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RESEARCH ARTICLE
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Understanding the Potential of Soil-Originated Bacteria and Root Nodules of Legume and Alfalfa Plants in Dairy Farm Environments in West Java, Indonesia

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ABSTRACT

Microbes play a vital role in sustaining dairy farm ecosystems by supporting soil fertility and plant growth. The nutrient supply from microbes directly influences the quality of soils and the productivity of forage crops such as alfalfa and legumes, which serve as essential feed for livestock. Alfalfa and legume plants, being important feeds for the livestock, are mostly planted around the farming area. Studying in microbial diversity and their soil-enriching potential is crucial. This study aimed to identify beneficial soil microorganisms and root nodule bacteria from alfalfa and legume plants for potential use as biofertilizers in dairy farming. Soil samples were collected from a dairy farm and dried, while nodules were collected from alfalfa and leguminous plants. Microbial enumeration of soil and root nodule microbes, followed by qualitative and quantitative screening of isolates degrade cellulose, indole-3-acetic acid (IAA) production, nitrogen fixation and phosphate solubilization. Molecular identification of selected strains was conducted using 16S rRNA sequencing. Results showed revealed that a higher bacterial and microbial population in legume-cultivated soil compared to alfalfa. Among the isolates, 34 isolates exhibited cellulolytic activity, while eight isolates demonstrated potential nitrogen fixing ability and IAA production. Molecular analysis identified 18 bacterial strains from soil and nodules with significant cellulolytic potential. In conclusion, this study successfully isolated and characterized bacterial strains with promising and great potential applications in sustainable dairy farm programs.

Keywords: Agriculture, Farming, Microorganism, Nutrient cycle

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INTRODUCTION

Microorganisms in the soil play a major role sustaining nutrient availability. Soil microbiomes are essential for ecosystem functioning, particularly in agricultural systems, where they soil fertility, nutrient cycling, plant health, and productivity (Bier et al., 2024). Without microbes, soil weathering processes would not occur. Environmentally friendly agricultural systems reduce reliance on chemical fertilizers and pesticides by instead utilizing organic and

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biological fertilizers, which recycle agricultural waste and sustain output. Biofertilizers derived from soil microorganisms provide essential nutrients in plant-available forms, enhancing soil fertility and crop productivity (Daniel et al., 2022). Beneficial microbes play a crucial role in nutrient cycling, organic matter decomposition, and plant growth promotion by fixing nitrogen, solubilizing phosphate, and producing plant growth regulators like indole-3-acetic acid (IAA). To develop effective biofertilizers, it is essential to harness superior bacterial strains with multiple plant-growth-promoting traits, including cellulose degradation, nitrogen fixation and phosphate solubilization (Shoukat et al., 2024).

In dairy farm ecosystem, maintaining soil health and plant productivity is vital not only for crop production, but also for sustaining livestock nutrition. Legumes and alfalfa are particularly important in sustainable agriculture due to their symbiotic relationships with nitrogen-fixing bacteria in root nodules, which reduce the need for synthetic fertilizers (Zhao et al., 2020). This symbiosis is characterized by the growth of root nodules on the root system (Battenberg & Hayashi, 2022). In these nodules, nitrogen fixation occurs, allowing plants to meet most of their nitrogen needs. Nitrogen fixation from the air takes place in the root nodules, which are symbiotic organs. Beyond nodule-associated bacteria, other beneficial microbes such as cellulose degraders, phosphate solubilizers and IAA producers also contribute to soil fertility and plant growth. Most existing research on plant-microbe interaction has focused on isolated bacterial strains in controlled laboratory ecosystems. Plant–soil–microbe interaction is an intricate, continuous and dynamic process, playing a critical role in shaping rhizosphere ecology, nutrient cycling, and overall plant health (Chauhan et al., 2023). To bridge the gap between controlled studies and real-world applications, this study aims to characterize native microbial communities and identify key bacterial strains, to assess their potential as an effective strategy in enhancing soil fertility and crop productivity (Flater, 2020). The purpose of this study is also to identify beneficial soil microorganisms and root nodule bacteria from legume and alfalfa plants in dairy farm environments for potential use as biofertilizers. We aim to evaluate bacterial strains with capabilities such as cellulose degradation, nitrogen fixation, phosphate solubilization, and IAA production to enhance nutrient availability and support sustainable agriculture. Our findings are expected to provide valuable insights into the role of microbial diversity in sustainable dairy farming, with for improving plant growth, reducing dependence on chemical fertilizers, and promoting ecosystem health.

MATERIALS & METHODS

Soil and Root Nodule Sampling

Eight polybags were used, containing 4 legume plants and 4 alfalfa plants. The samples were taken from around the dairy farms of South Bandung-West Java, Indonesia. Two polybags from each plant group were taken for analysis. Soil samples were taken at 5 sampling points: 4 points at the corners of the polybag and 1 point in the middle of the polybag. The soil samples were dried at room temperature for 3 days (Fig. 1).

(a) (b)

Fig. 1: (a) Soil being drained, (b) Dried soil.

The roots of legume and alfalfa plants were removed from the soil to collect their root nodules. Root nodules were collected by cutting part of the roots because they were too small. Furthermore, the root nodules were cleaned from the attached soil using sterile distilled water for TPC. Root nodules were collected from legume and alfalfa plants (Fig. 2). The root nodules of legume plants were larger than those of alfalfa plants (Fig. 3).

(a) (b)

Fig. 2: Legume plant (a) and alfalfa plant (b).

Fig. 3: Left is a root nodule of a legume plant, and right is a root nodule of an alfalfa plant.

Enumeration of Soil Microbes and Root Nodules Microbial Growth Medium

There were 4 media used, i.e. NA (1.3% NB and 1.6% agar) for general bacterial growth; HVA (Humic Acid

Vitamin Agar) (Humic acid, Na_2HPO_4, KCl, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, nalidixic acid, cyclohexamide, vitamin solution and agar) pH 7.2 for Actinomycete growth (Hayakawa and Nonomura, 1987), PSVA (Pikovskaya agar) (Glucose, NaCl, $Ca_3(PO_4)_2$, $(NH_4)2SO_4$, KCl, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, $MnSO_4$, yeast extract, and agar) pH 7 for the growth of phosphate-solubilizing bacteria (Nautiyal 1999), JA (Jensen agar) (KH_2PO_4, K_2HPO_4, $MgSO4._7H_2O$, $CaSO4.2H_2O$, $FeCl_3$, $Na_2MoO_2.2H_2O$, yeast extract, sucrose and agar) for the growth of soil bacteria of the Azotobacter type (Shomi et al., 2021).

Microbial Enumeration

The number of microbes was counted by dilution. Dilution was done serially from 10^{-1} to 10^{-6}. Samples (1mL) diluted to 10^{-4}, 10^{-5}, and 10^{-6} were inoculated into NA medium using the pour plate method, then incubated at 30°C for 1-2 days. Samples (0.1mL) at dilutions of 10^{-4}, 10^{-5}, and 10^{-6} were inoculated into HVA medium using the spread method, then incubated at 30°C for 4-5 days. Samples (1mL) at dilutions of 10^{-4}, 10^{-5} and 10^{-6} were inoculated into Pikovskaya agar medium using the pour plate method, then incubated at 30°C for 4-5 days. Samples (1mL) at dilutions of 10^{-4}, 10^{-5}, and 10^{-6} were inoculated onto Jensen agar medium using the pour plate method, then incubated at 30°C for 3-4 days.

Purification and Screening of Isolates from Soil and Root Nodules

Purification of soil and root nodule bacteria isolates was carried out by taking several colonies from the TPC results that looked morphologically different. Screening was carried out to obtain isolates that had the potential to degrade cellulose, fix nitrogen, dissolve phosphate, and produce IAA.

Qualitative and Quantitative Tests of Cellulose-Degrading Isolates (Avellaneda-Torres et al., 2014)

Qualitative test of cellulolytic activity Pure isolates were spotted on CMC media in the middle to see the ability of bacteria to degrade cellulase enzymes with the formation of a clear zone on the media incubated for 72 hours by giving 0.1% Congo red solution in distilled water for 30min, then washing it with 0.2M NaCl 3 times. After incubation for 48 hours, bacteria that were able to decompose cellulase enzymes would form a clear zone on the edge of the colony. Then calculate the Cellulolytic Index (IS) value from the clear zone formed using the formula:

IS (Cellulolytic Index) = (Ø Clear zone colony- Ø bacterial colony)/Ø bacterial colony

Quantitative Test of Cellulolytic Activity

The process of measuring enzyme activity by taking a culture of bacterial isolates that have been grown in CMC liquid media and putting it into a centrifuge tube. Then centrifuged at a speed of 8000rpm for 10min. A total of 0.5mL of the supernatant to measure the activity of the dissolved enzyme in the crude extract enzyme. 0.5mL of CMC 1% then vortexed and incubated at room temperature for 30min. One milliliter of DNS was added, then heated at a temperature of 100°C for 15min. The sample was cooled and measured using a spectrophotometer at a wavelength of 540nm. The working procedures of the sample, control, and blank were carried out simultaneously. The preparation of a standard glucose curve was to make a glucose stock solution; 1g (1000mg) of glucose was dissolved in 100mL of sterile H_2O, meaning that 1mL of stock solution contains 10mg of glucose. The solution was needed to make a standard glucose curve with a concentration of 1mg/mL glucose. A 100µL of stock solution was diluted with 900µL of sterile H_2O. Each solution was added with 1mL of DNS reagent, then incubated at 100^{o}C for 15min. absorbance reading at a wavelength of 540nm.

Screening of Nitrogen Fixer Isolates

A total of 1 loop of bacterial colonies grown on solid Nitrogen Free Bromthymol Blue (NFB) media was grown on semi-solid NFB media and incubated at room temperature for 5-10 days. Solid and semi-solid NFB media were made from the same ingredients. A total of 5g of DL-malic acid, KOH 4g, K_2HPO_4 0.5g, $MgSO_4.7H_2O$ 0.1g, $MnSO_4.H_2O$ 0.05g, NaCl 0.02g, $CaCl_2$ 0.01g, $FeSO_4.7H_2O$ 0.05g, $Na_2MoO_4.2H_2O$, 0.002g, and 30g of bacto agar for solid NFB media, while for semi-solid NFB media, 1.75g of bacto agar and 2mL of 0.5% bromthymol blue (BTB) were added. The color change of the medium from yellow to blue and the presence of a white ring under the surface of the medium were observed (Shi et al., 2023).

Phosphate-solubilizing Bacteria Screening

One pure bacterial colony was spotted on the Pikovskaya medium, then incubated at a temperature of 27-30°C for 4-5 days. Then the formation of a clear zone around the bacterial colony was observed (Nautiyal 1999).

IPF (Phosphate Solvent Index) = (Ø Clear zone colony- Ø bacterial colony)/Ø bacterial colony

Qualitative and Quantitative Testing of IAA Production Capabilities

Qualitative Test of IAA Production Capability

Pure bacterial isolates were streaked on LB + L-tryptophan medium, then incubated for 48-72 hours. For the test, the petri dish containing the isolate was dripped with Salkowski reagent and incubated in a dark room for 30min. After that, the formation of a red color in the bacteria was observed, indicating that the isolate could produce the IAA hormone.

Quantitative Test of IAA Production Capability

Each isolate was grown in LB broth enriched with tryptophan and the culture was incubated. One percent of the isolate culture was inoculated into fresh LB media enriched with tryptophan and then incubated at 28°C. Measurements were taken at 24, 48 and 72 hours. The cultured sample was centrifuged, and the supernatant was mixed with Salkowski reagent and incubated at room temperature. Absorbance was then measured with a spectrophotometer at λ = 530nm. The amount of IAA

produced was calculated by converting the absorbance to the IAA standard curve regression. The IAA standard curve was created with a concentration range of 5-50µg/mL (Gang et al., 2020).

Molecular Identification with 16S rRNA

Sample Preparation

The bacteria isolate was cultured in 5mL liquid medium and under shaking conditions for 1 night. A 5µL aliquot of the culture was centrifuged at 13,000rpm for 5min. The pellet was collected for genomic DNA extraction (Adeleke, 2024).

Genomic DNA Extraction and Amplification

The pellet was washed with 500µL TE Buffer (10mM Tris-HCl pH 8, 1mM EDTA). The pellet suspended with 50µL TE Buffer and then lysed by adding 300µL Extraction Buffer (200mM Tris-HCl pH 8, 250 mM NaCl, 25mM EDTA, and 0.5% SDS), followed by vortexing and mechanical grinding. The mixture was incubated at 65°C for 15min, cooled to room temperature, and then treated with 150µL Na acetate (pH 5.2) for 10min. After centrifugation (13,000rpm, 5min), the supernatant was transferred to a new microtube and DNA was precipitated with an equal volume of isopropanol (1:1). Then DNA precipitation was carried out by centrifugation at a speed of 13,000rpm for 10min. The DNA pellet was washed with 70% ethanol, air-dried, and resuspended in 50µL TE buffer. Extracted DNA was stored at -20°C until further analysis.

PCR Amplification of 16S rRNA Gene

PCR Master Mix Preparation (50µL total volume): 25µL DreamTaq Green PCR Master Mix, 20µL sterile dH_2O, 2µL Primer 27F (10pmol; 5'-AGAGTTTGATCCTGGCTCAG-3'), 2µL Primer1492R (10pmol; 5'-CGGTTACCTTGTTACGACTT-3'), and 1µL template DNA PCR Conditions (30 cycles): Pre-denaturation: 95ºC (1 min, 30s), denaturation: 95ºC (30s), annealing at 55ºC (1min, 30 sec), extension at 72ºC (10min), and hold 4ºC (∞) (Lisdiyanti et al., 2011). PCR products were electrophoresis on 1% agarose gel, (100 V, 35min) in 1× TAE buffer. Gel visualized under UV light and documented.

DNA Sequencing and Analysis

The PCR products were sent to 1st Base, Malaysia, for Sanger sequencing. The sequencing data were analyzed using BioEdit (ver. 7.2.6) (http://www.softpedia.com/get/Science-CAD/BioEdit.shtml) and aligned against the NCBI database via BLAST for homology identification (https://blast.ncbi.nlm.nih.gov).

RESULTS & DISCUSSION

Enumeration of Soil Microbes

Table 1 shows that the bacterial population in the soil of legume plants is higher than that of alfalfa plants. The microbial population is also seen to be greater in the root nodules of legume plants than alfalfa plants (Table 2).

Table 1: Soil TPC results for legumes and Alfafa

Sample Code	Medium	TPC Result (CFU/gram)
K3 (Beans)	NA	2.10×10^6
	Jensen	7.50×10^4
	Pikovskaya	5.98×10^5
	HV	1.75×10^6
K4 (Beans)	NA	3.02×10^6
	Jensen	8.75×10^4
	Pikovskaya	6.37×10^5
	HV	1.85×10^6
P3 (Alfafa)	NA	2.56×10^6
	Jensen	1.97×10^5
	Pikovskaya	8.67×10^5
	HV	5.50×10^5
P4 (Alfafa)	NA	1.83×10^6
	Jensen	1.05×10^5
	Pikovskaya	8.00×10^4
	HV	4.30×10^5

Table 2: TPC Results of Root Nodules of Legumes and Alfalfa Plants

Sample Code	Medium	TPC Result (CFU/g)
K3 (Beans)	NA	TBUD
	Jensen	1.96×10^6
	Pikovskaya	3.04×10^6
	HV	1.85×10^5
K4 (Beans)	NA	$2.45x\ 10^7$
	Jensen	5.13×10^5
	Pikovskaya	8.02×10^6
	HV	$7.10x\ 10^5$
P3 (Alfafa)	NA	TBUD
	Jensen	7.67×10^5
	Pikovskaya	7.23×10^4
	HV	1.30×10^5
P4 (Alfafa)	NA	1.69×10^7
	Jensen	5.48×10^5
	Pikovskaya	5.42×10^5
	HV	2.80×10^5

Purification and Screening of Isolates from Soil and Root Nodules

Qualitative Test of Cellulolytic Activity

The presence of cellulolytic activity can be seen by the formation of a clear zone around the colony, as in Fig. 4. The results of screening soil isolates in CMC agar medium with Congo red staining obtained 22 bacterial isolates from soil and 12 isolates from root nodules (Fig. 5 and 6). In addition to common bacteria, 8 actinomycete isolates were also obtained that had cellulolytic activity (Fig. 7).

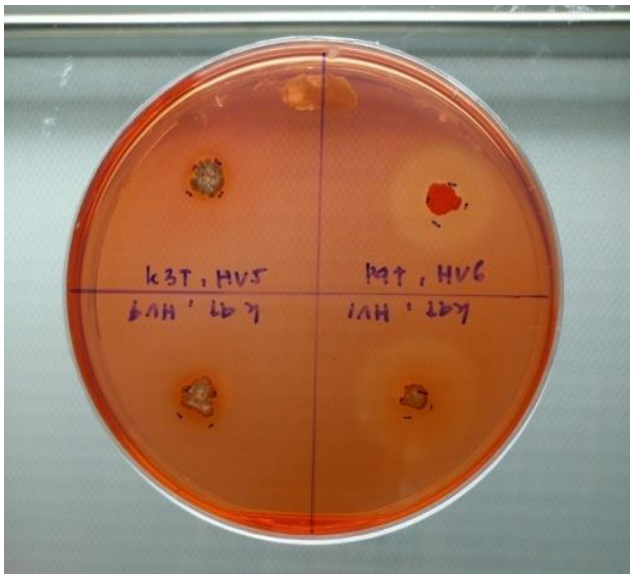

Fig. 4: Results of qualitative cellulase activity tests.

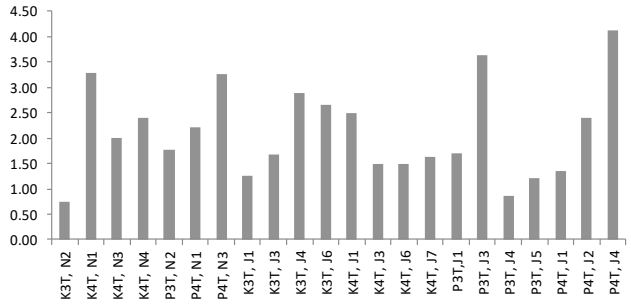

Fig. 5: Cellulolytic index of bacteria isolates from soil.

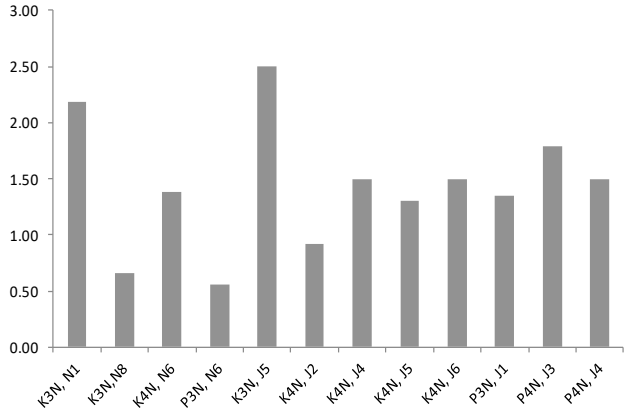

Fig. 6: Cellulolytic index of bacteria isolates from root nodules.

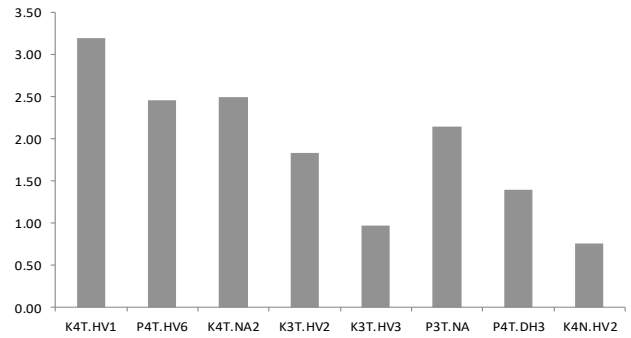

Fig. 7: Cellulolytic index of actinomycetes isolates.

The presence of a clear zone is caused by the cellulose degradation process by cellulolytic bacteria. Cellulose hydrolysed in the agar medium, when flooded by Congo red, will produce a clear zone. This is because Congo red cannot bind to the medium without the β-1,4-glycosidic bond contained in the cellulose polymer. This is due to the presence of the cellulase enzyme so that the cellulose polymer bond is hydrolyzed (Kognou et al., 2022). Rinsing with NaCl will dissolve Congo red especially in the area around the colony containing hydrolyzed cellulase derivatives such as cellodextrin, cellobiose, and glucose, because Congo red is not strongly bound, so that a clear zone is visible. Cellulolytic bacterial isolates produce clear zones of varying diameters, which can differ from one isolate to another. This can be caused by several factors, one of which is because the colony size is different between one isolate and another. Based on the results of the cellulolytic index calculation, 15 isolates (12 from soil and 3 from root nodules) were taken had high IS values to continue quantitative testing. In addition, 8 actinomycete isolates that tested positive for cellulolytic activity were also selected for quantitative testing.

Quantitative Test of Cellulolytic Activity

A total of 15 isolates (from soil and root nodules) and 8 actinomycetes isolates were selected that had high cellulolytic indexes to be tested quantitatively using the DNS method. Total cellulase activity was determined by measuring the activity of a mixture of enzymes that hydrolyze materials containing cellulase and produce glucose as the final product. Cellulase is a multienzyme complex that works to hydrolyze cellulase into glucose (Ejaz et al. 2021). Enzyme activity is influenced by several factors, including the type of bacteria, incubation time, interaction of bacteria with the type of substrate, and interaction of substrate and time (Hapsoh et al., 2021). Cellulase enzyme activity is calculated in units/mL. Cellulolytic activity in units per milliliter (U/mL) is defined as the activity of bacterial isolates that produce glucose as cellulose monomers per minute. The results of the enzyme activity test showed that isolate K3T.J4 had the highest activity of 1.123U/mL, and isolate K3N.N1 was in second place with an activity of 0.923U/mL, while the lowest enzyme activity was produced by isolate P3T.N2 of 0.156U/mL (Fig. 8).

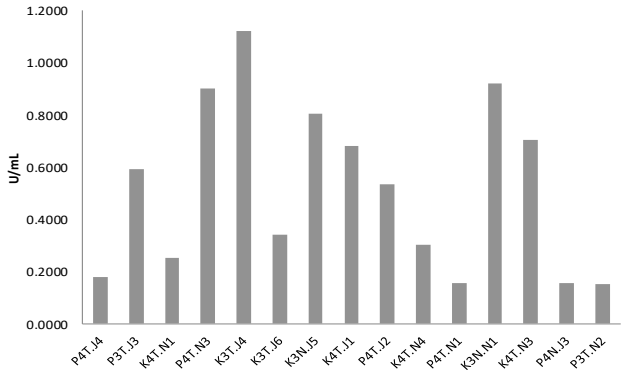

Fig. 8: Results of measuring cellulase enzyme activity from soil bacteria and root nodules.

Fig. 9 shows that the highest cellulase enzyme activity was produced by the actinomycetes isolate P4T.HV6 at 0.598U/mL and the lowest enzyme activity was produced by the isolate P4T.DH3 at 0.051U/mL. Based on the qualitative test of the cellulase enzyme and the quantitative test, there were differences because they were influenced by several factors, i.e. differences in the type of organism, the growth rate of each isolate in agar and liquid media, the amount of inoculum given to both media and the type of enzyme produced. Enzyme activity values and clear zones can also occur in the same species due to differences in strains because differences in bacterial strains can also be followed by differences in enzymatic ability (Piotrowska-Długosz et al., 2022).

Nitrogen Fixation Isolate Screening

The results of the nitrogen fixation isolate screening are indicated by a color change in the NFB media from

yellow to blue (Fig. 10). A total of 8 isolates were found to have potential as nitrogen-fixing bacteria on semi-solid NFB media with the addition of bromthymol blue indicator compounds (Table 3). According to Salsabila & Nur Rahmawati (2022), the color changes in NFB media occurs because the nature of the bromthymol blue indicator changes to blue at higher pH levels due to nitrogenase activity. This result also supported by Ghorai & Gosh (2023) result, which states that semi-solid NFB media is able to provide the nutrients needed by non-symbiotic nitrogen-fixing bacteria; the blue color change in semi-solid NFB media indicates that there is nitrogenase activity carried out by non-symbiotic nitrogen-fixing bacteria.

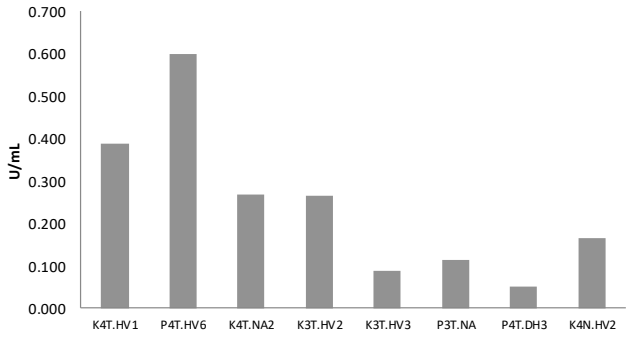

Fig. 9: Results of measuring cellulase enzyme activity from actinomycetes.

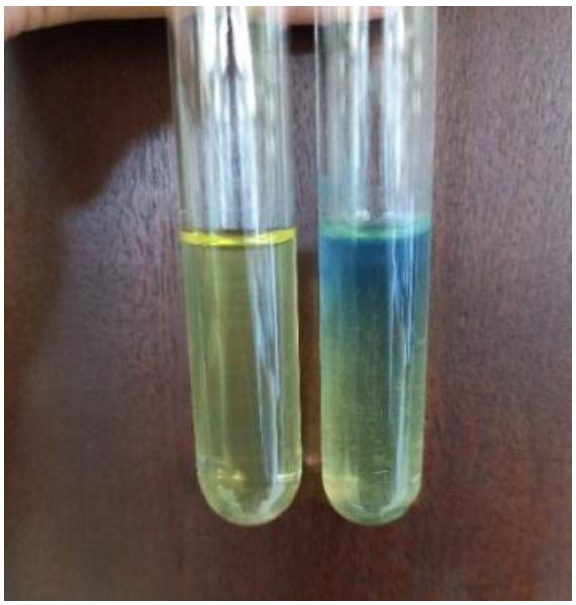

Fig. 10: Results of the nitrogen-fixing bacteria test: the left is the control and the right is the positive test result.

Table 3: Results of screening of nitrogen-fixing bacteria

No	Isolate Code	Result
1	K3N, J2	++++
2	K3N, J4	+++
3	K4N, J2	+++
4	K4N, J5	++
5	K4N, J6	+++
6	P3N, J2	+++
7	P3N, J4	++
8	K3T, J6	+

Note: (+) low, (++) mid, (+++) high, (++++) very high

Isolates that can fix nitrogen almost all come from root nodules. The isolate K3N.J2 exhibited high nitrogenase activity, as it was able to change the semi-solid NFB medium more rapidly than the others. In addition, most of them come from legume plants. These bacteria also only have a symbiosis with legume plants by infecting plant roots and forming nodules. Most nitrogen-fixing bacteria are heterotrophic, which requires a small amount of carbon, such as species in the genera *Azotobacter* and *Azospirillum*. Some others are autotrophic, capable of reducing carbon dioxide (Liang et al., 2020). Generally, nitrogen fixation can only occur anaerobically, with only a few strains of certain species able to perform this process under such conditions (Nurhakiki et al., 2025).

Phosphate-solubilizing Bacteria Screening

Phosphate-solubilizing bacteria are soil bacteria that can dissolve phosphate, making it available for plant absorption. In addition to increasing phosphate in the soil, these bacteria can also play a role in enhancing vitamin D metabolism, promoting plant root growth, and increasing nutrient absorption (Pang et al., 2024). The results of phosphate-solubilizing bacteria screening are indicated by the presence of a clear zone around the colony (Fig. 11). This clear zone forms because the bacterial isolates growing on Pikovskaya medium are able to dissolve phosphate present in the medium, specifically $Ca_3(PO_4)_2$. This dissolution occurs due to the activity bacterial isolates that produce organic acids. This clear zone forms because the bacterial isolates growing on Pikovskaya medium are able to dissolve phosphate present in the medium, which is found in the media, specifically $Ca_3(PO_4)_2$. Organic acid has been suggested as the principal mechanism of inorganic P solubilization (Rawat et al., 2021).

Fig. 11: Phosphate-solubilizing bacteria screening results; (Pikovkaya medium showing halo zones by phosphate-solubilizing bacteria).

Fig. 12 shows the potential index of isolates that can dissolve phosphate, isolate K3N. N3 has the highest IP of 1.4. The higher the phosphate solubilization index, the higher the amount of phosphate that can be dissolved. The formation of a clear zone is due to the presence of the organic acids produced by bacteria. The release of phosphate is related to the formation of organic acids by

bacteria to dissolve bound phosphate in the soil. So that phosphate will be released to form $H_2PO_4^-$ and HPO_4^{2-} ions, which are forms of phosphate that can be absorbed by plants (Pang et al., 2024). Bacteria that act as phosphate solvents in the soil have been widely found, including from the genera *Pseudomonas, Micrococcus, Bacillus, Azotobacter, Microbacterium* and *Flavobacterium* (Timofeeva et al., 2022).

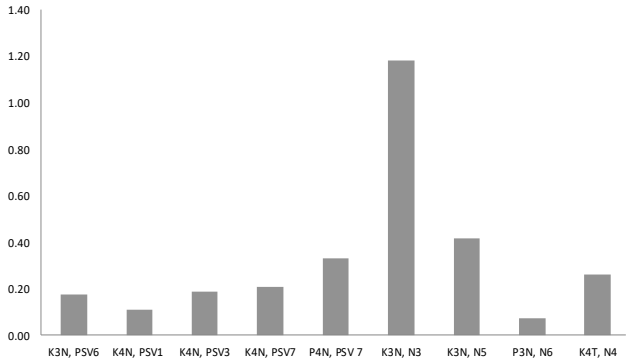

Fig. 12: Potential index of phosphate solubilizing isolates.

Qualitative and Quantitative Testing of IAA Production Capabilities

Qualitative Test of IAA-producing Isolates

The results of screening IAA-producing isolates obtained 8 isolates that were able to produce IAA, marked by the formation of a red color on the nitrocellulose membrane. The red color is an indication of the detection of indole from IAA (Etesami & Glick 2024). The redder the color formed, the higher the concentration of IAA produced (Fig. 13). Furthermore, 8 isolates that were positive for producing IAA were continued with quantitative testing consisting of K3N. J4, P3N. J3, P3N. J4, K3N. N6.

Fig. 13: Qualitative test results of IAA-producing isolates.

Quantitative Test of IAA Production Capability

The quantitative test is based on the ability of isolates to produce a red color on a nitrocellulose membrane, indicating IAA production. Eight tested positive for IAA production, measured in µg/mL units. The test results showed that the highest IAA production was by isolate P3N.N6 at 72 hours, with a result of 12472.73 µg/mL (Fig. 14). This isolate produces IAA during the stationary phase, as 72 hours marks the end of the logarithmic or stationary phase. Generally, bacteria produce maximum IAA at 24 or 48 hours, with a decrease by 72 hours. This is because at this time the bacteria are in the final logarithmic phase, during which the enzyme that converts tryptophan into IAA is produced in large quantities (Rohmah et al., 2020). Excess IAA concentration is regulated by bacteria through the production of IAA oxidase and peroxidase enzymes (Lata et al. 2024).

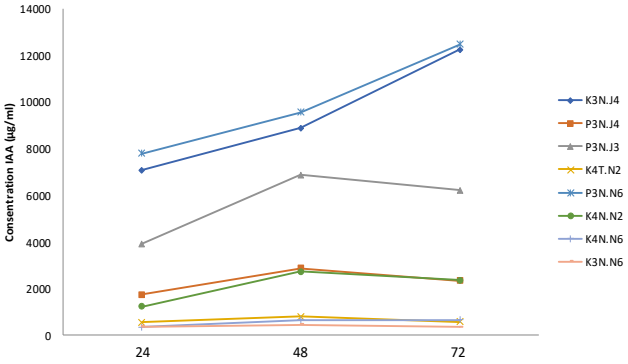

Fig. 14: IAA production at 24, 48, and 72 hours.

Molecular Identification with 16s rRNA

Genomic DNA Extraction Results

Genomic DNA from a total of 57 potential bacterial isolates was carefully extracted, and concentration and purity were measured. Almost all samples had quite high DNA concentrations, with only a few isolates having DNA concentrations below 100ng/µL. Meanwhile, DNA purity was assessed by the OD 260/280 values, which ranged between 1.8-2.0.

The absorbance ratio between 260 and 280nm is used to assess DNA quality. A DNA sample is generally considered "good" if the ratio is around 1.8±0.5. A ratio that is significantly lower (≤1.6) may indicate the presence of phenol, proteins, or other contaminants that absorb at or near 280nm. A common secondary indicator of DNA purity is the 260/230 ratio, with expected values typically ranging from 2.0 to 2.2. A lower than expected 260/230 ratio may suggest the presence of impurities that absorb at 230nm, such as proteins, guanidine HCl (used in DNA extractions), EDTA, carbohydrates, lipids, salts, or phenol (Versmessen et al. 2024). A DNA concentration of more than 20 ng/µL is generally considered sufficient for DNA isolation purposes (Pineda-Rodriguez et al. 2023).

Identification of 16S rRNA

A total of 57 isolates were amplified (Fig. 15). Of the 57 isolates, 20 isolates were selected that had the ability to degrade cellulose, fix nitrogen, solubilize phosphate, and produce IAA to continue sequencing (Table 4).

Table 4: BLAST Analysis result

No	Isolate Code	Potential activity	BLAST Result	Identity (%)	Acc. Number
1	K4T.N1	Cellulase	*Bacillus licheniformis* strain Nor3075	99.44	EU871707.1
2	P4T.N3	Cellulase	*Bacillus velezensis* strain SKC/YLP-2	99.86	MT229334.1
3	K4T.N4	Cellulase	*Bacillus stratosphericus* strain SN1-1	99.43	MT071669.1
4	P4T.J4	Cellulase	*Bacillus licheniformis* strain SR5-11	99.79	MN421512.1
5	K3N.N1	Cellulase	*Bacillus* sp. (in: firmicutes) strain 0725LM100198	99.57	OQ874297.1
6	K3N.J4	N Fixation	*Enterobacter asburiae* strain AFS093380	98.66	OP986188.1
7	K4N.J5	N Fixation	*Pseudomonas koreensis* strain PgBE27	99.79	MH144250.1
8	K4N.J6	N Fixation	*Pseudomonas* sp. WPCB008	99.57	FJ006865.1
9	K3N.PSV6	Phosphate Solubilizing	*Klebsiella* sp. strain 192_a_42_10	99.50	ON845406.1
10	K4N.PSV1	Phosphate Solubilizing	*Herbaspirillum* sp. DUT-1	99.50	KU359250.1
11	K4N.PSV3	Phosphate Solubilizing	*Klebsiella* sp. strain BT1MT	99.65	OL662826.1
12	K4T.HV1	Cellulase	*Streptomyces* sp. E3N146	99.49	KX279567.1
13	P4T.HV6	Cellulase	*Streptomyces zaomyceticus* strain QMA47	99.64	MT525265.1
14	K3T.HV2	Cellulase	*Streptomyces olivochromogenes* strain 20-3	99.71	EU367973.1
15	K3T.HV3	Cellulase	*Streptomyces* sp. strain YJ-FR-4	99.49	OQ439115.1
16	K4N.HV2	Cellulase	*Streptomyces turgidiscabies* strain T5R3	99.42	KT363057.1
17	P3N.J4	IAA	*Pantoea agglomerans* strain Ns13	87.67	MG544110.1
18	P3N.N6	IAA	*Bacterium* strain AGE_KM_C04	99.22	MW037353.1

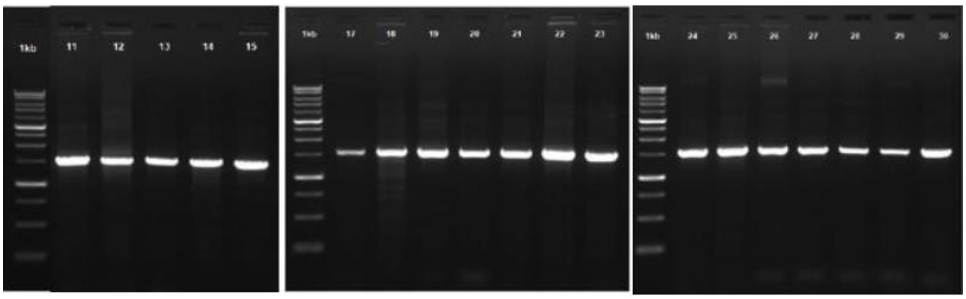

Fig. 15: Electrophoresis of PCR Products.

The identification results based on the 16S rDNA gene are shown in Table 4. Partial 16S ribosomal RNA (rRNA) gene sequencing is considered a more accurate method for identifying bacteria compared to traditional phenotypic techniques (Bertolo et al., 2024). A >99% identity in 16S rRNA gene sequence is used to identify an isolate at the species level. A 97 to 99% identity in 16S rRNA gene sequence is the criterion used to identify an organism at the genus level, while a <97% identity in 16S rRNA gene sequence was the criterion used to define a potentially new bacterial species (Hackmann 2025).

Of the 20 isolates sent for sequencing, 18 isolates were able to proceed with the sequencing process. The results of the sequencing analysis showed that bacteria with cellulolytic activity belong to the genus *Bacillus*, namely *Bacillus subtilis, Bacillus licheniformis,* and *Bacillus altitudinis*. Several cellulolytic bacteria species have been reported to originate from soil, including members of the genera *Cellulomonas, Cytophaga, Pseudomonas, Bacillus,* and several actinomycetes (Bautista-Cruz et al., 2024).

In addition to their cellulolytic activity, the genus *Bacillus* is often used as a biological control agent against soil-borne diseases. *B. subtilis* has been proven to be a good biological control agent (Zhang et al., 2023). *Bacillus altitudinis* has the ability to produce growth-promoting hormones. These bacteria in plant roots can produce plant growth regulators, or phytohormones, such as IAA, cytokinins, and gibberellins, which support plant growth (Zhang et al., 2021). In addition to *B. subtilis* and *B. altitudinis*, *B. lincheniformis* was also identified, a soil microorganism that forms spores, contributing to the nutrient cycle and exhibiting antifungal activity (Ramirez-Olea et al., 2022). Several bacteria are known to fix nitrogen and supply it to host plants, including *Arthrobacter, Azoarcus, Azospirillum, Azotobacter, Bacillus, Enterobacter, Klebsiella, Pseudomonas, Herbasprillum* and *Gluconobacter* (Gómez-Godínez et al., 2023). In addition to the above bacteria, the analysis results showed that there were five actinomycetes isolates identified with cellulolytic activity: *Streptomyces zaomyceticus, Streptomyces sp., Streptomyces olivochromogenes,* and *Streptomyces turgidiscabies.* Numerous species of Streptomyces have a variety of activities that promote plant growth. These activities contribute to a wide range of biological processes by increasing the availability of nutrients in the soil to plants, producing phytohormones, and/or suppressing plant disease by preventing the growth of soil-borne plant pathogens. *Streptomyces* affect the biogeochemical cycles, community structure, and/or other microbes in their surroundings (Chouyia et al., 2022; Donald et al., 2022).

Microbial interactions in the rhizosphere—the soil region influenced by plant roots—are critical for nutrient cycling, manure management, and the advancement of sustainable agriculture (Das et al., 2022). In legumes and alfalfa, these interactions are especially important due to their ability to host symbiotic nitrogen-fixing bacteria. The complexity of soil microbial and their ability to promote soil fertility, including phosphate solubilizers and IAA producers, can reduce dependency on chemical inputs. Harnessing native or locally-adapted microbes through inoculation or organic amendments may reduce input costs for smallholder farmers (Arellano-Wattenbarger et al. 2025). IAA produced by the bacteria will also help plants recover from abiotic stresses, such as heat and drought (Mal & Panchal, 2024), which are prone to happen in tropical countries like Indonesia.

Conclusion

The results indicate that legume-associated soils and nodules harbor more diverse and abundant microbial communities than those of alfalfa. These microbial populations include key functional groups: cellulolytic bacteria useful in manure degradation, nitrogen-fixers that contribute to natural soil fertility, phosphate solubilizers that increase nutrient availability, and IAA producers that promote plant growth. Together, these findings highlight the potential for applying such indigenous microbial strains as biofertilizer agents to support a more sustainable dairy farming system. Notably, the diversity of cellulolytic bacteria may also enhance manure management practices, contributing to improved animal health and pasture quality.

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Ethics Statement: This research does not require ethical review because it does not involve data with human or animal subjects.

Author's Contribution: Ellin Harlia: developed the idea, conceived and planned the experiments, analysed the results, also supervised the project, Mieke Rochimi Setiawati: analysed the microbiology results. Roni Ridwan: carried the molecular laboratory works and analysed. Miranti Nurindah Sari: Carried the microbiology laboratory works. Shanti Ratnakomala: Carried the microbiology laboratory works and analysed. Annisa: writing the manuscript draft, analysed the molecular result and analysed. All authors discussed the results and contributed to the final manuscript.

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