO) and was significantly higher than bacterial

numbers of the other remaining IMO. The IMO collected from maize, vegetables, and sugarcane cultivated fields together shared second place with the same numbers of bacteria for all three treatments (32 x 10^7cfu/g IMO). The lowest number of bacteria was found in IMO collected from crop rotation system, banana, and shallot cultivated fields with several 3.70 x 10^7, 2.15 x 10^7 and 4.75 x 10^7cfu/g IMO, respectively while the other remaining IMO had higher numbers of bacteria and ranged from 8.45 x 10^7 to 28.40 x 10^7cfu/g IMO.

Table 2

The microbial density of fifteen indigenous microorganism communities

Number	Origin of samples (Farming system)	Microbial density		
		Bacteria (10^7cfu/g IMO)	Fungus (10^6cfu/g IMO)	Actinomyces (10^5cfu/g IMO)
1	Bamboo	13.30^{bcd}	143.5^{a}	3.67^{d}
2	Crop rotation	3.70^{cd}	20.50^{cd}	2.80^{d}
3	Banana	2.15^{d}	91.50^{b}	1.93^{d}
4	Shallot	4.75^{cd}	27.00^{cd}	2.93^{d}
5	Lettuce	13.60^{bcd}	2.05^{d}	51.33^{bc}
6	Rice	13.15^{bcd}	51.50^{bc}	113.3^{a}
7	Watermelon	24.90^{bcd}	140.0^{a}	118.0^{a}
8	Grassland	21.05^{bcd}	2.05^{d}	61.33^{b}
9	Maize	32.50^{b}	3.00^{d}	30.00^{cd}
10	Vegetables	32.35^{b}	3.55^{d}	24.67^{cd}
11	Oranges	28.40^{bc}	2.65^{d}	2.60^{d}
12	Grapefruit	8.45^{bcd}	24.00^{cd}	2.60^{d}
13	Guava	212.50^{a}	93.50^{b}	3.27^{d}
14	Sugarcane	32.45^{b}	32.50^{cd}	18.00^{d}
15	Mix	11.75^{bcd}	30.50^{cd}	24.00^{cd}

*Note: Values in the same column having the same letters are not a significant difference at 5% level ($p<0.05$)
Source: The researcher's data analysis

3.2.2. Fungi

The results presented in Table 2 show that the fungal density in all IMO was lower than the bacterial density and among different IMO, the fungal density was significantly different from each other. The fungi's numbers of IMO collected from bamboo plantation was highest with the number of 143 x 10^7cfu/g IMO, and next, the number of fungi in IMO collected from

bananas and guava farms were 91.5 x 10^6 and 93.5 x 10^6cfu/g IMO and significantly higher than those of the remaining IMO. Followed by IMO collected from the rice field with a fungal number of 51.5 x 10^6cfu/g IMO. In contrast, the other five IMOs collected from lettuce, grassland, maize, vegetables, and oranges farms had the lowest density of fungi with a variation between 2.05 x 10^6 and 3.55 x 10^6cfu/g IMO while the remaining IMO had fungal numbers ranged from 20.5 x 10^6 to 30.5 x 10^6cfu/g IMO.

3.2.3. Actinomyces

The numbers of actinomyces in fifteen different IMO are presented in Table 2. The results illustrated that the numbers of actinomyces were significantly lower than that of bacteria and fungi and between IMO collected from different soil ecosystems; there was also a strongly significant difference among each other in terms of bacterial numbers. Particularly, two IMO collected from rice and watermelon farms had the highest numbers of actinomyces with several 113.0 x 10^5 and 118.0 x 10^5cfu/g IMO, respectively. While the lowest density of actinomyces was found with a range between 1.93 x 10^5 and 3.67 x 10^5cfu/g IMO in IMO from bamboo, crop rotation, banana, shallot, oranges, grapefruit, and guava farms. Other remained IMO had numbers of actinomyces varied between 18.0 x 10^5 and 61.33 x 10^5cfu/g IMO.

In a comparison with other previous studies, Chiemela et al. (2013a) indicated that in the bamboo forest IMO there was a richness of bacterial population with a number at 2.8 x 10^9cfu/g IMO, followed by the fungi at 4.2 x 10^4cfu/g IMO. Also, according to this study, the growth of microorganisms in IMO depended on various factors such as origin, temperature, pH, incubation period, carbon, moisture, etc. Similarly, Abu-Bakar and Ibrahim (2013) evaluated the numbers of bacteria in composts and showed that the number of bacteria was increased very, from 3.1 x 10^7 cfu/g to 3.1 x 10^8cfu/g while the numbers of fungi were decreased drastically from 1.8 x 10^8cfu/g to 1.6 x 10^6cfu/g after day 10 incubation days. The numbers of actinomycetes were slightly dropped during the composting process. However, it is shown that the total of the actinomycetes population remained high throughout the entire composting process until the curing phase.

This result revealed that there was a presence of high numbers of three main microbial groups in all fourteen studied IMO and this result implies that almost all IMO could be considered as a good source of beneficial microbes for soil improvement as well as plant growth promotion. According to Reddy (2011), indigenous microbes are good sources of microbes for farming because they are very powerful and effective. They can survive better under the extreme climatic conditions of the local environment than under artificial cultures and environments. Since they have resided and already adapted to the local conditions, they are considered to be the best survival source of microbes for soil and plant improvement effectively.

3.3. Coliforms, E. coli, Salmonella, Shigella spp. in the collected IMO2

The results of the experiment showed that there wasn't any presence of *Salmonella* and *Shigella* in all fifteen collected IMO2 samples. Thus, no *Salmonella* and *Shigella* colonies were detected in SS agar media (Figure 3). It could be that the temperature and pH of the fermentation process to create IMO samples limited the survival of these two microorganisms in IMO.

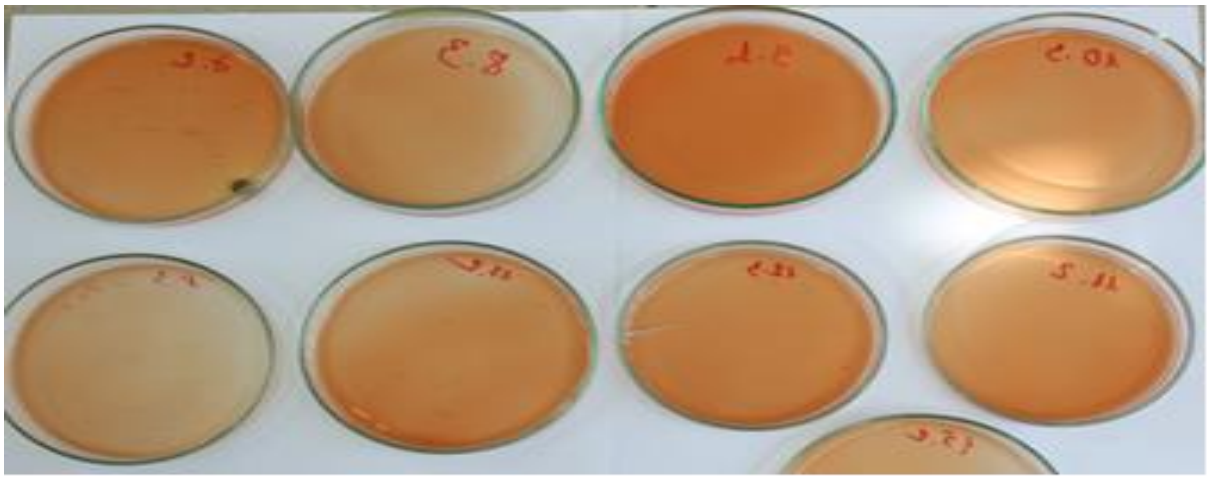

Figure 3. SS agar medium after 48 hours of incubation

Similarly, for *Coliform* and *E.coli*, the results of the survey experiment showed that there was no presence of *Coliform* and *E.coli* in all fifteen collected IMO samples since all the tested samples did not have any turbidity and bubble in Durham tubes in the samples (Figure 4). Therefore, it can be concluded that *Coliform* and *E.coli* don't exist in our 14 collected IMO samples. This could be explained by the fact that the environmental conditions like temperature, pH of samples and other factors during the fermentation process were not good and suitable for these groups of bacteria to survive and develop.

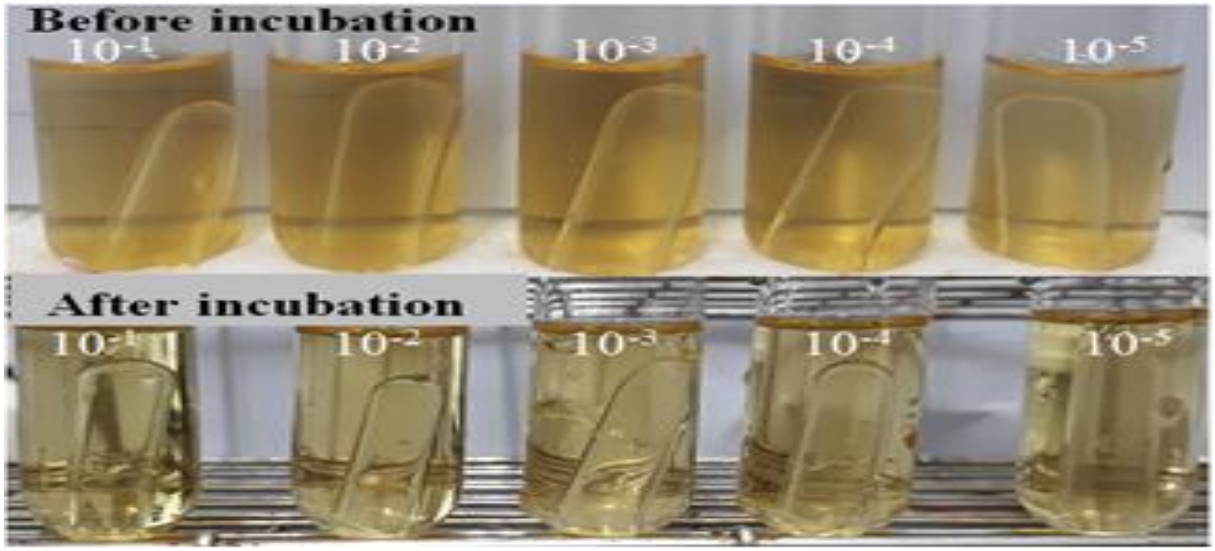

Figure 4. The test tube containing LSB medium with no air bubbles in Durham tubes both before and after 48 hours of incubation

It is very interesting that although bacteria are detected with a high density in each IMO, there are not any harmful bacteria in it. According to Molina et al. (2015), public health protection requires the prompt evaluation of microorganisms in drinking water, raw and processed foods as well as bio-fertilizer to prevent outbreaks of microbial contamination. Therefore, the evaluation of harmful bacteria such as *Salmonella*, *Shigella*, *Coliforms*,

particularly *Escherichia coli* cause sickness for the human is necessary. In short, all surveyed IMO samples did not have any contamination of human disease pathogens like *Salmonella*, *Shigella*, *Coliforms*, and *E.coli*. These results imply that collected IMO contains the high diversity of major groups of microbes and can be used as safe bio-stimulants for clean vegetable production.

4. Conclusion

A big community of bacteria, fungi, and actinomyces were detected in all fourteen collected IMO samples from many diverse origins of habitats by a tool of biological molecular technology. The largest number of microbes was recorded for bacteria with a range between 1.36×10^7 and 2.13×10^9cfu/g IMO sample whereas the numbers of fungi and actinomyces varied from 2.05×10^5 to 1.40×10^7 and 1.80×10^5 to 1.18×10^7cfu/g IMO sample, respectively. Especially, all surveyed IMO samples have not been found to have any contamination of human disease pathogens such as *Salmonella*, *Shigella*, *Coliforms*, and *E.coli*. These high diversities of all collected IMO can be exploited for enhancing soil fertility and plant growth. Moreover, using IMO for plants as a bio-fertilizer source is safe for human health. IMO is a great potential source of beneficial microbes that can be used for the isolation of beneficial microbes for bio-fertilizers to enhance soil health, quality and fertility and for plant growth promotion and sustainable and clean agricultural development. It is obvious to accept the fact that the study on the functions of the IOM for agricultural application has still lacked and many outstanding and good results in this research field are still waiting.

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