Isolation, Characterization, and Efficacy Assessment of Microbial Flora from Fish Amino Acid Fertilizer for Sustainable Biofertilizer Application in Enhancing Plant Growth

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ABSTRACT

A key component of sustainable agriculture, fish amino acid fertilizer (FAA) is an organic input that is abundant in vital nutrients and advantageous microbes. In this study, the microbial flora found in FAA will be isolated, characterized, and their potential as biofertilizers and biopesticides assessed. Using accepted microbiological techniques, microorganisms were separated and then described according to their morphological and biochemical characteristics. Plant growth-promoting characteristics such nutrient solubilization, phytohormone synthesis, and antagonistic action against plant diseases were further evaluated for the isolated isolates.

The findings demonstrated the existence of a variety of advantageous microorganisms that can increase plant development, improve soil microbial activity, and increase nutrient availability. Furthermore, a few isolates had inhibitory effects on dangerous pathogens, suggesting a possible use for them in biological pest control. Applying microbial flora obtained from FAA can boost crop yield, enhance plant health, and lessen reliance on chemical herbicides and fertilizers. As a result, FAA is a viable, economical, and environmentally beneficial resource for creating sustainable biofertilizer and biopesticide formulations for use in agriculture**.**

Keywords: Fish aminno acid (FAA), Bio-fertilizer, Fermentation, Plant Growth Promoting Factors

INTRODUCTION

Fish amino acid (FAA) is an organic liquid fertilizer produced by fermenting fish waste with brown sugar. It provides the essential nutrients, especially nitrogen,promoting plant growth,chlorophyll formation and improved flowering and fruiting.FAA is applied as a foliar spray or oil dench after dilution (commonly 1:100) to prevent phototoxicity.fermentation enhances nutrient availability and stability,making it an eco-friendly fertilizer.this study evaluates its potential role in sustainable agriculture.

MATERIAL AND METHODOLOGY

Preparation process of Fish Amino Acid (FAA) Fertilizer:

Fish Amino Acid (FAA) fertilizer was prepared using the fish. The fish that used in this experiment was Tilapia. The making process for this liquid organic fertilizer was the fish was mixed with the brown sugar with ratio 1:1 and the mixture was left for Four weeks, fermentation process breaks down the protein and turns it into amino acids. After Four weeks, the solution was filtered and 1 ml & 2ml of the solution was added with 1000 ml of water. This solution was used with different volume as the treatments for the study.

The process of producing FAA (Fish Amino Acids) includes the following steps:

1. Collection of Fish Waste: Gather fish by-products such as heads, bones, and entrails.
2. Mixing with Fermenting Agent: Combine the collected fish waste with a fermenting agent, such as sugar or molasses.

3. Fermentation: Allow the mixture to ferment for several weeks, ensuring to stir periodically.
4. Straining and Storage: Once fermentation is complete, strain the liquid and store it in a cool, dark environment.

Experimental Design:

The effects of Fish Amino Acid (FAA) on the growth and yield of Green chilli(*Capsicum annuum*),Brinjal (*Solanum melongena*), Black gram (*Vigna mungo*) , Red spinach (*Amaranthus dubius*). The study was conducted over a growing season with the following treatments:

- **Control:** No foliar application
- **FAA:** Foliar application of Fish Amino Acid
- **AA:** Each treatment was replicated two times for these crops

Application Schedule Of FAA Fertilizer: Foliar applications were conducted at [specify growth stages, e.g., 7 day stage, 14 day stage] at [specify interval, e.g., 7 days]. Applications were performed early in the morning or late in the afternoon to minimize the risk of foliar burn and to maximize absorption.

Isolation And Identification

The primary step in the development of liquid biofertilizer involved isolating bacteria from spoiled and fermented fish waste with brown sugar, which served as a microbial source. The methodology was designed to ensure the recovery of diverse beneficial bacterial strains

Sample Preparation:

Take 1 mL of the fermented fish aminoacid (FAA) sample and add it to a sterile test tube containing 9 mL of sterile distilled water

Seriel Dilution:

Label six additional sterile test tubes containing 9 mL of sterile distilled water as 10^{-2} to 10^{-6}. Transfer 1 mL from the 10^{-1} tube to the 10^{-2} tube using a sterile micropipette and mix well. Repeat this process sequentially until the 10^{-6} dilution is prepared, ensuring that each dilution is mixed properly before transferring.

Spread Plating on Nutrient Agar:

Label the nutrient agar plates with the corresponding dilution factor (10^{-3} to 10^{-6}, as higher dilutions reduce colony crowding).Take 100 µL (0.1 mL) of the selected diluted sample using a micropipette and dispense it onto the center of a labeled nutrient agar plate.Use a sterile glass spreader (previously dipped in ethanol and flame-sterilized) to evenly spread the sample across the surface of the agar.Allow the plates to absorb the liquid for a few minutes before incubation.

Morphological Identification:

Isolated colonies were observed for color, shape, and size. Gram staining was performed to determine the Gram reaction of the bacterial isolates.

Biochemical Characterization

Catalase Test

A clean glass slide was taken to which a loopful of the test culture was placed on it and one drop of H_2O_2 was placed over it. Release of oxygen was noted.

Oxidase Test

From the 48 hours broth culture, a loopful of the test inoculum was taken and placed on an oxidase disc. Formation of violet colour was recorded as positive and no colour formation indicated the negative result.

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Indole Test

Tryptone broth tubes were prepared and sterilized at 121ºC for 15 minutes. The culture was then inoculated into the tubes containing sterilized tryptone broth medium and incubated at 37ºC for 24-48 hours. After the incubation period, 0.2ml of Kovac's reagent was added into the tubes and the results were observed.

Methyl Red Test

The MR-VP broth tubes were prepared and sterilized at 121ºC for 15 minutes. The culture was then inoculated into the tubes containing sterilized MR-VP broth medium and inoculated at 37ºC for 24-48 hours. After the incubation period, about 5-6 drops of Methyl Red indicator solution was added.

Voges-Proskauer Test

The MR-VP broth tubes were prepared and sterilized at 121ºC for 15 minutes. The test culture was then inoculated into the tubes containing sterilized MR-VP broth medium and incubated at 37ºC for 24-48 hours. After the incubation period, about 3ml of 5%, α-naphthol in absolute ethanol (Barrit's Reagent A) and 1ml of 40% Potassium hydroxide (Barrit's Reagent B) were added. The tubes were allowed to remain undisturbed for 15-20 minutes. The development and absence of red colour was noted and recorded.

Citrate Utilization Test

Simmon's Citrate Agar was prepared and dispensed on test tubes and sterilized at 121ºC for 15 minutes and allowed to set as slant. The culture was then inoculated into the tubes containing Simmon's Citrate Agar slants (stabbed into the bud and streaked on the surface of slants) and incubated at 37ºC for 24-48 hours.

Triple Sugar Iron Test

It is based on the ability of the organism to ferment sugar and to produce hydrogen sulphide. The Triple Sugar Iron was prepared and sterilized at 121°C for 15 minutes and was dispensed on test tubes and allowed to set as slants. The test culture was later inoculated into the tubes containing Triple Sugar Iron slant (stabbed into the bud and streaked of the surface of slants) and incubated at 37°C for 24-48 hours.

Starch Hydrolysis Test

Starch Agar medium was prepared and sterilized. The test organisms were inoculated under sterile condition. The plates were incubated at 37°C for 24 hours.

Instrumentation Analysis

Molecular analysis will be done using Gas Chromatography – Mass Spectroscopy(GC-MS) and Fourier-Transform Infrared (FTIR)

RESULT AND DISCUSSION

This study focuses on the formulation of a liquid biofertilizer using fish waste and brown sugar, leveraging their natural fermentation process to enrich microbial populations beneficial for plant growth. The fermentation process promotes the proliferation of plant growth-promoting bacteria (PGPB), which aid in Numerous L-form amino acids (the form that is biologically active) can be found in abundance in fish amino acid, which is frequently a form of fish protein hydrolysate.These amino acids are used as; Building Blocks: These are the basic building blocks of proteins, which are necessary for the synthesis of hormones, enzymes, and new plant tissue.

Direct Nutrient Source: Plants can avoid the energy-intensive process of synthesising some amino acids from inorganic nitrogen by directly absorbing L-amino acids through their leaves and roots.

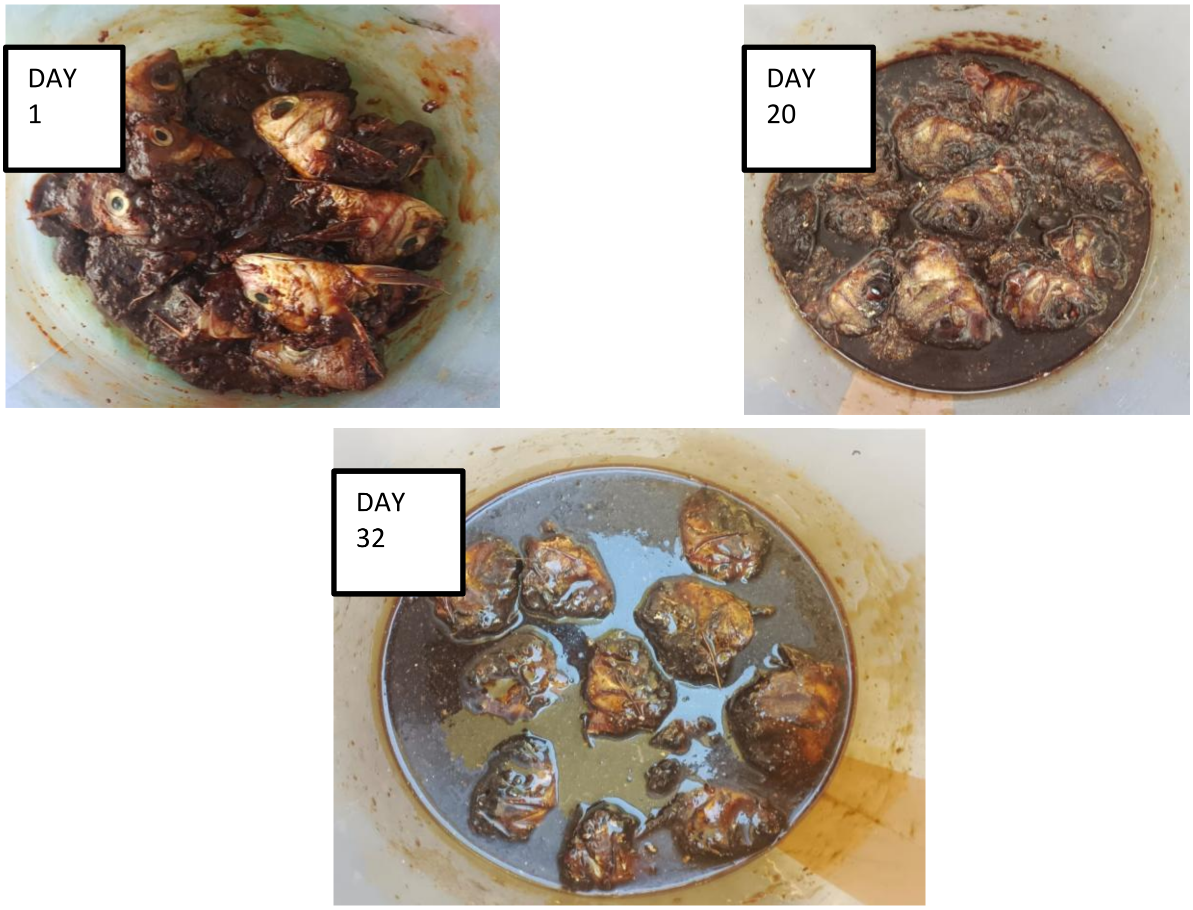

Fig.1 Shows the fermentation of Fish amino acid Biofertilizer

Isolation of bacteria from Fish amino acid biofertilizer

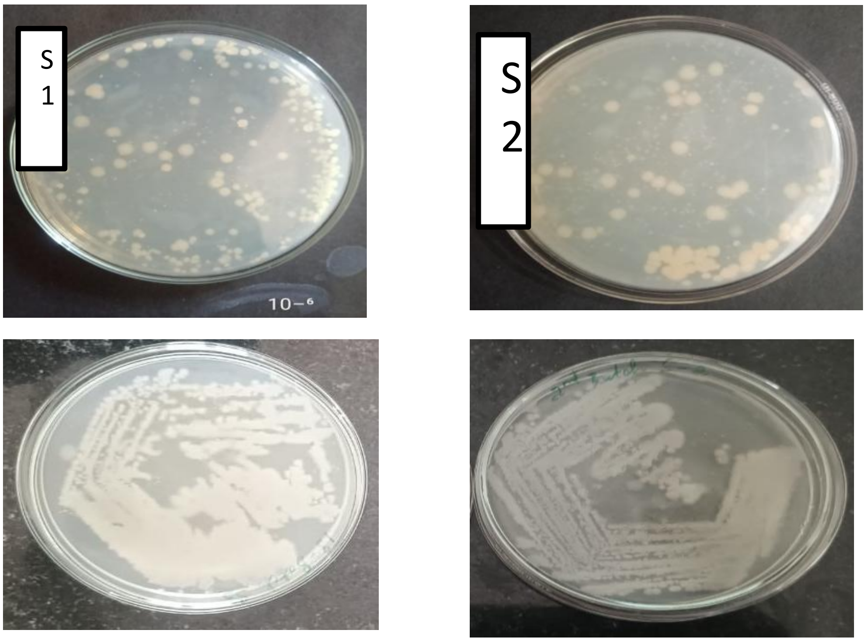

Fig.2 Bacterium isolated from Fish amino acid biofertilizer

Gram's Staining

The Gram staining was done to identify the morphological characteristics of the isolated microbes . The smear was examined under the microscope, bacterium S1 retained violet colour and it was confirmed as Gram-positive which appeared as rod shaped bacterium. Bacterium S2 also retained violet colour and it was confirmed as Gram-positive with the culture as rod shaped bacterium.

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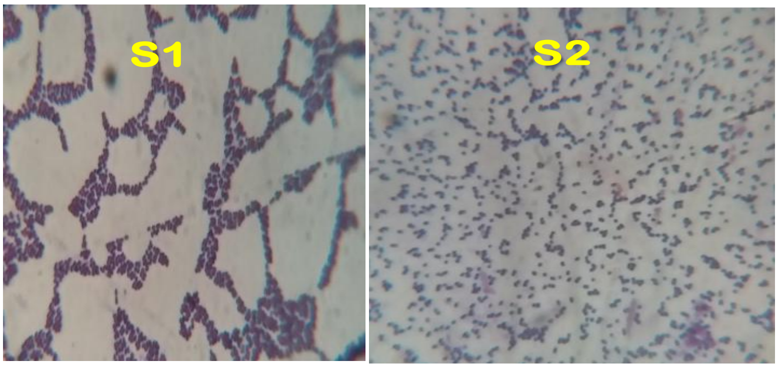


Fig.3 shows the Gram stained images of isolated bacterial cultures. (S1) Gram positive clustering cocci (S2) Gram positive single cocci shaped bacterium

Biochemical Test

A Series of Biochemical tests were performed to identify the cultures.The entire test were carried out with positive and negative controls.

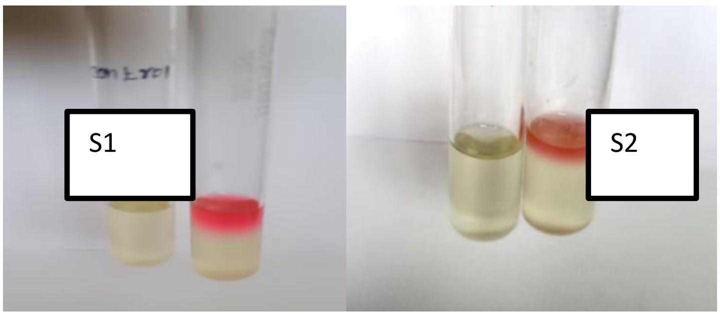
Table 1 Biochemical characterization of the isolated bacterial cultures.

Fig	Tests	S1	S2
a.	Methyl Red	+	+
b.	Voges proskauer	-	-
c.	Indole	-	-
d.	Catalase	-	+
e.	Oxidase	+	+
f.	Starch hydrolysis	-	-
g.	Trible suger iron	+	+
h.	Citrate	+	+

+: Positive -: Negative

Table 1 represents the biochemical characteristics of the two bacterial cultures S1, S2 Isolated from the Fish aminoacid fertilizer

a.)



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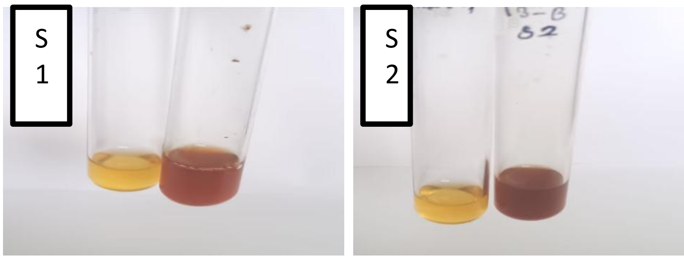

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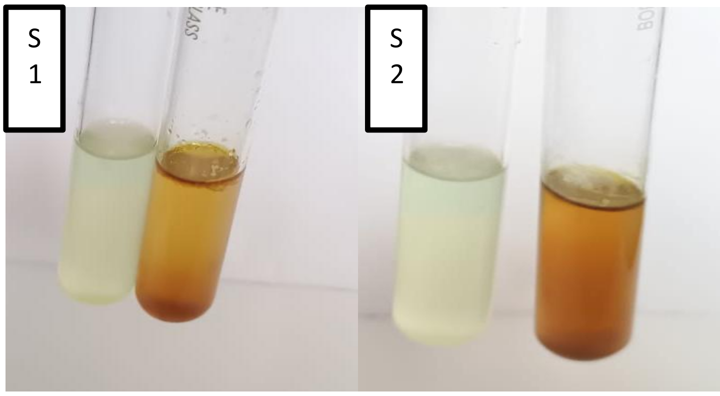
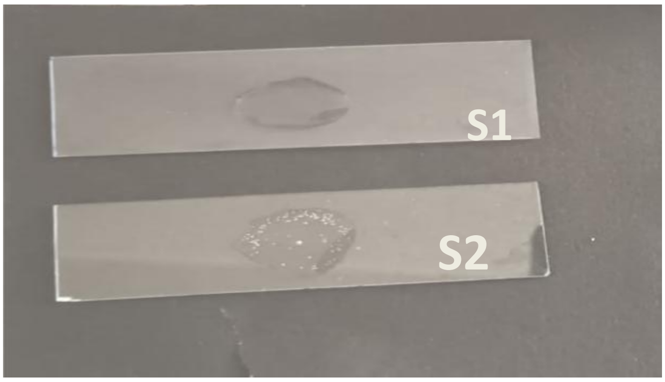
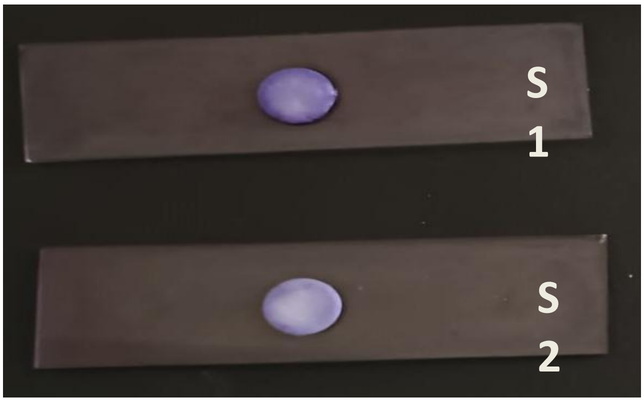
c.)



d.)



e.)



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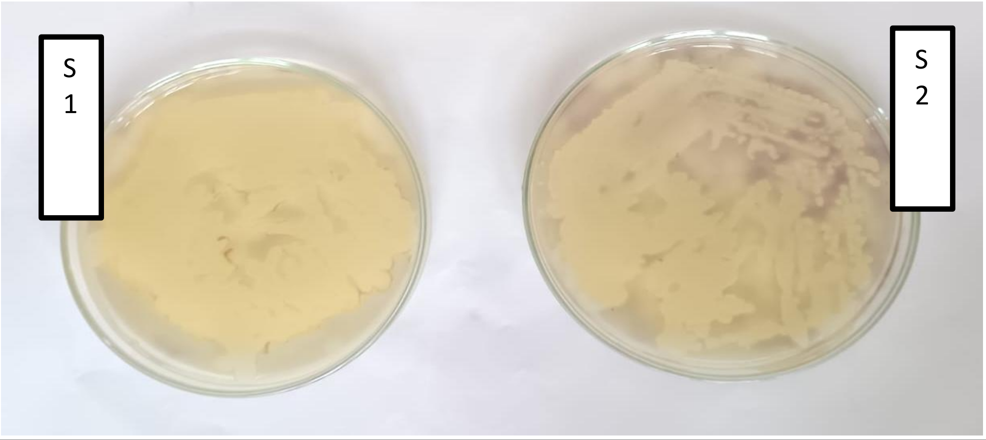

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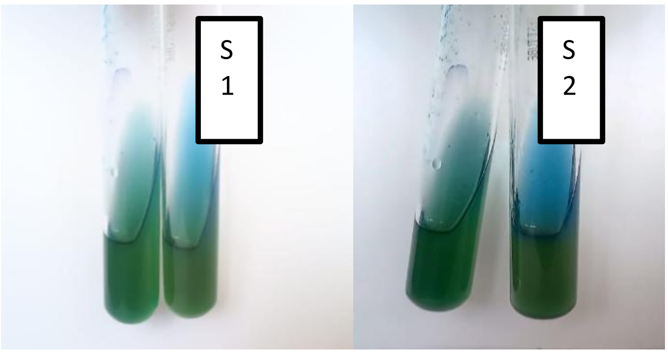
g.)

h.)



Biofertilizer Formulation

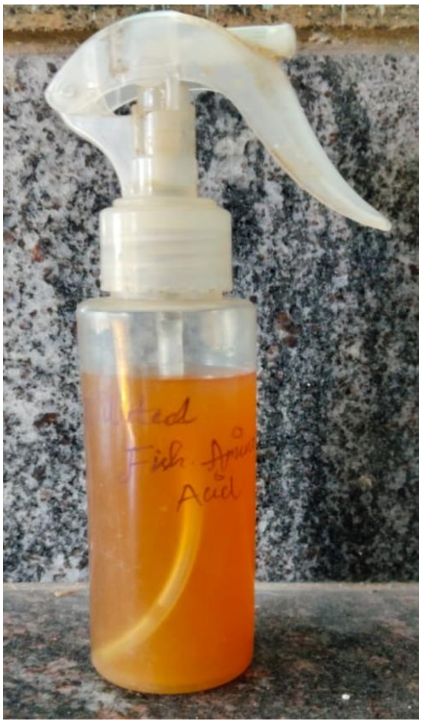


Fig.4 Biofertilizer was formulated into 1:5 ratio for better plant growth

Biofertilizer : sterile water

Using a 1:5 dilution for biofertilizer application optimizes microbial activity, prevents phytotoxicity, enhances nutrient absorption, and ensures uniform application, making it a practical and effective strategy for sustainable agriculture.The diluted biofertilizer promotes microbial diversity and nutrient cycling, leading to sustainable soil fertility. It act as the Easier foliar spraying a less viscous solution ensures even coating on leaves, increasing nutrient absorption through stomata and leaf surfaces.

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Field trial

This field trial was conducted to evaluate the effect of liquid biofertilizer derived from Fish waste and brwon suger on the germination, seedling vigor, and early growth of Green chilli(*Capsicum annuum*),Brinjal (*solanum melongena*), Black gram *(vigna mungo)* , Red spinach (*Amaranthus dubius)* plants. The trial was set up in green grow bags under open field conditions, using normal soil without any compost to assess the standalone impact of the biofertilizer.

Experimental Setup:

- Control Group (T0): Green chilli ,Brinjal,Black gram ,Red spinachseeds irrigated with plain water (no biofertilizer).
- Treatment Group (T1): Green chili ,Brinjal ,Black gram, Red spinachseeds treated with a 1:5 biofertilizer solution (1 part biofertilizer + 5 parts water).

Table; 2 shows result of the measurements of plant growth

Seed	Overall plant growth		Root length		Shoot length		Biomass	
	Sample	Control	Sample	Control	Sample	Control	sample	control
Green chilli	14.8	7.9	3.5	1.1	11.3	6.8	1.2	0.8
Brinjal	78.8	30	11.8	10.9	67	19.1	30.5	15.1
Blackgram	32.5	20.4	7.5	6	25	16.2	13.7	9
Red spinach	12.5	8.2	2.5	3	10	5.2	2.2	1.3

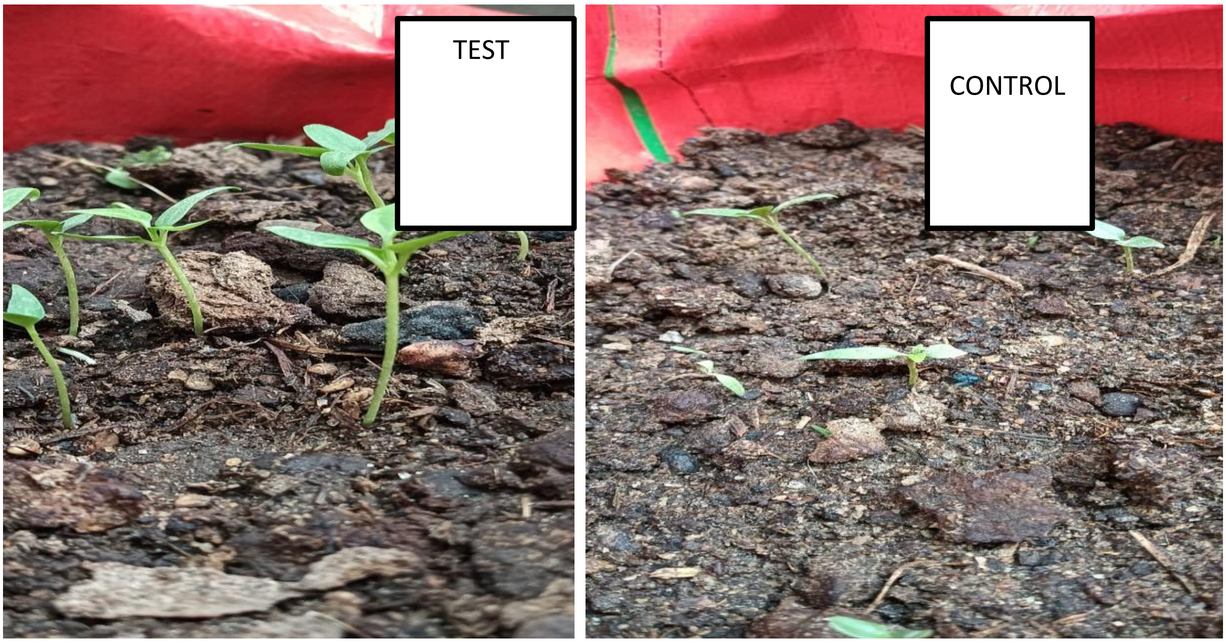

Fig. 5 shows the result of the Green chilli plant growth

The Green chilli plant's maximum root length was 3.5 cm and stem length was11.3 cm respectively, The plants overall length was 14.8 cm. while the control group's root length was 1.1cm and stem length was 6.8 cm respectively, The control plant measured7.9 cm in total length. (Fig6) . By using fish amino acid (FAA) and indigenous microorganisms (IMO), on the other hand, Natural Farming techniques increase soil nitrogen availability, increase crop yields, and preserve water quality. It is known that fish emulsions stimulate the growth of seedlings (Murray and Anderson 2004), fruiting (Aung and Flick 1980), and soil microbial activity(El-Tarabily *et al*. 2003).

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Fig. 6 Shows result of root and shoot measurements of green chilli plant

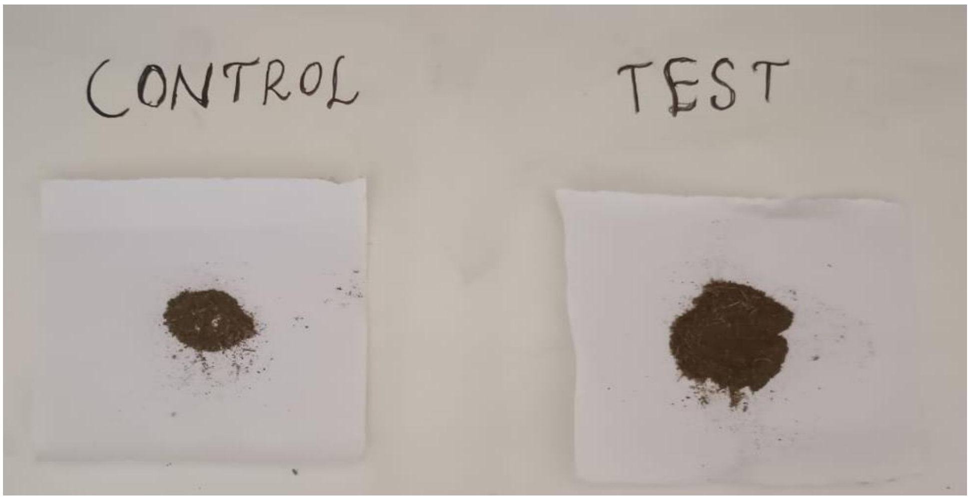

Fig.7 shows the result of the chilli plant Biomass

Fig. 8 shows the result of the Brinjal plant growth

The Brinjal plant's maximum root length was 11.8 cm and stem length was 67 cm respectively, The plants overall length was 78.8 cm, while the control group's root length was 10.9 cm and stem length was 19.1 cm respectively, The control plant measured 30 cm in total length. (Fig.9). Fish-based fertilizers have been demonstrated in studies to improve seedling development, stimulate fruiting, and encourage beneficial microbial activity .This natural fertiliser is administered via foliar mist or soil drench to give vital nitrogen (N) to plants, supporting healthy growth and chlorophyll production while minimising waste and environmental effect (Aung, L.H., and G.J. Flick, 1980).

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Fig.9 Shows the result of Root and Shoot measurements of the Brinjal plant

Fig.10 shows result of the brinjal pant biomass

Fig.11 Show the result of the Black gram plant growth

The Black gram plant's maximum root length was 7.5 cm and stem length was 25 cm respectively, The plants overall length was 32.5 cm. while the control group's root length was 6 cm and stem length was 16.2 cm respectively, The control plant measured 20.4 cm in total length. (Fig12.)Essential macro and micronutrients such as nitrogen, phosphorous, potassium, calcium, magnesium, sulphur, iron, zinc, copper, boron, manganese, and molybdenum (Siddique *et al.*, 2023) are also what set FAA apart. These nutrients are essential for plant physiology, including vegetative growth, fruit and flower maturation, and fortification against pathogenic challenges (Hung-Chang Huang *et al.*, 2005).

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Fig.12 shows result of Root and Shoot Measurements of Black gram

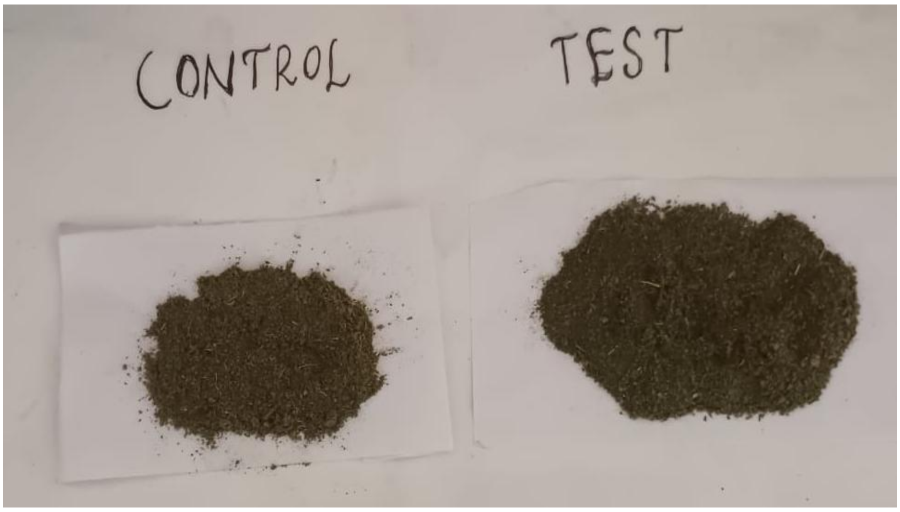

Fig .13 shows result of black gram plant Biomass

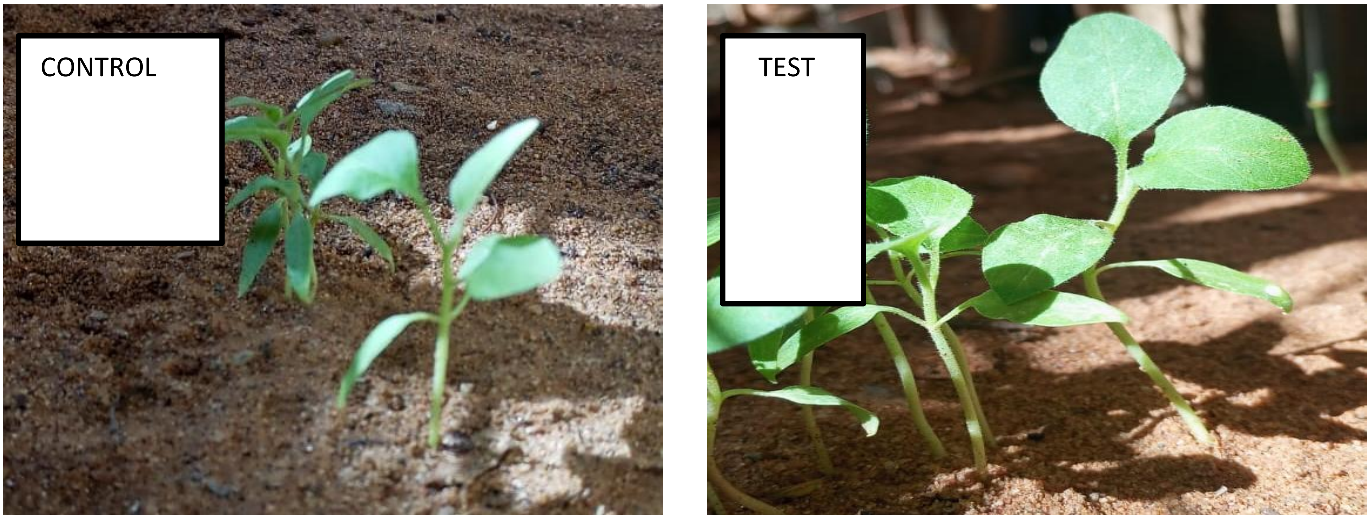

Fig.14 Shows the result of the Spinach plant growth

The Red spinach plant's maximum root length was 2.5 cm and stem length was 10 cm respectively, The plants overall length was 12.5cm. while the control group's root length was 3cm and stem length was 5.2 cm

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respectively, The control plant measured 8.2 cm in total length. (Fig.15)FAA helps to maintain water quality and improve crop yields by increasing the amount of nitrogen available in soils (T. Ramesh, 2020).

Fig.15 shows result of Root and shoot measurements of Spinach plant

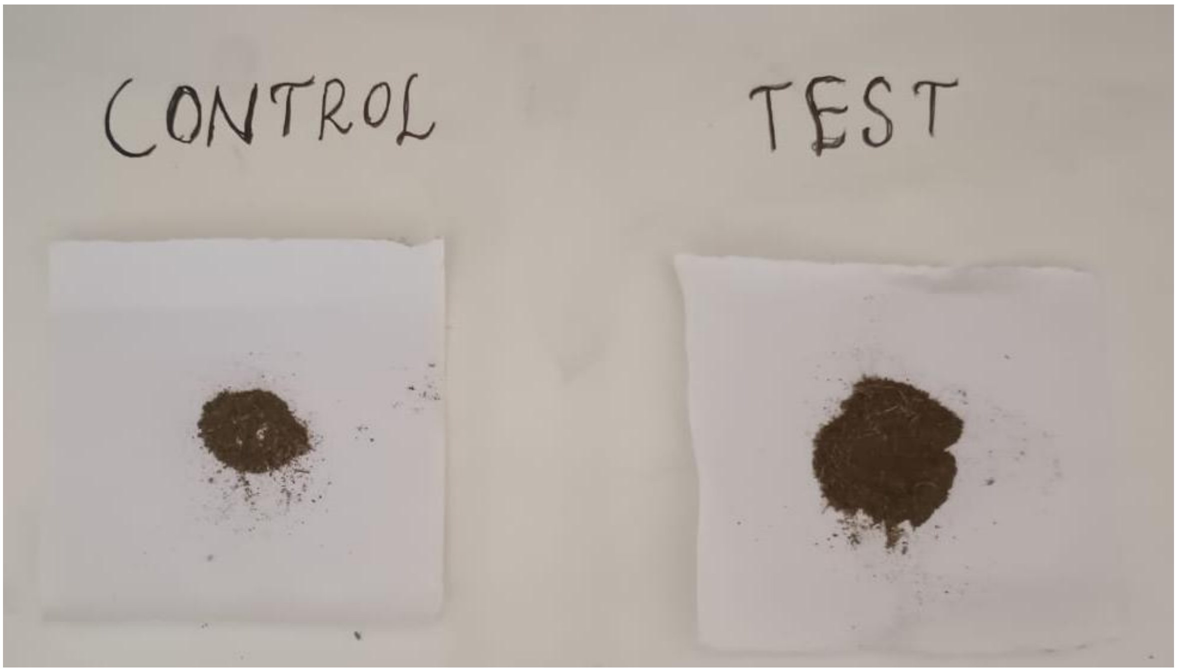

Fig.16 shows result of spinach plant Biomass

Plant Growth and Soil Health

Treated plants had better leaf expansion, higher chlorophyll content, and increased root biomass, indicating improved nutrient uptake.

Microbial analysis showed increased populations of phosphate-solubilizing bacteria, nitrogen-fixingbacteria,and beneficial fungi in biofertilizer-treated plots.

FTIR Analysis of Biofertilizer Components

The Fourier-Transform Infrared (FTIR) spectrum exhibits distinct absorption peaks that strongly indicate the presence of organic and biological components, which are characteristic of biofertilizers .

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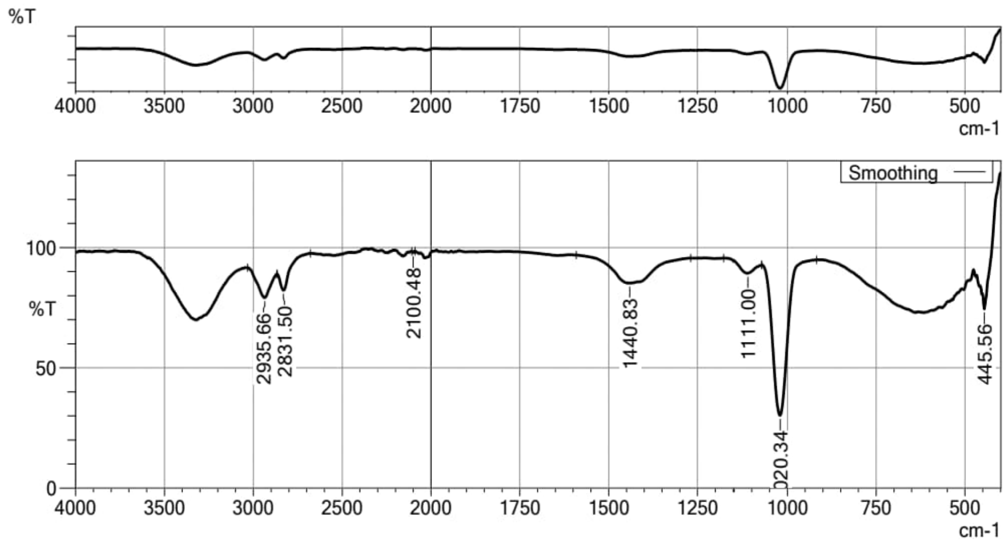

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	Sample name	
	Sample ID	
	Option	
	Comment	
	No. of Scans	45
	Resolution	4 [cm-1]
	Apodization	Happ-Genzel

	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area	Comment
1	445.56	74.48	11.95	451.34	399.26	-85.651	224.853	
2	1020.34	30.22	63.30	1072.42	918.12	3762.352	2814.608	
3	1111.00	89.35	4.49	1176.58	1072.42	796.984	193.458	
4	1440.83	85.26	11.07	1591.27	1269.16	2616.746	1421.292	
5	2100.48	97.88	0.60	2108.20	2090.84	30.661	4.397	
6	2831.50	82.25	8.35	2866.22	2679.13	1506.828	254.765	
7	2935.66	79.21	10.91	3032.10	2866.22	2456.151	853.125	

Fig.17shows result of FTIR Analysis

Wavenumber (cm−1)	**Functional Group Assignment**	**Interpretation and Relevance**
2935.51	C-H Stretching	**Aliphatic Hydrocarbons:** Represents the C-H bonds in **lipids, fatty acids, and cellular membranes**. This confirms a significant content of general organic matter from the base biological material.
2831.33	C-H Stretching	**Aliphatic Hydrocarbons:** Further supports the presence of long-chain organic molecules.
1440.85	C-HBending	**MethylCH_2 and Methylene CH_2**groups: A common signature in all organic materials and biological compounds.
1111.45	C-O-C or C-O-P Stretching	**Carbohydrates and Phosphates:** This region is typical for **polysaccharides** (sugars) that make up microbial cell walls (e.g., Exopolysaccharides/EPS) or plant structure. C-O-Pindicates the presence of **phosphate groups** (essential nutrients and part of DNA/RNA).
1020.80	C-O-Cor C-O-P Stretching	**Carbohydrates and Phosphates:** Another prominent peak in the carbohydrate region, often associated with the backbone structure of organic matter derived from biological sources.

Table. 3 Shows result of FTIR Analysis of Biofertilizer Components

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The detected peaks confirm the presence of complex organic compounds associated with biological materials such as plant extracts and microbial cells. Strong C–H and C–O signals indicate lipids and carbohydrates, suggesting a biological origin.

In biofertilizers, these functional groups are characteristic of microorganisms such as Rhizobium, Azotobacter, and phosphate-solubilizing bacteria, along with their organic carrier materials.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

Analysis using gas chromatography-mass spectrometry (GC-MS), which is frequently used in biological research of this kind: The sample contains a number of chemicals with known biofertilizer (plant growth/health enhancement) and/or biopesticide (antimicrobial/insecticidal) properties, according to the Gas Chromatography-Mass Spectrometry (GC-MS) data you supplied.

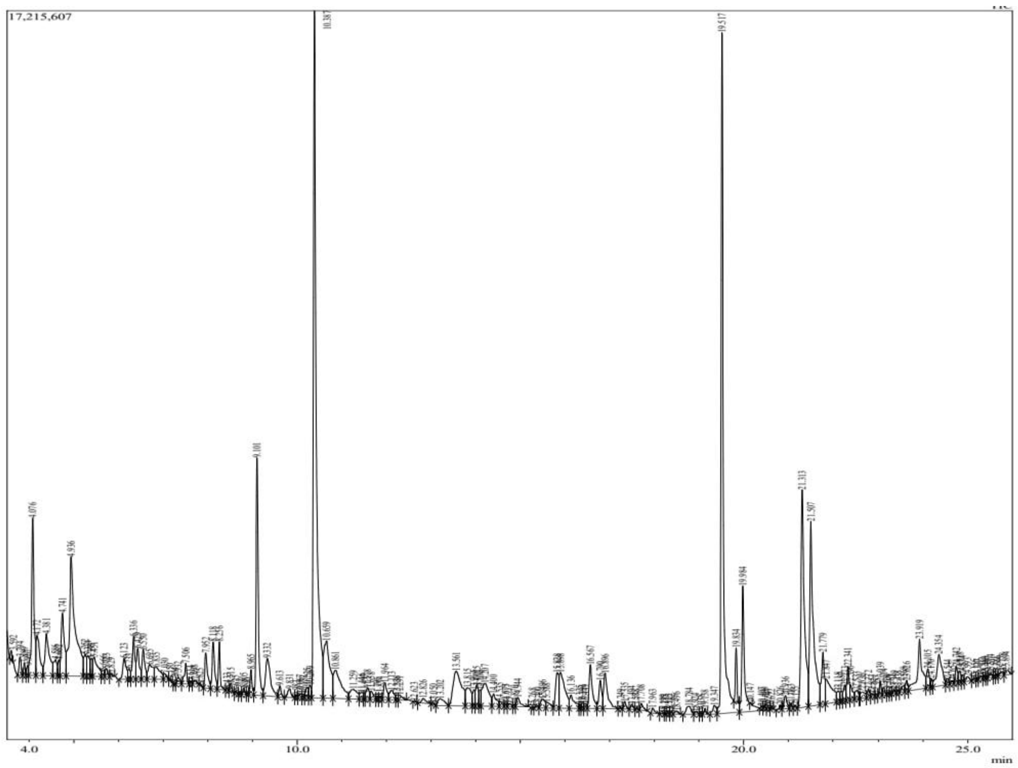

Fig. 18 shows result of Gas Chromatography-Mass Spectrometry (GC-MS) analysis

Component Name	Peak # (Approximate)	Area % (Concentration)	Primary Bio-Activity
gamma-Butyrolactone Derivatives	23, 44, 127	0.63% (and others)	**Biofertilizer/Biocontrol:** Related to quorum sensing molecules that regulate the gene expression and virulence of bacteria, influencing **plant-microbe interactions** and **growth promotion**.
Phenol Derivatives	78, 46	Various	Phenolic compounds are generally excellent **antimicrobial** and **antifungal** agents.

Table. 4 shows result of Gas Chromatography-Mass Spectrometry (GC-MS) analysisComponents of biofertilizer, often known as growth-promoting:

Compounds such as squalene, phytol, oleic acid, and hexadecanoic acid are common secondary metabolites derived from plants or microorganisms involved in biofertilizer production. Although not biofertilizers themselves, these compounds can support plant growth and serve as precursors for essential biomolecules.Additionally, organic compounds from nitrogen-fixing and phosphate-solubilizing organisms, including gamma-butyrolactone and butanoic acid derivatives, indicate the presence of microbial metabolites with potential roles in plant growth regulation and beneficial soil microbial activity.

SUMMARY AND CONCLUSION

Fertilizer made from fermented fish waste, known as fish amino acid (FAA) fertilizer, is a sustainable organic input enhanced with helpful microbes. The purpose of this study was to separate, describe, and assess the effectiveness of the microbial flora found in FAA for the application of biofertilizer and the promotion of plant development. Standard morphological and biochemical techniques were used to analyze the microbial isolates, which showed the presence of microorganisms that promote plant growth and aid in nutrient mineralization and soil fertility enhancement.Efficacy assessment demonstrated enhanced seed germination, root and shoot growth, and biomass production compared to control plants, indicating the positive role of FAA-associated microbial communities in improving nutrient availability and plant development. According to the research, FAA supports sustainable farming methods by acting as a microbial biofertilizer and a source of nutrients. To confirm its widespread use, more field-level research and molecular characterisation are advised.

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