JETIR.ORG ISSN: 2349-5162 | ESTD Year : 2014 | Monthly Issue
JOURNAL OF EMERGING TECHNOLOGIES AND INNOVATIVE RESEARCH (JETIR)
An International Scholarly Open Access, Peer-reviewed, Refereed Journal

ISOLATION AND SCREENING OF PLANT GROWTH-PROMOTING BACTERIA FROM SPOILED, FERMENTED *Cocos nucifera* AND PREPARATION OF LIQUID BIO FERTILIZER USING ISOLATED BACTERIA

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Abstract : The excessive application of chemical fertilizers has prompted significant environmental concerns, necessitating the exploration of sustainable alternatives. This research investigates the potential of plant growth-promoting bacteria (PGPB) derived from spoiled, fermented *Cocos nucifera* combined with wild turmeric (*Curcuma aromatica*) to create an environmentally friendly liquid biofertilizer. Various bacterial strains were evaluated for essential plant growth-promoting (PGP) characteristics, such as phosphate solubilization, nitrogen fixation, and ammonia production. Morphological and biochemical analyses confirmed the presence of beneficial microorganisms, with certain strains exhibiting high efficacy in nutrient mobilization. A liquid biofertilizerwas developed using the most effective isolates and subjected to field trials on crop plants, measuring its effects on germination, root growth, and overall plant health. The findings indicated a notable enhancement in growth parameters, underscoring the potential of this biofertilizer as a cost-effective and sustainable substitute for synthetic fertilizers. This research not only promotes waste valorization but also advances biofertilizer technology to enhance agricultural productivity.

IndexTerms **- Phosphate solubilization, Plant growth promoting factors, Biofertilizer, fermentation.**

I. INTRODUCTION

Agriculture is one of the human activities that contributes most to the increasing amount of chemical pollutants via excessive use of synthetic chemical fertilizers and pesticides, which cause further environmental damage with potential risks to human health. Nitrous oxide (N_2O) is an example of chemical pollutant produced by excessive use of nitrogen fertilizer and is a major source of greenhouse gases causing global warming (Pravin Vejan et al. Molecules. 2016). And The presence of waste around us that has not been treated properly yet, environmental issues and economic factors, provides a potential and at once opportunity regarding them. One way is to utilize waste from the production of porridge, in the form of old coconut water and side products from a sugar factory, in the form of molasses. Old coconut water is believed, it's still containing enough nutrients and micro elements that can be used as a medium as well as a source of microorganisms that can be added in the process of making liquid fertilizer (R Darmawan et al 2020). The existence of untreated trash around us, as well as environmental and economic challenges, creates both a potential and an opportunity. One method is to use ruined coconut, which still has enough nutrients and microelements to serve as a medium as well as a source of microorganisms that may be produced throughout the liquid fertilizer production process. BPS Kabupaten Ponorogo (2015). A nutrient-rich substrate for the growth of beneficial microorganisms, especially lactic acid bacteria and yeast, is provided by fermented coconut milk. By reducing organic matter to simpler forms and enhancing plant absorption of nutrients, these microbes support soil health (Bashan et al., 2014). This article reviews the isolation and screening of PGPB from spoiled fermented coconut milk and explores their potential as biofertilizers.

II. RESEARCH METHODOLOGY

1.1 Preparation of liquid biofertilizer

The liquid biofertilizer was prepared by fermenting spoiled coconut flesh with turmeric, using selected plant growth-promoting bacteria.

1.1.1 Substrate preparation

Spoiled coconut flesh was blended with sterile distilled water in a 1:1 ratio to form a slurry. Wild Turmeric powder(*Curcuma aromatica*) was added to the slurry as a natural antimicrobial and nutrient source.

1.1.2 Fermentation

The mixture was transferred to airtight jars and incubated at 30°C for 4-5 days. Stirring was performed daily to ensure even fermentation.

1.1.3 Viability Testing

Samples were taken at intervals during fermentation and tested for microbial viability using serial dilution and plating.

1.1.4 Storage

After fermentation, the liquid biofertilizer was filtered and stored in sterilized bottles at 4°C.

2.1 Isolation and Identification

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

2.1.1 Sample preparation

Take 1 mL of the fermented spoiled coconut milk sample and add it to a sterile test tube containing 9 mL of sterile distilled water.

2.1.2 Serial dilution

Label nine additional sterile test tubes containing 9 mL of sterile distilled water as 10^{-2} to 10^{-9}.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^{-9} dilution is prepared, ensuring that each dilution is mixed properly before transferring.

2.1.3. Spread Plating on Nutrient Agar

Label the nutrient agar plates with the corresponding dilution factor (10^{-3} to 10^{-9}, 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.

2.1.4 Morphological Identification

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

2.1.5 Gram's staining

A loopful of the overnight culture was subjected to Gram's staining. The culture was smeared and heat fixed on a clean glass slide and stained using crystal violet for 1 minute followed by a water wash. Gram's iodine was added to the smear and was kept undisturbed for 1 minute after which water wash was done. The smear was later stained with Gram's decolourizer for 15 seconds after which it was water washed. The smear was counterstained with saffranin for 1 minute and it was finally water washed with tap water. The smear was air dried and examined under 100X oil immersion microscope and the results were noted.

3.1 Biochemical characterization

3.1.1 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. Bubble formation indicates the presence of catalase enzyme. No bubble formation indicates the absence of catalase enzyme. Results were noted.

3.1.2 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.

3.1.3 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. Results were noted for the development and absence of bright red colour at the interface of the reagent and the medium.

3.1.4 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. The development of stable bright red colour of the indicator indicates sufficient acid production which constitutes a positive reaction. A weekly positive test will be red orange. Yellow orange colour indicates a negative reaction.

3.1.5 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.

3.1.6 Citrate utilization test

Simmons 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 Simmons Citrate Agar slants (stabbed into the bud and streaked on the surface of slants) and incubated at 37ºC for 24-48 hours. After the incubation period, the development of intense blue colour from the original green colour and of the medium indicates the ability of the organism to utilize citrate as carbon source and constituted the positive reaction. The absence of blue colour indicates negative reaction.

3.1.7 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 121ºC 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 37ºC for 24-48 hours. After the incubation period, the development of intense red colour constitutes positive reaction. The absence of the red colour indicates negative reaction.

3.1.8 Gelatin hydrolysis

In the gelatin hydrolysis test, a nutrient gelatin medium is inoculated with the test organism and incubated at 25–37°C for 24–48 hours. After incubation, the medium is observed for liquefaction. If the gelatin remains liquid, it indicates gelatinase production positive result, while solidification indicates a negative result.

3.1.9 Urease test

A loopful of the 24 hour bacterial test culture was inoculated into Sterile Urea broth and it was incubated at 37°C for 24 hours. After incubation, the pink colour change was observed indicating positive result.

4.1 Screening of isolated strains for plant growth-promoting traits

To identify potential plant growth-promoting bacteria, isolates were tested for specific biochemical traits known to enhance plant growth and soil fertility.

4.1.1 Phosphate solubilization

Phosphate solubilization potential was evaluated using pikovskaya's medium, which contains insoluble tricalcium phosphate. Isolates were spot inoculated on the medium and incubated at 28–30°c for 5–7 days. The formation of clear halo zones around bacterial colonies indicated phosphate solubilization. The solubilization index was calculated by measuring the diameter of the halo zone relative to the colony size.

4.1.2 .Nitrogen fixation

The ability of bacterial isolates to fix atmospheric nitrogen was assessed using nitrogen-free ashby's medium. Isolates were inoculated into the medium and incubated at 30°c for 48–72 hours. Growth in the absence of external nitrogen sources indicated nitrogen-fixing potential.

4.1.3 Ammonia production

Ammonia production was tested by inoculating bacterial isolates in peptone water and incubating them at 30°C for 48 hours. After incubation, 0.5 mL of Nessler's reagent was added to the culture broth, and the development of a yellow to brown coloration indicated the presence of ammonia. This test helped determine the nitrogen-releasing potential of the bacterial isolates, which contributes to plant nutrient availability.

5.1 Field trials

The efficacy of the prepared biofertilizer was evaluated through pot-based experiments under controlled conditions.

5.1.1 Experimental Setup

Pots were divided into two groups:

- Control: No fertilizer applied.
- Biofertilizer: Liquid biofertilizer applied.

5.1.2 Seed Sowing and Fertilizer Application

Seeds were sown in the paper cups later transferred into grow bags and biofertilizer was applied weekly to the respective treatment group.

5.1.3 Monitoring

Growth parameters (germination rate, plant height, number of leaves) were recorded every weeks.

III. RESULTS AND DISCUSSION

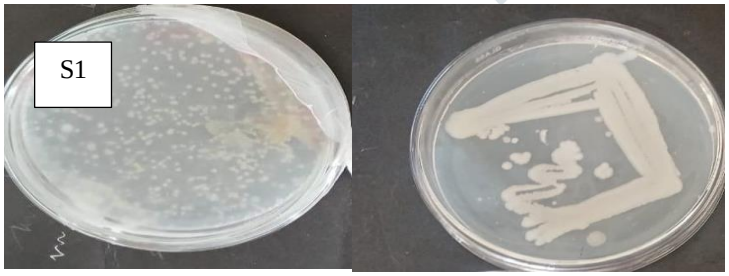
6.1 Natural Fermentation of Biofertilizer

Fig.1 Natural fermentation of spoiled coconut milk with wild turmeric, day 1 and day 3

Fig.2 Day -4 and Day- 5

This study focuses on the formulation of a liquid biofertilizer using spoiled coconut milk and wild turmeric (*Curcuma aromatica*), 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 nitrogen fixation, phosphate solubilization, and plant hormone production.

6.2 Isolation of bacteria from spoiled and fermented coconut milk with turmeric



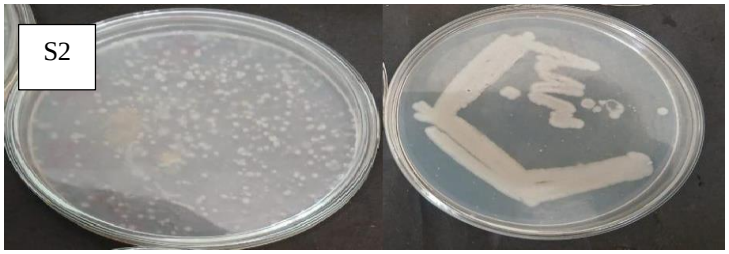


Fig.3 Bacterium isolated from spoiled and fermented coconut sample .

6.3 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.

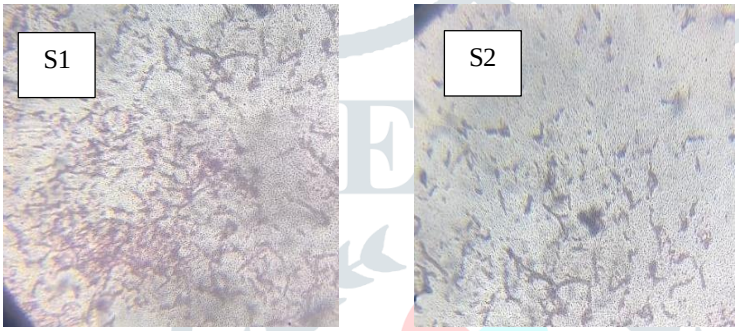
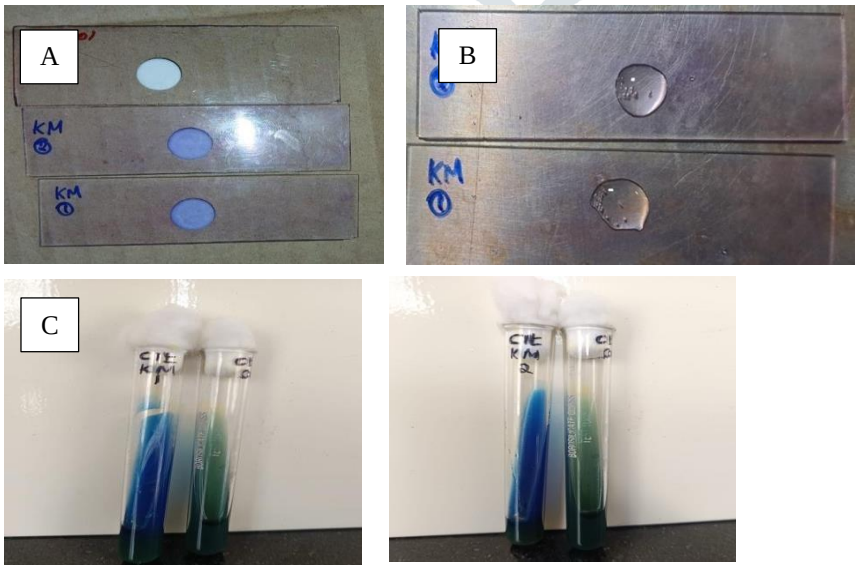


Fig.4 shows the Gram stained images of isolated bacterial cultures S1- Gram positive Rods and S2 – Gram positive Rods

6.4 Biochemical tests

Tests	S1	S2
Methyl Red	+	+
Voges proskauer	-	-
Indole	+	+
Catalase	+	+
Oxidase	+	+
Gelatin hydrolysis	+	+
Citrate	+	+
Urease	-	-



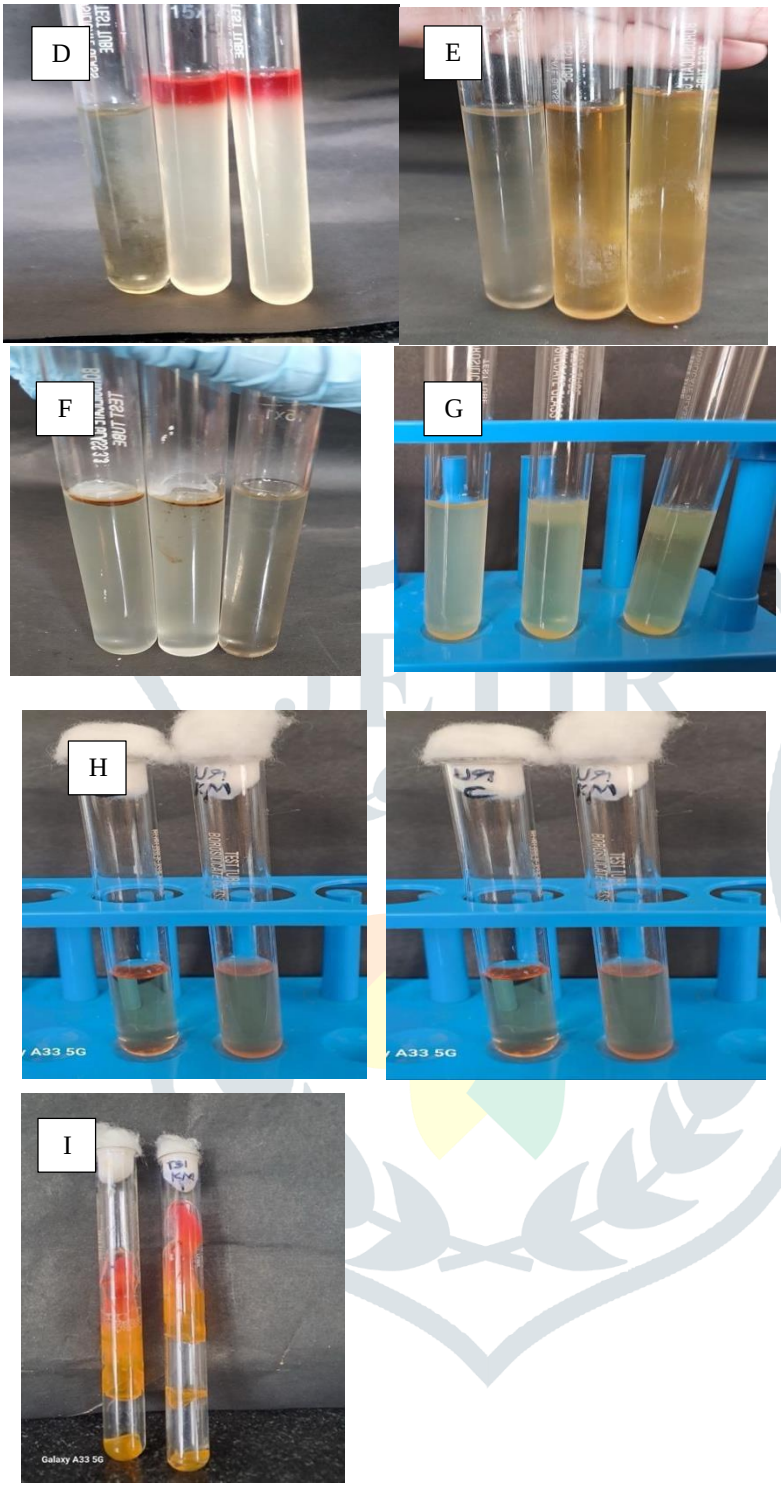


Fig.5 shows the biochemical tests of isolated cultures,
A. Oxidase test, B. Catalase test,C.Citrate test, D.Methyl red test,E.Voges proskauer test,F.Indole test, G.Gelatin test, H.Urease test,I. Triple sugar iron test.

6.5 Screening of isolated strains for plant growth-promoting traits

6.5.1 Phosphate solubilization test

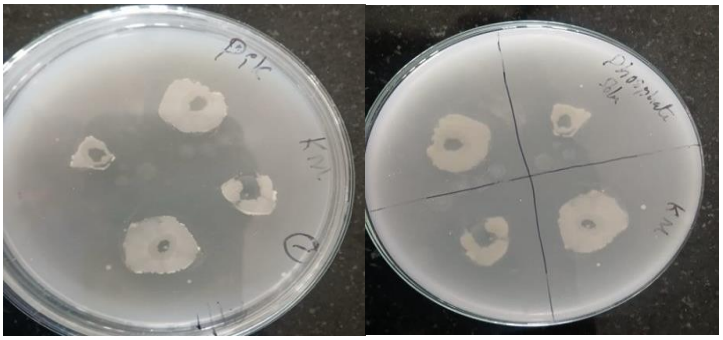


Fig.6 Phosphate solubilization test on pikovskaya agar plates

Gyaneshwar et al. (2002) observed that bacterial strains capable of producing organic acids, such as Pseudomonas and Bacillus, play a critical role in breaking down insoluble phosphate compounds . The formation of halo zones suggests that the bacterial isolates shows positive results and similar mechanisms may be responsible for phosphate solubilization in plants.

Chen et al. (2006) demonstrated that phosphate-solubilizing microbes enhance plant root development by increasing phosphorus uptake . The positive results from our isolates suggest their potential application as biofertilizers to improve crop yield. This is likely due to the production of organic acids (such as gluconic acid, citric acid, and lactic acid), which lower pH and release bound phosphate. Phosphate-solubilizing bacteria play a crucial role in improving plant phosphorus uptake, enhancing root development, and promoting early seedling establishment.

6.5.2 Nitrogen fixation

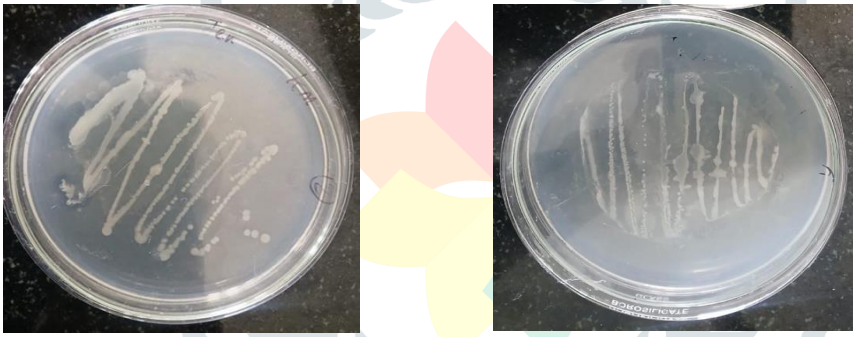


Fig.7 Nirogen fixation test on Jensen's nitrogen free medium.

Bhattacharyya & Jha (2012) found that free-living nitrogen-fixing bacteria, can grow in nitrogen-free media and enhance plant growth by producing phytohormones. The bacterial isolates grown on the Jensen's nitrogen free media shows the positive results and indicate that S1 and S2 bacterial isolates might possess similar plant growth-promoting traits.The isolates suggesting their potential use as biofertilizers to reduce dependency on synthetic nitrogen fertilizers. Nitrogen-fixing bacteria convert atmospheric nitrogen (N_2) into bioavailable ammonia (NH_3), which plants can absorb. The possible mechanism are the Activity of the nitrogenase enzyme complex and Symbiotic or associative interactions with plant roots and the Release of ammonia and nitrates into the rhizosphere.

6.5.3 Ammonia production

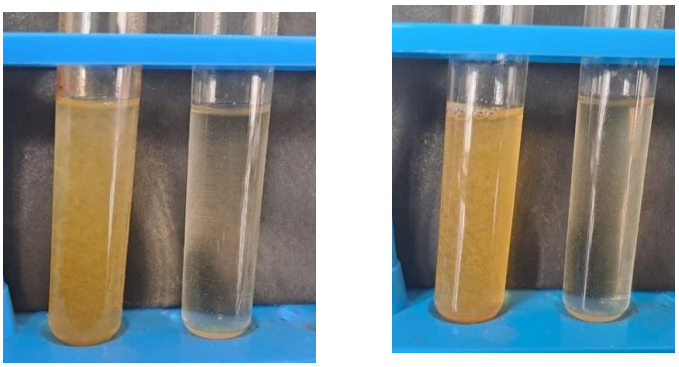


Fig.8 Ammonia production test

Joseph et al. (2007) demonstrated that ammonia production by PGPR is a key mechanism of plant growth promotion, as ammonia produces by adding nessler's reagent it changes into bright yellow to brown color.

The bacterial isolate S1 and S2 possess positive ammonia production and confirms their potential as biofertilizers. which is a key indicator of plant growth-promoting rhizobacteria (PGPR) activity. Ammonia released by bacteria can serve as an immediate nitrogen source for plants, promoting vegetative growth and chlorophyll synthesis. The possible mechanism are Ammonification

of organic nitrogen compounds and Deamination of amino acids and the Production of extracellular enzymes aiding in nitrogen cycling.

7.1 BIOFERTILIZER FORMULATION.

Fig.9 Biofertilizer was formulated into 1:3 ratio for better plant growth

Biofertilizer : sterile water

Using a 1:3 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.

8.1 Field trail

This field trial was conducted to evaluate the effect of liquid biofertilizer derived from spoiled coconut milk and wild turmeric on the germination, seedling vigor, and early growth of tomato (*Solanum lycopersicum)* plants. The trial was set up in green grow bags under open field conditions, using normal soil without any compost to assess the stand alone impact of the biofertilizer.

Fig.10 Seeds were grown on the paper cup later transferred into the grow bags.

8.1.1Experimental Design

Location: Green grow bags placed in an open field area.

Soil Type: Normal soil (without compost or organic amendments) with a pH of 6.5–7.0.

Crop Selection: Tomato (*Solanum lycopersicum*).

8.1.2 Experimental Setup:

Control Group (T0): Tomato seeds irrigated with plain water (no biofertilizer).

Treatment Group (T1): Tomato seeds treated with a 1:3 biofertilizer solution (1 part biofertilizer + 3 parts water).

Weeks after planting	Biofertilizer (cm) T1	Control (water) T0
1	3.8	2.5
2	7.6	5.2
3	12.3	9.1
4	19.8	14
5	27.5	21.1
6	36.1	26.6
7	42.8	32.7

Fig.11 pictures of plants

Fig.12 Flower in the T1 plant

8.1.3 Plant Growth and Soil Health observation

- 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-fixing bacteria, and beneficial fungi in biofertilizer-treated plots.
- Tomato plants treated with biofertilizer showed earlier flowering compared to control plant.

8.1.4 Bio pesticide

Wild turmeric (*Curcuma aromatica)* is rich in curcuminoids, essential oils, and phenolic compounds, which possess antifungal, antibacterial, and insecticidal properties. During fermentation, these bioactive compounds are released and interact with microbial metabolites, enhancing the biopesticidal activity of the biofertilizer.

Fig.13 Pest Attacks on plants before the application of Biofertilizer.

Fig.14 Healthy leaves after the application of biofertilizer

CONCLUSION

This study successfully harnessed the power of natural fermentation to develop a cost-effective, eco-friendly liquid biofertilizer from spoiled coconut milk and wild turmeric. By isolating and enriching plant growth-promoting bacteria (PGPB), the formulated biofertilizer demonstrated remarkable potential in enhancing soil fertility, root development, and overall plant health. The fermentation process not only optimized microbial diversity but also transformed agricultural waste into a valuable resource, reinforcing the principles of sustainable and organic farming.

Field trials confirmed that this biofertilizer significantly improved germination rates, plant vigor, and nutrient availability, making it a viable alternative to synthetic fertilizers. Its ability to enhance phosphate solubilization, nitrogen fixation, and disease resistance positions it as a game-changer in sustainable agriculture.

By turning waste into wealth, this project bridges the gap between biotechnology and regenerative farming, paving the way for greener, more resilient agricultural practices. With further optimization and large-scale application, this innovative biofertilizer holds immense potential to revolutionize organic farming, reduce chemical dependency, and promote environmental sustainability.

IV. ACKNOWLEDGMENT

We would like to express our heartfelt gratitude to all those who have contributed to the completion of this review. We extend our sincere thanks to Velumanoharan Arts and Science College for Women for its unwavering support and valuable insights, which have greatly enhanced the quality of this work. Our deepest appreciation goes to our Secretary, Mrs. P. Shakuntala Parthasarathy, our Principal, Dr. A.E.G.C. Rajani and our HoD Ms. R. Mohana Priya for their constant encouragement and for facilitating the successful completion of this research. We also wish to acknowledge the invaluable support and feedback from our colleagues and peers, whose input has been instrumental throughout the process.

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