Isolation, Characterization, Evaluation and Comparative Study of Beneficial Microorganisms among the Agricultural Soil, Saline Intruded Soil and Commercial Biofertilizers

Bipasha Sur[1], Md. Kawser Ahmed[2], Seema Rani[1]*, Md. Latiful Bari[3], Md. Jobaer Alam[2], Abdullah Al Mamun Siddiqui[1] and Rupak Loodh[1]

[1]Bangladesh Oceanographic Research Institute (BORI), Cox's Bazar, Bangladesh
[2]Department of Oceanography, University of Dhaka, Dhaka 1000, Bangladesh
[3]Center for Advanced Research in Sciences, University of Dhaka, Dhaka 1000, Bangladesh

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ABSTRACT : Degradation of soil health is a growing concern as both human health and soil microbial community are directly related to this. One of the main hazards to soil deterioration in Bangladesh is soil salinization. In this study, the microbiological status of two commercial biofertilizers, an agricultural soil, and saline soil in Bangladesh were analyzed and compared. Five saline soil samples from various agricultural fields in Satkhira, two commercial biofertilizer samples, and one agricultural soil from Hajee Danesh Agricultural University were collected to carry out this work. The saline soil sample's physicochemical properties, such as pH and salinity, were examined. Saline soil had a salinity range of 0.17-1.60 ppm and a pH range of 6.20-7.22. Five beneficial bacteria, three types of food-borne pathogen indicators, three types of soil quality indicators, and one plant pathogen indicator bacterium were selected for the microbiological investigation. The microbial status of the five beneficial bacteria was observed; for Rhizobium, the range was not detected to 7.62 log CFU/g, for Azotobacter, it ranged from 3.11 log CFU/g to 5.72 log CFU/g, for phosphate-solubilizing bacteria, it ranged from 3.87 log CFU/g to 4.69 log CFU/g, and for Bacillus, it was not detected to 4.65 log CFU/g. The helpful bacteria *Pseudomonas* spp. and the plant pathogen inhibitor *Trichoderma* spp., which are deemed important markers for soil health, were found to be absent in all the samples.

Keywords: Biofertilizer; Beneficial Microorganisms; Plant Pathogen; Soil Fertility; Salinity

INTRODUCTION

Bangladesh's economy is driven by agriculture, which accounts for the majority of private-sector employment and makes a higher contribution to the GDP of the nation. According to an official assessment, this industry is in danger because the amount of productive land is decreasing by 1% per year. The factors responsible for this downward trend include a decline in soil fertility, an increase in soil erosion, and in soil salinity (Rahman, 2017). Agriculture has been contributing to our economy since the 1971 Liberation War. In 2021, it accounted, for 13.02% of our GDP and employee ~40% of the working force (Bangladesh Bureau of Statistics, 2022). It is a matter of concern that the agriculture industry heavily relies on synthetic inputs rather than on natural resources. As a result, the sustainability of agriculture is under threat and a move towards resource-conserving agriculture is imperative (Shannon et al., 2016; Stockdale et al., 2002).

*Corresponding Author: Seema Rani
Email: seema@bori.gov.bd
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Soil quality, a significant natural resource, is one of the primary factors affecting agricultural yield and sustainability. As quality soil ensures plant, animal, and human health, it is vital that soil quality be maintained for a good agricultural output (Fageria, 2002). However, many parts of our country's soils are woefully low in important macro- and micronutrients such as potassium, zinc, and boron. The majority of the country's representative soils have been examined by several national institutes, but no soil quality index (SQI) has been developed (Bishwas et al., 2019) (Doran & Parkin,1994). In systems that produce arable crops, regular applications of inorganic fertilizer and manure are crucial to soil management. Although the main purpose of these amendments is to make more nutrients available to plants, they might also have an impact on the soil's microbes (Wardle, 1992). The application of organic amendments by reducing the application of inorganic fertilizers may be an economically viable and ecologically sound strategies to achieve sustainable agriculture. Excessive use of inorganic fertilizers may have negative effects on soil quality

(Ning et al., 2017). In order to maintain healthy levels of soil organic material, which in turn maintains soil structural and biological fertility, researchers must find reliable and sustainable ways to increase soil agricultural productivity. Biofertilizers could be very good alternative for maintaining soil health.

Environmental risks and sustainable agriculture issues have gained a lot of attention in the recent past. Given the increased expense of chemical fertilizers and their detrimental impact on soil health, the function of microbial biofertilizer in agriculture is expected to be more important (Kumar et al., 2017). Microbial biofertilizers are biological preparations of enough strains of microorganisms that aid in the development of plants in rhizospheres (Farfour et al., 2015). Preparations include active (metabolically active) or inactive (living) cells. They are used to treat seeds or the soil. Biofertilizer emboldens all the natural processes requires for nitrogen fixation, phosphorus solubilization, and plant growth stimulation via the manufacture of necessary chemicals that promote growth by supplying natural nutrients (Saeidet et al., 2019).

Study Area

Five saline soil samples (Buri Goalini, Gabura, Ishwaripur, Kashimari, Munshiganj) were collected from different agricultural fields located at Satkhira in Khulna Division. Soil samples were collected at a range of 0 to 30 cm vertically using sterile spatula and placed sterile plastic bag. Non-saline (control) soil samples were collected from the field of Hajee Danesh Agriculture University. The samples were instantly carried to the laboratory in an icebox by maintaining a constant temperature 4°C. These samples were analyzed for various sensitive parameters (salinity, temperature, and pH) within 24 hours.

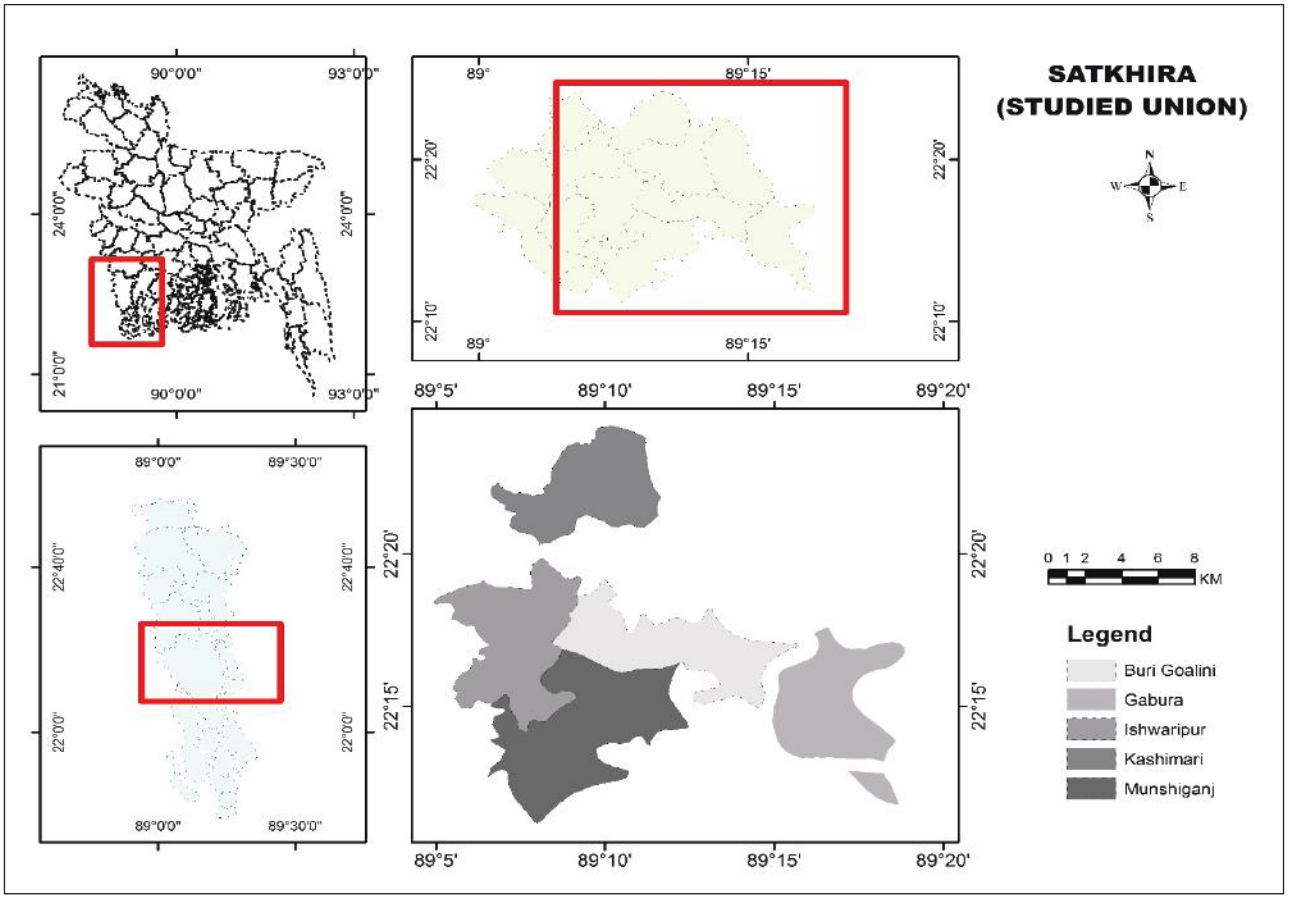

Figure 1: Location of Study Area

METHODOLOGY

Measurement of Physical and Chemical Parameters

By making a 10% soil solution and measuring the pH using a glass electrode pH meter, soil pH was determined (using Jenway, 3305). A saline measuring meter was dipped in the soil for 30 minutes to measure the salinity of the soil (Devatha et al., 2019)

Microbiological Analysis

Five (5g) soil samples from each type were weighed and added to a sterile stomacher bag with 45 ml of sterile normal saline for the purpose of isolating bacteria. With a stomacher machine set to 230 rpm (rotation per minute) for 90 seconds, each soil sample was separately homogenized before being serially diluted with sterile saline water. Both original and diluted samples were spread with sterile spreaders onto petri plates containing selective and non-selective media. One colony forming unit (CFU) was assigned to each colony that emerged on the plates (Sau *et al*., 2017). All plates were incubated for the requisite number of hours at the appropriate temperature, and once the incubation time was through, final counts of CFU were made. C.F.U. was determined as:

$$\text{No of Bacteria}\left(\frac{CFU}{gram\ of\ soil}\right) = \frac{No.\ of\ colonies}{Inoculum\ size\ (mL) \times dilution\ Factor}$$

On the basis of colony features, microorganisms were separated from their unique selective medium under various optimal conditions. Table.1 lists the isolated microorganisms.

Table 1: Isolation of Microbes using Standardized Colony Characterization, Selective Culture Medium, and Incubation Conditions

Microorganisms	Media	Colony	Incubation condition
Total aerobic bacteria	Trypticase Soy Agar	Creamy white, yellow, green color	37 ºC, 24 hours
Coliform	Chromocult Agar	Creamy white	
E. coli	Chromocult Agar	Dark blue to violet	
Pseudomonas spp.	Cetrimide Agar	Yellow green to blue green colonies	
Salmonella spp.	Xylose lysine deoxycholate agar	Black centre yellow color	
Rhizobium spp.	Congo Red Yeast Extract Mannitol Agar	Pink	(30-32) ºC, 4-7 days
Azotobacter and Nitrogen fixing fungus	Nitrogen free Agar	Whitish or cream color	
Phosphobacteria and Phosphate solubilizing fungus	National Botanical Research Institute Phosphate Bromo Phenol Blue (NBRIP-BPB) medium	Blue and white	
Total fungus count	Soya Dextrose Agar	White, Creamy white colony	30 ºC, 24-48 hours
Trichoderma spp.	Soya Dextrose Agar	Greenish to Black colonies	(30-32) ºC, 4-7 days
Bacillus spp.	NGKG	Pink	30 ºC, 24-48 hours

Purification of Isolates

The selected isolates were purified through repeated plating (by streak plate when a plate yielded only one type of colony the organism was considered as pure isolate based on biochemical and morphological characteristics. Though in a few cases, phenotypic tests can result in confusing biochemical profiles and for "difficult-to-identify" isolates, 16s confirmation could be performed for better characterization (Valenzuela-Tovar et al., 2005). However, bacterial isolates were grown on selected bacterial media and for further confirmation API 50 CHB test was followed.

Maintenance of and Preservation of Isolates

For long term storage glycerol stocks were prepared from the pure culture of isolates. To prepare glycerol stock, pure culture of all isolates were inoculated into Tryptic Soy Broth (TSB) and incubated overnight. After overnight growth, 700µL liquid culture was taken into an eppendorf tube supplemented with 300 µL of 20% autoclaved glycerol and gently mixed. The stock was kept at 4^0 for 24-48 hours followed by preservation at -20^0C.

Identification of Microorganisms

- ***Primary Identification:*** Observing standard colony characterization on their particular selective culture media under various optimal incubation conditions was the main method used to identify microorganisms.

- ***Secondary Identification:***

Identification of Bacteria by Analytical Profile Index: Identification of bacteria was confirmed by the analytical profile index. Pure colonies were inoculated into the analytical profile index (API) kit followed by incubation under optimum conditions. After identification, the bacterial colonies were stocked in tryptone soy broth medium at -20 ºC

Analytical Profile Index:

The Analytical Profile Index (API) is a speedy and advance technique for performing biochemical tests and closely related bacteria can be easily identified through this method of quick approach (Al-Mossawi et al., 2018) For that an API kit is needed which in needed to be composed of a base or tray and a cover. A base containing multiple chambers assist in holding the water for the test. A API strip entails of 20 discrete compartments. Individual compartment is made up of a small cupule and tube which holds specific dehydrated media for 20 different biochemical test able to perform at a time.

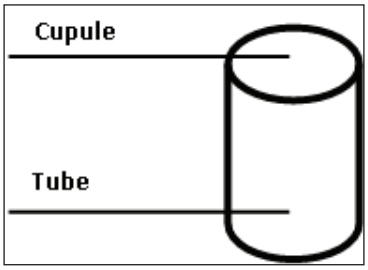

Figure 2: One Compartment with Cupule and Tube

A saline suspension of pure cultured bacteria was inoculated in the compartment of the strip containing the dehydrated media. After the inoculation process the cupule received the suspension of bacteria and allowed it to flow into the tube of the medium where the dehydrated medium was reconstituted with this suspension. For creating the anaerobic condition, sterile mineral oil was added to some compartments. The final reading was taken after incubation (24 h or less depending which API is being used. The color reactions were observed (some of the tubes will have color change due to pH differences and some with the aid of added reagent to detect end metabolic products). After separately performance of oxidase reaction a seven-digit code being found. A seven-digit code was identified followed by oxidase reaction test. Identification of the bacterium usually as genus and species was then easily carried out from the database with the relevant cumulative profile code book or software. With API 50 CHB panel, the identification of Bacillus spp. was done. The confirmation of the specific bacterial spp. was ensured using the database.

Identification of Microorganisms by API 50 CHB:

- API 50 CHB medium and API NaCl 0.85% medium were suspended with previously isolated identical bacterial colonies with different morphological entity. After that, the tubes with bacterial inoculation were vortexed to form a turbid bacterial suspension

which was equivalent to 2 McFarland units and then approximately 5 mL of distilled water was dispersed on the base for maintaining the moisture content of the specific medium.

- API strips of 50 CHB and 20 E from the sealed pouch were removed and placed onto the base. Additionally, the tubes (not the cupules) of the API 50 CHB strip were inoculated with API 50 CHB medium. If needed mineral oil can be added to hold the moisture content but it is not recommended for any aerobic bacterial isolation or any aerobic bacteria.
- Only the first 12 tests of the API 20 E strip were inoculated, as the last 8 tests were duplicated on the API 50 CHB strip. The GLU test was inoculated to reveal the NIT reaction.
- At 55° C ± 2° C for 3 - 3 ½ hours, 6 - 6 ½ hours and 24 hours (±2 hours) thermophiles bacteria were incubated. The same incubation technique and conditions were followed for API 20 E strip to further observe the result.

For the API 50CHB Strip:

-All tubes except tube number 25(esculin test) turned yellow from phenol red indicator contained in the medium.

-Tube number 25 turned black from red and considered as positive reaction.

-If a positive test turned negative at the second reading, only the positive result should be taken into account as this can be caused by alkalization due to the production of ammonia from peptone.

- The results were recorded on the result sheet

RESULT AND DISCUSSION

Microbial Analysis of Control Soil and Commercial Biofertilizer

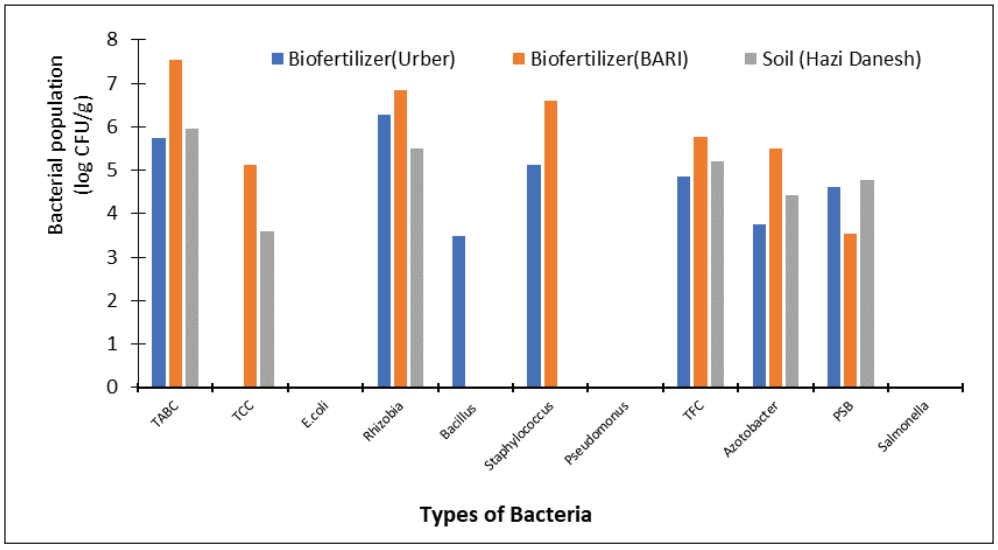

Figure 3: Comparison of Microorganisms in Different Commercial Biofertilizers and the Control Soil

Table 2: Comparison of Soil Samples from Commercial Biofertilizers and Controls for Soil Quality Bacteria, Foodborne Pathogens, Soil-beneficial Bacteria, and Plant Pathogen Inhibitor Bacteria

Types of Bacteria	Name of the Organism	Microbial Medium used	Biofertilizer (Urber) Log CFU/g	Biofertilizer (BARI) Log CFU/g	Soil (Hazi Danesh) Log CFU/g
Soil quality indicator	TABC	PCA	5.7 ± 0.40	7.5± 0.44	5.9± 0.54
	TCC	CHR	0	5.1± 0.14	3.6±0.20
	TFC	SDA	4.8 ± 0.45	5.8 ± 0.43	5.2± 0.04
Foodborne pathogen indicator	*E.coli*	CHR	<1.0	<1.0	<1.0
	Salmonella	BSA	<1.0	<1.0	<1.0
	Staphylococcus	MSA	5.1 ± 0.14	6.6±0.20	0
Soil beneficial bacteria	*Rhizobium* spp.	YECRA	6.4 ± 0.27	6.8±0.38	5.5 ± 0.13
	Azotobacter spp.	Ashby	3.7 ± 0.48	5.5±0.13	4.4± 0.31
	PSB	NBRIP	4.6 ± 0.20	3.5± 0.44	4.8 ± 0.38
	Bacillus spp.	NGKG	3.5 ± 0.37	<1.0	<1.0
	Pseudomonus spp.	CTD	<1.0	<1.0	<1.0
Plant pathogen inhibitor	Trichoderma spp	SDA	<1.0	<1.0	<1.0

*Biofertilizer (Urber) = Indian commercial biofertilizers supplied by AgriPlus Ltd.

**Biofertilizer (BARI) = Bangladeshi commercial biofertilizers supplied by Chemicon

***Soil (Hajee Danesh) = Agricultural plot for research soil of Hajee Danesh Agri University

Microbial Analysis of Saline Soil Sample

Table 3: Existing Correlation Among Types of Bacteria

	TABC	*TCC*	*TFC*	*Staphylococcus*	*Rhizobium spp*	*Azotobacter spp*	*PSB*	*Bacillus spp*
TABC	1.00							
TCC	0.34	1.00						
TFC	0.35	-0.25	1.00					
Staphylococcus	0.66	0.31	0.67	1.00				
Rhizobium spp	0.28	0.89	0.07	0.59	1.00			
Azotobacter spp	-0.07	-0.03	-0.16	0.36	0.18	1.00		
PSB	0.19	0.36	0.12	0.73	0.63	0.84	1.00	
Bacillus spp	0.54	0.07	-0.45	-0.21	-0.30	-0.12	-0.30	1.00

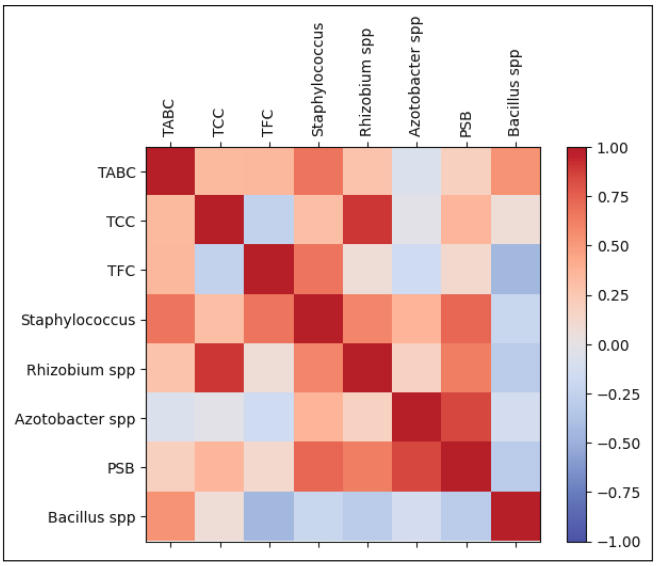

Figure 4: Comparison of Microbial Analysis of Different Saline Soil Sample from Satkhira

From the correlation analysis, it is clearly observed that relation among the bacterial species are highly correlated and vice versa. For instance, TCC, Rhizobium spp and *Staphylococcus,* PSB are highly positive correlated (0.89 and 0.73 respectively). In contrast, TFC, Bacillus spp and TFC, *Rhizobium* spp. are highly negative correlated *(*-0.45 and -0.30 respectively). No correlation study could be established and interpreted among *E. coli, Salmonella, Pseudomonas* and *Trichoderama* spp as the presence of these bacterial species were too less to be considered for statistical exploration.

For microbial analysis, 12 types of bacteria have been chosen. The microbial load in different agricultural soil samples varied from sample to sample. The aerobic bacterial count ranging from 6×10^4 Log CFU/g to 2.3×10^6 Log CFU/g. None of these soil samples contained *E. coli.* Numerous types of helpful bacteria have been discovered, with Azotobacter, Phosphate Solubilizer, Rhizobium, Baccillus, and Staphylococcus standing out.

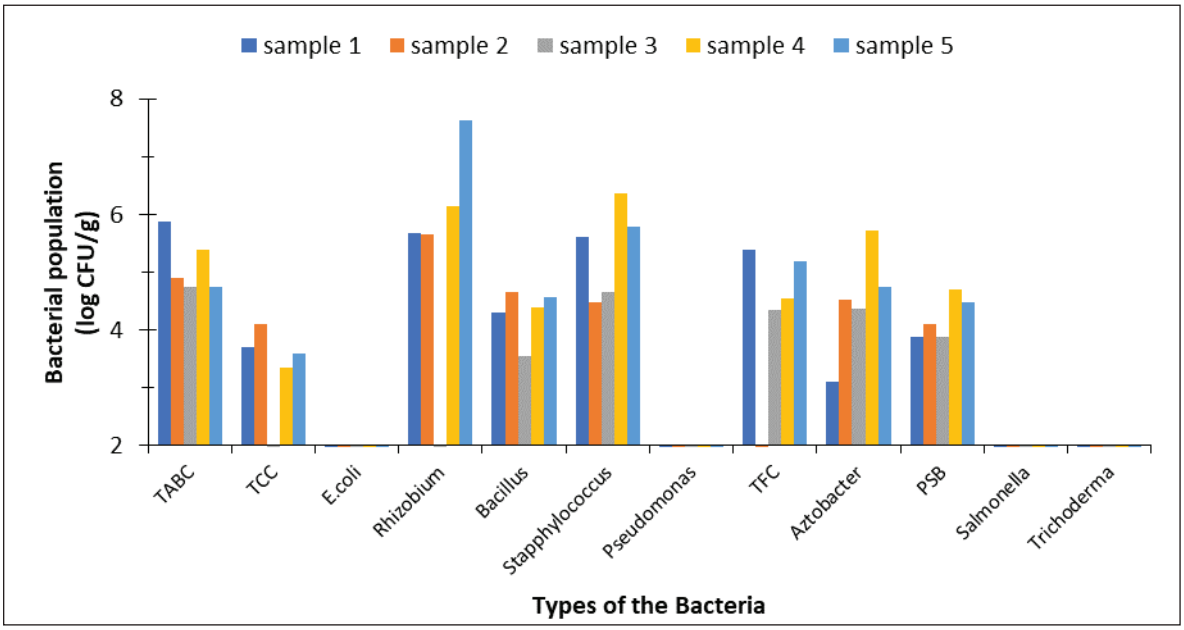

Figure 5: Comparison of Microbial Analysis of Different Saline Soil Sample from Satkhira

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Table 4: Comparison of Soil Quality Bacteria, Foodborne Pathogens, Soil Beneficial Bacteria and Plant Pathogen Inhibitor Bacteria in Salt Intruded Soil Samples in Different Agricultural Fields of Satkhira

Name of the Organism	Microbial Medium used	Saline soil sample 1	Saline soil sample 2	Saline soil sample 3	Saline soil sample 4	Saline soil sample 5
TABC	PCA	5.88 ± 0.68	4.90 ± 0.09	4.74 ± 0.06	5.9± 0.12	4.74 ± 0.01
TCC	CHR	3.71 ± 0.02	4.11 ± 0.05	<1.0	3.35±0.03	3.59 ±0.01
TFC	SDA	5.38± 0.02	<1.0	4.35 ± 0.07	4.54± 0.09	5.19 ± 0.02
E.coli	CHR	<1.0	<1.0	<1.0	<1.0	<1.0
Salmonella	BSA	<1.0	<1.0	<1.0	<1.0	<1.0
Staphylococcus	MSA	5.60 ± .01	4.48 ±0	4.65± 0.07	6.36 ± 0	5.78±0.8
Rhizobium spp	YECRA	5.68 ± .03	5.65 ±0.07	<1.0	6.15 ± 0.04	7.62± 0.70
Azotobacter spp	Ashby	3.11 ± 0.05	4.52 ±0.03	4.37 ±0.04	5.72± 0.71	4.75± 0.86
PSB	NBRIP	3.87 ± 0.02	4.10 ± 0.02	3.87 ±0.04	4.69 ± 0.36	4.48 ± 0.14
Bacillus spp	NGKG	4.32 ± 0.01	4.65 ± 0.75	3.54±0.09	4.39±0.12	<1.0
Pseudomonas spp	CTD	<1.0	<1.0	<1.0	<1.0	<1.0
Trichoderma spp	SDA	<1.0	<1.0	<1.0	<1.0	<1.0

No *Escherichia coli* or *Salmonella* spp. was observed in 5 saline soil samples. However, moderate levels of Staphylococcus spp. (4.48-5.78 log CFU/g) was observed in the soil samples. On the other hand, Total aerobic Bacterial count, total coliform count, total fungal count was recorded as 4.7 - 5.9 log CFU/g; coliform count ranged from non-detected to 4.11 log CFU/g was observed. Total fungal count was recorded as 4.3 - 5.38 log CFU/g, respectively. This value indicates the substantial reduction of non-pathogenic bacteria which consequently decrease the soil functional activity. Furthermore, substantial decrease in all the beneficial microorganisms was observed, that could limit the uptake of N, P, K by the plant. Although *Pseudomonas* spp. and *Trichoderma* spp. were not available in the saline soil, this soil was unable to protect the crop from plant pathogens.

In the presence of the appropriate bacterial strains, the micro biome of the rhizosphere is also altered, which is thought to be particularly advantageous for the enhancement of plant health. It has been suggested and shown that the use of Azotobacter and Rhizobium spp. in a variety of agricultural field crops reduced plant-induced stressors originating from a variety of sources. Through nitrogen fixation, phosphate solubilization, induced synthesis of growth hormone, and solubilization of organic molecules, these organisms can greatly contribute and be employed as potential bio fertilizers to improve the health of the soil. With the integrated use of bio fertilizer, organic manure, and chemical fertilizer systems, the bacterial population of Azotobacter and Azospirillum in soil after harvest was significantly enhanced, while it was decreased with the exclusive use of chemical fertilizers.

Microbial Assessment of Different Beneficial Bacteria in Saline Soil Sample and in Bio-fertilizer

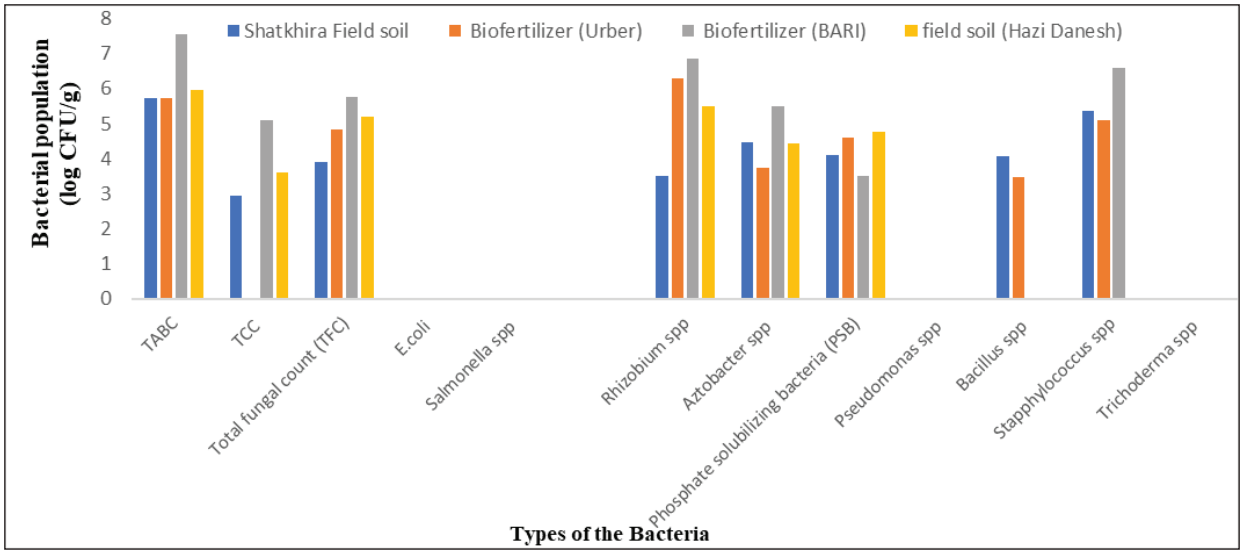
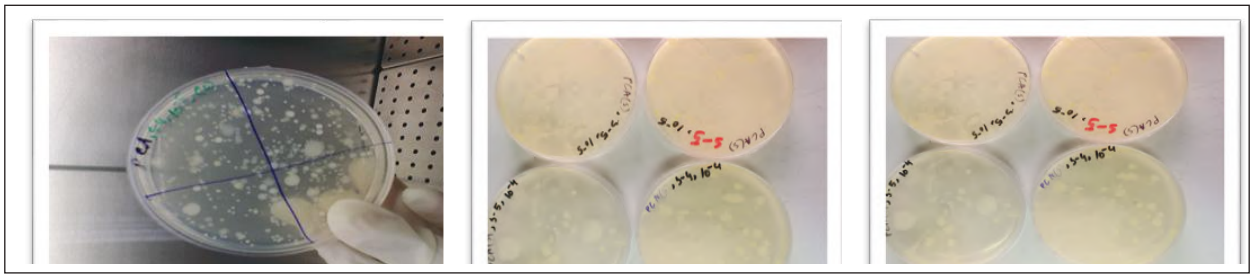


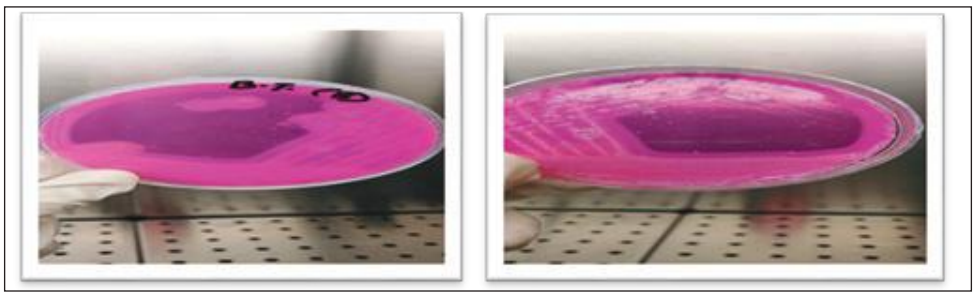
Figure 6: Comparison of Beneficial Bacteria in Satkhira Soil Sample, Commercial Biofertilizer and Control Soil Sample

Isolation and Identification

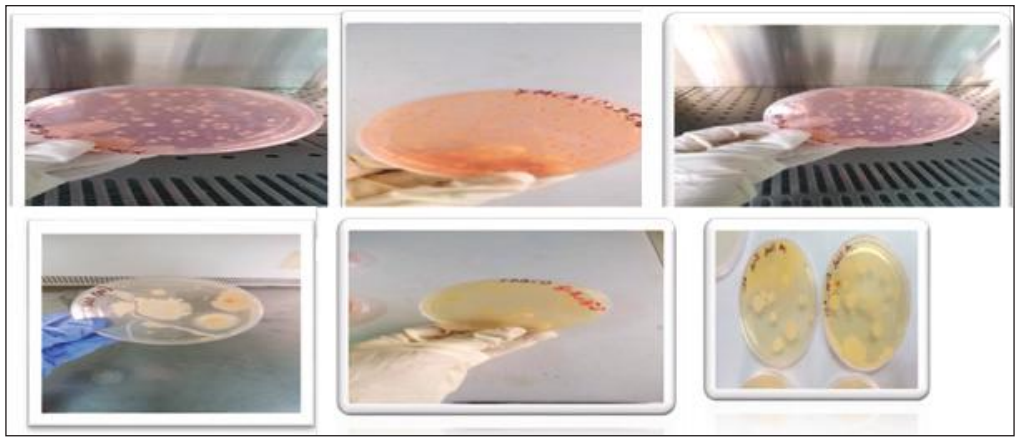
Among the twelve organisms, five beneficial bacteria were selected for characterization and further used in the field to increase soil fertility, the selected bacterial strains were *Rhizobium, Azotobacter,* Phosphate solubilizing bacteria, *Bacillus* spp., and *Trichoderma* spp. Individual organisms were grown in different and specific culture media and single isolated colony was observed. For each organism two pure culture colonies were isolated and preserved at -20 ^{0}C for further study.



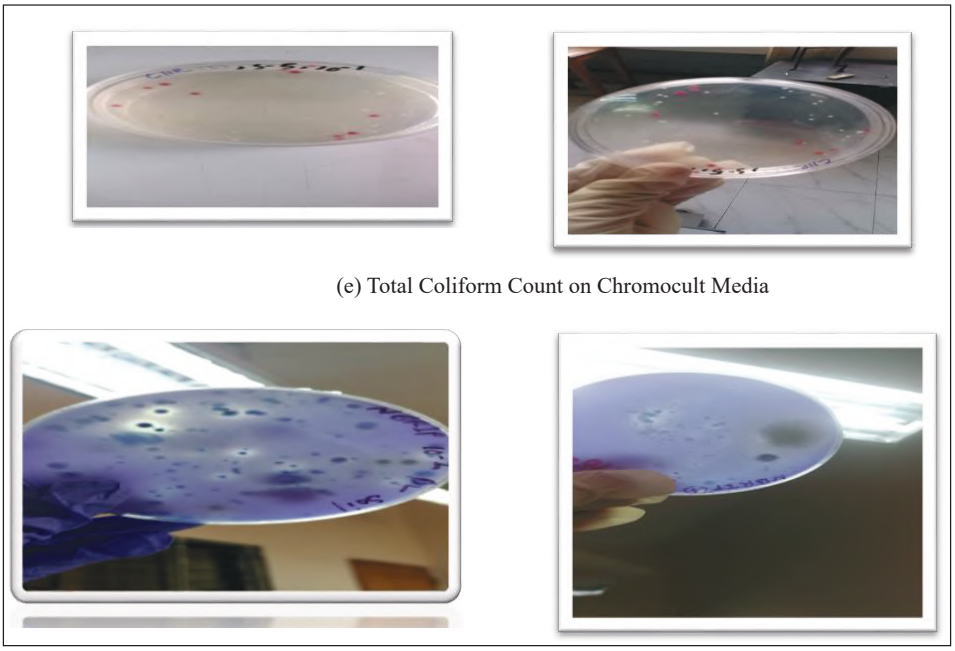
(a) Total Aerobic Bacterial Count on PCA Plate



(b) Bacillus spp on the NGKG Agar Media

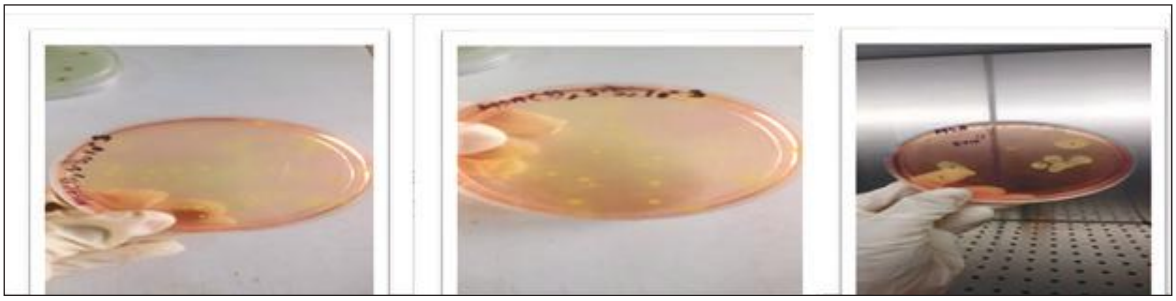


(c) Rhizobium on the YERCA Agar Media & (d) Total Fungal Count on SDA Media



(e) Total Coliform Count on Chromocult Media

(f) Phosphate Solubilizing Bacteria on NBRIP Media



(g) Staphylococcus spp on the Mannitol Salt Agar Media

Figure 7: Observation of Bactarial Colopies on different Selective Media

CONCLUSIONS

In this study, a total of 12 distinct bacteria were chosen to be studied from different agricultural field soil and from different biofertilizer samples. Among them four distinct isolates were identified from the saline intruded field soil samples based on their morphological, cultural, biochemical, and immune assay technique. Those isolates were identified as Azotobacter spp., Rhizobium spp., Bacillus spp. and phosphate solubilizing bacteria. The four found beneficial bacterial strains will be able to reduce a variety of unanticipated environmental stress in the soil biome. The result showed that among the different agricultural saline intruded soil sample and control soil sample, the microbial health of the saline intruded soil sample was lower than that of the control soil sample as salinity could provide stress to the resident bacteria or inhibitory to facilitate other beneficial microorganisms. The present study demonstrated that the commercial biofertilizer BARI contains more adjuvant bacteria than that of other bio-fertilizers (Urber) available in the market. In addition, nitrogen fixing bacteria including Rhizobium spp. and Azotobacter spp. were found higher in the BARI biofertilizers sample.

According to the findings, integrated nutrient management with bio fertilizers (Azotobacter and Azospirillum) in combination with 50% inorganic N through organic manure (VC or FYM) and the remaining N and PK through chemical fertilizer is thought to be the most useful for obtaining the highest yield with the highest fertility status (Jayathilake et al., 2006). We require an eco-friendly atmosphere for sustainable crop production. Thus, employing these bacterial strains as biofertilizer to improve soil fertility with additional production would be a highly beneficial strategy for our agriculture.

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