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ISOLASI DAN KARAKTERISASI RIZOBAKTERI PENAMBAT NITROGEN DARI EKOSISTEM PADI SAWAH ORGANIK

ISOLATION AND CHARACTERIZATION OF NITROGEN FIXING RHIZOBACTERIA FROM RICE FIELDS ORGANIC ECOSYSTEMS

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ABSTRACT

Rhizobacteria are capable of nitrogen fixation through mutualism, converting atmospheric nitrogen (N) into nitrogen compounds available for plant use, thereby promoting plant growth. The aim of this research is to isolate and characterise N-fixing bacteria in the lowland organic rice ecosystem of Poso Regency, Central Sulawesi. Bacteria were obtained from the rhizosphere of healthy rice plants and subsequently cultured in free Burk-N media for further analysis. Twelve bacterial isolates were able to grow on free Burk-N media. Each isolate had a unique morphology and biochemistry. The RFN7 isolate exhibited the highest total nitrogen content at 0.41%, while the RFN4 isolate exhibited the lowest at 0.16%. These free Burk-N media grown isolates possessed the capacity to bind free nitrogen, which promotes plant growth and development. Therefore, these isolates display considerable potential as biofertilisers to advance sustainable agriculture that is environmentally sound.

KATA KUNCI:
Fiksasi N2, pertanian berkelanjutan pupuk hayati, rizobakteri,

ABSTRAK

Rhizobakteri mampu mengfiksasi nitrogen (N) secara mutualisme, mengubah N dari atmosfer menjadi senyawa nitrogen yang tersedia bagi tanaman, sehingga meningkatkan pertumbuhan tanaman. Tujuan dari penelitian ini yaitu untuk mengisolasi dan mengkarakterisasi bakteri penambat N pada ekosistem padi sawah organik di Kabupaten Poso, Sulawesi Tengah. Bakteri diisolasi dari rizosfer tanaman padi yang sehat, untuk selanjutnya dikulturkan dengan menggunakan media Burk N-bebas. Hasil yang diperoleh sebanyak 12 isolat bakteri memiliki kemampuan tumbuh pada media Burk N-bebas dengan karakter yang berbeda-beda setelah diidentifikasi secara morfologi dan biokimiawi. Isolat RFN7 menghasilkan kadar nitrogen total sebesar 0.41%, sedangkan yang terendah pada isolat RFN4 sebesar 0.16%. Isolat rizobakteri yang mampu tumbuh pada media Burk-N bebas mempunyai kemampuan untuk mengikat nitrogen bebas yang berguna bagi pertumbuhan dan perkembangan tanaman, sehingga isolat-isolat tersebut berpotensi sebagai biofertilizer dalam upaya mendukung pertanian berkelanjutan yang ramah terhadap lingkungan.

1. INTRODUCTION

Intensive use of inorganic fertilizers exceeding the recommended dosage limits in various agricultural businesses is a major issue in realizing sustainable agriculture. This behavior impacts decreasing soil fertility, such as damage to soil texture and structure, loss of microorganisms, and pollution of the surrounding environment in the form of environmental health problems and living things. Such a large impact due to the entry of chemicals indirectly raises the awareness of some people to switch to using biological fertilizers. Biofertilizers contain biologically active microorganisms that can provide the elements needed by plants for their growth and development.

Utilization of alternative nitrogen-fixing rhizobacteria as nutrient providers for plants with their ability to fix free nitrogen from the air (Glick, 2012) and have the ability to increase the efficiency of using available nitrogen in the soil. Nitrogen is an essential nutrient most needed by plants. The nitrogen content in the atmosphere is around 78% but cannot be used directly by plants, while the nitrogen content in the soil is limited depending on the factors that influence it. Nitrogen in the soil is easily lost by various mechanisms such as evaporation, nitrification, denitrification, or even leaching by water or erosion (Whetton et al., 2022; Pashaei et al., 2022; Farzadfar et al., 2021). Uddin et al., (2021) suggested that in some areas, the total N content of the soil is still relatively low, ranging from 0.06-0.17%, so efforts are needed to utilize nitrogen from unavailable forms to available forms for plants through a biological nitrogen fixation process.

Nitrogen fixation is one of the main mechanisms used by soil microbes to promote plant growth. Nitrogen fixation biology is carried out either by non-symbiotic microorganisms that can stand alone or certain bacteria that live symbiotically with higher plants (Bangkele et al., 2020; Bekele et al., 2021). Still, the use of non-symbiotic nitrogen-fixation bacteria is wider than symbiotic bacteria. The process of biological nitrogen fixation will change air N_2 into ammonia due to the presence of the enzyme nitrogenase (Harris et al., 2018; Lindström & Mousavi 2020; Bloch et al., 2020). This enzyme is only possessed by free-living (non-symbiotic) nitrogen-fixation rhizobacteria such as *Enterobacteriaceae, Bacillus, Azotobacter, Clostridium, Chlorobium, Chromatium, Rhodomicrobium, Azospirillum*, and *Herbaspirillum* (Arfarita et al., 2018; Aasfar et al., 2021; Soumare et al., 2020). Several research results regarding the use of nitrogen-fixation rhizobacteria as biological fertilizers have been carried out. Aryanto et al., (2015) research results using bacterial inoculants *Bacillus* sp, *Pseudomonas* sp, *Azospirillum* sp, and *Azotobacter* sp loading lowland rice and upland rice can reduce the dosage of inorganic fertilizers by 50% and improve the quality of acidic soil. Growth-promoting nitrogen-fixation rhizobacteria (RPN-PT) can increase rice plants' growth and potentially be developed as biological fertilizers in saline ecosystems (Tarigan et al., 2021; Khumairah et al., 2022).

Using rhizobacteria as potential biofertilizers can minimize chemical input and be effective and efficient (Etesami and Alikhani 2016; Basu et al., 2021; Nosheen et al., 2021; Gashash et al., 2022). Therefore, a study was conducted to explore nitrogen-fixation rhizobacteria obtained from organic paddy ecosystems for further isolation, identification, and analysis of their ability to fix free nitrogen (non-symbiotic). Organic paddy from this site, grown by the surrounding community, has the ability to grow under a variety of environmental stresses. This is attributed not only to genetic factors, but also to environmental elements, in particular the presence of abundant soil microbes in the region. As a result, the researchers focused on the study of bacteria isolated from the nearby Kamba rice plantation in the Bada valley.

2. MATERIAL AND METHODS

2.1 Source Isolate

The bacterial isolates were obtained from a healthy soil sample in the rhizosphere of rice with superior growth characteristics in an organic rice ecosystem in West Lore District, Poso Regency, Central Sulawesi. Random methods (purposive random sampling) and composite sampling were used by determining 5 sample points in each location for a total of 20 points from 4 different locations. The samples were only the rice plants' roots and soil, while the plants' tops were cut. The sample is put in a sterile brown envelope, labeled according to its location, and then stored in a more excellent box. Then the sample was taken to the laboratory for immediate testing.

2.2 Isolation of Nitrogen-Fixation Rhizobacteria

The initial step for exploring non-symbiotic nitrogen-fixation rhizobacteria is selecting bacterial isolates using specific Burk's N-free solid media. The ingredients in 1 liter of media consist of sterile aquadest, plain agar, sucrose, $MgSO_4.7H_2O$, NaCl, K_2HPO_4, KH_2PO_4, $CaSO_4.2H_2O$, $Na_2MoO_4.2H_2O$ and $FeSO_4.7H_2O$ (Park *et al.,* 2005; Sulaiman *et al.,* 2019).

Bacterial isolates capable of fixing nitrogen were qualitatively tested by their ability to grow on Burk's N-free media. A total of 1 gram of rhizosphere soil sample was weighed and then pulverized using a sterile mortar, then added 10 mL of sterile aquadest stirring until well blended. Then the serial dilution method was carried out with a concentration of 10^{-1} to 10^{-9}. A fine and homogeneous soil sample was taken as much as 1 mL and put into the first dilution tube (already containing 9 mL of sterile water), a 1/10 or 10^{-1} dilution. The suspension homogenized used vortex to be retaken as much as 1 ml and transferred to the second dilution tube (10^{-2}). This transfer process is repeated the same way until the last dilution tube. Furthermore, 0.1 mL of each dilution tube 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} was taken to be spread on Burk's N-free solid media using drigalsky rods aseptically. This technique is called the spread plate method.

Bacterial cultures were incubated for 3-7 days at 28^0C. Bacterial colonies that grew on the media were subcultured to obtain pure isolates. Isolates that were able to grow on N-free Burk media showed that these bacteria could fix nitrogen (Hadija *et al.,* 2021; Saputri *et al.,* 2021). The single colonies were morphologically and biochemically identified to determine their character.

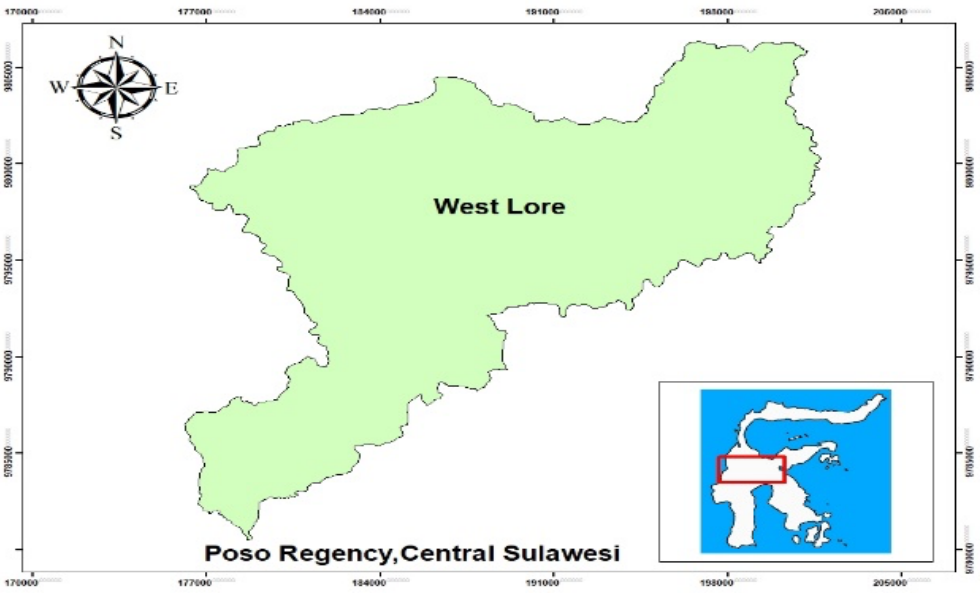

Figure 1. Sampling Area Rhizobacteria in West Lore, Poso Central Sulawesi

2.3 Characterization of Morphology and Biochemical Properties of Rhizobacteria Isolates

Single colonies resulting from a pure culture of nitrogen fixation rhizobacterial isolates were visually characterized according to the method offered by Borkar (2018), based on colony size, colony shape, colony pigment, colony margins, and elevation. Gram reaction test and catalase enzyme production test were carried out to determine the biochemical characteristics of bacterial isolates.

Testing of biochemical properties with the general Gram reaction is conducted to provide convenience in identifying large groups of these bacteria (Hardiansyah *et al.,* 2020). Nitrogen fixation rhizobacterial isolates were placed on a glass object that had previously been dripped with a 3% KOH (*Potassium Hydroxide*) solution. The indicator for the Gram reaction test is positive if the isolated bacteria looks slimy like threads when lifted from the surface of the suspension on the glass slide, and vice versa (Suslow *et al.,* 1982). The catalase test was carried out to test the ability of catalase-producing bacterial isolates to degrade Hydrogen peroxide (H_2O_2). About one full use of a pure culture of a single colony of bacterial isolates is smeared on a slide with two drops of 3% hydrogen peroxide. The emergence of gas bubbles from free oxygen indicates a positive reaction, while an adverse reaction does not indicate the presence of gas bubbles.

2.4 Hemolysis Activity Test and Pathogenicity of Nitrogen fixation Rhizobacteria Isolates

Bacterial isolates potentially pathogenic in humans and animals can be identified by carrying out a hemolysis test. One ose of the bacterial isolate was cultured on a blood agar medium consisting of 40g L^{-1} blood agar base mixed with 5% L^{-1} sterile fresh sheep blood that had been defibrinated. Bacterial isolates were cultured on the blood agar medium and incubated for 18-24 hours at room temperature. Isolates that have been cultured and form clear zones around their colonies show positive hemolysis or are pathogenic for humans and animals, and vice versa. Negative hemolysis indicators do not produce clear zones around their colonies when cultured on blood agar media (Zimbro *et al.,* 2009).

Hypersensitivity reaction tests with pathogenicity methods used tobacco plants (*Nicotiana tabacum* L.). Bacterial isolates to be tested were first cultured in NA media for 24 hours at room temperature 28^0C. After 24 hours, dilution was carried out at (10^8 CFU/mL with OD = 0.06 UV-VIS spectrophotometer) to obtain a suspension of bacterial isolates. 1 mL of the bacterial isolate suspension was injected into the leaves of the plants through the secondary leaf veins. Injections were made on each isolate with three replications. The negative control isolates in this test used sterile aquadest PA, while the bacterial isolate *Burkholderia glumae* served as the positive control. Observations 12 - 72 hours after application to see any hypersensitivity reactions to the tobacco plants that had been injected. A positive hypersensitivity reaction is characterized by the appearance of brown necrotic spots and dryness on the leaf tissue after being injected with bacterial isolates. In contrast, a negative hypersensitivity reaction means no necrosis change or appearance is seen on the tobacco leaf surface (Balint-Kurti 2019).

2.5 Nitrogen Fixation Ability Test By Bacterial Isolates

The ability of bacterial isolates to fix nitrogen quantitatively refers to the method proposed by Park *et al.,* (2005) by culturing bacterial isolates in Burk N-free liquid media in sterile glass vials for 24 hours at 28 ± 2^0C and placing them in an orbital shaker. The digestion stage was carried out by taking 5 mL of the supernatant and then putting it into a digestion tube by adding 1 g of a mixture of selenium and 3 ml of concentrated sulfuric acid (H_2SO_4) to be digested at 35^0C for 4 hours until white steam came out and the extract became clear. Furthermore, the cooled extract was diluted with distilled water (50 mL) and then shaken for 24 hours until well mixed and produced a precipitate of particles.

The distillation process begins by transferring the precipitated extract into a boiling flask and then preparing a reservoir to accommodate the evaporated NH_3. The container is an Erlenmeyer flask

containing 10 ml of 1% boric acid and three drops of Conway's indicator (red). Connect the reservoir to the distillation apparatus, add 10 ml of 40% NaOH to the boiling flask containing the sample extract and close it immediately. Perform distillation until the volume of the container reaches 50 – 75 ml (indicator is green). Bacterial culture measures the total nitrogen content according to the Kjeldahl method (Narsing-Rao *et al.,* 2017) using the formula (Wiyantoko *et al.,* 2017; Haerani *et al.,* 2021).

$$\text{Nitrogen Level (\%)} = \frac{\{(Vb - Vs) \times N \times \text{bst N})\}}{w} \times 100 \quad (1)$$

where: Vs = Distillation titration example (mL), Vb = Blanco titration volume (mL), N = Standard solution normality H_2SO_4 , bst N = Nitrogen equivalent weight (14.008), w = sample weight (g), 100 = conversion to %

3. RESULTS AND DISCUSSION

3.1 Isolation and Morphological Characterization of Nitrogen fixation Rhizobacteria

Based on the initial selection process, rhizobacteria isolated from organic lowland rice rhizosphere samples by culturing isolates on Burk N-free medium found that 12 isolates could grow on this medium (Fig. 2). N-free Burk media is a selective media generally used to select bacterial isolates capable of fixing free nitrogen. This media has a composition of materials needed by bacteria during the growth process, such as *Sucrose*, *Magnesium sulfate*, *Calcium carbonate*, *Sodium chloride*, *Sodium molybdate*, etc (Lubis *et al.,* 2020; Nafisah *et al.,* 2022).

The results of the exploration of four sampling locations in the organic lowland rice ecosystem found only 12 isolates of bacteria that can fix nitrogen. This number is still relatively low compared to the results of research by Susilowati & Setyowati (2016), who successfully isolated 50 isolates of rhizosphere bacteria obtained from coastal paddy soil in West Java. It is suspected that there is intense competition from various microorganisms in using carbon sources produced by plant root exudates as energy in carrying out nitrogen fixation activities.

Table 1. Morphological characterization of nitrogen fixation rhizobacterial isolates

No	Isolate code	Size	Colony form	Colony Edge	Elevation	Colony color
1	RFN1	Small	Circular	Entire	Flat	Yellow
2	RFN2	Small	Circular	Undulate	Flat	Cream
3	RFN3	Moderate	Irregular	Filamentous	Flat	Cream
4	RFN4	Small	Irregular	Undulate	Umbonate	White
5	RFN5	Moderate	Circular	Undulate	Flat	Cream
6	RFN6	Small	Circular	Entire	Umbonate	Cream
7	RFN7	Small	Circular	Entire	Raised	Yellow
8	RFN8	Moderate	Circular	Undulate	Flat	Yellow
9	RFN9	Moderate	Circular	Entire	Flat	Beige
10	RFN10	Moderate	Circular	Undulate	Flat	Beige
11	RFN11	Small	Circular	Entire	Flat	Beige
12	RFN12	Moderate	Circular	Entire	Flat	Yellow

Notes : *RFN (Nitrogen-fixation rhizobacteria)

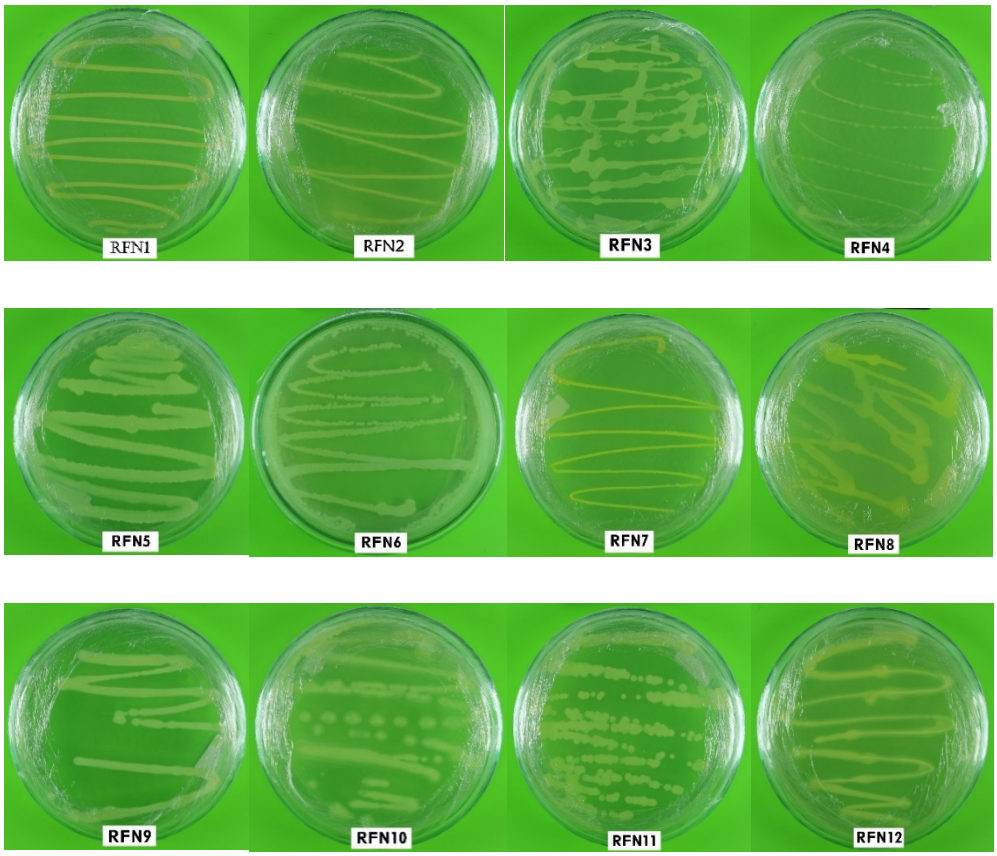

Figure 2. Pure culture of nitrogen fixation rhizobacterial isolates on Burk N-free medium

The results of the exploration of four sampling locations in the organic lowland rice ecosystem found only 12 isolates of bacteria that can fix nitrogen. This number is still relatively low compared to the results of research by Susilowati & Setyowati (2016), who successfully isolated 50 isolates of rhizosphere bacteria obtained from coastal paddy soil in West Java. It is suspected that there is intense competition from various microorganisms in using carbon sources produced by plant root exudates as energy in carrying out nitrogen fixation activities.

In morphological characterization in the research conducted, rhizobacterial isolates with various morphological characters were obtained. The small colony size was six isolates, and the medium was six. The form of the colony is dominated by a circular shape, while two isolates have an irregular shape. Colonies with undulate edges 5 isolate, entire edges 6 isolate, and only 1 isolate with a filamentous edge. The elevation of the nitrogen fixation isolates is dominated by flat elevation (9 isolates), umbonate elevation (2 isolates), and raised elevation (1 isolate). Colony pigments produced from each nitrogen fixation rhizobacteria isolate cream, beige, yellow and white (Table 1). Different morphological characterizations indicate a diversity of different types of bacteria. Although this observation is still at the morphological identification stage, which has not been able to determine the genus of the bacteria, at least this observation is needed as the first step for further molecular identification.

3.2 Biochemical Characterization, Hemolysis Activity, and Pathogenicity Test of Nitrogen Fixation Rhizobacteria Isolates

The biochemical properties (Gram reaction test) of nitrogen fixation rhizobacterial isolates showed the results of 9 isolates of gram-positive bacteria. In comparison, three isolates were included in the Gram-negative bacteria. Gram reaction testing on bacterial isolates using 3% KOH. A

positive Gram reaction is characterised by the absence of mucus threads in the suspension when lifted from the surface of the glass object that has been dabbed with KOH solution. Gram positive bacteria that do not form mucus are caused by the bacteria having a thick peptidoglycan layer and an inner membrane in the cell wall so that it is not easily broken. Gram negative bacteria are characterised by the presence of clear mucus produced after the ose is removed. The cell wall of Gram-negative bacteria will break easily when tested with KOH solution which has a high alkali. The cell wall of Gram-negative bacteria contains high lipids in the form of liposaccharides and lipoproteins (Hardiansyah *et al*., 2020).

The biochemical characters in their ability to produce catalase enzyme were seven isolates, a five others were unable to produce the enzyme (Table 2). The catalase enzyme that can be produced by bacteria plays a role in breaking down hydrogen peroxide (H2O2) into water and oxygen. Under certain conditions, bacteria will produce H2O2 compounds that can interfere with the bacterial metabolic system, damage cells and even cause the death of bacteria if the compound is not broken down. Bacterial isolates that are able to produce catalase enzymes are characterised by the formation of air bubbles when the isolate is dripped with H2O2, while isolates that do not produce catalase enzymes cannot form air bubbles because the isolate is unable to break down H2O2 (Pulungan & Tumangger, 2018).

Hemolytic activity from the test results on nitrogen fixation rhizobacterial isolates showed negative hemolysis results. All tested isolates did not form a clear zone when streaked on blood agar media. The clear zone formed is an extracellular product produced by bacteria that can lyse red blood cells, making it dangerous for living things. However, in this study, all isolates tested for use were safe for humans and animals, as was the case with the hypersensitivity reaction test on tobacco leaves. Nitrogen fixation rhizobacterial isolates do not have the potential to become plant pathogens. It can be seen from the injected isolates that did not show necrosis symptoms on tobacco leaves after being observed for five consecutive days. Isolates that showed negative reaction (no symptoms of necrosis but the leaves remained green) in the inoculated tobacco leaf area (Table 2; Figure 3).

Table 2. Biochemical properties, hemolytic activity, and hypersensitivity reactions of bacterial isolates

No	Isolat Code	Gram's reaction	Catalase reaction	Hemolysis Activity	Hypersensitivity Reactions
1	RFN1	+	+	-	-
2	RFN2	+	-	-	-
3	RFN3	+	+	-	-
4	RFN4	+	-	-	-
5	RFN5	+	+	-	-
6	RFN6	+	+	-	-
7	RFN7	-	+	-	-
8	RFN8	+	+	-	-
9	RFN9	-	+	-	-
10	RFN10	+	+	-	-
11	RFN11	+	+	-	-
12	RFN12	-	+	-	-

Note: (+) = positive (-) = negative

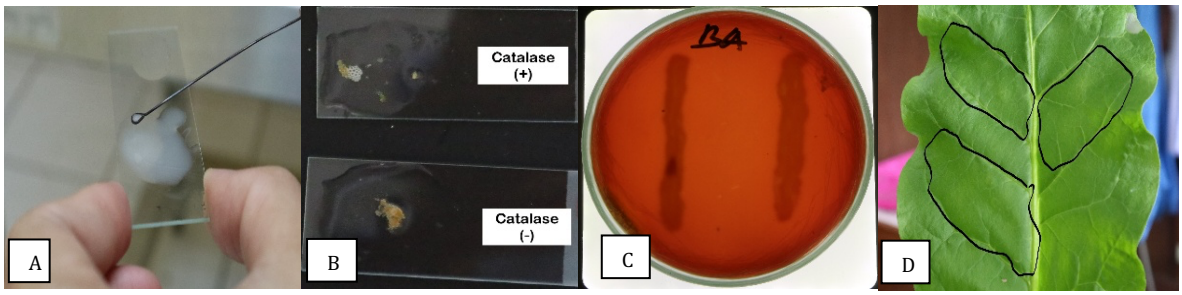

Figure 3. (A) Gram-positive reaction test (does not form threads when lifted from the surface of the slide), (B) Catalase reaction test (C) Test of hemolytic activity on blood agar media (negative hemolytic activity without clear zone), (D) Negative hypersensitivity test (no necrosis appears on tobacco leaves).

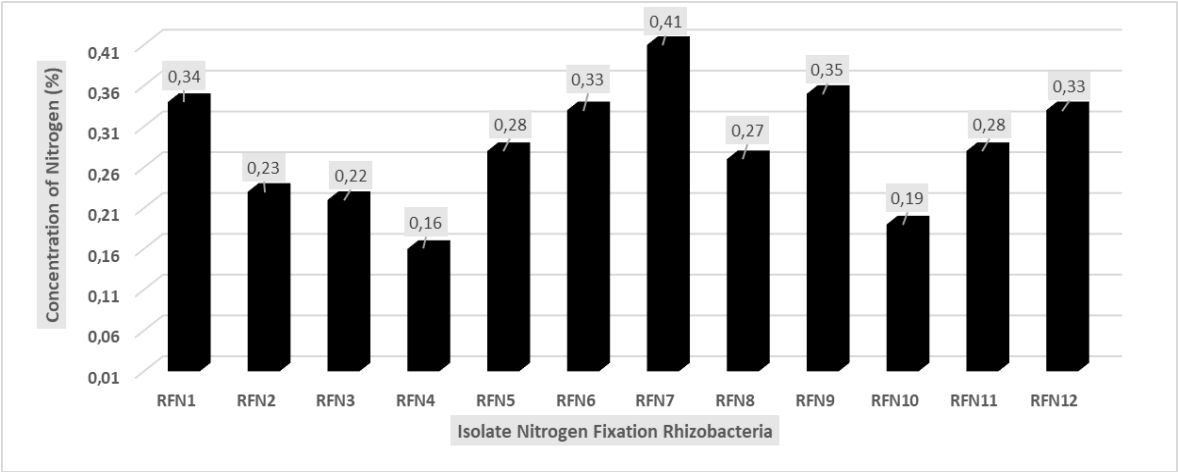

Figure 4. Total nitrogen content produced by nitrogen fixation rhizobacteria isolates

3.3 Nitrogen-fixation ability by rhizobacterial isolates

Quantitatively, the ability of bacterial isolates to fix nitrogen was determined by looking at the total nitrogen content they were able to produce. The total nitrogen obtained from the test results using the Kjeldahl method showed RFN7 isolate to give the highest content of 0.41%, followed by isolate RFN9 of 0.35%. In comparison, the lowest total nitrogen content was produced by isolate RFN4 (0.16%) which was not much different from isolate RFN10 of 0.19% (Figure 3). The difference in total nitrogen concentrations produced is thought to be due to differences in nitrogenase activity by each bacterial isolate in excreting ammonium. It is in line with Widawati & Suliasih (2019) research, which reported that eight isolates were identified as nitrogen fixation rhizobacteria. Still, only 20% had nitrogenase activity due to differences in the bacterial isolates in excreting ammonium.

However, the composition of this media does not contain nitrogen elements, so isolates that can grow on this media are isolates that must have the ability to bind nitrogen in the air (Nuraini *et al.,* 2015). In line with research (Santoso *et al.,* 2019), bacteria that grow on media without nitrogen can fix non-symbiotic nitrogen by carrying out nitrogenase activity. Nitrogenase activity increases with Sodium molybdate in the medium (Farhangi-Abriz *et al.,* 2017). Susilowati & Setyowati (2016), explain to nitrogenase activity is also influenced by the concentration of nitrogenase in the sample. The higher the action, the higher the concentration of nitrogenase produced, so there is a linear relationship between the two. Although not symbiotic with plants, non-symbiotic nitrogen fixation rhizobacterial isolates can fix free nitrogen from the air (Mus *et al.,* 2016). The fixation of N from the

atmosphere of bacteria will be converted into NH_3 with the help of nitrogenase enzymes, so that nitrogen from bacterial fixation can be absorbed by plants in the form of NO_3 and NH^{4+} (Leghari *et al.,* 2016).

The same was expressed by Yoneyama *et al.,* (2017) the roots of rice plants in paddy soil ecosystems will release some O_2 and N_2, glucose, organic acids and amino acids through aerenchyma tissue (root exudate) as a source of energy for growth, microbial metabolism including nitrogenase activity in nitrogen fixation. However, this is a source of competition between microorganisms. In anaerobic conditions, microorganisms break most of the root exudate into methane gas through a decomposition process (Sudewi *et al.,* 2021; Saleh *et al.,* 2022).

The fundamental difference between Gram-negative and Gram-positive bacteria lies in the peptidoglycan layer. Gram-negative bacteria have a thin Peptidoglycan layer, breaking easily when reacted with a 3% KOH solution. This causes DNA to come out of the cell and form slimy threads when removed from the surface of the slide. In contrast, Gram-positive bacteria do not form threads when removed from the surface of the slide because of their thick Peptidoglycan layer, so these bacteria do not lyse easily when dropped with a 3% KOH solution (Sudewi *et al.,* 2020). The catalase reaction test yielded positive catalase results of 83.33%, while catalase negatives were 16.66% (Table 2). The catalase reaction is positive, as indicated by the appearance of air bubbles when H_2O_2 is dropped. Bacterial isolates can produce the catalase enzyme, which breaks down Hydrogen peroxide into water and oxygen.

Nitrogen fixation rhizobacterial isolates in this study produced relatively high levels of total nitrogen. It is presumably because the growing environment from which the rhizosphere samples we obtained is an organic lowland rice ecosystem in which farmers only use organic inputs as inputs from generation to generation so that the organic matter content in the soil is abundant. This organic material is a source of energy for soil microbes in carrying out metabolic activities, one of which is nitrogenase activity so that bacteria can bind free nitrogen from the air. In line with Zulfarina *et al.,* (2017), microorganisms can break down the abundance of organic matter contained in the soil as a source of carbon and energy in the processes of metabolism and growth. In addition, the ability of microbes to fix nitrogen also depends on the type of plant. Host plants can supply a source of carbon and energy from the exudate they produce to be utilized by microbes (Rosenblueth *et al.,* 2018) so that there are indirect interactions between plants and soil microorganisms. More nitrogen content in the soil is produced from the ability of fixation by soil microbes. Before use, the N-organic formed is first converted into ammonia by a deamination process. The ammonia produced can be directly assimilated by microbes, but it can also convert into nitrate compounds through nitrification (Sari & Prayudyaningsih 2015).

Plants cannot directly absorb the nitrogen availability in diatomic N_2, so a process is needed to make it available in the form of ammonium (NH^{4+}) and nitrate (NO_3). Can obtain this element from the soil with the help of certain microorganisms, namely diazotrophs or microbes capable of biologically fixing nitrogen (Permatasari & Nurhidayati 2014). Some of the results of research on the ability of bacteria to fix nitrogen include (Akintokun *et al.,* 2019) reporting that they obtained the ability to fix nitrogen mainly from the bacterial isolate Alcaligenes faecalis and the least from *Bacillus mojavensis*, *Pseudomonas, Klebsiella, Azotobacter,* and *Agrobacterium* isolated from the rhizosphere of rice plants can fix nitrogen freely (Aloo *et al.,* 2022; Adedayo *et al.,* 2022; Kaur *et al.,* 2022). Corn plants, as a result of research (Gómez-Godínez *et al.,* 2019), showed a beneficial effect obtained through N_2 fixation, namely that it could increase plant root growth, increasing nutrient uptake.

The mechanism of biological nitrogen fixation from the atmosphere is divided into two: symbiotic and non-symbiotic nitrogen fixation. Rhizobium bacteria live symbiotically and infect legume plant roots, forming nodules. In contrast, non-symbiotic bacteria (*Azotobacter, sp, Azospirillum sp, etc.*) live freely in various types of soil and plant rhizosphere. The ability of bacteria to fix N_2 can increase the availability of N in the soil so that it affects the increase in crop production

and does not pollute the environment (Bangkele *et al*., 2019; Anggrainy *et al*., 2021; Sembiring *et al*., 2021). Rhizobacteria non-symbiotic contributes nitrogen which reaches 10-15 kg N ha^{-1} per year of the total nitrogen requirement of plants, depending on the available carbon source as energy in carrying out nitrogenase activity by microbes (Roper & Gupta 2016).

4. CONCLUSION

Twelve rhizobacterial isolates could grow on Burk N-free media with different morphological characters. Result identification of Gram-positive bacteria showed ten isolates capable of producing catalase enzymes. All isolates of nitrogen fixation rhizobacteria were safe against living things and were not potentially pathogenic to plants based on the hemolysis and hypersensitivity tests performed. RFN7 isolate produced the highest total nitrogen content of 0.41% and the lowest by isolate RFN4 of 0.16%. Rhizobacteria nitrogen fixation can be used as a biofertilizer agent, reducing the use of chemical fertilizers. The application of this technology is very efficient, effective, and environmentally friendly, so it has a vital role in supporting sustainable agriculture.

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