Amplification of 16S rRNA primer on rhizosphere bacteria from reclamation area and natural forest of PT. Vale

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Abstract. Open-pit mining activities cause land degradation; therefore, post-mining land recovery or reclamation is essential. An indicator to rate the success of mine reclamation activities is the diversity of soil microbial populations. Molecular bacteria identification requires prior information about the amplification of universal primers for molecular analysis. This study analyzes the amplification of 16S rRNA primers on rhizosphere bacteria isolates from reclamation and forest areas. The research activities encompassed sample preparation, isolation of bacteria isolates, isolation of DNA isolate, quantitative test, qualitative test, and amplification. The findings showed that pure isolates of rhizosphere bacteria from reclamation and natural forest areas that could be used were five and ten isolates, respectively. One isolate (20%) from the reclamation area and four isolates (40%) from natural forest generated DNA band, which were amplified using 16S rRNA.

1. Introduction

Mining is a vital industry for several countries, which provides considerable economic benefits. Indonesia is known as a country with high mineral potential. One of the utilization of nickel mineral resources is carried out at PT. Vale Indonesia Tbk. located on the island of Sulawesi[1]. Nickel are is obtained by applying the open-pit mining method or stripping the soil and nickel cover rock. Based on the Minister of Environment Regulation No. 9 of 2006, nickel are mining includes excavation, transportation, and stockpiling in both open pit and underground mines. This activity causes land degradation, which includes changes in physical properties (opening of forest vegetation, changing in topographic, changing the composition of the soil layer, soil compaction, erosion, and sedimentation), chemical (pollution, loss of nutrients, and soil organic matter), and soil biology (decrease in the amount of soil organic matter).

Restoration of environmental carrying capacity, especially on post-mining land through mine reclamation, is the obligation of the mining business unit means that the activity is crucial. The reclamation land must be able to function again as a medium for plant growth. One indicator that can be used as a success of mine reclamation is the diversity of soil microbial populations, increasing along with the reclamation efforts.
Soil is a dynamic medium in which plants and microorganisms live together and in symbiosis. One part of the soil that contains many microbes that are beneficial for plant growth is the rhizosphere. Rhizosphere has an essential role in nutrient cycles and soil formation processes, plant growth, influence microbial activity, and biological controllers against root pathogens [2].

The currently developing technology uses microorganisms (non-pathogenic saprophytic bacteria) explored from the plant rhizosphere (rhizobacteria), which can stimulate plant growth. Rhizobacteria are bacteria that live saprophytically in the rhizosphere or root areas. Several species can act as plant growth promoters or as biocontrol agents against disease.

The development of microbial identification begins with identification through morphological, physiological, and metabolic characteristics [3]. Morphological and physiological characterization in the bacterial identification stage can describe the observed isolates that can provide bacterial identity [4]. However, this method lacks accuracy and requires a long time for identification.

A faster molecular-based identification method has been developed with high sensitivity and specificity, such as analyzing the 16S rRNA (16S ribosomal ribonucleic acid) gene. The 16S rRNA gene is widely used because it is considered fast and practical, multi-copy, universal in bacteria, and conservative. It can be used as a differentiator between species, and the bacterial database is based on 16S rRNA primer amplification which is extensive, making comparisons easier [5].

Amplification is the doubling step of DNA usually done by the Polymerase Chain Reaction (PCR) method. DNA is denatured, annealed, and extended in several cycles using a thermal cycler machine. Molecular identification of bacteria requires more accurate information on bacterial isolation techniques to amplify universal primers and become a reference for further molecular research. Based on the description above, it is necessary to research the isolates of rhizosphere bacteria of pure microbial isolation from reclamation land and natural forest with 16S rRNA primer amplification using a molecular approach.

2. Research method

2.1. Research procedure
The research started by the isolation of bacteria from the soil, rejuvenation of bacteria, DNA isolation, and amplification of the 16S rRNA primer. The samples were the collection at the Laboratory of Biotechnology and Tree Breeding, Hasanuddin University. Makassar, which was taken from PT. Vale area in Sorowako, South Sulawesi.

2.2. Purification process
Purification was conducted to obtain the desired pure culture without any contaminants from other microbes. The selection of purified microbial colonies was based on differences in the macroscopic appearance of the colonies. Thus, pure isolates were obtained by transferring the bacterial isolates using the line method, which was then regrown on NA media.

2.3. DNA isolation
DNA isolation was carried out using the DNeasy the DNA extraction KIT

2.4. DNA quantity test
DNA quantity testing was done using the Qubit 3.0 fluorometer (Thermo Fisher Scientific). We used qubit ds DNA BR buffer, qubit dsDNA standard 1 and standard 2, dsDNA reagent, and working solution for this test.

2.5. DNA quality test
The next step of DNA isolation was the DNA quality test. This stage provided information about the master DNA. It was showing the DNA quality with agarose with the concentration of 0.8% using horizontal electrophoresis.
2.6. Data analysis
The data analysis used was descriptive by looking at the bands that appear on the gel. Research on the presence or absence of bands was done by observing the photo of the electropherogram manually. Each DNA band formed on the KIT marker indicates the position of the allele at the locus.

3. Results and discussion

3.1. Rhizosphere microbial isolation
Isolation of bacteria from soil samples on reclamation land and natural forest obtained 18 isolates each. The results of macroscopic observations on bacterial isolates in reclamation land obtained five isolates, while in the natural forest, ten different pure isolates were not contaminated. The results of the dilution of bacterial isolates are shown in Figure 1.

![Figure 1. Bacterial isolate dilution (1) reclamation land (2) natural forest. Note: a) dilution $10^{-3}$, (b) dilution $10^{-4}$, (c) dilution $10^{-5}$](image)

The dilution of bacterial isolates in Figure 1 shows that some isolates did not grow. Some isolates were overgrown with fungi and contaminated because they were not sterile at the time of dilution. The dilutions taken were $10^{-3}$, $10^{-4}$, and $10^{-5}$ dilutions. Bacterial isolates that were successfully purified from the three dilutions were dilutions $10^{-4}$ and $10^{-5}$, which produced several different bacteria in color and shape. Bacterial colonies on reclamation land and natural forest showed similarities in a circular shape and were predominantly white.

3.2. Molecular analysis of rhizosphere bacterial isolates
Isolation is a technique to obtaining the desired microorganism from a group of microorganisms in the same habitat. The purpose of isolation is to get pure isolation consisting of one species thus can be used for molecular analysis [6].

The isolates obtained were then isolated using the KIT isolation method. The isolated DNA was tested for its purity and concentration using a spectrophotometer and electrophoresis. The results of the quantity test can be seen in Table 1 and the quality test in Figure 2.
Based on the measurement results of the DNA quantity test of bacterial isolates on the Qubit 3.0 fluorometer (Table 1), the average concentration of dsDNA in DNA isolates on reclamation land was 112.6 ng/µl, and the natural forest was smaller, it was 49.13 ng/µl. According to [7], DNA concentrations that are too high may still contain contaminants such as phenols and other secondary metabolites, thus interfere with the primer attachment process to DNA and the subsequent amplification process. The value obtained from this quantity test can affect the amount of dilution conducted in the PCR stage. The greater the value obtained at the time of the quantity test, the greater the dilution to be carried out, and vice versa, the smaller the value obtained in the quantity test, the smaller the dilution to be used for PCR analysis [8].

![Figure 2](image-url)

**Figure 2.** Electropherogram of bacterial isolate DNA quality test (A) reclamation land (B) natural forest. Note: M: 100 bp marker (base pair); A1-A5: samples of bacterial isolates of reclamation land; B1-B10: natural forest bacterial isolate sample

Figure 2 shows that the isolation results of bacterial DNA isolate contained DNA fragments with band lengths from the overall sample with an average of 800 bp – 1500 bp. DNA fragments showed that DNA with bright, thick, and thin bands or less bright in each bacterial isolate came from reclamation land and natural forest. The thick band indicates a high concentration, and the total extracted DNA is intact [9]. In addition, there were smears on several samples of the resulting bands. Smear is the residue from the solutions that are still carried away during the isolation process or in the form of DNA degraded in the isolation process [10].

3.3. **Amplification using 16S rRNA primer**

The results of the primer amplification showed that the primers tested had different characteristics and amplification abilities. 16S rRNA primers are widely used as marker genes in bacterial identification [5]. According to [11], primer is the most crucial point in determining success in the amplification process. The results of amplification using 16s rRNA primer on bacterial isolate samples in reclamation land and natural forest can be seen in Figure 3.
Figure 3 shows the presence of DNA bands or the ability to be amplified from bacterial isolate samples in reclamation land and natural forest. However, not all of these samples produced DNA bands. The amplification of bacterial isolate samples using 16s rRNA primer showed that there was only one band or 20% of the total five samples that were amplified in the reclamation land bacterial isolate sample. As for the natural forest bacterial isolate samples, four DNA samples were successfully amplified, namely samples 4, 5, 6, and 8, or 40% of the total ten samples amplified with a band length of about 100-600 Bp. The success of the primer amplification is based on the suitability of primer, efficiency, and optimization of the PCR process [12][13]. If the primer is not specific, it can cause amplification in another area of the genome that is not targeted or has no amplified genomic area [14].

4. Conclusion

Based on the study results, it was concluded that the pure isolates of rhizosphere bacteria on reclamation land and natural forest that could be used in the primer amplification test of 16s rRNA were five isolates in reclamation land and ten isolates in natural forest. There was one sample with a band or 20% amplified on reclamation land, while in the natural forest, four samples had a band or 40% amplified.

Reference
[1] Larekeng S H, Nursaputra M, Nasri, Hamzah A S, Mustari A S, Arif A R, Ambodo A P, Lawang Y and Ardiansyah A 2021 An assessment of high carbon stock and high conservation value approaches in mining area IOP Conference Series: Earth and Environmental Science vol 807 (IOP Publishing Ltd)
[2] Jufri S W, Restu M and Gusmiaty G 2017 Identifikasi dan karakterisasi mikroba rhizosfer pada hutan rakyat tanaman bitti (vitexcofassus reinw), jati (tectona grandis) dan jabon merah (anthocephalus macrophyllus) (Universitas Hasanuddin)
[3] M Iksan, L Aba, F I Taharu, A Alffian, D P I Ardyati, Jumiati, W O D Alzarliani H and S H L 2019 The diversity of mangrove forests in Kumbewaha , Buton Island , Indonesia IOP Conf. Ser. Earth Environ. Sci. 343 1–6
[4] Nuranto A and Agusta A 2015 Identifikasi molekular dan karakterisasi morfo-fisiologi actinomycetes penghasil senyawa antimikroba (molecular identification and morphophysiological characterization of actinomycetes with antimicrobial properties) J. Biol. Indones. 11 195–203
[5] Akihary C V and Kolondam B J 2020 Pemanfaatan gen 16S rRNA sebagai perangkat identifikasi bakteri untuk penelitian-penelitian di indonesia pharmacon 9 16–22
[6] Istiqifarin N 2017 Identifikasi Secara Molekuler Menggunakan Gen 16s Rrna, Dan Uji Aktivitas Antibakteri Bakteri Simbion Endofit Yang Diisolasi Dari Alga Halimeda Opuntia
Occup. Med. 53 130

[7] Joko T, Kusumandari N and Hartono S 2011 Optimasi metode PCR untuk deteksi pectobacterium carotovorum, penyebab penyakit busuk lunak anggrek J. Perlindungan Tanam. Indones. 17 54–9

[8] Syafaruddin, Randriani E and Santoso T J 2011 Efektivitas dan Efisiensi Teknik Isolasi dan Purifikasi DNA pada Jambu Mete J Ristri 2 151–60

[9] Sholihah S M 2014 Hubungan kekerabatan beberapa kultivar pisang (musa sp.) uuntuk sifat ketahanan terhadap penyakit berdasarkan resistance gene analog (RGA) (UIN Malang)

[10] Mulyani Y, Purwanto A and Nurruhwati I 2011 Perbandingan Beberapa Metode Isolasi DNA untuk Deteksi Dini Koi Herpes Virus (Khv) Pada Ikan Mas (Cyprinus Carpio L.) J. Akuatika Indones. 2

[11] Peloa A, Wullur S and Sinjal C A 2015 Amplifikasi Gen Cytochrome Oxidase Subunit I (Coi) Dari Sampel Sirip Ikan Hiu Dengan Menggunakan Beberapa Pasangan Primer J. Pesisir Dan Laut Trop. 3 37

[12] Larekeng S H, Paelongan R, Cahyaningsih Y F, Nurhidayatullah and Restu M 2020 Primer screening and genetic diversity analysis of jabon putih (Anceocephalus cadamba (roxb) miq.) based on random amplified polymorphic dna (rapd) markers Int. J. Curr. Res. Rev. 12 173–8

[13] Larekeng S., Dermawan R, Iswoyo H and Mustari K 2019 RAPD primer screening for amplification on Katokkon pepper from Toraja, South Sulawesi, Indonesia IOP Conference Series: Earth and Environmental Science vol 270 p 012023

[14] Aris M, Sukenda, Harris E and Sukadi M F 2013 Identifikasi Molekular Bakteri Patogen Dan Desain Primer Per Budid. Perair. 1 43–50