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Assessment of the biological and chemical activities of organic ethno-biofertilizer formulations for maize growth enhancement

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ABSTRACT

Organic ethno-biofertilizer (OEB) is a low-cost, eco-friendly agricultural input derived from locally available materials, aligning with sustainable and smallholder farming practices. By combining agricultural residues with beneficial microorganisms, OEB offers an environmentally responsible alternative to restore soil health and improve crop productivity. This study aimed to assess and characterize OEB formulations based on local wisdom, evaluate their biochemical activities, and determine the effectiveness of plant growth-promoting bacteria (PGPB) in enhancing maize growth through bioassays. Six OEB formulations were tested, from which 12 PGPB isolates were obtained and evaluated to identify superior strains. A randomized block design (RBD) with 13 treatments (one control and 12 bacterial isolates) and three replications was employed. The highest bacterial and nitrogen-fixing contents were observed in the OEB formula combining plant-based (bamboo root) and animal-based (rabbit urine) components. Bioassay results showed that all 12 isolates significantly enhanced maize growth and phytohormone production. Based on performance rankings, two superior isolates (GPNF9 and GPNF12) were identified, which significantly increased plant height, root length, and fresh weight compared to the control. Biochemical analyses revealed that GPNF9 and GPNF12 produced indole acetic acid (IAA) at 1.153 and 1.394 ppm, and nitrogenase activity at 0.560 and 1.381 μM mL^{-1} g^{-1} h^{-1}, respectively. Molecular identification classified GPNF9 as *Stenotrophomonas* sp. HH10 and GPNF12 as *Enterobacter* sp. strain YF3. These findings suggest that integrating plant- and animal-based OEB with selected PGPB isolates can effectively support sustainable maize production.

Keywords: appropriate technology, ecofriendly fertilizers, local wisdom, organic extract, soil health.

INTRODUCTION

Ecofriendly sustainable agriculture and appropriate technology are vital strategies for building climate-resilient farming systems. Within this framework, ethno-biofertilizers have emerged as innovative inputs that integrate traditional ecological knowledge with modern microbial biotechnology. Derived from locally available organic extracts such as compost teas, fermented plants, and manures and rich in beneficial microbes that improve nutrient availability, nitrogen fixation, phosphorus solubilization, and phytohormone production, thereby enhancing soil fertility and crop productivity (Sharma et al., 2021; Singh et al., 2022). As context-appropriate technologies, ethno-biofertilizers are low-cost, ecofriendly, and culturally accepted, making them highly adaptable for farmer adoption and long-term sustainability (Sihombing et al., 2022). Beyond serving as nutrient inputs, they play a central role in restoring soil health, defined as the soil's capacity to function as a living ecosystem that sustains plants, animals, and humans (Lal, 2020).

In Indonesia, where smallholders manage over 90% of agricultural land, such innovations are essential to reduce reliance on chemical fertilizers, rehabilitate degraded soils, and strengthen food security under climate stress (Zhao, 2024).

Ethno-biofertilizers refer to a class of biofertilizers that combine indigenous agricultural wisdom with modern microbial biotechnology, serving as a bridge between traditional ecological knowledge and scientific innovation. They are usually formulated from locally available organic substrates such as composts, plant extracts, animal manures, or fermented biomass, which are inoculated or naturally enriched with beneficial microorganisms including nitrogen-fixing bacteria, phosphate-solubilizing microbes, mycorrhizal fungi, and plant growth-promoting rhizobacteria (PGPR) (Singh et al., 2020; Sharma and Rai, 2022). These inputs not only supply essential nutrients but also enhance soil health, stimulate plant growth, and improve resilience against abiotic stresses (Patra et al., 2019). As emphasized by Kumar et al. (2021), the concept is deeply rooted in centuries of farmer-led soil fertility management, where traditional communities developed effective organic amendments long before the advent of synthetic fertilizers. Today, ethno-biofertilizers are being validated, standardized, and refined through ecological and microbiological research, positioning them as sustainable and culturally grounded innovations for future farming systems.

Combined ethnological practices with microbial technologies, ethno-biofertilizers not only function as nutrient providers but also revitalize traditional ecological knowledge, empower smallholder farmers, and enhance agroecosystem resilience. Their reliance on diverse microbial consortia offers significant opportunities for building climate-resilient farming systems, particularly in regions vulnerable to soil degradation and variability (Singh and Gupta, 2022; Yadav et al., 2023). Ethno-biofertilizers represent both a scientific advancement and a sociocultural pathway toward sustainable agriculture. Ethno-biofertilizer extracts play a significant role in enhancing soil fertility and crop performance by integrating traditional organic inputs with beneficial microbial consortia. Their effectiveness lies in supplying bioavailable nutrients, improving soil microbial diversity, and enhancing biochemical processes such as nitrogen fixation, phosphorus solubilization, and organic matter decomposition. Quantitative studies have shown that ethno-biofertilizer applications can increase soil organic carbon by 18–25% and microbial biomass carbon by up to 30% compared to untreated soils (Singh et al., 2020). In rice-based systems, extracts derived from fermented plant and animal residues enriched with nitrogen-fixing bacteria improved grain yield by 12–20% under saline and rainfed conditions (Kumar et al., 2021). Similarly, in maize and wheat ecosystems, ethno-biofertilizers enhanced nutrient use efficiency, leading to yield gains of 10–18% while reducing dependency on synthetic fertilizers by nearly 25% (Sharma and Rai, 2022). Beyond yield improvements, their application improved soil enzyme activities such as dehydrogenase and phosphatase, which are critical indicators of soil biological health (Patra et al., 2019).

The benefits stem from beneficial microbes such as *Azospirillum*, *Azotobacter*, *Pseudomonas*, and *Bacillus*, which enhance nutrient cycling, root colonization, and pathogen suppression, especially when delivered via local biomass carriers (Sharma et al., 2021; Singh et al., 2022; Sari et al., 2021). Enterobacter sp. is another well-documented plant growth-promoting bacterium (PGPB) that enhances the growth of crops such as groundnut and maize (Ludueña et al., 2019). The genus Enterobacter are known to possess various PGP traits, including nitrogen fixation, phosphate solubilization, antibiotic production, secretion of siderophores, chitinase, ACC deaminase, and other hydrolytic enzymes, as well as the production of exopolysaccharides that contribute to improved soil porosity (Jha et al., 2011). Biofertilizers can also be derived from organic sources such as animal urine and plant residues. Ethno-biofertilizers therefore serve as a reservoir of beneficial microbes, including Azotobacter, decomposers, Bacillus pantothenticus, *Lactobacillus* sp., *Staphylococcus aureus*, and *Bacillus alvei* (Usdar et al., 2021).

Experimental evaluation. This study aims to evaluate the effectiveness of organic ethno-biofertilizer (OEB) formulations in improving microbial activity, nutrient availability, and plant performance under saline soil conditions. We hypothesize that the integration of functional microbes with organic enrichment materials enhances microbial resilience and biofertilizer efficacy compared to conventional formulations. This study evaluated six OEB (organic ethno-biofertilizer) formulations and tested 12 growth promotor and nitrogen fixer (GPNF) isolates, through bioassays to identify the most effective candidates. The results revealed superior isolates, molecularly identified, which served as active ingredients of ethno-biofertilizer formulations. These strains demonstrated high potential in enhancing nutrient availability, improving soil fertility, and supporting crop productivity. Their integration into OEB formulations bridges traditional organic amendments with scientifically validated microbial inoculants, offering a sustainable alternative to chemical fertilizers. Beyond boosting yields, these biofertilizers improve microbial biodiversity, strengthen ecological resilience, and reduce farming costs. The findings underscore the future prospects of ethno-biofertilizers as innovative, ecofriendly, and culturally rooted solutions for promoting sustainable agriculture across diverse ecosystems.

MATERIALS AND METHOD

The research was conducted in two main stages: the first involved the formulation and extraction of OEB, while the second comprised the isolation, characterization, and identification of isolates (macroscopic, microscopic, and biochemical), followed by bioassays and biochemical analyses (IAA and nitrogenase).

Formulation and extraction of organic ethno-biofertilizer)

The first stage of the study focused on the selection and preparation of six formulations of OEB. Each formulation was developed using a combination of organic substrates, carrier materials, and microbial inoculants. The selection was based on nutrient content, compatibility of components, and potential for microbial growth. The six formulations were systematically designed, and their compositions are presented in Table 1. This research encompasses a process that commences with the creation of six OEB formulas through anaerobic extraction methods, along with the preparation of the necessary tools and materials.

The materials were subjected to fermentation using a 50 L anaerobic fermentor for 30 days under controlled conditions. Homogeneous mixing was ensured by the use of turbo mixers, and after fermentation, the materials were filtered through a 100-mesh screen to obtain the extract. The equipment utilized in this study included local biofertilizer production tools: measuring cups (ranging from 100 mL to 1000 mL), a weighing scale (with a range of 0–5 kg), a stirring bucket, a stirrer, a knife, a funnel, a drum (30–50 L capacity), jerrycans (10–20 L capacity), a small hose (1 m in length), and six small bottles (e.g., 600 ml mineral water bottles). Laboratory analysis equipment and tools for biological testing were also employed. The materials employed in this research comprised bamboo roots, banana corms, rabbit urine, goat urine, goldenen apple snails, tajin water (rice washing water), coconut water, molasses (sugarcane syrup) (Table 1). The fermentation process was conducted under controlled conditions to ensure optimal microbial activity and product stability, including: (1) fermentation duration of 14–21 days, depending on stabilization of pH and odor; (2) incubation temperature maintained at 28–32 °C under ambient laboratory conditions; (3) pH conditions monitored regularly and maintained within the range of 6.5–7.5; (4) semi-anaerobic fermentation conditions using loosely closed containers to allow limited gas exchange; (5) microbial inoculation procedure involving the addition of a starter culture (10^7–10^8 CFU/mL) consisting of functional microbes (e.g., nitrogen-fixing and phosphate-solubilizing bacteria); and (6) use of sterile plastic containers (20–30 L capacity) equipped with gas-release valves to ensure controlled fermentation.

Measured respon

Microbial diversity in OEB formula

Comprehensive microbiological analyses demonstrated the diversity and functional capacity of microorganisms present in the OEB formula. Selective media were applied to target specific groups, including nitrogen-fixing bacteria on N-free Jensen's medium, phosphate-solubilizing bacteria (PSB) on Pikovskaya's agar, and potassium-solubilizing bacteria (KSB) on Aleksandrov's medium. The characterization process involved assessing the microbial colony population using the total plate count (TPC) method, which aims to determine the number of bacterial colonies grown on agar media (Yunita et al., 2015). Colony enumeration, and functional assays provided clear evidence of both the abundance and the potential activity of these microbial communities.

Table 1. Composition s and formulas for producing ethnobiological fertilizers involve a simple fermentation process aimed at cultivating a benefyicial microbial community to obtain the microbial isolates

No.	Materials	Ingredient formulation					
		BR	BW	RU	GU	GS	FC
1	Bamboo roots (kg)	1					1
2	Banana weevils (kg)		5				5
3	Rabbit urine (L)			10			10
4	Goat urine (L)				10		10
5	Golden snail (kg)					5	5
6	Rice washing water (L)	10	10	10	10	10	10
7	Molasses (L)	1	1	1	1	1	1

Note: BR – bamboo root, BW – banana weevils, RU – rabbit urine, GU – goat urine, GS – golden snail, FC – formula combination.

Bioassay of OEB formula on maize seedlings

Bioassays were conducted to evaluate the biological activity of the OEB formulations using maize seedlings. The assays were performed under controlled conditions on sterile Murashige and Skoog (MS) medium, prepared with a composition of 90% medium and 10% OEB formulation. Maize variety BISI-2 type of maize was utilized in this study. Before treatment, the maize seeds were air dried and sterilized by washing them three times with Aquadest steril. After three days of sowing in straw paper, the maize seedlings were ready for the bioassay test. The procedure for growing maize seedlings involved using a 20 × 300 mm sterile test tube. The seeds were then planted in 100 mL test tubes containing a mixture of OEB formula and Fahraeus' medium, combined at a volume ratio of 1:9. The respons of maize plant including (a) plant height, (b) root length, (c) plant wet weight, (d) root dry weight, (e) shoot dry weight and (f) plant dry weight. The responses were compared across all formulations, and a ranking system was applied to determine the best-performing OEB formulation.

Bioassay on maize seedlings and biochemical of selected superior GPNF and PSB isolates from OEB formula

Identification and characterization isolates

Phenotypic characterization of growth promotor and nitrogen fixer (GPNF) was carried out using morphological (macroscopic and microscopic). Cellular morphology was determined through microscopic (colony form and colony colour) and microscopic (cell shape) observation, and Gram staining was performed to differentiate between Gram-positive and Gram-negative isolates. These characterizations were used to GPNF the growth promotor and nitrogen fixer potential of plant growth-promoting traits.

Ashby's medium contains mannitol (1.000 mg) as the principal carbon source, calcium carbonate (200 mg) as a buffering agent, and mineral salts such as dipotassium phosphate (20 mg), magnesium sulfate (20 mg), sodium chloride (20 mg), ferric chloride (0.5 mg), and agar (1.500 mg) for solidification.

Okon medium is composed of malic acid (500 mg) as the primary carbon source, potassium hydroxide (400 mg) for pH adjustment, ammonium chloride (20 mg) as a nitrogen source, dipotassium phosphate (60 mg), magnesium sulfate (20 mg), sodium chloride (10 mg), calcium chloride (2 mg), and trace amounts of micronutrients.

The maize variety BISI-2 was used in this study. Prior to treatment, seeds were air-dried and surface-sterilized by triple washing with sterile distilled water. After three days of germination on sterile straw paper, seedlings were transferred into 20 × 300 mm sterile test tubes containing 100 mL of a mixture of GPNF inoculum suspension and Fahraeus medium (1:9, v/v). Growth responses of maize, including plant height, root length, fresh weight, root dry weight, shoot dry weight, and total dry weight, were recorded. The performance of each formulation was evaluated comparatively using a ranking system to identify the most effective OEB treatment. The treatment used randomized block design (RBD) with 13 treatments and 3 replicates. The treatment consisted of the folowing different isolates: GPNF = Control, GPNF1, GPNF2, GPNF3, GPNF4, GPNF5,

GPNF6, GPNF7, GPNF8, GPNF9, GPNF10, GPNF11, and GPNF12. The experiment was designed using a completely randomized design (CRD) with three replicates per treatment, conducted under controlled greenhouse conditions with temperature maintained at 28 ± 2 °C, relative humidity of 70–80%, and a natural photoperiod of approximately 12 h light and 12 h dark, over an experimental period of 21 days after planting, with detailed consideration of the growth media composition and watering regime.

Biochemical assay of superior GPNF

Nitrogenase activity of selected GPNF isolates (GPNF9 and GPNF12) was determined using the acetylene reduction assay (ARA) following the method of Hawkes (2010) with slight modifications. Bacterial isolates were cultured on Okon's slant agar medium, sealed with rubber caps, and injected with 1 mL acetylene (C_2H_2) gas. After 1 h of incubation, 1 mL of headspace gas was collected and analyzed for ethylene (C_2H_4) production using gas chromatography. Nitrogen, hydrogen, and air were used as carrier gases, and ethylene concentrations were quantified by reference to a standard calibration curve (0–225 µg mL^{-1}).

Indole acetic acid (IAA)

IAA production using a GPNF spectrophotometer and PSB isolates was quantified following Lebrazi et al. (2020). Bacterial suspensions (10^7 CFU mL^{-1}, 10 mL) were incubated for 24 h, centrifuged at 5.500 rpm for 10 min, and 5 mL of the supernatant was mixed with Salkowski reagent (4:1). After 20 min incubation in the dark, absorbance was measured at 535 nm using a spectrophotometer, and IAA concentration was estimated from a standard curve.

Biomolecular identification

The species identification of three selected nitrogen fixing bacteria isolates was determined through the 16S rRNA gene sequencing method, employed oligonucleotide primers 27F (5'-AGAGAGTTTGATCCTGGCTCAG 3'), 785F (5'-GGATTAGATACCCTGGTA 3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (1st base). The 16S rRNA sequences of nitrogen fixing bacteria isolates were subjected to further analysis using Bioedit software, version 7.0.5.3 (Hall, 1999), and MEGA (Molecular Evolutionary Genetics Analysis), version X (Kumar et al., 2019), with the objective of obtaining the species identity of the bacterial isolates.

Statistical analysis

The data obtained was entered into Microsoft Office Excel and subsequently analyzed using analysis of variance (ANOVA) with SPSS version 26. In the event of a significant effect, a further test was conducted using the Duncan Test (Duncan multiple distance test) at a significance level of 5%. Data were analyzed with statistical software (SPSS version 26), and results were expressed as mean ± standard error (SE).

RESULT AND DISCUSSION

Microbial diversity in the OEB formula

The population of microorganisms within different biofertilizer formulations can vary significantly due to the specific microorganisms included in the formulation and the quality of the manufacturing process. For instance, biofertilizers containing Rhizobium bacteria can achieve a robust microorganism population when produced and stored under optimal conditions. This is primarily due to the mutualistic relationship between Rhizobium bacteria and the roots of leguminous plants. In this symbiotic partnership, Rhizobium bacteria plant roots, forming nodules that house bacteria capable of nitrogen fixation from atmospheric nitrogen, thus enhancing nitrogen availability for the plants.

Ethnobiological fertilizer formulations comprising multiple types of microorganisms, such as Rhizobacteria, *Azotobacter* sp, *Pseudomonas* sp, *Bacillus* sp and etc. or a blend of bacteria and fungi, tend to exhibit a greater microorganism population than formulations containing only a single type of microorganism. The subsequent table presents the findings from assessing microbial colony numbers using the total plate count method (TPC) across various local biofertilizer formulations. The microbial diversity within ethnobiological fertilizer formulations is detailed in Table 3. It is notable that the highest TPC values for total bacteria and fungi are observed in GU and GS, respectively. Furthermore, formula GS exhibits the highest TPC value for *Azotobacteria*, while FC is indicative of *Azospirillum*, RU of *Pseudomonas,* and FC of *Bacillus*. The elevated TPC values signify the abundance of microbial

Table 2. Microbial population (total bacteria, total fungi, *Azotobacteria, Azospirillum, Pseudomonas, Bacillus*) in various formulations of ethnobiofertilizer

No.	Formula	Total bacteria (10^9 CFU/ml)	Total fungi (10^6 CFU/mL)	*Azotobacteria* (10^7 CFU/mL)	*Azospirillum* (10^7 CFU/mL)	*Pseudomonas* (10^{-7} CFU/mL)	*Bacillus* (10^7 CFU/mL)
1.	BR	1.10	1.58	11	53	75	61
2.	BW	1.00	1.84	7	25	57	80
3.	RU	1.12	1.59	8	67	125	80
4.	GU	1.99	1.56	7	22	74	75
5.	GS	1.62	2.50	13	56	108	93
6.	FC	1.62	1.75	4	68	77	115

colonies within the respective materials. These findings underscore the distinct microbial compositions across the various formulations, highlighting that each formula contains a unique set of microorganisms.

Effect of application OEB formula on maize seedlings growth

The growth characteristics of maize, namely plant height, root length, and dry weight were significantly influenluenced by the ethnobiological fertilizer formulations (Table 2). The combined formula containing all ingredients (FC) and the goat urine formula (GU) exhibited pH values of 7.00 and 7.17, respectively, indicating that their solutions are mildly alkaline. In contrast, the bamboo root (BR), banana weevil (BW), rabbit urine (RU), and goldenen snail (GS) formulations were neutral in pH, while the control without additives was slightly acidic. The pH of these formulations is a critical determinant of both their effectiveness for plant application and the microbial habitat they create. For instance, the application of cyanobacterial biofertilizer consortiums has been shown to increase soil pH, alongside improvements in fertility and plant growth (Zeljković et al., 2022). Additionally, combining organic amendments (e.g., compost, biochar) with inorganic materials effectively raises soil pH and improves soil organic carbon, especially under acidic conditions (Guo et al., 2024). These findings underscore how formulation pH directly influence biological activity and soil chemical dynamics.

The average value followed by the same letter (superscript) indicates that it is not significantly different based on Duncan's Multiple Range Test at the 95% level. All data were analyzed using one-way ANOVA followed by Duncan test at $p < 0.05$. Results are now presented as mean ± standard deviation (SD)

The treatment of OEB formulas BR, BW, RU and GS showed a good effect on plant height, root length, plant fresh weight, root dry weight, shoot dry weight and plant dry weight. This is thought to be due to the nutrient and bacterial content contained in the formula being able to provide optimal growth for corn plants. The research results of Sharma et al. (2021) reported that biological fertilizer derived from bamboo roots is known to contain PSB, and these bacteria can form IAA as a nutrient that will be utilized by plants. Singh et

Table 3. Effect OEB formula on pH medium, and maize seedlings growth traits

Formulation	pH	Plant height (cm)	Root length (cm)	Wet weight (g)	Root dry weight (g)	Shoot dry weight (g)	Plant dry weight (g)
F0 (control)	5.17 ± ^{a}	41.83 ± ^{b}	12.57 ± ^{ab}	10.86 ± ^{b}	0.46 ± ^{b}	0.65 ± ^{b}	1.10 ± ^{b}
BR	6.33 ± ^{ab}	40.77 ± ^{b}	14.33 ± ^{b}	11.24 ± ^{b}	0.33 ± ^{b}	0.68 ± ^{b}	1.01 ± ^{b}
BW	6.33 ± ^{ab}	37.63 ± ^{b}	6.97 ± ^{ab}	8.14 ± ^{ab}	0.31 ± ^{b}	0.45 ± ^{ab}	0.76 ± ^{ab}
RU	5.83 ± ^{ab}	38.33 ± ^{b}	12.97 ± ^{ab}	10.03 ± ^{b}	0.41 ± ^{b}	0.52 ± ^{ab}	0.93 ± ^{b}
GU	7.17 ± ^{b}	31.17 ± ^{b}	6.13 ± ^{a}	7.50 ± ^{ab}	0.27 ± ^{ab}	0.40 ± ^{ab}	0.67 ± ^{ab}
GS	6.00 ± ^{ab}	40.57 ± ^{b}	8.17 ± ^{ab}	9.44 ± ^{b}	0.33 ± ^{b}	0.49 ± ^{ab}	0.82 ± ^{ab}
FC	7.00 ± ^{b}	11.73 ± ^{a}	5.80 ± ^{a}	0.58 ± ^{a}	0.03 ± ^{a}	0.04 ± ^{a}	0.09 ± ^{a}

Note: BR – bamboo root, BW – banana weevils, RU – rabbit urine, GU – goat urine, GS – golden snail, FC – formula combination.

al. (2022) also confirmed that IAA is a phytohormone that functions in the formation of plant canopies, elongation of plant stems and development of plant roots. The research results of Uddin et al. (2021) reported that biological fertilizer made from banana pseudostem contains many nitrogen-fixing microbes. The research results of Yetunde et al. (2022) reported that rabbit urine contains nitrogen, magnesium, calcium, potassium and phosphate which can increase plant growth and yield. Similarly, Puspitasari et al. (2023) reported that the use of rabbit urine as a biofertilizer can improve the appearance of agronomic characteristics in tomato plants.

The research results of Siregar et al. (2020) and Nugroho et al. (2021) reported that fertilizer formulations made from goldenen snails contain protein, phosphate, potassium and microbes which are useful in stimulating plant growth. Based on these ingredients in Formula BR, BW, RU, and GS, it will have a positive effect on plant growth. Drawing upon the analysis results presented in Table 2, it is evident that formulas BR, RU, and GS exhibit a favorable influence on maize plant growth. Positive impact can be attributed to the nutrient-rich composition of these formulas, which contain beneficial microbes that supply essential nutrients, such as nitrogen (N) and phosphorus (P), and enhance their availability to plants and the microbial community.

Characterization and bioassay of selected growth promotor and nitrogen fixer on maize seedlings

Characterization isolates of nitrogen-fixer bacteria

Characterization of nitrogen-fixing bacterial isolates involves a series of phenotypic tests to identify the bacterial species associated with nitrogen fixation. The characterization procedures include both macroscopic and microscopic examinations. Macroscopic analysis provides information on colony morphology, such as form and pigmentation, while microscopic examination reveals cellular characteristics, including Gram reaction and cell shape. In addition, the presence of nitrogen-fixing traits can be observed, offering further evidence of the functional capacity of the isolates. Nitrogen-fixing bacteria are predominantly Gram-negative, although certain taxa belong to Gram-positive groups. Gram staining serves as a fundamental technique to distinguish between these bacterial types within nitrogen-fixing isolates, thereby supporting their taxonomic characterization. Following staining, Gram-positive bacteria retain the crystal violet-iodine complex and appear purple to dark blue, whereas Gram-negative bacteria take up the counterstain and appear red to pink. The characterization results of the nitrogen-fixing isolates, comprising 12 isolates, including 6 from Ashby's medium and 6 Okon medium., yielded diverse outcomes in micro-morphological tests (as determined through Gram staining) (Table 4).

Based on the conducted observations (as presented in Table 4), the isolation process on Ashby and Okon media resulted in twelve isolates. These isolates were designated as Azotobacter when using the selective media Ashby GPNF1, GPNF2, GPNF3, GPNF4, GPNF5, and GPNF6, and as Azospirillum sp. when obtained from selective media Okon GPNF7, GPNF8, GPNF9, GPNF10, GPNF11, and GPNF12. These isolates exhibited distinctive macroscopic and microscopic characteristics, including specific shapes, colony forms, and oval or vibroid cell shapes. Additionally, the Gram staining results confirmed that isolates were Gram-negative.

Bioassay of growth promotor and nitrogen fixer on maize seedlings

Bioassay were conducted to assess the effectiveness of locally developed biofertilizer on maize. These tests were designed to evaluate the impact of plant growth stimulants contained in the biofertilizer, including measurements of plant height, leaf area, and root length. Additionally, the testing aimed to determine the efficiency of nutrient uptake by corn plants, specifically concerning nitrogen and phosphorus. Bioassays are utilized to assess the effects of substances or materials on living organisms, both directly and indirectly. These assessments are based on the observable consequences they produce. The application of nitrogen-fixing bacteria demonstrated a significant positive effect on increasing plant height (cm), root length (cm), and plant fresh weight (g). The results of the Bioassay tests revealed notable differences between the control group and the GPNF9 and GPNF12 isolates in terms of plant height response. The control had an average height of 19.5 cm, those treated with GPNF9, and GPNF12 isolates reached heights of

Table 4. Characteristics of nitrogen-fixing bacteria

Code	Macroscopis		Microscopic	
Ashby's medium	Colony form	Colony colour	Gram	Cell shape
GPNF1	Round	Red	negative	*cocci*
GPNF2	Round	Red	negative	*cocci*
GPNF3	Round	Red	negative	*cocci*
GPNF4	Round	Red	positive	*cocci*
GPNF5	Round	Red	negative	*cocci*
GPNF6	Round	Red	negative	*cocci*
Okon medium				
GPNF7	Round	Red	positive	*bacili*
GPNF8	Round	Red	negative	*cocci*
GPNF9	Round	Red	negative	*cocci*
GPNF10	Round	Red	positive	*cocci*
GPNF11	Round	Red	negative	*cocci*
GPNF12	Round	Red	negative	*cocci*

Note: Microbiology laboratory analysis results (2022).

29.12 cm and 28.93 cm, respectively. These findings suggest that specific nitrogen-fixing isolates can substantially enhance maize growth parameters compared to untreated controls (Table 5).

Average value followed by the same letter (superscript) indicates that it is not significantly different based on Duncan's Multiple Range Test at the 95% coGPNFidence level.

The observed improvements in maize growth may be explained by multiple mechanisms associated with nitrogen-fixing bacteria, particularly the production of indole-3-acetic acid (IAA) and nitrogenase activity, which were confirmed in this study. Nitrogenase activity contributes to biological nitrogen fixation, thereby enhancing nitrogen availability for plant uptake, while IAA plays a key role in stimulating root elongation, cell division, and overall root system architecture, ultimately improving nutrient and water absorption. In addition, nitrogen-fixing bacteria are widely reported to exhibit other plant growth-promoting traits, including enhanced atmospheric nitrogen fixation, increased solubilization of phosphorus, and the synthesis of phytohormones such as gibberellins and cytokinins that stimulate root and shoot development. Plant growth-promoting rhizobacteria (PGPR) are well known to exert these direct mechanisms, thereby enhancing nutrient acquisition and improving plant vigor (Bhattacharyya and Jha, 2012; Bashan et al., 2020). Phosphate-solubilizing bacteria, Rhizobium strains not only mobilize soil phosphorus but also produce auxin-like compounds, resulting in significant gains in maize productivity under reduced phosphorus inputs (Wahbi et al., 2023). Similarly, the application of a native rhizobacterial consortium was shown to improve maize performance, with reported increases of up to 25% in root length and 32% in plant height, highlighting the role of biochemical traits and nutrient uptake efficiency in promoting growth (Sood et al., 2025). These findings reinforce the potential of nitrogen-fixing and plant growth-promoting microbial inoculants as effective biofertilizer candidates for sustainable maize cultivation. Inoculation with plant growth-promoting rhizobacteria (PGPR), including *Azotobacter* and *Azospirillum*, significantly improves nutrient acquisition and biomass accumulation in maize and other cereal crops. Therefore, the present results highlight the potential of indigenous nitrogen-fixing isolates, particularly GPNF9 and GPNF12, as effective biofertilizer candidates for sustainable maize production, primarily through their ability to produce IAA and exhibit nitrogenase activity.

Biochemical activity of superior selected GPNF

Nitrogen is an essential element for living things in every ecosystem but it cannot be absorbed by plants directly from the atmosphere,

Table 5. Bioassay results of nitrogen-fixing bacterial isolates on maize growth

Treatment	Plant height (cm)	Root length (cm)	Plant wet weights (g)
Control	19.5^{a}	12.97^{a}	2.07^{a}
GPNF1	25.67^{c}	23.13^{cdef}	2.97^{ab}
GPNF2	28.73^{c}	14.67^{ab}	2.67^{ab}
GPNF3	25.4^{bc}	14.00^{ab}	2.19^{a}
GPNF4	21.5^{ab}	19.63^{bcde}	2.78^{ab}
GPNF5	28.23^{c}	18.43^{abcd}	2.70^{ab}
GPNF6	25.5^{c}	17.43^{abc}	2.05^{a}
GPNF7	27.47^{c}	27.63^{f}	3.26^{ab}
GPNF8	27.13^{c}	22.27^{cdef}	2.97^{ab}
GPNF9	29.12^{c}	27.67^{f}	4.13^{b}
Increament than control	49.33%	113.35%	99.5%
GPNF10	27.33^{c}	23.80^{def}	2.91^{ab}
GPNF11	25.07^{bc}	25.83^{ef}	2.45^{a}
GPNF12	28.93^{c}	22.43^{cdef}	3.11^{ab}
Increament than control	48.31%	72.9%	50.24%

so for nitrogen, plants depend on free living and microbial symbiosis present in the soil. The potential of soil nitrogen fixation indicates the fertility of the soil with respect to nitrogen fixation. Nitrogenase is a key indicator of the capacity of bacteria that have the ability to fixate nitrogen and release nitrogen in the soil, so that it can be transformed by Nitrosomonas and Nitrobacter bacteria into available to plants. The nitrogenase enzyme catalyzes the reduction of N_2 to NH_3 and the reaction is sensitive to the presence of O_2 (Figure 1).

The biochemical assay results show that among the three tested isolates, GPNF12 demonstrated the highest potential as a biofertilizer candidate, producing the greatest amount of indole-3-acetic acid (IAA, 1.394 ppm) and exhibiting markedly superior nitrogenase activity (1.381 $\mu M \cdot mL^{-1} \cdot g^{-1} \cdot h^{-1}$) and GPNF9 (1.153 ppm; 0.560 $\mu M \cdot mL^{-1} \cdot g^{-1} \cdot h^{-1}$).

The findings indicate that GPNF12 is the most promising isolate for promoting plant growth through both phytohormone production and nitrogen fixation, while GPNF9 may serve as supportive strains in biofertilizer consortia, which is consistent with recent studies highlighting the importance of microbial IAA and nitrogen-fixing activity in enhancing nutrient uptake and plant growth efficiency (Gang et al., 2021; Pongsilp and Nimnoi, 2024). Bacteria can produce phytohormones, especially IAA can be beneficial for plant growth and development. Based on the IAA phytohormone testing presented in Table 6. showed that GPNF12 isolates produced the highest IAA phytohormone of 1.380 ppm, then isolates with the second highest

Table 6. Biochemcial activity of selected isolate of Etbiofertilizers to IAA and to fix the nitrogen quantitatively

Isolate code	IAA (ppm)	Nitrogenase µM mL^{-1} g^{-1} h^{-1}
GPNF9	1.153	0.560
GPNF12	1.394	1.381

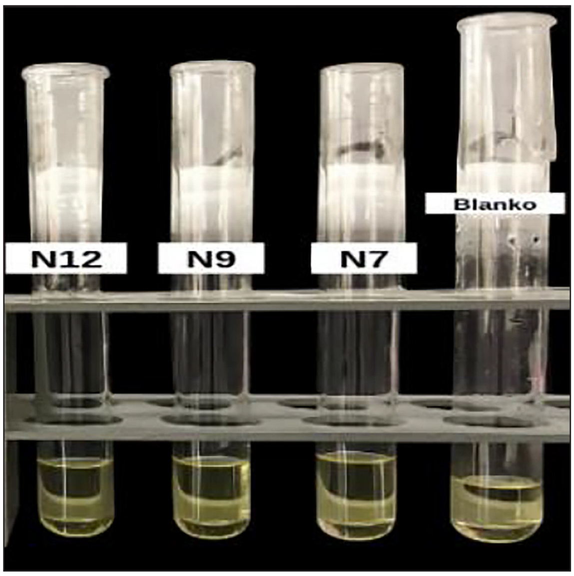

Figure 1. Analysis of indole acetic acid concentration value – the more yellow the color, the higher the value

Table 7. Blast analysis sequencing 16S rRNA fitogrowth promotor and nitrogen fixing bacteria

Code	Bacteria	Homology	Accession number
NF9	*Stenotrophomonas* sp. HH10	99.06%	2312.09183
NF12	*Enterobacter* sp. strain YF3	81.28%	2312.09184

IAA concentration value were GPNF9 of 1.146 ppm. The highest 95.81 nmol mL^{-1}h^{-1} of nitrogenase activity was reported in strain A02, and thus more nitrogen fixation was observed in strain A02. In conclusion, A02 is a newly discovered endophytic nitrogen-fixing bacteria in cassava that can be further used in the research of biological bacterial fertilizers (Zhang et al., 2022).

Biomolecular analysis of selected superior NFB and PSB

Biomolecular analysis and taxonomic identification of selected superior GPNF isolates were performed to validate their classification beyond preliminary phenotypic characterization. The isolates were initially screened through bioassays based on their functional traits, including biofilm formation, indole-3-acetic acid production, organic acid secretion, and nitrogenase activity, indicating their potential as plant growth-promoting bacteria. For molecular identification, 16S rRNA gene sequencing was conducted on representative superior isolates (GPNF9 and GPNF12). Sequence data were processed and analyzed using appropriate bioinformatics tools, and taxonomic affiliation was determined through comparison with reference sequences in the NCBI database using BLAST (Table 7). Sequencing analysis was performed at the ICBB Laboratory of PT Biodiversity Biotechnology Indonesia, Bogor, Indonesia (Figure 2).

The results showed that isolate GPNF9 exhibited high sequence similarity (99.06%) to *Stenotrophomonas* sp. HH10, indicating a close phylogenetic relationship at the species level. In contrast, isolate GPNF12 showed a lower similarity value (81.28%) to *Enterobacter* sp. strain YF3, suggesting a more distant relationship. According to established criteria, 16S rRNA gene sequence similarity values above 97% generally indicate species-level identity, whereas lower similarity may reflect taxonomic divergence or the potential presence of a novel strain (Janda and Abbott, 2007). Therefore, GPNF12 may represent a distinct or potentially novel taxon, warranting further molecular and genomic characterization.

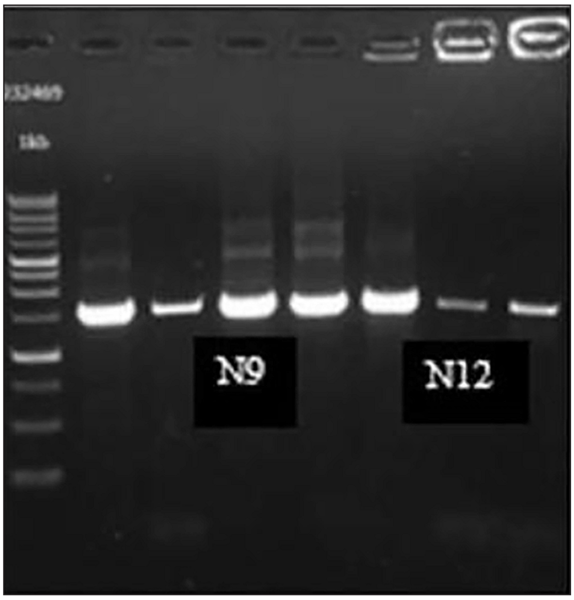

Figure 2. Molecular characterization of hormone producer and nitrogen fixer

CONCLUSIONS

The potential of local wisdom-based biofertilizers (OEB formulations) enriched with plant growth-promoting bacteria to enhance maize growth is highly promising. Developed from locally available plant- and animal-based materials, OEB functions as an effective carrier medium that supports microbial survival and activity in the soil. PGPB isolates, particularly hormone producer and nitrogen-fixer, play a vital role in improving crop performance by producing beneficial bioactive compounds such as indole-3-acetic acid, nitrogenase, and phosphatase. These metabolites increase nutrient solubility and uptake, thereby supporting better plant growth and productivity. Experimental trials demonstrated that OEB formulations derived from bamboo roots, banana weevil, and golden snails effectively stimulated maize development. Among them, the bamboo root formulation was the best treatment, producing the longest root length (14.33 cm, an increase of 13.9% over the control) and the highest plant fresh weight (11.24 g, slightly higher than the control at 10.86 g). This confirms its ability to enhance nutrient uptake and root system development. The isolates tested, GPNF9 and GPNF12 displayed strong nitrogen-fixing ability and high IAA production. These microbial traits translated into substantial improvements in plant growth parameters. Notably, the highest increments reached 113.35% in root length from GPNF inoculation compared to the uninoculated control. The results highlight the potential of OEB as a sustainable and eco-friendly alternative to chemical fertilizers. By enhancing soil fertility and crop resilience, these bioformulations can empower smallholder farmers while supporting climate-smart and resilient agricultural practices.

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