Identification of lead (Pb(CH₃COO)₂)-reducing bacteria using 16S rRNA Gene

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Abstract. In recent years bacteria have been used as bioremediation agents to repair polluted environments. The purpose of this research is to discover the types and capabilities of bacteria in reducing lead (Pb(CH₃COO)₂) concentration from sediments in Karangsong Coast, Indramayu. The method used is exploratory, and the results are analyzed descriptively. In the molecular identification method, 16S rRNA Gene, a universal primer for a bacterium, is used. Bacterial sequences obtained from GenBank data are analyzed by NCBI sites. The results of this research show that 11 bacterial isolates from porewater sediments have the highest lead (Pb(CH₃COO)₂) reduction capability from a concentration of 1.5 mg l⁻¹ to 0.364 mg l⁻¹ with an efficiency value of 75.7%. Four bacterial isolates with the highest density are identical to Albirdodobacter marinus strain N9 (ACC. No. NR 126203.1) with 98% identity value and 100% genetic relationship, Pseudoalteromonas tetraodonis strain NBRC 103034 (ACC. No. NR 114187.1) with 81% identity and 84% genetic relationship, Pseudoalteromonas lipolytica strain LMEB 39 (ACC. No. NR 116629.1) with 93% identity and 87% genetic relationship.

1. Introduction
Pollution in coastal areas occurs frequently, and the sources of pollution and damages in these areas come from land and sea activities. One of the pollutants that contaminate coastal regions is lead (Pb). It is a non-essential metal, which means that its presence does not produce any positive effects. When lead concentration in water bodies exceeds a standard amount, deaths occur in aquatic biota [1].

To minimize lead (Pb) concentration levels, bioremediation agents are needed, and one of them is bacteria. These bacteria are known as indigenous bacteria or bacteria from ecosystems that have adapted to their native environment. Therefore they are likely to have the ability to lead reduction in lead-contaminated environments [2]. To figure out whether the bacteria have this ability, experimental testing can be performed. In addition, its type and genetic relationship can be known through molecular identification with PCR (Polymerase Chain Reaction) technique using 16S rRNA Genes.
The purpose of this research is to obtain the bacteria’s type and its lead-reduction capability from sediments in Karangsong Beach, Indramayu.

2. Methodology
2.1. Sampling
This research was conducted from April to November 2018. Sediments and water data samplings were carried out by direct measurement (in situ). Sediment samples were taken from the docks of Karangsong Beach on April 4, 2018. The coordinates of sediment sampling are 6°15'–6°40' S and 107°52'–108°36' E. Porewater sampling in contaminated sediments was performed by using a piston core at a depth of 30-60 cm for 3 repetitions. Pore water samples were taken using 3 ml injection, then put in a sterile bottle and stored in a cool box. Afterward, data of water quality parameters were collected: temperature, salinity, pH, and DO (Dissolved Oxygen), and dissolved lead (Pb) in Karangsong Beach.

2.2. Lead (Pb) reduction test
Bacteria in porewater sediments were put into Pb(CH₃COO)₂ solution with concentrations of 0.5 mg l⁻¹, 1.0 mg l⁻¹, and 1.5 mg l⁻¹. Each concentration was made into five solutions and observed at 0-hour (T0), 6-hour (T2), 12-hour (T3), 24-hour (T4), and 48-hour (T5). Metabolism termination was done by injecting a 95% alcohol solution at each predetermined observation time. Then the concentration of Pb(CH₃COO)₂ lead was measured by using AAS (Atomic Absorption Spectrophotometry), and the calculation of bacterial density by TPC (Total Plate Count) method.

2.3. Culture and purification of Pb-reducing bacteria
The culture and purification of lead-reducing bacteria were carried out to obtain pure bacterial isolates to be identified molecularly. The source came from pore water in lead solutions of Pb(CH₃COO)₂ at the 48th hour of observation. Bacteria in this source are the ones that are able to grow at the determined lead concentrations. Bacterial culture was carried out by pour plate method, and the source was diluted to 10⁻⁵ at 37ºC for 24 hours. Purification was carried out by the streak plate method using NA (Nutrient Agar) solid medium.

2.4. Bacteria gram staining
Bacteria Gram Staining determines the nature of bacterial gram (gram-positive or gram-negative) and views the bacteria’s cell morphological shape. The substances used were violet crystals as gram-positive dyes and fuchsin water as gram-negative dyes. Gram-positive bacteria are shown in blue, while gram-negative properties are shown in purple.

2.5. Bacterial challenging test for Pb(CH₃COO)₂
This challenge test observes the bacteria’s resistance and growth towards solutions containing Pb(CH₃COO)₂. It was carried out by inoculating the bacteria into a liquid medium of Nutrient Broth (NB), which already contained Pb(CH₃COO)₂ with a concentration of 1.5 mg l⁻¹. The method used in this challenging test refers to the research conducted by Lewaru et al. [3]. Bacterial isolates were inoculated in a shaker incubator at 37°C for 18 hours, followed by measurements using a TECAN spectrophotometer at the 0th and 18th hour with a wavelength of 600 nm.

2.6. Bacterial DNA extraction
The purpose of bacterial DNA extraction is to separate DNA from other cell components. The method used is phenol-chloroform with a homogenization stage (cell lysis stage) using reagents from TRIsure. The procedure is based on the bacterial DNA extraction protocol from TROLURE BIOLINE. The stages include homogenization, phase separation, DNA precipitation, DNA washing, and DNA re-processing. Furthermore, DNA purity and concentration were measured using spectrophotometry at wavelengths (λ) 260 nm and 280 nm.
2.7. DNA amplification (PCR)
The isolated DNA template was then amplified by PCR (Polymerase Chain Reaction). This amplification is required to increase the amount of DNA in vitro. The method used in DNA amplification refers to research conducted by Mihdir et al. (2016). The PCR process begins with amplification using primers 9F (5' -GAGTTTGATCCTGGCTCAG-3') and 1542R (5' -AGAAAGGAGGTGATCCAGCC-3').

2.8. Electrophoresis
In this process, agarose gel with a concentration of 1% was used for electrophoresis after DNA amplification. Syber Safe staining gel Thermofisher was used in a ratio of 1:10,000. A volume of 2 µl 1 kb (10,000 bp) DNA ladder with a DNA band size target of ± 1500 bp was used. The amplified DNA sample (2.5 µl) was electrophoresed and mixed with 0.5 µl loading dye (ballast). The electrophoresis process was carried out for 30 minutes with a voltage of 100 volts and a current of 100 mA. Afterward, the agarose gel was transferred to UV transilluminator to observe the band that was formed.

2.9. Sequencing & BLAST
The purpose of DNA sequencing (or DNA base sequencing) is to determine the order of nitrogen bases (adenine, guanine, cytosine, and thymine) in a sample. Sanger method was carried out by ‘First Base’ Company in Singapore. The sequencing results will be processed using Bioedit software. Furthermore, the results of data processing were carried out by BLAST on the NCBI (National Center of Biotechnology Information) website.

2.10. Phylogenetic analysis
Phylogenetic analysis of nucleotide sequences determines the ancestors and genetic relationship of organisms [4]. Construction of a phylogenetic tree is done using MEGA X software.

3. Results and discussion
3.1. General water conditions
The sampling locations have a mud-type sediment texture, gray in color, have a pungent odor, and traces of oil spills in the waters of disposed of vessels. This condition is similar to sediments in the western part of Jakarta Bay, which contain high levels of heavy metals. The sediments there consist of smooth mud with a black-gray surface and foul smell [5].

In addition to these conditions, the water quality parameters at Karangsong Beach have a temperature of 32°C; a fair value for tropical waters. The range of tropical water temperature is considered normal between 25.6-32.3°C [6]. Temperature measurements in this research were carried out on April where the transition season occurred, which caused a high-temperature value. The salinity is an average of 29.7 ppt, indicating that the location is an area that is still influenced by land, such as the mixing of freshwater from river flows [6]. The pH is 6.8 and is considered a safe limit for aquatic life [7], whereas the DO value is 5.62. Low oxygen levels may occur due to the stations being close to estuaries that are tightly related to sea water turbidity and are also thought to be caused by increased microorganism activities to break down organic matter into inorganic substances that use dissolved oxygen (bioprocess) in these waters [6].

3.2. Lead-reduction test and growth of bacteria density
Reduction test is carried out to find out the bacteria’s capability in reducing the concentration of Pb(CH₃COO)₂. The reduction test results are shown in Figure 1 below. Based on Figure 1, it is revealed that bacteria from porewater sediments are able to reduce Pb(CH₃COO)₂ from 0-hour to 48-hour; starting from treatment A with a concentration of 0.5 mg l⁻¹ to 0.202 mg l⁻¹, treatment B from 1.0
mg l$^{-1}$ to 0.277 mg l$^{-1}$, and treatment C from 1.5 mg l$^{-1}$ to 0.364 mg l$^{-1}$. The decrease in concentration showed that the bacteria in these porewater sediments are able to reduce the concentration of lead.

![Figure 1. Test results for Lead-reduction.](image)

This is in accordance with research conducted by Satya and Larashati [8], which proves that lead-resistant bacteria can also reduce lead concentration from 15 mg l$^{-1}$ to 0 mg l$^{-1}$ with decreasing levels of efficiency up to 100%.

![Figure 2. Graph of bacterial colony growth.](image)

Based on Figure 2, it can be seen that bacterial growth occurs over time with an increasing Pb(CH$_3$COO)$_2$ concentration. At a lead concentration of 0.5 mg l$^{-1}$, bacterial growth occurred from 10 cfu x 10$^5$ ml to 91 cfu x 10$^5$ ml whereas at a concentration of 1.0 mg l$^{-1}$, growth occurred from 4 cfu x 10$^5$ ml to 62 cfu x 10$^6$ ml, and at a concentration of 1.5 mg l$^{-1}$, growth occurred from 3 cfu x 10$^5$ ml to 89 cfu x 10$^5$ ml.
In reference to Khoiroh [9], one of the lead-reducing bacteria, i.e. *Pseudomonas pseudomallei*, is known to have increased growth in Pb(CH$_3$COO)$_2$ solution whereon the 20$^{th}$ day, the number of bacteria is 6 cfu x 10$^{-8}$ ml. It then decreased on the 30$^{th}$ day into 1.72 cfu x 10$^{-8}$ ml and increased again on the 40$^{th}$ day to 2.43 cfu x 10$^{-8}$ ml. However, the death phase was not clearly visible.

3.3. Culture and purification of lead-reducing bacteria

The culture results from three treatments of Pb(CH$_3$COO)$_2$ with concentrations of 0.5 mg l$^{-1}$, 1.0 mg l$^{-1}$ and 1.5 mg l$^{-1}$, were eleven bacterial isolates that have different morphological characteristics. The bacteria were not known yet to be pure before cell shape observation from the gram test results.

![Figure 3. Results of bacterial culture and purification.](image)

3.4. Gram stain bacteria

The 8 bacterial isolates were gram-negative. In addition, the shape of the cells varies, i.e. round and filamentous. The results of bacterial gram staining are shown in Figure 4.

![Figure 4. Results of Bacterial Gram stainin.](image)

3.5. Bacterial challenge test towards Pb(CH$_3$COO)$_2$

Bacteria that were tested were chosen bacteria that were truly pure, i.e, isolates of bacteria Pb 1, Pb 2, Pb 3, Pb 4, Pb 5, Pb 6, Pb 10, and Pb 11. Eight of these bacterial isolates were subsequently tested towards Pb(CH$_3$COO)$_2$. Afterward, the Optical Density (OD) value and cell density were measured at 0 and 18-hour. The challenge test results are listed in Table 1.
The results of this test were measured using a spectrophotometer to determine the bacteria’s turbidity value. The turbidity results (Optical Density) were then converted into cells/ml to determine cell density. Based on the results, 4 isolates with the highest Optical Density (OD) are Pb 3 (1.8944), Pb 4 (1.8038), Pb 10 (1.6704), and Pb 11 (1.8779). Additionally, these four bacterial isolates have the highest bacterial cell density; Pb 3 (1.52 x 10^9), Pb 4 (1.44 x 10^9), Pb 10 (1.34 x 10^9) and Pb 11 (1.5 x 10^9).

**Table 1.** Optical density (OD) values at t = 0 and t = 18.

| Sample | Initial Value (OD) | Final Value (OD) | Value Differences (OD) |
|--------|-------------------|------------------|------------------------|
| Control | 0.0000            | 0.0000           | 0.0000                 |
| Pb 1   | 0.0714            | 1.4088           | 1.3374                 |
| Pb 2   | 0.0821            | 1.1470           | 1.0649                 |
| Pb 3   | 0.0823            | 1.9767           | 1.8944                 |
| Pb 4   | 0.0716            | 1.8754           | 1.8038                 |
| Pb 5   | 0.0821            | 1.0508           | 0.9687                 |
| Pb 6   | 0.0861            | 1.1734           | 1.0873                 |
| Pb 10  | 0.0737            | 1.7441           | 1.6704                 |
| Pb 11  | 0.0863            | 1.9642           | 1.8779                 |

Optical Density (OD) measurements and bacterial cell density calculations show interrelated results. The higher the OD is, the larger the number of bacteria. According to Lizayana et al. [10], the greater the Optical Density (OD), the more light is absorbed by the bacteria, and very little light is missed. Therefore, from these data, it can be said that the bacterial isolates were able to adapt and be resistant towards lead (Pb).

This is reinforced by Hughes and Rolle [11] who stated that the presence of bacteria which is capable of absorbing heavy metals in their cell walls, and production of extracellular compounds or enzymatic systems.

Four of the bacterial isolates with the highest density proceeded to the identification stage with the molecular method using the 16S rRNA gene. The bacterial isolates are Pb 3, Pb 4, Pb 10 and Pb 11.
3.6. DNA extraction

Bacterial genomic DNA extraction is the first step to identify bacteria. According to Madigan [12], molecular identification is an accurate method in identifying bacteria. DNA extraction is required to separate DNA from other components such as protein and RNA. This process is a quantitative test in the molecular analysis because it shows the purity and concentration of the extracted DNA. DNA with decent purity is determined by protein and RNA contamination in the solution. The results of DNA purity and concentration are listed in Table 2.

Table 2. DNA concentration and purity.

| Sample | A260  | A280  | Concentration ng/µL | Purity (260/280) |
|--------|-------|-------|---------------------|------------------|
| Pb 3   | 0.1569| 0.0921| 156.9               | 1.70             |
| Pb 4   | 0.2087| 0.1086| 208.7               | 1.92             |
| Pb 10  | 0.2048| 0.1192| 204.8               | 1.72             |
| Pb 11  | 0.1520| 0.0850| 152                | 1.79             |

The purity value of 4 DNA samples showed that 3 DNA samples were not decent, and only 1 DNA sample that was in accordance with the determined DNA quality value of 1.8 - 2.0. According to Fatchiyah et al. [13] DNA purity can be measured by calculating the absorbance values of 260 nm divided by 280 (A260 / A280), with values ranging from 1.8 to 2.0. DNA samples that are not good enough mean that they are not pure and still contain protein components. This is consistent with Termos Fisher Scientific [14], which stated that DNA with a value below 1.8 means that it is contaminated with protein while a value above 2.0 means that it is contaminated with RNA. The presence of protein contamination in the DNA of this sample is thought to be due to a poor process during the precipitation of the DNA sample.

The results of the DNA concentration samples varied between 152 - 208.7 ng/µl. It is suspected that the presence of a mixture in a product affects the DNA concentration. According to War dani et al. [15] the materials added in processed products cause the extracted DNA to be mixed with other contaminants such as oligopeptides, protein polysaccharides, and other organic materials.

3.7. DNA amplification

Amplification is an advanced step in identifying bacteria. The purpose of amplification, according to Nursyirwani [16] is to multiply the number of DNA molecules exponentially in a relatively short time. This amplification was done by 29 cycles of Polymerase Chain Reaction (PCR). The success of the bacterial DNA genome amplification process depends on the primer sequences and annealing temperature. The primer used in this PCR process is 16S rRNA (9F & 1542R) with a target DNA band size of 1500 bp. The DNA marker was 1 kb DNA ladder and electrophoresed with an agar concentration of 1%. The DNA results of electrophoresis were visualized with UV light and revealed positive results, i.e., the formation of DNA bands in agarose gels with the right target size.

Based on Figure 5, it is known that the 16S rRNA gene amplification process shows positive results and went well as it can be seen that the DNA band formed is at a size of ± 1500 bp. According to Nikunjkumar and Dhruvil [17] this 16S rRNA gene has a size that is quite long if used for bioinformatics (± 1500 bp) and can amplify the 16S rRNA region of all bacteria. In addition, the annealing temperature that has been optimized in the PCR program for DNA amplification process also went well.
Figure 6. Results of DNA electrophoresis with 1% agarose gel after amplification.

In Pb 11 DNA sample, the formed band is not too thick compared to Pb 3, Pb 4, and Pb 10. This occurred because the concentration results in Pb 11 DNA sample are lower than the others. This is consistent with the opinion of Weeden et al. [18], who stated that smears can be a contaminant of organic materials such as protein and RNA as well as the remnants of the solutions that are still carried during the extraction process. Additionally, although DNA bands are well-formed, smears are found under the DNA band. The presence of this smear is due to the presence of protein and other material contaminants during the extraction process [19].

3.8. Sequencing analysis of nucleotide sequencing results using NCBI’s GenBank database

A total of 4 PCR 16S rRNA gene samples were sequenced by 1st BASE in Singapore to successfully identify the nucleotide base sequences. Data from this sequencing process are in the form of nucleotide sequences, forward and reverse sequences. Forward and reverse sequence data is processed using BioEdit software, which is then identified using the BLAST (Basic Alignment Search Tools) program on the NCBI (National Center for Biotechnology Information) website. The data sequencing results are listed in Table 3.

| Sample | Query Cover | E-value | Identity | Species | Accession |
|--------|-------------|---------|----------|---------|-----------|
| PB 3   | 96%         | 0.0     | 98%      | Albirhodobacter marinus strain N9 16S ribosomal RNA gene, partial sequence | NR 126203.1 |
| PB 4   | 87%         | 0.0     | 81%      | Pseudoalteromonas tetraodonis strain NBRC 103034 16S ribosomal RNA gene, partial sequence | NR 114187.1 |
| PB 10  | 95%         | 0.0     | 98%      | Pseudoalteromonas lipolytica strain LMEB 39 16S ribosomal RNA gene, partial sequence | NR 116629.1 |
| PB 11  | 92%         | 0.0     | 93%      | Pseudoalteromonas lipolytica strain LMEB 39 16S ribosomal RNA gene, partial sequence | NR 116629.1 |
The results from Blast showed that the bacterial species for Pb 3 isolate has similarities with *Albirhodobacter marinus* strain N9 with a 96% Query Cover and 98% Identity. These percentages indicate that of all 16S rRNA gene sequences, Pb 3 samples that were successfully matched are as many as 96% and from this, 98% are similar to the species *Albirhodobacter marinus* strain N9. The Pb 4 isolate has similarity with the bacteria *Pseudoalteromonas tetraodonis* strain NBRC 103034 with 87% Query Cover and 81% Identity. Pb 10 isolate has similarities with *Pseudoalteromonas lipolytica* strain LMEB 39 with 95% Query Cover and 98% Identity. Pb 11 isolate is similar to *Pseudoalteromonas lipolytica* strain LMEB 39 with 93% Query Cover and 92% Identity.

In addition, the E-value of each sample is 0.0, indicating more significant alignment with the BLAST results. According to Sabbathini et al. [20], a value of 0.0 means that the isolate sequences have no other possibility on the chance of sequences alignment that was performed. So if the E-value is higher, it indicates a lower level of homology between sequences, whereas a lower E-value indicates a higher level of homology between sequences.

From the BLAST results, two of the four isolates have identification values above 97%. Therefore the data can be interpreted as proper and has a significant alignment. According to Stackebrandt and Goebel [21], bacterial species that have homology or Identity more than or equal to 97% can be considered to be the same species. Pb 4 isolate and Pb 11 isolate have a low identity of 81% and 92%. According to Drancourt et al. [22], based on the 16S rRNA gene sequence data, ≤ 97% identity means that the isolates are in the same genus, while an identity between 89-93% shows a different family. Pb 4 isolate is suspected to be a new species with a different family, and Pb 11 is a new species with the same genus as *Pseudoalteromonas lipolytica* strain LMEB 39.

According to Janda and Abbott [23], identification of bacterial taxonomy with more validation and scientific accuracy needs to be done based on a polyphasic approach, which is a combination of phenotype testing methods (biochemical tests, fatty acid analysis, and numerical analysis) and genotype testing methods.

3.8.1. *Albirhodobacter marinus* strain N9. Nupur et al. [24] first discovered *Albirhodobacter marinus* in Vishakhapatnam sea, Andhra Pradesh, India. The species *Albirhodobacter marinus* strain N9 belongs to the genus *Albirhodobacter*, order *Rhodobacteraceae*, class *Alphaproteobacteria*. The *Alphaproteobacteria* class includes a lot of oligotrophic proteobacteria, which are bacteria that can grow at low nutrient levels. *Alphaproteobacteria* can utilize nutrients in various ways, such as nitrogen fixation, chemoheterotrophs, or chemolithotrophs. Some of these bacteria are able to use hydrogen, ammonia, methane, volatile fatty acids as their substrates [25].

*Albirhodobacter marinus* is a gram-negative bacteria, has the form of bacilli (rods) and facultatively anaerobic. It's cell width is about 0.5 - 0.8 μm and the length is about 2-3 μm. Its colony is circular, 2-4 mm in diameter, and yellowish-white. This bacteria grows at temperatures ranging from 30°C - 37°C with an optimum temperature of 30°C and an optimum pH of 7.5. *Albirhodobacter marinus* can live at a salinity of 2-9%. Based on these characteristics, the *Albirhodobacter marinus* bacteria correspond to bacterial isolates, which were observed for morphology and gram staining.

3.8.2. *Pseudoalteromonas tetraodonis* strain NBRC 103034. *Pseudoalteromonas tetraodonis* bacteria was first discovered by Simidu et al [26] on the mucus of pufferfish’s that produces tetrodotoxin. The bacterial species *Pseudoalteromonas tetraodonis* strain NBRC 103034 belonged to the genus *Pseudoalteromonas*, order *Alteromonadales*, class *Gammaproteobacteria* phylum *Proteobacteria*. The *Gammaproteobacteria* class are mostly chemolithoautotrophs, meaning that these bacteria obtain energy using inorganic compounds. *Gammaproteobacteria* have the potential to oxidize carbon, sulfur, iron, and nitrogen [27].

*Pseudoalteromonas tetraodonis* is a gram-negative bacteria, has the form of bacilli (stem), measuring 1.0 μm - 2.4 μm. It grows at 4 - 35°C [26]. In addition, these bacteria are able to produce tetrodotoxin, gelatinase, and lipase enzymes. According to Deskawati [28], tetrodotoxin is a
neurotoxin that has a low molecular weight (319.27), which is toxic but can be used mainly in the pharmaceutical field. The genus *Pseudoalteromonas* was proposed by Gauthier et al. [29] to represent a group of marine bacteria grouped by phylogenetic relationships based on the 16S rRNA gene. The genus *Pseudoalteromonas* is a gram-negative bacteria, aerobic, stem-shaped cells and has a chemoheterotrophic metabolism [30].

3.8.3. *Pseudoalteromonas lipolytica* strain LMEB 39. According to Wu et al. [31], *Pseudoalteromonas lipolytica* was first discovered in the estuary of Yangtze River near the East China Sea. The bacterial species *Pseudoalteromonas lipolytica* strain LMEB 39 belonged to the genus *Pseudoalteromonas*, order Alteromonadales, class Gammaproteobacteria, and phylum Proteobacteria. It is a gram-negative bacteria, has the form of bacilli (stem), and is facultatively anaerobic. It is cell width is about 0.5 - 0.8 mm and has a length of about 1-2 mm. The colonies are circular and smooth, 1-2 mm in diameter and yellowish-white. This bacterium grows at temperatures ranging from 30°C - 37°C with an optimum temperature of 30°C and an optimum pH of 7.0 - 8.0. *Pseudoalteromonas lipolytica* is able to live at a salinity of 0.5-15%. The biochemical characteristics of *Pseudoalteromonas lipolytica* give it the ability to hydrolyze fat, oxidize and catalase, and reduce nitrate.

3.9. Phylogenetic analysis
This phylogenetic tree was obtained by the neighbor-joining method with Bootstrap 1000 repetitions in MEGA (Molecular Evolutionary Genetics Analysis) software Version 6.0 [32]. According to Muzzazinah [33], the neighbor-joining method is the basis for making phylogenetic trees based on differences between two sequences where phylogenetic trees with high bootstrap values of at least above 70% are decent phylogenetic trees.

![Phylogeny trees of bacterial isolates Pb 3, Pb 4, Pb 10 and Pb 1 based on the 16S rRNA sequence neighbor-joining method, bootstrap 1000x.](image-url)
Based on the phylogenetic tree, Pb 3 sequence formed a close phylogenetic lineage with *Albirhodobacter marinus* strain N9T. This is proven by the high bootstrap value between the two sequences which is 100% and max identity of 98%. A 100% bootstrap value indicates that from 1000 times reconstruction of this phylogenetic tree, the sequence MI.P1.B has a 100% genetic relationship with the species *Albirhodobacter marinus* strain N9.

The sequence of Pb 4 does not show a close genetic relationship with *Pseudoalteromonas tetraodonis* strain NBRC 103034. The bootstrap value between the two sequences is 84% with 81% identity, meaning that from 1000 times phylogenic reconstruction, Pb 4 has 84% genetic relationship with *Pseudoalteromonas tetraodonis* strain NBRC 103034.

The Pb 10 sequence has a close genetic relationship with *Pseudoalteromonas lipolytica* strain LMEB 39. The bootstrap value between the two sequences is 90% with 98% identity, meaning that from 1000 times phylogenic reconstruction, Pb 10 has a 90% genetic relationship with *Pseudoalteromonas lipolytica* strain LMEB 39.

Pb 11 has a sequence which shows a close relationship with *Pseudoalteromonas lipolytica* strain LMEB 39. The bootstrap value between the two sequences is 83% with 92% identity, meaning that from 1000 times phylogenic reconstruction, Pb 11 has 83% genetic relationship with *Pseudoalteromonas lipolytica* strain LMEB 39.

4. Conclusion

Bacteria from porewater sediments from Karangsong Beach were able to reduce lead (Pb) from a concentration of 1.5 mg l$^{-1}$ to 0.364 mg l$^{-1}$ with a 75.7% efficiency. There are four lead-reducing bacterial isolates from Karangsong beach sediments. Pb 3 isolates are identical to *Albirhodobacter marinus* strain N9 (Acc. No. NR 126203.1) with 98% identity and 100% genetic relationship, Pb 4 isolates with *Pseudoalteromonas tetraodonis* strain NBRC 103034 (Acc. No. NR 114187.1) with 81% identity and 84% genetic relationship, Pb 10 isolates with *Pseudoalteromonas lipolytica* strain LMEB 39 (Acc. No. NR 116629.1) with 98% identity and 96% genetic relationship, and Pb 11 isolate with *Pseudoalteromonas lipolytica* strain LMEB 39 (Acc. No. NR 116629.1) with 93% identity and 87% genetic relationship. Suggestions from the results of this research is the need for further research to find out the mechanisms of lead reduction. In addition, the bacteria reducing lead (Pb) concentration -in which the type and genetic relationship are known- need to undergo a consortium test.

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