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CONCENTRATION OF HEAVY METALS IN GREEN MUSSELS (*Perna viridis*) OF LAMPUNG BAY AND THEIR SYMBIONE BACTERIAL RESISTANCE

KONSENTRASI LOGAM BERAT PADA KERANG HIJAU (Perna viridis) TELUK LAMPUNG DAN RESISTENSI BAKTERI SIMBIONNYA

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ABSTRACT

Green mussels are marine organisms that are threatened due to heavy metal pollution such as lead and copper in marine waters. In addition, to threatening shell organisms heavy metals are also a threat to symbiont organisms. Bacteria exposed to heavy metals continuously will later be able to adapt (resistance) to heavy metal contamination. This study aims to determine the concentration of heavy metals lead (Pb) and copper (Cu) in green mussels from Lampung Bay and to test the resistance of symbiotic bacteria to Pb and Cu as well as to conduct molecular identification to determine the type of selected symbiotic bacteria. This research was conducted by analyzing the content of heavy metals in water samples and green mussels then isolated the symbiotic bacteria and selected using Luria Bertani agar by adding heavy metal concentrations, then tested the level of resistance to Pb and Cu which were added continuously from concentrations of 100 ppm to 1000 ppm to bacteria can no longer grow optimally. After that, molecular identification was carried out to determine the type of bacteria and reconstructed to see the molecular proximity. The results showed that the water and green mussels had exceeded the quality standard and were classified as polluted. Bacterial isolates were resistant to Pb in the range of 100-1000 ppm and Cu in the range of 100-700 ppm. Molecular identification of the selected samples, namely STL09 and STL11, showed that the symbiont bacteria were a type of bacterial species *Bacillus* sp.

Keywords: bacteria ability, bacterial resistance, bioaccumulation

ABSTRAK

Kerang hijau merupakan organisme laut yang terancam karena kontaminasi logam berat seperti timbal dan tembaga yang ada pada perairan Teluk Lampung. Selain mengancam organisme kerang, logam berat juga menjadi ancaman bagi organisme simbion. Bakteri yang terpapar logam berat secara terus menerus nantinya akan bisa beradaptasi (resistansi) dengan cemaran logam berat. Penelitian ini bertujuan untuk mengetahui kemampuan bioakumulasi kerang hijau asal Teluk Lampung dan menguji kemampuan resistensi bakteri simbion terhadap logam berat timbal (Pb) dan tembaga (Cu) serta melakukan identifikasi molekular untuk mengetahui jenis dari bakteri simbion yang terpilih. Penelitian ini dilakukan dengan menganalisis kandungan logam berat pada sampel air dan kerang hijau kemudian diisolasi bakteri simbionnya dan diseleksi menggunakan media Luria Bertani agar dengan menambahkan konsentrasi logam berat, kemudian diuji tingkat ketahanannya terhadap logam berat Pb dan Cu yang ditambahkan terus menerus dari konsentrasi 100 ppm sampai 1000 ppm hingga bakteri tidak dapat tumbuh lagi secara maksimal. Setelah itu, dilakukan identifikasi molekular untuk mengetahui jenis bakteri dan direkonstruksi untuk melihat kedekatan molekular. Hasil penelitian menunjukkan bahwa air dan kerang hijau telah melebihi baku mutu dan isolat bakteri resisten terhadap logam berat Pb pada kisaran 100-1000 ppm dan logam berat Cu pada kisaran 100-700 ppm. Identifikasi molekular terhadap sampel terpilih yaitu STL09 dan STL11 menunjukkan bahwa bakteri simbion merupakan jenis dari spesies bakteri Bacillus sp.

Kata kunci: *bioakumulasi, kemampuan bakteri, resistensi bakteri*

I. INTRODUCTION

Shellfish is one of the largest cultivation fisheries products and commodities in Indonesia. Indonesian fishery export data show that, Indonesia has exported 5.354 tons shellfish in 2021 (KKP, 2022). Green mussel (*Perna viridis*) is one of the common shellfish exploited since it contain a high nutritional value reaching 7.97% to 9.17% protein. One of Indonesia's centers of green mussel cultivation is the waters of Lampung Bay (Noor, 2014; Putri *et al*., 2018). However, heavy metal contamination in these waters causes accumulation in the body of green mussels, making green mussels dangerous for human consumption.

Heavy metals are natural constituents of the earth's crust. Some metal ions are essential for the normal functioning of living organisms such as copper (Cu) (Nies, 1999). Copper is an essential heavy metal needed by organisms in small concentrations (Große *et al.*, 2004). Beside, heavy metals at high concentration can be hazardous pollutants, especially for water areas (Sabdono, 2012) and are carcinogenic if they enter the human body (Ramakritinan *et al.,* 2014). The waters of the bay of Lampung is one of the waters contaminated by pollution of Pb and Cu (Safitri *et al*., 2018). The heavy metal content in Lampung Bay has exceeded those of water quality provided set by the government regulation. The research conducted in Lampung Bay and show that the levels of Pb and Cu on Pasaran 47,82 mg/kg (Rahmah *et al*., 2014).

One of the organisms directly affected by this dangerous pollution is the green mussel because it is directly exposed to water and sediment. Green mussels accumulate heavy metals in their tissues because they are filter feeders. In addition, green mussels are also associated with other organisms such as bacteria which are also indirectly exposed to heavy metal content. Bacteria exposed to heavy metals continuously can become resistant. The high resistance ability of bacteria can cause bacteria to accumulate more heavy metals so that it can cause many problems for green mussel organisms. However, some microorganisms also have tolerance for heavy metal levels such as mercury (Hg) (François *et al.,* 2012). Based on these problems, this study was conducted to determine the bioaccumulation ability of green mussels from Teluk Lampung bay and the resistance ability of their symbiotic bacteria to heavy metals Pb and Cu and then to conduct molecular identification to determine the type of selected symbiont bacteria which can later be used as information in aquaculture and marine biotechnology.

I. RESEARCH METHODS

2.1. Sampling Time and Location

Sampling of green mussels and water was carried out in September 2020 in Lampung Bay's green mussel cultivation area. Green mussels and water samples taken at the sampling location were placed an ice box. Then, blue ice gel and ice cube was added to keep the sampel at the cool temperature. The prepared samples were transported to the marine microbiology laboratory of IPB University.

2.2 Water Quality and Heavy Metal analysis

The water sample was filtered using Whattman filter paper with a filter diameter of 0.45 m then 1 ml of HNO_3 was given until the pH of the water sample became acidic. The filter results are stored in an ice box with a temperature of -4°C (Wang *et al.,* 2012) and then distributed to the Environmental Productivity Laboratory to measure the value of heavy metal levels in the sample water. Before analysis for heavy metal levels, green mussel samples should clean the shellfish using water flow and separate the mantle, gills, and intestine. Each piece is weighed and dried for 12 hours in an

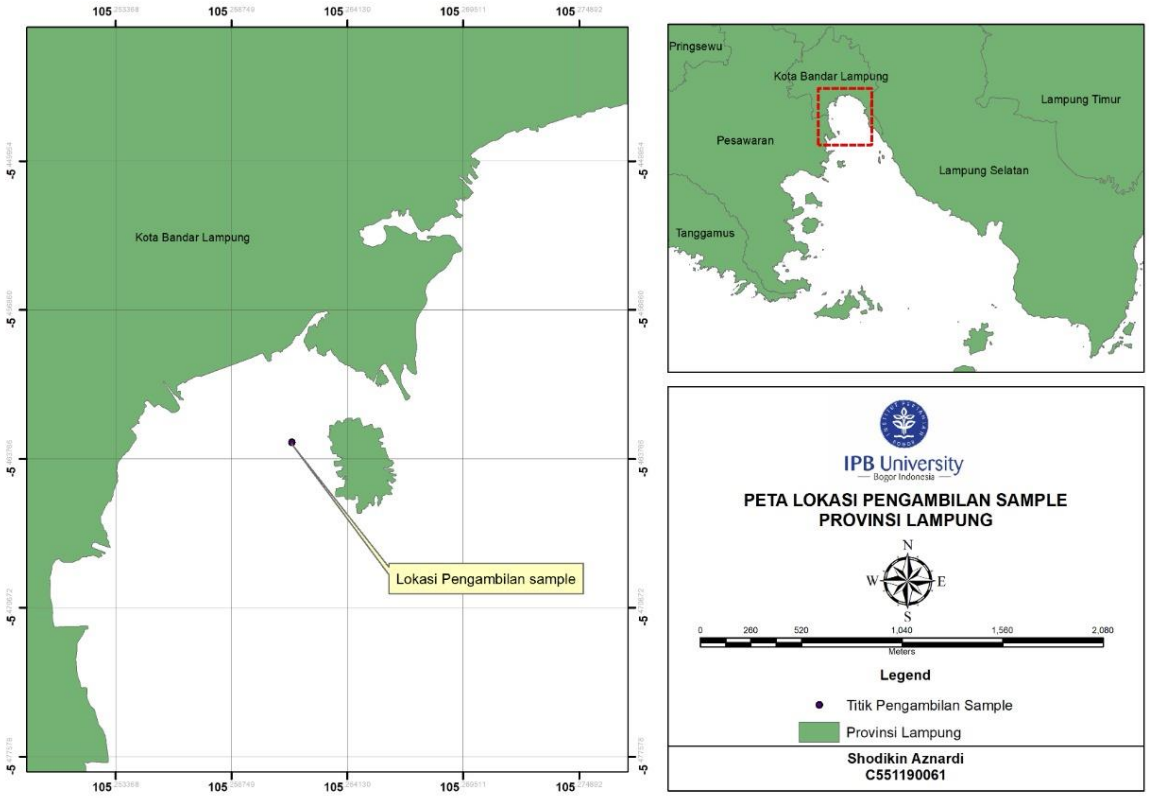

Figure 1. Map of sampling locations in Lampung Bay, Lampung Province

oven at 80°C until the sample is dry. The dried samples were then ground until smooth using a mortar and weighed 5 grams to distribute to the Environmental Productivity Laboratory of the Department of Water Resources Management of IPB for heavy metal analysis.

2.3 Screening of Bacterial Isolates from Green Mussels

Green mussel sample (gills, intestine and mantle) were separated and then crushed and isolated on Luria Bertani cultured mixed with sterile seawater (Marzan *et al.*, 2017). The dilutions used were 10^{-5}, 10^{-6}, and 10^{-7} dilutions. The bacteria, growing on the initial bacterial isolation, were then filtered using Luria Bertani cultured media by adding 10 ppm $PbNO_3$. Then the bacterial isolates were incubated for 24 hours in an incubator.

The green mussel organs to be isolated were separated between the gills, stomach and mantle and then ground until smooth. The results of bacterial isolation from green mussels obtained as many as 32 bacterial isolates. The bacterial isolates were divided into 13 isolates from the gills, 8 isolates from the stomach and 11 isolates from the mantle. After that, the 32 bacterial isolates were screened using the same medium by adding as much as 10 ppm of heavy metal $PbNO_3$ so that 28 bacterial isolates were obtained that could survive. The bacterial isolates obtained were representative of 13 isolates from the gills, 5 isolates from the stomach and 10 isolates from the mantle.

2.4 Bacterial Resistance Test

The bacterial isolates passing through the bacterial screening process were then tested using Luria Bertani (LB) cultured media added by 100 ppm PbNO3 and 100 ppm CuSO4. The concentration of heavy metals was added to 100 ppm continuously until no bacterial isolates could grow on the

media. The results of the resistance test were used as the MIC (Minimum Inhibitory Concentration) standard by the European Food Safety Authority (EFSA) in 2010 (EFSA, 2010).

2.5 Molecular Identification

Selected bacterial isolates that have been tested for heavy metal resistance ability, analyzed molecularly using Presto^{tm} Mini gDNA Bacteria kit. Molecular analysis of PCR using template 16S Ribosomal RNA universal primer using forward primer 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and reverse primer 1492r (5'-TACGGHTACCTTGTTACGACTT-3') (De Fretes *et al.*, 2019). Amplification used a temperature profile of 1 min at 95˚C followed by 30 cycles of denaturation (95˚C, 1 min), annealing (55˚C, 45 s) and extension (72˚C, 1 min), with final extension at 72˚ C for 10 minutes. The results of the analysis sent to Genetica Science for analysis on the sequence and then a phylogenetic tree is made.

The results of the sequence analysis were then reconstructed into a phylogenetic tree to see the genetic proximity of the bacterial isolates obtained. The sequences were processed on the NCBI (National Center for Biotechnology Information) genbank using a device (http://www.ncbi.nlm.nih.gov/BLAST) to see the proximity of nucleotide bases and the results then reconstructed using Clustal W in the MEGA program with the method Maximum likelihood (Kumar *et al.*, 2016).

II. RESULTS AND DISCUSSION

3.1. Water Quality and Heavy Metal Analysis

Measurement of water quality aims to determine the quality of water in which these green mussel is cultivated (Table 1). Some measured parameters show a similarity to those established in quality standards. The results of these measurements of water quality support the previous study in the waters of Lampung Bay for aquaculture conducted by Safitri *et al.* (2018).

Table 1. The water quality condition of the green mussel living area

Water quality parameters	Value	Quality standards
Temperature (°C)	28	28 - 30
pH	7.9	7 – 8.5
Salinity (‰)	31	33 - 34
Total Suspended Solid (mg/L)	< 8	20 - 80

* The Regulation of Ministry of Environment no 22 year 2021

In addition to measuring water quality, Pb and Cu were also measured in water and green mussel samples. The heavy metals in the green mussels and water originating from the waters of Lampung Bay found that the levels of heavy metals in the green mussel show very significant higher than those in water.

Table 2. Heavy metal analysis (Pb and Cu) content of waters and green mussels of lampung bay

Sample Type	Concentration (ppm)		Quality standards (ppm)*	
	Pb	Cu	Pb	Cu
Water sample	0.016	0.013	0.008	0.008
Gill	1.09	6.56	0.008	0.008
Intestine	0.9	4.13	0.008	0.008
Coat	0.84	3.66	0.008	0.008

*The Regulation of Ministry of Environment no 22 year 2021
** The results of the analysis in 5 g dry weight

The concentrations of Pb and Cu are 0.016 ppm and 0.013 ppm, respectively, and has exceeded the quality standard limits according to the regulation of Ministry of Environment. This condition is also similar to the previous studies conducted by Permata *et al.* (2018) and Safitri *et al.* (2018). The

analysis Pb and Cu in 3 organs (gills, mantle and intestine) of green mussels are high concentration. This is probably related to the feeding mechanism of the green mussels a filter feeder (Sijabat *et al.*, 2014).

The high content of Pb and Cu in water and green mussels maybe due the impact of antropogenic activities such as marine transportation and run off from the mainland through small rivers around these waters. Research conducted by Tugiyono (2007) explained that the Pb could also derivered from fuel burning and corrosion of pipes on land carried subsequently to the sea. Cu in the waters of Lampung Bay may come from activities of coal industry in the Bandar Lampung area. The process of transporting coal in the port is one of the causes of the high Cu content in these waters (Rahmah *et al.*, 2014).

3.2. Bacterial Isolate Screening

The results of bacterial isolation from green mussels obtained as many as 32 bacterial isolates. The bacterial isolates were divided into 13 isolates from the gills, 8 isolates from the stomach and 11 isolates from the mantle. After that, the 32 bacterial isolates were screened using the same medium by adding as much as 10 ppm of heavy metal $PbNO_3$ so that 28 bacterial isolates were obtained that could survive. The bacterial isolates obtained were representative of 13 isolates from the gills, 5 isolates from the stomach and 10 isolates from the mantle.

The results of Figure 2 show that not all bacterial isolates were able to survive the conditions added with 10 ppm Pb of heavy metal. Bacteria that were able to live and survive were then tested to see their resistance to heavy metals Pb and Cu using Luria Bertani media.

3.3. Bacterial Resistance

Bacterial resistance test was carried out to determine the ability of symbiotic bacteria in green mussels to Pb and Cu. The results of the heavy metal Pb resistance test showed that 13 were able of living up to a concentration of 900 ppm and 14 bacterial isolates that could live at a concentration of 1000 ppm and there was 1 bacterial isolate that could not grow, namely bacteria with isolate code STL12. The results of the study showed that the bacteria in symbiosis with green mussels had high resistance to Pb. Heavy metal Pb is one of the non-essential

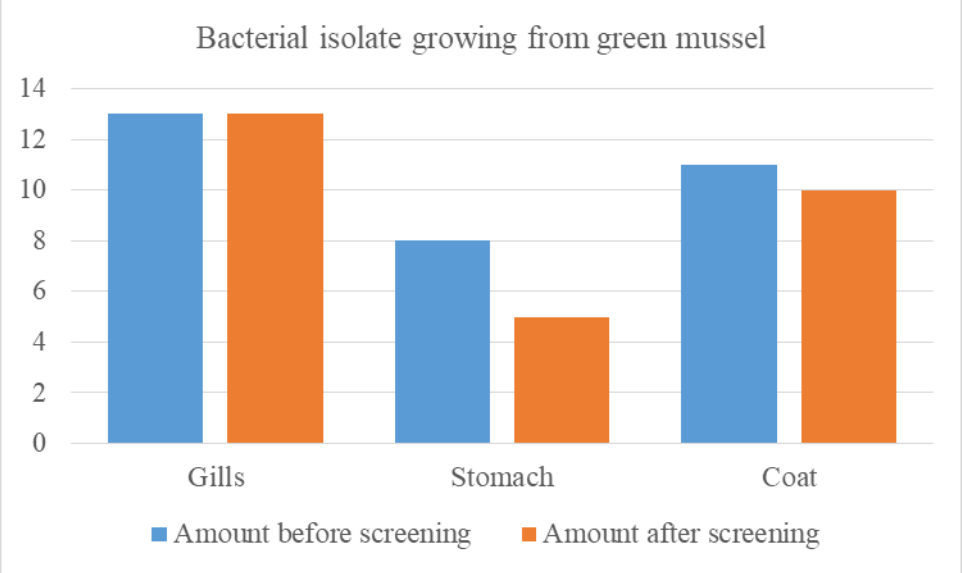

Figure 2. Number of bacterial isolates growing from green mussel organs

metals that can be tolerated by bacteria in high concentrations. The Pb-tolerable bacteria has also been reported by De Fretes *et al.* (2019). In addition, there are bacteria that can be tolerate Pb heavy metal levels up to a concentration of 2000 ppm (Imamuddin, 2001) The results of the Cu resistance test showed that bacterial isolates with both isolate codes of STL11 and STL25 could survive up to a concentration of 400 ppm toward Cu and that of isolates code STL09 had the highest level of resistance, up to 700 ppm against Cu. Previous research stated that bacteria have resistance to heavy metals up to 800 ppm copper (Cu) (De Fretes *et al*., 2019).

Bacteria have resistance mechanisms in the form of extra cellular barriers and active transport of metal ions called efflux. Essential metal ions such as Cu can enter cells through systems that function for the absorption of essential elements (Ianeva, 2009). This mechanism was developed by bacteria in order to protect themselves against continuous exposure to heavy metals. In addition, another bacterial resistance mechanism in anticipating excessive heavy metal exposure is to create a mechanism using gene chromosomes that determine copper heavy metal resistance (Cervantes & Guiterrez-Corona, 1994).

The results of the bacterial isolate resistance test were then tested statistically to see the correlation between the concentrations of Pb and Cu on the number of resistance bacteria obtained in green mussels. The results showed that the concentration of heavy metal Pb had no effect on the resistance of symbiont bacteria. Meanwhile, Cu heavy metal shows that the concentration of heavy metal affects the resistance of symbiont bacteria.

3.3. Bacterial Isolate Phylogenetic Tree Reconstruction

Reconstruction of the phylogenetic tree of selected bacterial isolates using MEGA (Kumar *et al.*, 2016) and Bioedit software. The results of the reconstruction of the phylogenetic tree showed that the bacterial isolates STL09 and STL11 were close to bacteria belonging to the genus Bacillus. However, the isolate with the code STL25 after molecular identification was

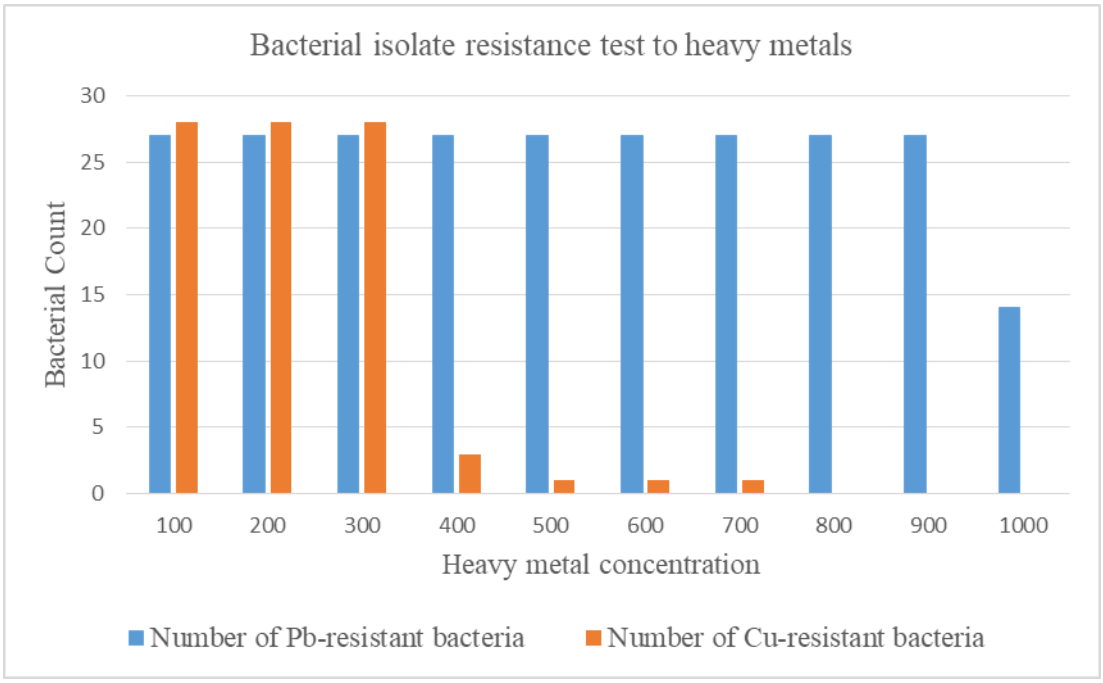

Figure 3. Number of resistance bacterial isolates to heavy metals Pb and Cu test

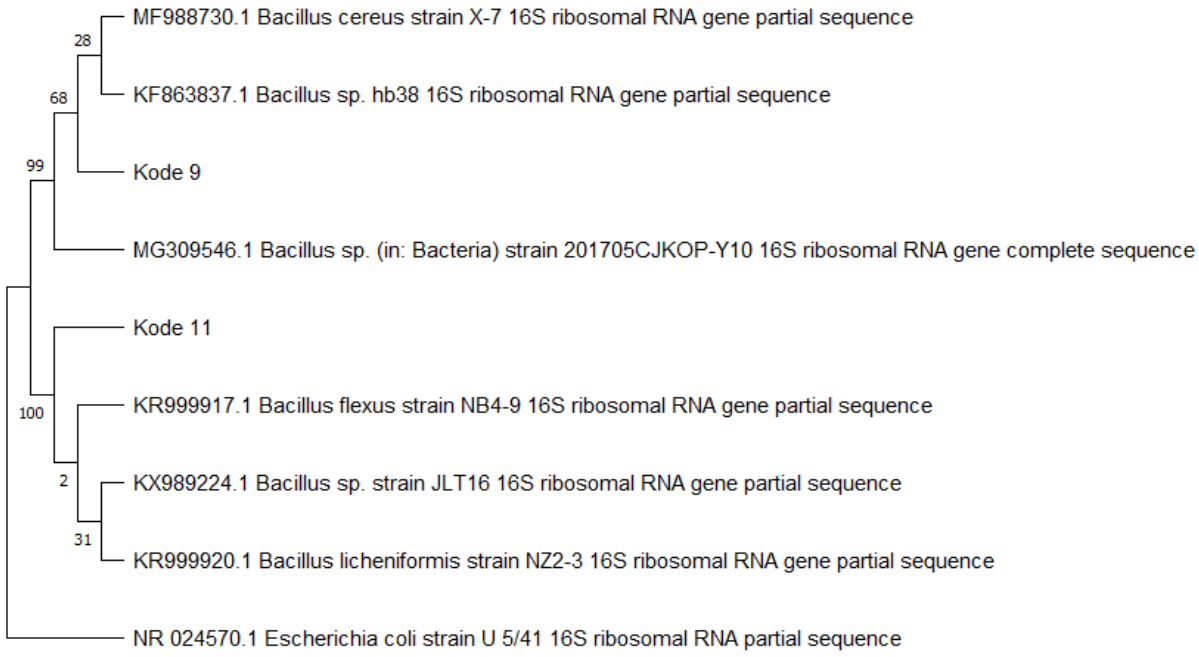

Figure 4. Reconstruction of the phylogenetic tree of selected bacterial isolates using the maximum likelihood method

unable to reconstruct the isolate because the bacterial isolate STL25 had a very small sequence of nucleotide bases.

Reconstruction of the phylogeny tree using the maximum likelihood method aims to find the highest probability of the selected bacterial isolates (Figure 4). From this reconstruction, several bacteria in the gene bank have the highest probability of proximity based on their nucleotide base sequences. The results of sequence analysis using NCBI showed that the STL09 isolate had a query cover value and percent identification 100%. Meanwhile, the bacterial isolate STL11 had a query cover value of 100% and a percent identity value of 98.43% with the bacteria in the gene bank. This indicated that the selected bacterial isolates had relatively the same coverage as the bacteria in the gene bank and had high compatibility (Table 5).

The results of the reconstruction of the phylogeny tree showed that the bacterial isolates STL09 and STL11 had close proximity to bacteria from the genus Bacillus. Research conducted by De Fretes *et al.* (2019) stated that bacteria from the genus Bacillus have high resistance to heavy metals Pb, Cu and Cd. In another study, it was stated that Bacillus bacteria had resistance to arsenic and boron (Nithya & Pandian, 2010). In addition, Bacillus bacteria are widely used in bioremediation and industrial fields because of their ability to be easily developed (Hatmianti, 2000). Bacillus is one of the bacteria that has the ability to degrade heavy metals. Research conducted by Ayangbenro & Babalola (2020) stated that bacteria from the genus Bacillus have the ability to degrade heavy metals. Bacillus is a bacterium that has the ability in various ways, including the potential for biodegradation of heavy metal pollutants regardless of their pathogenic nature.

III. CONCLUSION

Tests of heavy metal levels in water samples and green mussels showed high values, so it can be explained that the waters around green mussels have a high concentration of heavy metals so that they

Table 5. Results of sequence analysis on the NCBI blast device

Bacteria name	sciencetific name	Query cover	per ident	acc len	code	sample origin
Bacillus sp. (in: Bacteria) strain 201705CJKOP-Y10 16S ribosomal RNA gene, complete sequence	*Bacillus* sp.	100%	100.00%	1460	MG309546.1	the first institue of oceanography shangdong China
Bacillus cereus strain X-7 16S ribosomal RNA gene, partial sequence	*Bacillus cereus*	100%	100.00%	1462	MF988730.1	yanan university, China
Bacillus sp. hb38 16S ribosomal RNA gene, partial sequence	*Bacillus* sp. hb38	100%	100.00%	1456	KF863837.1	Honey bee farm Guangxi Province of China
Bacillus sp. strain JLT16 16S ribosomal RNA gene, partial sequence	*Bacillus* sp.	100%	98.43%	1442	KX989224.1	Shallow water hydrothermal vent, Taiwan
Bacillus licheniformis strain NZ2-3 16S ribosomal RNA gene, partial sequence	*Bacillus licheniformis*	100%	98.43%	1443	KR999920.1	*Lepidium perfoliatum* plant
Bacillus flexus strain NB4-9 16S ribosomal RNA gene, partial sequence	*Priestia flexa*	100%	98.43%	1444	KR999917.1	*Lepidium perfoliatum* plant

exceed the quality standard of sea water which causes metal pollution in green mussels and is included in the polluted category. The symbiont bacteria from green mussels are resistant to Pb up to 1000 ppm and Cu up to 700 ppm so they are classified as bacteria that have high resistance capabilities. The results of molecular identification showed that the isolates of bacteria STL09 and STL11 were bacteria belonging to *Bacillus* sp.

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