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Exploration of bacteria from Setigi Lake, Gresik District, Indonesia as a candidate for industrial microalgae production

SITORESMI PRABANINGTYAS[1,2,♥], AGUNG WITJORO[1], ALFIA NUR LAILI[1], AMALIA SHITA DEVI[1], CHALIMATUS SA'DIYYAH[1], MUSHOFATUL FITRIA[1], ZELA LIA QOMARIA[1], FATCHUR ROHMAN[1], HERU SURYANTO[2,3], IDA MAWADAH[1], DITA AYU EKA SAPUTRI[1], WASIATUS SA'DIYAH[1,4]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Malang. Jl. Semarang 5, Malang 65145, East Java, Indonesia. Tel./fax.: +62-341-551312, ♥email: sitoresmi.prabaningtyas.fmipa@um.ac.id
[2]Center of Advanced Materials for Renewable Energy, Universitas Negeri Malang. Jl. Semarang 5, Malang 65145, East Java, Indonesia
[3]Department of Industrial Mechanical Engineering, Faculty of Engineering, Universitas Negeri Malang. Jl. Semarang 5, Malang 65145, East Java, Indonesia
[4]Department of Plant-Microbe Interaction, Institute of Plant Science and Resources, Okayama University. 2-20-1, Chuo, Kurashiki, 710-0046, Japan

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Abstract. *Prabaningtyas S, Witjoro A, Laili AN, Devi AS, Sa'diyyah C, Fitria M, Qomaria ZL, Rohman F, Suryanto H, Mawadah I, Saputri DAE, Sa'diyah W. 2025. Exploration of bacteria from Setigi Lake, Gresik District, Indonesia as a candidate for industrial microalgae production. Biodiversitas 26: 1135-1144.* The diversity of microorganisms leads to the utilization of biological resources. The aim of this study was to investigate the community structure of microalgae and identify and assess the microbial community and bacterial potential that were collected from Lake Setigi in Gresik District, East Java, Indonesia. The field sampling was done by collecting water from 0, 50, and 100 cm depths. Microalgae were identified according to the guidebook, while the bacterial genome was isolated for metagenomic analysis. The results revealed varying taxa numbers and density of microalgae in Lake Setigi, such as 54 species at a depth of 0-10 cm (35.63×10^8 cells/mL), 42 species at a depth of 50 cm (6.12×10^8cells/mL); and 24 species at a depth of 100 cm (1.56×10^8 cells/mL). Microalgal diversity was categorized as intermediate, well-distributed, or predominant. The three most abundant bacterial phyla were Proteobacteria (56%), Bacteroidetes (16%), and Actinobacteria (13%). The potential functions of the selected bacterial isolates and their species were determined based on the similarity in 16S rRNA gene phylogenetic reconstruction. Isolates IAA17 (*Delftia* sp.), P15 (*Pseudomonas libanensis*), S14 (*Bacillus subtilis*), and SG5 (*Lysinibacillus* sp.) produced IAA, phosphate solubilization, vitamin B12, and ammonium, respectively, as a result of nitrogen-fixing activity. These abilities of bacteria underline their potential roles in biogeochemical cycling and ecosystem processes.

Keywords: *Delftia* sp., exploration, microalgae, *Pseudomonas libanensis*, Setigi Lake

INTRODUCTION

Microalgae and bacteria dominate the aquatic environment and serve as bioindicators of aquatic ecosystems. Abiotic factors like oxygen, nutrition, and light intensity influence the diversity of microorganisms in the lake (Yadav et al. 2019). Aquatic ecosystems have many potential resources, as explored in Lake Setigi of Gresik District, East Java, Indonesia. This artificial lake is a chalk mining area (Asmoro and Azis 2020). Post-mining areas may release water-soluble chemical compounds from rocks or minerals (Putrawiyanta 2020). Lake Setigi experienced structural changes, which resulted in a shift in the community structure from terrestrial microorganisms to aquatic ones. As one of the primary producers with nutritional value and providing energy in the food chain, microalgae inhabit Lake Setigi (Samudra et al. 2013).

During the rainy season, rainwater carried into Lake Setigi waters dissolves mineral salts in the soil, increasing the Total Dissolved Solid (TDS) and Electric Conductance (DHL) values of the water (Hilal 2020). Limestone and leachate in the area can increase the pH of Lake Setigi air to become more alkaline, which was caused by the decomposition of proteins by microbes into ammonium and the release of OH−, thereby increasing the pH value. Monitoring Setigi Lake, a new tourist destination and former mining area, is needed to maintain the structure of the microalgae community. Physical factors (total dissolved solids, salinity, turbidity, electrical conductivity, brightness level) and chemical factors (pH, dissolved oxygen, nitrate, and phosphate test) are crucial parameters that must be monitored.

Bacteria are another component of aquatic ecosystems that have many potential functions. Aquatic bacteria play an important role in decomposing organic matter and mineralization (Feng et al. 2019). In addition to exchanging macronutrients and micronutrients, bacteria can secrete plant hormones that stimulate microalgae growth. Nitrogen is an abundant element in the atmosphere, primarily in the form of gas. The fixation of atmospheric nitrogen can be carried out by non-symbiotic or symbiotic nitrogen-fixing bacteria (Zulfarina et al. 2017). These bacteria, including cyanobacteria, autotrophic bacteria, and heterotrophic bacteria, can convert free nitrogen into ammonium and nitrate (Merlo et al. 2014). Microalgae require nitrogen in the form of ammonium and nitrate to synthesize amino acids and

genetic material (Sembiring and Sabrina 2021). The availability of nitrogen is essential for energy synthesis in microalgae, which accelerates their growth of biomass production. In addition to nitrogen-fixing bacteria, other bacteria that can enhance microalgae growth and biomass production produce the hormone Indole-3-Acetic Acid (IAA). These bacteria support the metabolic processes of microalgae by producing exogenous auxin hormones, such as IAA (Jusoh et al. 2015). The interaction between microalgae and IAA-producing bacteria involves the exchange of metabolites, including tryptophan, IAA phytohormone, thiamine, nitrogen, and carbon (Palacios et al. 2019).

Another group of microorganisms that can promote algal growth is phosphate-solubilizing bacteria. These bacteria decompose insoluble phosphorus compounds and release them into the environment that microalgae readily absorb. This interaction occurs because phosphate-solubilizing bacteria coexist with microalgae in aquatic environments (Liu et al. 2015; Dong et al. 2022). Phosphorus is a crucial element for algal growth. Phosphate-solubilizing bacteria can be classified as inorganic or organic phosphate solubilizers (Xie et al. 2024). They dissolve insoluble inorganic phosphate by producing organic acids and mineralize organic phosphate compounds with the help of enzymes (Li et al. 2024). In the symbiotic relationship between algae and phosphate-solubilizing bacteria, these bacteria produce organic acid, polysaccharides, and proteins as nutrients for algal growth (Dong et al. 2022). Another important group of microorganisms in this context are vitamin B12-producing bacteria, which can significantly enhance microalgae growth. Most of the vitamin B12 required by algae is supplied by these bacteria in aquatic ecosystems (Ramanan et al. 2016). Methionine, a type of amino acid serving as the initiation codon in protein formation, is essential for the growth and metabolism of microalgae. Vitamin B12 is a coenzyme for methionine synthase, an enzyme essential for methionine synthesis (Bromke and Hesse 2015). This study aimed to investigate the diversity of microalgae and bacteria in Lake Setigi and evaluate functional bacteria through several assays.

MATERIALS AND METHODS

Field sampling and measurement of abiotic factors

The water containing microalgae from five different locations in Lake Setigi of Gresik District, East Java, Indonesia (Figure 1) was collected using grab sampling with three repetitions. Water below 50 cm was collected in triplicate using a water grab sampler. The collected samples were stored in sterile bottles. Microalgae samples were collected using purposive sampling at different depths of 0, 50, and 100 cm, dripped with 30 drops of formaldehyde 2-5%, and placed in an ice box for further laboratory experiments.

The abiotic factors were measured at every depth, and an electrical conductivity meter was used to measure the temperature, total dissolved solids, and electrical conductivity. The salinity of water, dissolved oxygen, and pH were measured using a Secchi disk, DO meter, and pH meter, respectively. Nitrate nitrogen (NO_3-N) concentration and phosphate (PO_4) concentration were calculated by using the Kjeldahl method and the Molybdenum method, respectively, in Jasa Tirta (an Indonesian government company).

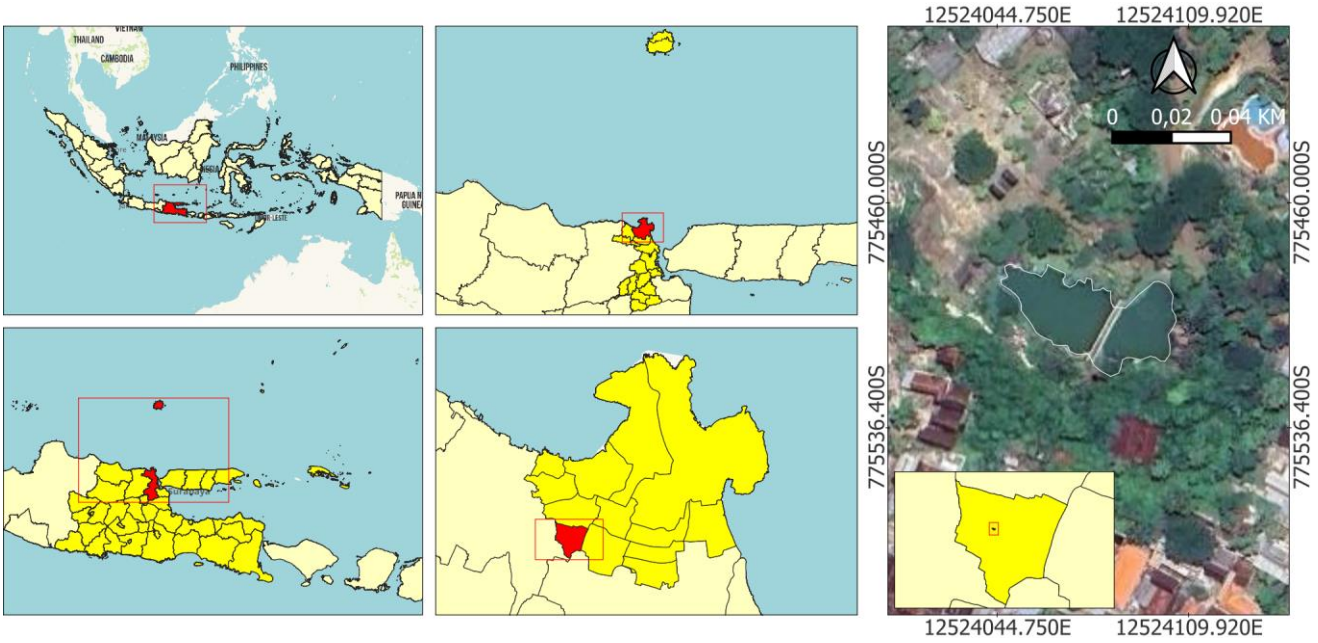

Figure 1. Study site in Setigi Lake, Sekapuk Village, Ujungpangkah Sub-district, Gresik District, East Java, Indonesia

Procedures

Microalgae identification

The water samples from 0, 50, and 100 cm depth were centrifuged at 2,000 rpm for 10 min. The number of algal species was calculated using the Sedgwick-Rafter counting chamber by filling the chamber with 1 mL of a homogenized water sample, followed by observation under a microscope at a magnification of 40× in triplicate. Counting was conducted systematically across multiple fields of view, recording the number of individuals for each species. Microalgae were identified based on a guidebook by Bellinger and Sigee (2010). The density of microalgae was measured using a hemocytometer. We analyzed several indices of microalgae that were measured, including H' (Shannon-Wiener Species Diversity Index), E (Pielou Species Evenness Index), R (Margalef Species Richness Index), and C (Simpson Dominance Index).

Isolation of bacteria

The water samples were diluted to a dilution level of 10^{-6}. Samples in the final dilution were plated on selective plate media and incubated at 20°C for 24 h. Bacterial colonies were selected and purified using selective media, and qualitative and quantitative methods were used to determine their potency.

Phenotypic characterization of bacteria

The potential bacteria were identified by observing the morphology of bacterial colonies (color, shape, edge, and elevation). Microscopic observations included bacterial cell shape, size, and Gram staining.

Metagenomic analysis

Bacterial samples were collected by filtering 1 L of water from each sampling spot using Whatman microfilter paper of 11 µm and 2.5 µm consecutively. The Whatman paper was cut into small pieces as samples for isolating the bacterial genome using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research, USA). The DNA samples were confirmed by 1% agarose gel electrophoresis in 1X TBE solution at 100 V for 30 min. DNA sequencing was performed at 1stBASE Laboratories Malaysia using the Illumina MiSeq platform.

Assessment of potential bacteria

IAA-producing bacteria

Salkowski reagent was used for qualitative assessment. Bacterial isolates were grown on tryptic soy agar + tryptophan medium at 20°C for 24 h. IAA-producing bacterial isolates were characterized by the formation of a pinkish color after adding the Salkowski reagent (Sukmawati et al. 2021). For the quantitative test, bacterial isolates were grown in a tryptic soy broth + tryptophan medium. Bacterial samples were collected at 0, 24, 48, and 72 hours, followed by centrifugation. The supernatant was added to Salkowski's reagent and incubated for 45 min in the dark. The absorbance value was measured using a spectrophotometer at 530 nm (Ramadhani et al. 2020). The standard solution was prepared using IAA (0, 5, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 65, and 70 ppm), with the addition of Salkowski reagent and distilled water at each concentration (Widawati and Muharam 2012).

Phosphate-solubilizing bacteria

The qualitative test was carried out by growing bacterial isolates on Pikovskayas agar medium and incubating at 25-28°C for 7 days, thereby showing a clear zone formed around the colonies identified as phosphate-solubilizing bacteria. For the quantitative test, isolates were cultured in Pikovskayas broth medium for 7 days. Centrifugation was performed at 10,000 rpm for 15 min. The stannous chloride method was used for total phosphate measurements by adding chloromolybdic acid and chlorostannous acid reagents to the supernatant. Absorbance was measured using a UV-Vis spectrophotometer at 600 nm of wavelength.

Vitamin B12-producing bacteria

Bacterial isolates were grown on selective plates of ½ PYBG (0.5% Trypticase peptone, 0.25% Phytone peptone, 0.12% Lab-Lemco powder, 0.1% Bacto yeast extract, 0.05% glucose, and 1% agar) at 20°C for 24 h (Prabaningtyas et al. 2021). The quantitative test was performed by culturing the cells in a 1/20 PYBG liquid medium. After 48 h of incubation at 20°C, the bacterial culture was centrifuged, and then distilled water was added to the supernatant, which was then sterilized. One drop of *Salmonella typhimurium* culture was added to the sterilized supernatant and incubated for 24 hours (Prabaningtyas et al. 2021). The growth of *S. typhimurium* was measured using a spectrophotometer at a wavelength of 546 nm. The standard solution was prepared using pure vitamin B12 with 0, 1.6, 3.2, 4.8, and 6.4 ppm concentrations.

Nitrogen-fixing bacteria

Bacterial isolates were grown in semi-solid NFB medium (5 g $C_4H_6O_5$; 0.5 g KH_2PO_4; 0.2 g $MgSO_4.7H_2O$; 0.1 g NaCl; 4.8 g KOH; 0.05 g yeast extract; 10 mL bromothymol blue; 7.5 g agar) at 20°C for 7 days (Nafisah et al. 2022). The white pellicle and color change of semi-solid NFB medium to blue indicated the presence of nitrogen-fixing bacteria. In the quantitative test, isolates were cultured in 14.5 mL of liquid medium at 20°C for 72 h, followed by centrifugation at 12,000 rpm. The supernatant was added by distilled water and Nessler's reagent. The sample absorbance was measured using a spectrophotometer at a wavelength of 425 nm wavelength (Nafisah et al. 2022). The standard solution was prepared using NH_4Cl with concentrations of 0, 1.5, 3, 4.5, 6, 7.5, 9, 10.5, and 12 ppm with the addition of Nessler's reagent at every concentration.

16S rRNA gene-based identification of bacteria

DNA isolation of potential bacteria was performed using the QIAamp®DNA Mini Kit (Qiagen, Germany). Bacterial DNA purity was measured using a NanoDrop 2000 spectrophotometer. Universal 16S rRNA primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1429R (5'-TACGGYTACCTTGTTACGACT-3') were used to amplify the gene under the following PCR conditions: initial denaturation at 94°C for 3 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 30 s, and extension

at 72°C for 90 s; and final extension at 72°C for 10 min. PCR products were confirmed using 1% agarose gel electrophoresis (50 V, 60 min) and visualized under a UV-transilluminator. Sequencing was performed at the 1st BASE Laboratories, Malaysia.

Data analysis

Data was statistically analyzed to compare the microalgae density between the depths of the lake (0, 50, and 100 cm) and the sampling spots. The 16S rRNA gene sequencing results of potential bacteria were examined using FinchTV (https://digitalworldbiology.com/FinchTV). DNA baser (www.dnabaser.com) was used to align DNA sequences and edit contigs. Determination of the similarity of the nucleotide sequence of the gene with the target gene was performed using Blastn (https://blast.ncbi.nlm.nih.gov). Phylogenetic trees were constructed using MegaX (https://www.megasoftware.net/).

RESULTS AND DISCUSSION

Microalgae community

The results showed that 86,605 individuals from 54 taxa in 33 microalgae families were identified in Lake Setigi. At a depth of 0-10 cm, there were 71,256 individuals of 54 taxa from 34 families; at a depth of 50 cm, 12,238 individuals of 42 taxa from 28 families, and 3,111 individuals from 24 taxa in 17 families at a depth of 100 cm. Based on Table 1, the microalgal diversity level was classified as moderate. The distribution of microalgae is uniform and predominant (Mahmudi et al. 2023). *Rhodomonas* sp. had the highest importance value index at 0-10 cm and 50 cm depth. *Aphanocapsa* sp. had the highest importance value at a depth of 100 cm. Significant differences in microalgae density were observed between depths and sampling spots.

Assessment of abiotic factor

Based on the current results, the microalgae density decreased with increasing lake depth. This might be caused by abiotic factors (Table 3) in aquatic ecosystems that can affect the microalgal diversity index. Most microalgae require a pH range of 7-8.5. The high pH of the water in Lake Setigi was due to the presence of limestone rocks and leachate. The decomposition of protein by microbes into ammonium and the release of OH^- can increase the pH value. The microalgal diversity index also affected decomposition, predatory animal activity, and natural mortality.

Bacterial metagenomics

The microbial community composition in samples can be investigated using Operational Taxonomic Units (OTU). Effective tags from all samples were used for OTU analysis. The groups of closely related individuals were clustered based on the 16S rRNA sequence similarity (97% similarity). The top 10 selected taxa from the samples were identified based on the taxonomic annotation results, such as Chloroflexi, Acidobacteria, Planctomycetes, Patescibacteria, Firmicutes, Verrucomicrobia, Cyanobacteria, Actinobacteria, Bacteroidetes, and Proteobacteria. Based on metagenomic analysis (Figure 1), ten bacterial phyla in Lake Setigi, of which the three most abundant phyla, Proteobacteria, dominated the microalgae in Lake Setigi (56%), followed by Bacteroidetes (16%) and Actinobacteria (13%) as the second and third dominant phyla.

Characterization of bacterial phenotypes

The potential bacteria were characterized and identified phenotypically and genotypically. Phenotypic observations included macroscopic and microscopic morphology of bacteria. The identified bacterial isolates were IAA17, P15, S14, and SG5. Morphological characterization of potential bacterial phenotypes, including colony morphology, cell morphology, and Gram staining, are shown in Table 4.

16S rRNA gene-based identification

PCR amplification of bacterial isolates S14, SG5, P15, and IAA17 resulted in products with a sequence length of 1500 bp. 16S rRNA gene sequence similarity values of <97% and <95% correspond to different species and genera.

Phylogenetic tree reconstruction

The phylogenetic tree was constructed using the Neighbor-Joining method with bootstrap 1000 in the MegaX program (Figure 3). The construction resulted in four main clades and one outgroup. The main clades included *Pseudomonas* sp., *Delftia* sp., *Lysinibacillus* sp., and *Bacillus* sp. Each of selected bacterial isolates belonged to different main clades: Isolate P15 in the clade of *Pseudomonas* sp. (bootstrap value of 62); Isolate IAA17 in the clade of *Delftia* sp. (bootstrap value of 100); Isolate SG5 in the clade of *Lysinibacillus* sp. (bootstrap value of 76); and Isolate S14 in the clade of *Bacillus* sp. (bootstrap value of 72).

The genetic distances between selected bacterial isolates and the bacterial species in each clade were also evaluated. Isolate IAA17 had the closest genetic distance to *Delftia* sp. (0.001), with 99.9% similarity. Isolate P15 had the closest genetic distance to *Pseudomonas libanensis* (0.000) with 100% similarity, while isolate S14 had the closest genetic distance to *Bacillus subtilis* (0.001), with 99.9% similarity. Isolate SG5 was genetically closest to *Lysinibacillus* sp. (0.000) with 100% similarity.

Assessment of potential bacteria

IAA-producing bacteria

The bacterial isolate IAA17 had the highest IAA production, with a concentration of 67.028 ppm. Several factors affect IAA production, including the length of bacterial incubation period and the addition of tryptophan.

Phosphate-solubilizing bacteria

Based on the total phosphate produced, bacterial isolate P15 could dissolve phosphate with the highest phosphate concentration of 21.43 ppm. Phosphate dissolution results can vary depending on the type of metabolism, dissolution rate, and distribution rate in the media.

Table 1. The species composition of microalgae in Lake Setigi, Gresik District, Indonesia

Depths of Lake	Species	Families
0 cm	*Oscillatoria* sp.	Oscillatoriaceae
	Microspora sp.	Microsporaceae
	Tribonema vulgare	Tribonemataceae
	Zygonema sp.	Zygonemataceae
	Geminella sp.	Chlorellaceae
	Ulothrix sp.	Ulotrrichaceae
	Asterionella sp.	Fragilariaceae
	Mougeotia sp.	Zygnemataceae
	Calothrix sp.	Rivulariaceae
	Elakatothirx sp.	Nostoceae
	Oocytis sp.	Oocytaceae
	Nitzcshia sp.	Bacillariaceae
	Penium sp.	Peniaceae
	Achnanthes sp.	Achnanthaceae
	Rhodomonas sp.	Pyrenomonadaceae
	Closterium sp.	Desmidiaceae
	Synura sp.	Synuraceae
	Chlamydomonas sp.	Chlamydiomonadaceae
	Cyclotella sp.	Stephanodiscaceae
	Gomphosphaeria sp.	Gomphosphaeriaceae
	Sphaerocystis sp.	Palmellaceae
	Cosmarium sp.	Desmideaceae
	Nostox sp.	Nostoceae
	Tetrastrum sp.	Scenedesmaceae
	Gloeocapsa sp.	Microcystaceae
	Merismopedia sp.	Merismopediaceae
	Snowella sp.	Coleosphaeriaceae
	Microcystis sp.	Microcystaceae
	Chroococcus sp.	Chroococcaceae
	Dictyosphaerium sp.	Dictyosphaeriaceae
	Chlorella vulgaris	Chlorellaceae
	Aphanocapsa sp.	Merismopediaceae
	Eudorina sp.	Volvoceae
	Westella sp.	Oocystaceae
	Pandorina sp.	Volvoceae
	Botryococcus sp.	Dictyosphaeriaceae
	Coelastrum astroideum	Scenedesmaceae
	Coelosphaerium sp.	Merismopediaceae
	Chlorogonium sp.	Chlamydomonadaceae
	Trachelomonas caudate	Euglenaceae
	Chaetoceros sp.	Chaetocerotaceae
	Scenedesmus quadricauda	Scenedesmaceae
	Scenedesmus opoliensis	Scenedesmaceae
	Scenedesmus obliquus	Scenedesmaceae
	Scenedesmus acuminatus	Scenedesmaceae
	Scenedesmus obtusus	Scenedesmaceae
	Fragilaria sp.	Fragilariaceae
	Tabellaria sp.	Tabellariaceae
	Tolypothrix sp.	Tolypothrichaceae
	Pediastrum tetras	Hydrodictyaceae
	Pediastrum duplex	Hydrodictyaceae
	Pediastrum boryanum var. *cornutum*	Hydrodictyaceae
	Pediastrum boryanum	Hydrodictyaceae
	Gloeotrichia sp.	Gloeotrichiaceae
50 cm	*Oscillatoria* sp.	Oscillatoriaceae
	Microspora sp.	Microsporaceae
	Tribonema vulgare	Tribonemataceae
	Zygonema sp.	Zygonemataceae
	Geminella sp.	Chlorellaceae
	Asterionella sp.	Fragilariaceae
	Mougeotia sp.	Zygnemataceae
	Calothrix sp.	Rivulariaceae
	Elakatothirx sp.	Nostoceae
	Oocytis sp.	Oocytaceae
	Nitzcshia sp.	Bacillariaceae
	Penium sp.	Peniaceae
	Achnanthes sp.	Achnanthaceae
	Rhodomonas sp.	Pyrenomonadaceae
	Synura sp.	Synuraceae
	Chlamydomonas sp.	Chlamydomonadaceae
	Cyclotella sp.	Stephanodiscaceae
	Sphaerocystis sp.	Palmellaceae
	Nostox sp.	Nostoceae
	Tetrastrum sp.	Scenedesmaceae
	Snowella sp.	Coleosphaeriaceae
	Microcystis sp.	Microcystaceae
	Chroococcus sp.	Chroococcaceae
	Dictyosphaerium sp.	Dictyosphaeriaceae
	Chlorella vulgaris	Chlorellaceae
	Aphanocapsa sp.	Merismopediaceae
	Eudorina sp.	Volvoceae
	Westella sp.	Oocystaceae
	Pandorina sp.	Volvoceae
	Botryococcus sp.	Dictyosphaeriaceae
	Coelastrum astroideum	Scenedesmaceae
	Chaetoceros sp.	Chaetocerotaceae
	Scenedesmus quadricauda	Scenedesmaceae
	Scenedesmus opoliensis	Scenedesmaceae
	Scenedesmus obliquus	Scenedesmaceae
	Scenedesmus acuminatus	Scenedesmaceae
	Scenedesmus obtusus	Scenedesmaceae
	Fragilaria sp.	Fragilariaceae
	Tabellaria sp.	Tabellariaceae
	Pediastrum tetras	Hydrodictyaceae
	Pediastrum duplex	Hydrodictyaceae
	Pediastrum boryanum var. *cornutum*	Hydrodictyaceae
	Pediastrum boryanum	Hydrodictyaceae
100 cm	*Oscillatoria* sp.	Oscillatoriaceae
	Zygonema sp.	Zygonemataceae
	Ulothrix sp.	Ulotrrichaceae
	Mougeotia sp.	Zygnemataceae
	Nitzchia sp.	Bacillariaceae
	Rhodomonas sp.	Pyrenomonadaceae
	Closterium sp.	Desmidiaceae
	Cyclotella sp.	Stephanodiscaceae
	Gomphosphaeria sp.	Gomphosphaeriaceae
	Sphaerocystis sp.	Palmellaceae
	Dictyosphaerium sp.	Dictyosphaeriaceae
	Chlorella vulgaris	Chlorellaceae
	Gomphospaeria sp.	Gomphosphaeriaceae
	Aphanocapsa sp.	Merismopediaceae
	Eudorina sp.	Volvoceae
	Pandorina sp.	Volvoceae
	Coelastrum astroideum	Scenedesmaceae
	Chlorogonium sp.	Chlamydomonadaceae
	Scenedesmus quadricauda	Scenedesmaceae
	Scenedesmus opoliensis	Scenedesmaceae
	Scenedesmus obliquus	Scenedesmaceae
	Scenedesmus acuminatus	Scenedesmaceae
	Scenedesmus obtusus	Scenedesmaceae
	Pediastrum duplex	Hydrodictyaceae
	Pediastrum boryanum	Hydrodictyaceae

Table 2. The density, index of species diversity, species evenness, species richness, and species dominance of microalgae in Lake Setigi, Gresik District, Indonesia

Depths of lake	Density (cells/mL)	Species Diversity Index (H')	Species Evenness Index (E)	Species Richness Index (R)	Species Dominance Index (C)
0 cm	35.63×10^8	2.95	0.74	4.74	0.08
50 cm	6.12×10^8	2.8	0.75	4.36	0.09
100 cm	1.56×10^8	2.37	0.74	2.86	0.15

Table 3. Average results of abiotic factor measurements at each sampling spot depth in Lake Setigi, Gresik District, Indonesia

Depths of lake	Turbidity (NTU)	TDS (ppm)	DHL (µs/cm)	Salinity (‰)	Temperature (°C)	pH	NO_3-N (mg/L)	PO_4 (mg/L)	DO (ppm)
0 cm	8.88	227.93	457.4	8.13	30.8	9.08	0.05	<0.01	25.03
50 cm	9.2	231.53	462.07	8.22	31.52	9.05	0.06	0.04	23.75
100 cm	9.51	229.47	458.53	8.45	31.15	9.08	0.04	0.03	25.89

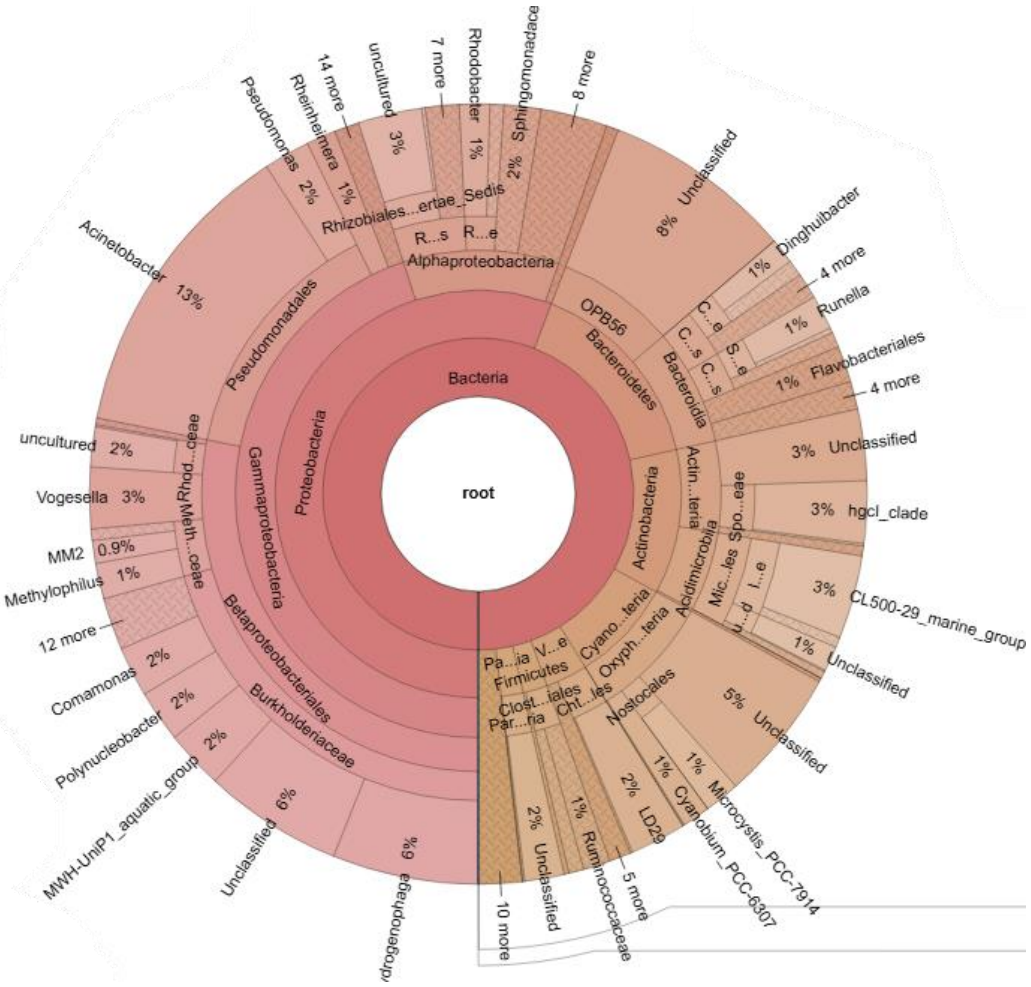

Figure 1. Metagenomic analysis of bacteria

Vitamin B12-producing bacteria

Results showed that only bacterial isolate S14 produced the highest amount of vitamin B12 (4.26 ppm).

Nitrogen-fixing bacteria

A total of 19 bacterial isolates could fix nitrogen through qualitative testing. Bacterial isolate SG5 had the highest nitrogen-fixing ability by producing ammonium of 10.38 ppm.

Discussion

Microbial diversity is critical in maintaining ecosystem functions and biogeochemical cycles, particularly in aquatic environments, where bacteria and microalgae contribute significantly to nutrient cycling, primary production, and ecological balance. This study investigates the diversity and functional potential of microalgae and bacteria in Lake Setigi. Based on microalgae identification, varying taxa numbers and density of microalgae were observed in Lake Setigi, such as 54 species at a depth of 0-10 cm (35.63×10^8 cells/mL); 42 species at a depth of 50 cm (6.12×10^8 cells/mL); and 24 species at a depth of 100 cm (1.56×10^8 cells/mL). The highest species richness was observed at a depth of 50 cm, while the lowest was recorded at 100 cm. Abiotic factors, including the physical and chemical characteristics of the waters in Telaga Setigi, influence this variation. One of the key physical factors affecting species richness is light intensity. Higher light intensity promotes phytoplankton growth by enhancing photosynthesis. At a depth of 0 cm or the water surface, Telaga Setigi receives the most sunlight as it is in the outermost layer of water. However, light intensity gradually decreases at 50 cm and 100 cm depths. Algal growth becomes limited when the average light intensity received is insufficient to reach the threshold required for photosynthetic saturation. In such conditions, reduced light availability restricts the photosynthetic efficiency of algae, leading to slower growth rates (Fettah et al. 2022).

Table 4. Morphological characterization of bacterial isolates

Characteristics	Isolates			
	IAA17	**P15**	**S14**	**SG5**
Colony color	White	White	White	White
Colony shape	Round	Irregular	Irregular	Irregular
Colony edge	Slippery	Undulate	Slippery	Undulate
Colony elevation	Arise	Umbonate	Flat	Raised
Cell length	± 3 µm	± 2 µm	± 3 µm	± 7 µm
Cell diameter	± 1 µm	± 1µm	± 1 µm	± 1 µm
Cell shape	Bacillus	Bacillus	Bacillus	Bacillus
Gram type	Gram-negative	Gram-negative	Gram-positive	Gram-positive

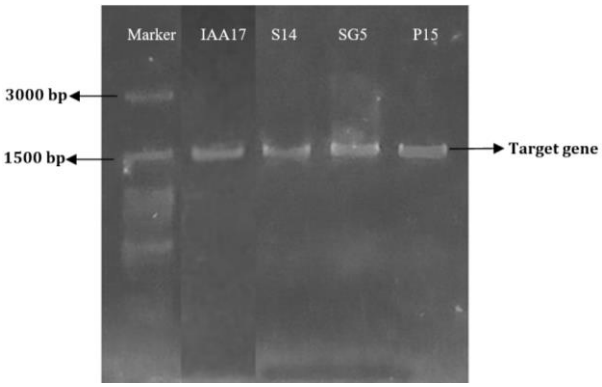

Figure 2. 16S rRNA gene amplification result

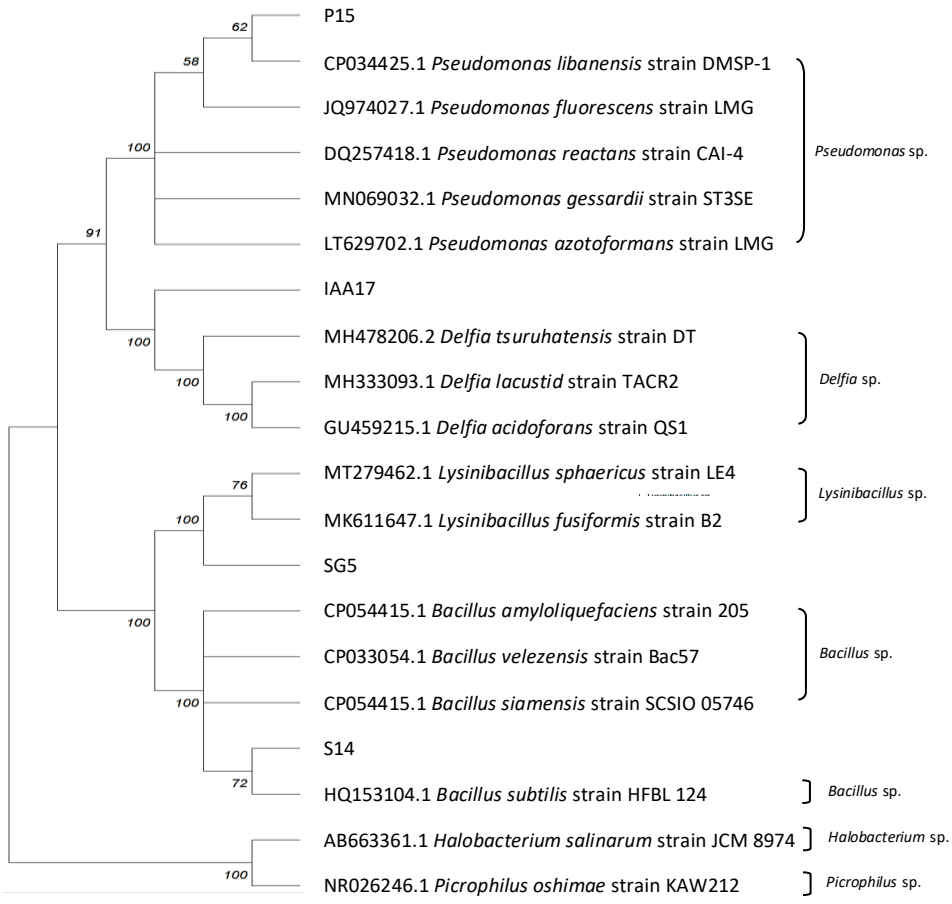

Figure 3. Phylogenetic tree reconstruction using the Neighbor-Joining method

Bacterial isolates from Lake Setigi have the potential to produce IAA (Isolate IAA17), dissolve phosphate (Isolate P15), produce vitamin B12 (Isolate S14), and fix nitrogen (Isolate SG5). Isolates IAA17 and P15 were Gram-negative bacteria, while isolates S14 and SG5 were Gram-positive. IAA production increases at 48 h of incubation and decreases at 72 h (Meza et al. 2015). A long incubation period causes a reduction in the amount of nutrients in the medium so that the number of IAA-producing bacteria decreases. As a precursor, tryptophan addition in high concentrations to the media will enhance IAA production (Imamuddin et al. 2015). Other environmental factors, such as –carbon, pH, and oxygen, can also influence IAA production by bacteria (Gusmiaty et al. 2019). The genus *Delftia* includes endophytic bacteria that live in water, plants, and soil (Han et al. 2017). The colony of the genus *Delftia* has a round shape with smooth edges, rod-shaped cells, and negative Gram staining (Khalifa and AlMalki 2019). Endophytic bacteria of this genus have the potential to produce IAA hormones and siderophores to stimulate plant growth (Woźniak et al. 2019).

The ability of potential phosphate-solubilizing bacteria to dissolve phosphate is characterized by clear zone formation around their colonies growing on Pikovskaya medium containing insoluble phosphate in the form of calcium phosphate ($CA_3(PO_4)_2$) (Kouas et al. 2024). Phosphate solubilization involves media acidification from organic acid production. The organic acids produced can chelate cations with their hydroxyl and carboxyl groups. The genus *Pseudomonas* belongs to the group of phosphate-solubilizing bacteria. *Pseudomonas* sp. can form a clear zone when tested using a solid Pikovskaya medium, indicating its ability to dissolve phosphate (Alori et al. 2017; Kumar et al. 2020). In the phosphate solubilization mechanism, phosphate-solubilizing bacteria produce organic acids that bind to cations. This mechanism was observed in a previous study that reported the production of citric acid and malonic acid by *P. libanensis* (Ma et al. 2019).

Isolate S14 was found similar to *B. subtilis*. *B. subtilis* can produce vitamin B_{12} through the salvage pathway (Balabanova et al. 2021). Cobalamin biosynthesis via the salvage pathway can occur if bacteria do not possess most genes involved in the de novo biosynthesis pathway (Shelton et al. 2019). A quantitative test of potential vitamin B12-producing bacteria was performed using *S. Typhimurium* (Torres et al. 2016; Prabaningtyas et al. 2021). *Salmonella typhimurium* uses vitamin B12 produced by bacterial isolates in the medium. Isolate SG5 has similarities to *Lysinibacillus fusiformis*, - a motile bacterium with rod-shaped cells, positive gram staining, and spore production ability (Passera et al. 2021). Several species of the genus *Lysinobacillus* (*Lysinibacillus fusiformis*, *Lysinibacillus xylanilyticus*, and *Lysinibacillus sphaericus*) have been confirmed as nitrogen-fixing bacteria (Aguirre-Monroy et al. 2019). Bacterial strains affect the concentration of ammonium produced due to differences in nitrogenase activity, an enzyme that converts nitrogen into NH_3 in bacteria (Wang et al. 2024).

Molecular identification of the 16S rRNA gene presents similarities between bacterial species with an accuracy rate of 99% (Yang et al. 2020). 16S rRNA gene sequence similarity values of <97% and <95% correspond to different species and genera. The 16S rRNA gene is a barcode gene commonly used to identify bacteria and their populations because it has an informative area and sufficient variation to differentiate between taxa within a taxon (Antil et al. 2023). Metagenomic analysis of 16S rRNA is based on isolating bacterial DNA from environmental samples to determine its composition (Ahmad et al. 2021). Phylogenetic reconstruction of the 16S rRNA gene revealed the similarity of selected bacterial isolates with certain species: isolate IAA17, similar to *Delftia* sp.; isolate P15, similar to *Pseudomonas libanensis*; isolate S14, similar to *Bacillus subtilis*; and isolate SG5, similar to *Lysinibacillus* sp. *Proteobacteria* was the most abundant phylum in the bacterial domain. Based on phylogenetic analysis of the 16S rRNA gene, *Proteobacteria* are divided into six classes: *Alphaproteobacteria*, *Betaproteobacteria*, *Deltaproteobacteria*, *Gammaproteobacteria*, *Epsilonproteobacteria*, and *Zetaproteobacteria* (Rizzatti et al. 2017). *Betaproteobacteria* are usually dominant in lakes and rivers (Shao et al. 2021), whereas *Alphaproteobacteria* and *Gammaproteobacteria* are detected in freshwater and seawater (Chen et al. 2019). *Proteobacteria* identified in Lake Setigi consisted of 81% *Gammaproteobacteria* and 19% *Alphaproteobacteria*. In seawater, *Alphaproteobacteria* and *Gammaproteobacteria* are often abundant (Lee and Eom 2016).

Members of the phylum *Bacteroidetes* are distributed in almost all types of habitats, including soil, ocean, freshwater, and various aquatic environments (Sun et al. 2019). In aquatic habitats, the abundance of *Bacteroidetes* is correlated with algal blooms. *Bacteroidetes* are considered to degrade High Molecular Weight (HMW) compounds and prefer growth attached to algal cells (Russo et al. 2016). The phylum *Actinobacteria* exists in the soil, rhizosphere, seawater, and freshwater. It was the most dominant phylum in the lake. More than 50% of *Actinobacteria* were found on the water surface. Bacteria in this phylum were also discovered at the bottom of the lake, but their abundance decreased as the oxygen concentrations declined. *Actinobacteria* are distributed in oligotrophic, mesotrophic, and eutrophic lakes; their dominance is spread globally (Zhu et al. 2019).

In conclusion, this study uncovered significant findings regarding the microalgal and bacterial communities in Lake Setigi. Fifty-four species were identified at 0-10 cm depth, 42 taxa at 50 cm, and 24 taxa at 100 cm, demonstrating a depth-dependent decline in species richness. The microalgal diversity in the lake was classified as intermediate, well-distributed, and dominant. Furthermore, this study revealed that the bacterial community was predominantly composed of three phyla: Proteobacteria (56%), Bacteroidetes (16%), and Actinobacteria (13%). Phylogenetic reconstruction of the 16S rRNA gene enabled the identification and characterization of key bacterial isolates with distinct functional potentials, including *Delftia* sp. (Isolate IAA17), *Pseudomonas libanensis* (Isolate P15), *Bacillus subtilis* (Isolate S14), and *Lysinibacillus* sp. (Isolate SG5). These isolates produced Indole-3-Acetic Acid (IAA), solubilize phosphate, synthesize vitamin B12, and fix nitrogen through ammonium production. These abilities of bacteria underline

their potential roles in biogeochemical cycling and ecosystem processes.

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