

Exploration non-symbiotic nitrogen-fixing bacteria from several lakes in East Java, Indonesia

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Abstract. Nafisah W, Prabaningtyas S, Witjoro A, Saptawati RT, Rodiansyah A. 2022. *Exploration non-symbiotic nitrogen-fixing bacteria from several lakes in East Java, Indonesia. Biodiversitas* 23: 1752-1758. Potential microorganisms, mainly nitrogen-fixing bacteria spread in almost lake areas in East Java. The biogeochemical processes that occur between biotic-biotic or biotic-abiotic factors can accelerate the growth of autotrophic microorganisms in waters as like microalgae. Non-symbiotic nitrogen-fixing bacteria in the water can supply nitrogen to support the growth of microalgae. This study is aimed to explore and calculate the ammonium concentration produced by non-symbiotic nitrogen-fixing bacteria that isolated from several lakes in East Java. The highest ammonium producing bacteria were characterized based on morphology, physiology, and genetic with 16S rRNA gene sequencing. The bacterial isolates were screened with a nitrogen-free bromothymol blue medium to select bacterial isolates capable of fixing nitrogen. The content of NH_3/NH_4 was produced by isolates of nitrogen-fixing bacteria was measured by using the prodac test kit NH_3/NH_4 . The ammonium concentration was calculated with Nessler's reagent by using the spectrophotometry method at a wavelength of 425 nm. The result was obtained 31 isolates of non-symbiotic nitrogen-fixing bacteria. The bacterial isolate B2 exhibited highest activity of fixing nitrogen that produced ammonium (14.46 mg/L) among the other screened bacteria. The morphological and physiological characterization from that isolate was identified as genus *Bacillus*, whereas the genetic characterization similar to *Bacillus paramycoides*, that included in the *Bacillus cereus* group.

Keywords: 16S rRNA, *Bacillus paramycoides*, East Java lakes, nitrogen-fixing bacteria

INTRODUCTION

Nitrogen (N_2) is an abundant element in the atmosphere in the form of gas. Plants and microalgae need nitrogen in ammonium (NH_4^+) and nitrate (NO_3^-) to synthesize genetic material and amino acids (Orr et al. 2011; Merlo et al. 2014; Sembiring and Sabrina 2021). The fixation process of free-nitrogen (N_2) to form ammonium (NH_4^+) and nitrate (NO_3^-) can be mediated by a group of bacteria, including cyanobacteria (blue-green algae), autotrophic bacteria, and heterotrophic bacteria. Those microorganisms almost accumulated in soils and roots (Orr et al. 2011; Merlo et al. 2014). Non-symbiotic nitrogen-fixing bacteria and symbiotic nitrogen-fixing bacteria are known as common organisms to reform free nitrogen in the atmosphere (Zulfarina et al. 2017). Non-symbiotic bacteria are able to fix nitrogen without associating with other organisms, while symbiotic bacteria are able to fix nitrogen by symbiotic and associate with other organisms. Some non-symbiotic nitrogen-fixing bacteria are included in the genus *Azotobacter* (Roper and Gupta 2016) and *Bacillus* (Jesmi et al. 2017). The non-symbiotic nitrogen-fixing bacteria such as *Azobacter* and *Bacillus* can fix nitrogen mediated by nitrogenase activity (Orr et al. 2011; Farnelid et al. 2013; Merlo et al. 2014).

Microalgae needs nitrogen for the energy synthesis. This process can accelerate the microalgae growth to

produce high biomass. Microalgae are recently widely considered as alternative biomass for biofuel production (Medipally et al. 2015). The biofuel produced by microalgae is important as renewable energy because due to limited resources for energy production (Jegathese and Farid 2014). Microalgae cultured in optimum conditions could produce high biomass, which is essential for biofuels-based microalgae production (Yao et al. 2019). *Chlorella vulgaris* is a common microalga used for co-culture study that lives in water, and they need essential elements like nitrogen. Microalgae and bacteria that live in the freshwater have specific characteristics influenced by conditions and environmental factors (Hou et al. 2017). This natural environment can be imitated and applied in the laboratory using the microbial consortium method. The limited number of bacteria that can fixed nitrogen non-symbiotically and many bacterial species that have not been explored as a whole make challenges for scientists to build the ideal environment for culturing microalgae (Roper and Gupta 2016).

This study is used to help scientists build an ideal environment for microalgae culture, because microalgae need nitrogen to optimize the synthesis process in their cells. A group of bacteria can mediate the nitrogen in the form of ammonium. The ability of bacterial isolate that fix nitrogen will be calculated qualitatively and quantitatively. Moreover, this study also aimed to identify the highest ammonium-producing bacteria based on morphology,

physiology, and genetics characteristics with the 16S rRNA gene sequence. Nitrogen-fixing bacteria can be used in the microalgae cultivation process by culturing with microalgae, such as research on co-culture with *Scenedesmus* sp. and *Azospirillum brasilense*, taking into account factors such as aeration, amount of oxygen, and carbon dioxide (Contreras-Angulo et al. 2019). The co-culture can increase the production of microalgae biomass so that it can be used in agriculture and waste treatment and biofuel production (Contreras-Angulo et al. 2019; Kang et al. 2021).

MATERIALS AND METHODS

Screening of non-symbiotic nitrogen-fixing bacteria

The eighty-nine bacteria were isolated from 4 lakes in East Java, Indonesia, namely Ranu Pani, Ranu Regulo, Ranu Grati, and Telaga Ngebel from previous study (data not published). These bacterial isolates were screened for non-symbiotic nitrogen-fixing bacteria selection with nitrogen free bromothymol blue (NFB) medium. The NFB medium contains K_2HPO_4 , $FeCl_3 \cdot 6H_2O$, $MgSO_4 \cdot 6H_2O$, NaCl, malic acid, KOH, yeast extract, bromothymol blue, and agar. All isolated bacterial were cultured in a semisolid NFB medium for 14 x 24 h. The fixation activities from each bacterial were determined by a pellicle on the surface of the medium and change in color at NFB semisolid medium from green to blue (Baldani et al. 2014).

Ammonium assay

The ammonium concentration was measured semi-quantitatively using the PRODACtest NH_4/NH_3 kit (Prodac, Italy) following the manufacturer procedures. The quantification of ammonium concentration was prepared with Nessler's reagent and was measured with spectrophotometry at 425 nm (Hartono et al. 2016). The bacteria exhibited nitrogen-fixing attributes were inoculated into liquid peptone 1% in a closed-test tube, then incubated for 3x24 h at room temperature. After incubation, those cultures were centrifuged at 12,000 rpm for 10 min then the pellets were removed. The 0.5 mL of supernatant was moved into a 5 mL test tube, and then 0.1 mL Nessler reagent and 4.5 mL distilled water also added. Those solutions were incubated at room temperature for 10 min. The quantification of ammonium was measured with Libra S12 UV-Vis spectrophotometer (Biochorm, UK) with a wavelength of 425 nm (Sumarno and Muryanto 2015). The concentration of ammonium was obtained with the linear regression of the absorbance from supernatant bacterial according to absorbance values from standard curve. The standard ammonium curve was made by NH_4Cl 0.3819 g and placed in the oven for 1-2 h at 105°C. NH_4Cl in the oven was moved to 100 mL volumetric flask and added up to 100 mL of distilled water to make a stock solution with a concentration of 1,000 mg/L. 1 mL from the stock solution was re-diluted with distilled water up to 100 mL to obtain a new standard solution with a concentration of 10 mg/L. This new standard solution was diluted with distilled water according to the desired concentrations for standard curve. The series standard solutions were added

0.1 mL Nessler's reagent, then the absorbance was determined with UV-Vis spectrophotometer at a wavelength of 425 nm.

Biochemical characterization

The biochemical characterization includes cell characterization, colony morphology, and physiology characterization. The variable of colony characterization consists of shape, color, elevation, border, thickness, and shiny or gloomy, while the cell characterization is as follows: Gram, spore, capsules, shape, and size (Holt et al. 1994; Ayitso and Onyango 2016; Yusra et al. 2014). The physiological characterization is based on the microbact biochemical identification kit (Oxoid, ThermoScientific).

Molecular characterization

The genomic DNA (gDNA) of the selected bacteria with the highest ability for fixing nitrogen was purified using QIAmp DNA Mini Kit (Qiagen, Germany). Firstly, the isolates were cultured in 5 mL NB medium for 1 x 24 h at 37°C. About 2.5 mL from that culture was centrifuged in the serial moments, the supernatant was discarded; then the pellets were used for gDNA purification that follows the manufacture protocols. The TopTaq Master Mix kit (Qiagen, Germany) was used for PCR reaction. The primers were used for 16S rRNA gene amplification as follows: 27F (5'-GAGTTTGATCMTGGCTCAG-3') and 1492R (5'-ACGGYTACCTTGTTACGACTT-3'). The reaction profile for PCR as follows: initial denaturation 94°C/3 min, denaturation 94°C/1 min, annealing 50°C/30 s, extension 72°C/1 min 30 s, final extension 72°C/10 min, and hold 4°C. The PCR product was checked on 1 % electrophoresis gel stained with ethidium bromide (EtBr). The 1 kb marker DNA from Geneaid was added in a gel to verify the length of amplicon. The gel was run in MupidX® one electrophoresis apparatus then the gel was visualized with UV transilluminator.

The PCR products were used for DNA sequencing at 1st BASE laboratories, Malaysia. The quality and the counting sequences were built with DNA baser (<https://www.dnabaser.com/>), and the consensus sequence was compared with the blast nucleotide program in NCBI (<https://blast.ncbi.nlm.nih.gov>). The phylogenetic tree and genetic distance were calculated using MegaX software (available at <https://www.megasoftware.net/>) with the Maximum Likelihood method, including the bootstrap method 1,000 replicates (Saitou and Nei 1987).

RESULTS AND DISCUSSION

Screening of non-symbiotic nitrogen-fixing bacteria

The selection of isolated bacterial from Ranu Grati, Telaga Ngebel, Ranu Pani, and Ranu Regulo showed thirty-one isolated bacterial exhibited nitrogen fixation activity (Table 1). Each lake has different conditions which are influenced by abiotic factors. The abiotic factors were measured in five stations from each lake, Ranu Regulo consists of DO around 8-11.6 mg/L, pH 8.65-9.4, Salinity 0-0.3, and Transparency 75-80 cm; Ranu Pani consists of

DO 4.2-11.6, pH 6.83-7.3, Salinity 0.2-0.4, Transparency 50-100; Ranu Grati consists of DO 7.1-8.5, pH 7.1-8.82, Salinity 0, Transparency 100-110 cm and the abiotic factors from Telaga Ngebel consist of DO 2.5-8.5, pH 6.98-7.69, Salinity 0.1-0.2, and Transparency 383-676 cm (Prabaningtyas et al. 2018).

Nitrogen-fixing bacteria or diazotrophic bacteria have a sensitivity level to oxygen and other aquatic environmental conditions such as inorganic nitrogen content, temperature, pH, humidity, light intensity, water depth, and nutritional trophic status (Leoni et al. 2018). These environmental conditions will affect the nitrogenase enzyme activity of bacteria in binding free nitrogen to form pellicle on the surface of the media. The one of the environmental conditions that affect nitrogenase activity is oxygen levels in the environment, nitrogenase activity takes place optimally when cells are in a low oxygen environment, so that the entry and exit of oxygen in the cells must be balanced, especially for aerobic bacteria (Stal 2017). Semisolid NFB is a medium that does not contain nitrogen but contains other components needed for the process of respiration, metabolism, and the formation of ATP (Baldani et al. 2014). The cellular respiration activity from bacteria associated with the presence of oxygen in the environment affects the nitrogenase enzyme activity, so it will also affect the process of nitrogen fixation (Merlo et al. 2014). The pellicle formation occurs because the nitrogen-fixing process by the nitrogenase enzyme that reached a balance point with the contribution of ATP; so the nitrogen that has been fixed will accumulate to the surface of the medium (Susilowati and Setyowati 2016; Inomura et al. 2018).

Ammonium semi-quantitative and quantitative assay

The ammonium concentration with a semi-quantitative test using the NH_3/NH_4 kit showed that the concentration of produced ammonium from the nitrogen-fixing process around 0.5 mg/L to 0.25 mg/L (Table 1). Twelve isolates with the highest fixation activity that produced ammonium from the semi-quantitative assay were then tested for ammonium quantitatively using UV-Vis spectrophotometry with standard curve equation $y = 0.121x + 0.049$ and R-value about 0.99. The quantitative assay for ammonium concentration showed that the isolate "B2" from Ranu Pani was the highest ammonium concentration of about 14.46 mg/L, while the lowest ammonium concentration was bacterial isolate "S" from Ranu Pani and Ranu Regulo, reaching 8.08 mg/L (Table 2).

The results of biochemical identification including physiological characterization also showed the correlation with the genus *Bacillus* (Table 4), which has a similarity with *Bacillus cereus* (66.67%), *Bacillus mycoides* (66.67%), and *Bacillus thuringiensis* (66.67%).

Non-symbiotic nitrogen-fixing bacteria can bind nitrogen then they convert it into other compounds like ammonium. Availability of ammonium compounds provides nitrogen accumulation for the growth of microalgae, such as *Chlorella* sp. These bacteria can be used for consortium with microalgae (Gonçalves et al. 2017; Makut et al. 2019), these two organisms can be

cultured together to increase the microalgae biomass (Fuentes et al. 2016). The consortia of bacteria and microalgae are beneficial because microalgae help provide oxygen through photosynthesis, used by bacteria for respiration. Bacteria help to provide a nitrogen source for microalgae metabolism and produce organic substances (Jia and Yuan 2016).

Table 1. Semi-quantitative of ammonium/ammonia measured with PRODACTest NH_4/NH_3 kit

Isolate code	Ammonium conc. (mg/L)	Source
14	0.50	Ranu Grati''
IN	0.50	Telaga Ngebel
L	0.50	Ranu Pani & Regulo
B2	050	Ranu Pani & Regulo
X	0.50	Ranu Pani & Regulo
B1	0.50	Ranu Pani & Regulo
W	0.50	Ranu Pani & Regulo
Y	0.25	Ranu Pani & Regulo
S	0.50	Ranu Pani & Regulo
D	0.50	Ranu Pani & Regulo
B	0.50	Ranu Pani & Regulo
N	0.50	Ranu Pani & Regulo
Q	0.50	Ranu Pani & Regulo
10B	0.25	Ranu Grati
13	0.25	Ranu Grati
33	0.25	Ranu Grati
15	0.25	Ranu Grati
6	0.25	Ranu Grati
2	0.25	Ranu Grati
30	0.25	Ranu Grati
34	0.25	Ranu Grati
20	0.25	Ranu Grati
AN	0.25	Telaga Ngebel
ON	0.25	Telaga Ngebel
HN	0.25	Telaga Ngebel
DN	0.25	Telaga Ngebel
FN	0.25	Telaga Ngebel
QN	0.25	Telaga Ngebel
RN	0.25	Telaga Ngebel
I	0.25	Ranu Pani & Regulo
J	0.25	Ranu Pani & Regulo

Table 2. Ammonium concentration from nitrogen-fixing bacteria measured with spectrophotometry method

Code	Average (mg/L)	Source
B2	14.46	Ranu Pani
X	12.78	Ranu Pani & Regulo
L	10.96	Ranu Pani & Regulo
B	10.90	Ranu Pani & Regulo
N	10.67	Ranu Pani & Regulo
B1	9.94	Ranu Pani & Regulo
IN	9.81	Telaga Ngebel
W	9.30	Ranu Pani & Regulo
Q	9.18	Ranu Pani & Regulo
D	8.85	Ranu Pani & Regulo
14	8.81	Ranu Grati
S	8.08	Ranu Pani & Regulo

The ammonium produced from non-symbiotic nitrogen-fixing bacteria was measured by the spectrophotometry method and using the Nessler reagent. This reagent could change the solution from light yellow to the orange-brown (Sumarno and Muryanto 2015; Ngibad 2019). The process of changing color solution occurs when ammonium (NH_4^+) reacts with Nessler's reagent (K_2HgI_4) so that it will form mercury (Hg-O), NH_2 and H_2O bonds. The reaction of ammonium formation depends on environmental conditions such as pH, oxygen content, and temperature. Ammonium (NH_4^+) will be formed at pH 6-9 and its formed by nitrogen compounds binding with H^+ ions in water (Luo et al. 2015). The trophic status, one of which is eutrophic, which is a condition that can influence the amount of ammonium in the waters and the nutrient content in waters also affect the amount of ammonium in the environment (Leoni et al. 2018). Some eutrophic waters, the ammonium content was found to reach high amounts (Leoni et al. 2018). The amount of ammonium correlated with dissolved organic matter and phosphorus matter (Pandey and Sreelu 2006). The concentration produced by the "B2" isolate is included in the high category. According to (Ngibad 2019) stated that the started concentration of ammonium in the water Ngelom's river is 1.61-14.8 mg/L. According to the study (Hartono et al. 2016), the ammonium concentration produced by bacteria in the soil is 256.7 μM up to 1027.77 μM (0.2567-1.0277 mg/L), while according to a study (Hendrawan et al. 2021), in the freshwater of Rawa Pening Lake, it shows that the ammonium produced by bacteria through the nitrification process reaches 0.2 mg/L/hour.

Ammonium can be synthesized by the nitrogen-fixing bacteria in the soil or the water. The environment affects the lives of these bacteria, especially those that live in water. According to the previous study Prabaningtyas et al. (2018), several water conditions in this lake, such as pH about 6.8-9.4, dissolved oxygen (DO), were between 4.2 and 11.6 mg/L, and salinity was around 0.00%-0.4%, which are estimated to have nitrogen-fixing bacteria. The range of pH about 6-9 is feasible for ammonium formation (Luo et al. 2015).

Bacterial identification

The characterization results showed that the bacterial isolates "B2" belong to the group of Gram-positive bacteria, non-conjoined bacteria, having endospores, aerobic, and several other morphological properties (Table 3). The cell of bacteria from "B2" isolate based on microscopy showed the gram-positive strain (Figure 1). The characterization of morphological characteristics identified as a genus of *Bacillus* is based on the analysis in the book Bergey's Manual (Holt et al. 1994).

The results of molecular identification using the 16S rRNA gene showed that amplicon product from PCR has a sequence size of about 1500 bp (Figure 2).

The sequences assembly of 16S rRNA gene sequence of B2 isolate has a length of 1444 bp. This sequence was aligned with the blast nucleotide program showed it has a genetic similarity with the 16S rRNA gene in the *Bacillus* species. The genetic distance of isolate B2 showed that the isolate "B2" was in one clade with *Bacillus paramycoides*.

This position has a high confidence score marked with bootstrap value of 100 (Figure 3). The 16S rRNA gene sequence in B2 isolate has a similarity with other *B. cereus* Group species up to 99%, indicating that the sequences are homogeny. The "B2" isolate was suspected to be a *B. paramycoides* strain LU6 with the highest genetic similarity up to 99.9%.

The bacterial identification based on morphology, biochemical characteristics, and molecular DNA was showing a correlation between bacterial isolate B2 in a genus *Bacillus*, specifically in the *B. cereus* group. This bacterial group is including Gram-positive bacteria, which have endospores, and have rod-shaped (basil), catalase activity, oxidase activity and others (Liu et al. 2017). Bacterial species in the genus *Bacillus* and the *B. cereus* group have the ability to fixed nitrogen, this species can produce IAA hormone and solubilizing phosphate (Ding et al. 2005; Jesmi et al. 2017; Osman and Yin 2018). According to the several studies, the genus of *Bacillus* can be fixing nitrogen and change it to ammonium that is useful for plants and other organisms (Kumar et al. 2012).

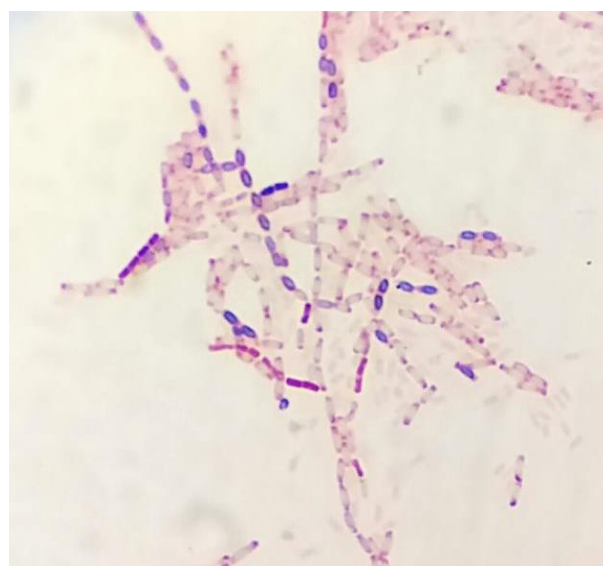


Figure 1. Cell morphology of "B2" isolate (Magnification: 1000x)

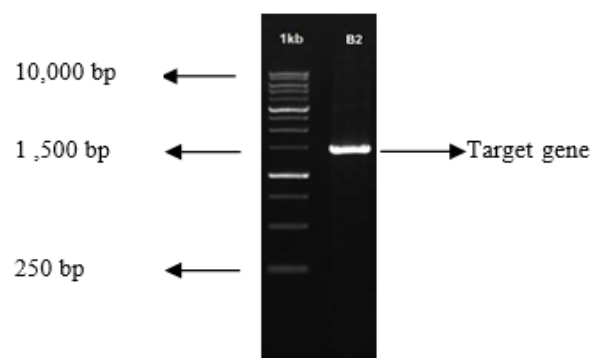


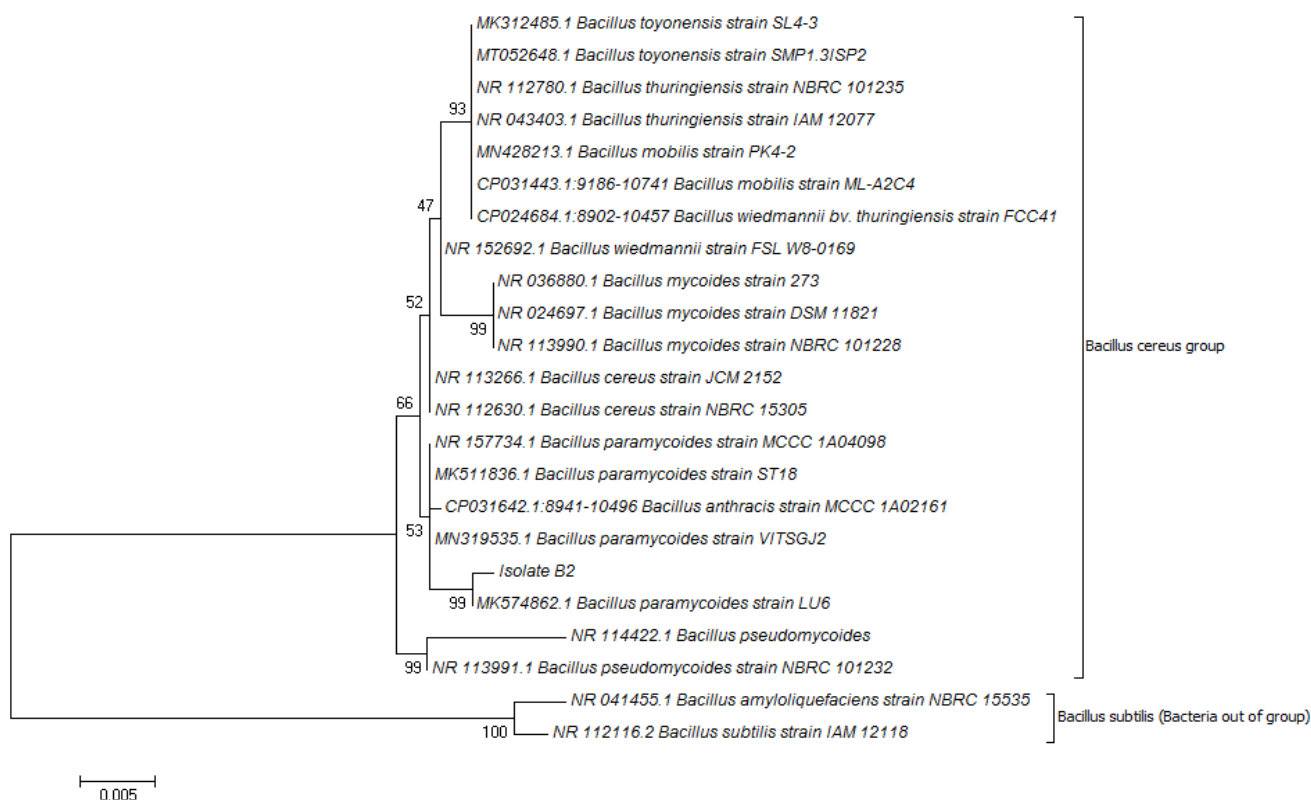
Figure 2. Visualization of 16S rRNA PCR product with 1 kb marker of 1% agarose Gel, 100 Volt, 200 A, for 30 min

Table 3. Characterization morphology of “B2” isolate

Characterization colony / cell	Results	Source (Bergey's book, 1994)
Colony color	With like milk	
Colony shape	Round with spread edges	
Edge of the colony	Irregular	
Elevation	Arise	
Colony density	High density	
Shiny / gloomy	Shiny	
Diameter of the colony	± 2-3 mm	
Gram staining	Gram-positive bacteria	Gram-positive bacteria
Cell shape	Basil	Basil
Cell color	Transparan	
Cell size	l: ± 3-4.5 µm d: ± 0.5-1 µm	l: 1.2-10 µm d: 0.5-2.5 µm
Motility	Motil with flagel	Motil
Capsule	Nothing	
Spores / endospores	Contained endospores	Endospora
Spore shape	Ovale	Ovale
Spore location	Subterminal	
Respiration	Aerob	Aerob

Table 4. Identification biochemical of “B2” isolate

Characteristic	Results
Adonitol	-
Arabinose	-
Arginine	-
Catalase	+
Citrate	-
Endospore	+
Gelatin	+
Glucose	-
Gram staining	Positive
H ₂ S	-
Indole	-
Inositol	-
Lactose	-
Lysine	-
Malonate	-
Mannitol	-
Motilitas	+
Nitrate	+
ONPG	-
Ornithine	-
Oxidase	+
Raffinose	-
Rhamnose	-
Salicin	-
Shape	rod straight shaped
Sorbitol	-
Sucrose	-
TDA	-
Urease	-
VP	+
Xylose	-

**Figure 3.** Phylogenetic tree of “B2” isolate with Maximum-likelihood method

In conclusion, the potential of non-symbiotic nitrogen-fixing bacteria from several lakes in East Java qualitatively and quantitatively was found 31 bacterial isolates. The bacterial isolate code "B2" was the highest activity to fixed nitrogen measured with its ability to produce ammonium about 14.46 mg/L. The bacterial isolate "B2" identification based on morphological, physiological, and genetic characterization with the 16S rRNA gene sequence was identified as *B. paramycoides* which is a member of the *B. cereus* group.

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REFERENCES

- Ayitso AS, Onyango DM. 2016. Isolation and identification by morphological and biochemical methods of antibiotic producing microorganisms from the gut of *Macrotermes michaelseni* in Maseno, Kenya. *J Appl Biol Biotechnol* 4 (01): 027-033. DOI: 10.7324/JABB.2016.4.0105.
- Baldani JJ, Reis VM, Videira SS, Boddey LH, Baldani, VLD. 2014. The art of isolating nitrogen-fixing bacteria from non-leguminous plants using N-free semi-solid media: a practical guide for microbiologists. *Plant Soil* 384: 413-431. DOI: 10.1007/s11104-014-2186-6.
- Contreras-Angulo JR, Mata TM, Cuellar-Bermudez SP, Caetano NS, Chandra R, Garcia-Perez JS, Muylaert K, Parra-Saldivar R. 2019. Symbiotic co-culture of *Scenedesmus* sp. and *Azospirillum brasilense* on N-Deficient media with biomass production for biofuels. *Sustainability* 11: 707. DOI: 10.3390/su11030707.
- Ding Y, Wang J, Liu Y, Chen S. 2005. Isolation and identification of nitrogen-fixing bacilli from plant rhizospheres in Beijing region. *J Appl Microbiol* 99: 1271-81. DOI: 10.1111/j.1365-2672.2005.02738.x.
- Farnelid H, Bentzon-Tilia M, Andersson AF, Bertilsson S, Jost G, Labrenz M, Jürgens K, Riemann L. 2013. Active nitrogen-fixing heterotrophic bacteria at and below the chemocline of the central Baltic Sea. *ISME J* 7: 1413-1423. DOI: 10.1038/ismej.2013.26.
- Fuentes J, Garbayo I, Cuaresma M, Montero Z, González-del-Valle M, Vilchez C. 2016. Impact of microalgae-bacteria interactions on the production of algal biomass and associated compounds. *Mar Drugs* 14: 100. DOI: 10.3390/md14050100.
- Gonçalves AL, Pires JCM, Simões M. 2017. A review on the use of microalgal consortia for wastewater treatment. *Algal Res* 24: 403-415. DOI: 10.1016/j.algal.2016.11.008.
- Hartono, Nurfitriani, Asnawati F, Citra H, Handayani NI, Junda M, Ali A, Hala Y, Jumadi O. 2016. Ability of ammonium excretion, indol acetic acid production, and phosphate solubilization of nitrogen-fixing bacteria isolated from crop rhizosphere and their effect on plant growth. *ARNP J Eng Appl Sci* 11: 11735-11741.
- Hendrawan AKF, Afati N, Rahman A. 2021. Laju nitrifikasi pada bioremediasi air limbah organik menggunakan *Chlorella* sp. dan bakteri nitrifikasi-denitrifikasi. *Jurnal Pengelolaan Sumberdaya Alam dan Lingkungan* 11 (2): 309-323. DOI: 10.29244/jpsl.11.2.309-323. [Indonesian]
- Holt JG, Krieg NR, Sneath PHA, Staely JT, Williams ST. 1994. *Bergey's manual of determinative bacteriology*, Ninth Edition. eds. Williams and Wilkins, USA.
- Hou D, Huang Z, Zeng S, Liu J, Wei D, Deng X, Weng S, He Z, He J. 2017. Environmental factors shape water microbial community structure and function in shrimp cultural enclosure ecosystems. *Front Microbiol* 8: 2359. DOI: 10.3389/fmicb.2017.02359.
- Inomura K, Bragg J, Riemann L, Follows MJ. 2018. A quantitative model of nitrogen fixation in the presence of ammonium. *PLoS One* 13 (11): e0208282. DOI: 10.1371/journal.pone.0208282.
- Jegathese SJP, Farid M. 2014. Microalgae as a renewable source of energy: A niche opportunity. *J Renew Energy* 2014: 430203. DOI: 10.1155/2014/430203.
- Jesmi Y, Thajudeen J, Rahiman M, Krishnankutty S, Alikunju A, Abdulla M.H. 2017. Nitrogen fixing potential of various heterotrophic *Bacillus* strains from a tropical estuary and adjacent coastal regions. *J Basic Microbiol* 57. DOI: 10.1002/jobm.201700072.
- Jia H, Yuan Q. 2016. Removal of nitrogen from wastewater using microalgae and microalgae bacteria consortia. *Cogent Environ Sci* 2: 1275089. DOI: 10.1080/23311843.2016.1275089.
- Kang Y, Kim M, Shim C, Bae S, Jang S. 2021. Potential of algae-bacteria synergistic effects on vegetable production. *Front Plant Sci* 12: 656662. DOI: 10.3389/fpls.2021.656662.
- Kumar, Ajay, Amit, Devi S, Patil S, Payal C, Negi S. 2012. Isolation, screening and characterization of bacteria from rhizospheric soils for different plant growth promotion (PGP) activities: an in vitro study. *Recent Res Sci Technol* 4: 1-5.
- Leoni B, Patelli M, Soler V, Nava V. 2018. Ammonium transformation in 14 lakes along a trophic gradient. *Water* 10: 265. DOI: 10.3390/w10030265.
- Liu Y, Du J, Lai Q, Zeng R, Ye D, Xu J, Shao Z. 2017. Proposal of nine novel species of the *Bacillus cereus* group. *Intl J Syst Evol Microbiol* 67: 2499-2508. DOI: 10.1099/ijsem.0.001821.
- Luo X, Yan Q, Wang C, Luo C, Zhou N, Jian C. 2015. Treatment of ammonia nitrogen wastewater in low concentration by two-stage ozonization. *Intl J Environ Res Public Health* 12: 11975-11987. DOI: 10.3390/ijerph120911975.
- Makut BB, Das D, Goswami G. 2019. Production of microbial biomass feedstock via co-cultivation of microalgae-bacteria consortium coupled with effective wastewater treatment: A sustainable approach. *Algal Res* 37: 228-239. DOI: 10.1016/j.algal.2018.11.020.
- Medipally SR, Yusoff FM, Banerjee S, Shariff M. 2015. Microalgae as sustainable renewable energy feedstock for biofuel production. *BioMed Res Intl* 2015: 519513. DOI: 10.1155/2015/519513.
- Merlo C, Reyna L, Abril A, Amé MV, Genti-Raimondi S. 2014. Environmental factors associated with heterotrophic nitrogen-fixing bacteria in water, sediment, and riparian soil of Suquia River. *Limnologia* 48: 71-79. DOI: 10.1016/j.limno.2014.06.004.
- Ngibad K. 2019. Penentuan konsentrasi ammonium dalam air sungai pelayaran Ngelom. *Afr J Microbiol Res* 2 (1): 37-42. DOI: 10.21070/medicra.v2i1.2071. [Indonesian]
- Orr CH, James A, Leifert C, Cooper JM, Cummings SP. 2011. Diversity and activity of free-living nitrogen-fixing bacteria and total bacteria in organic and conventionally managed soils. *Appl Environ Microbiol* 77: 911-919. DOI: 10.1128/AEM.01250-10.
- Osman NI, Yin S. 2018. Isolation and characterization of pea plant (*Pisum sativum* L.) growth-promoting Rhizobacteria. *Afr J Microbiol Res* 12: 820-828. DOI: 10.5897/AJMR2018.8859.
- Pandey PK, Sreelu NS. 2006. Nitrogen-fixing aerobic bacteria and their nitrogenase activity in north Mumbai coastal waters. *J Indian Fish Assoc* 95-102.
- Prabaningtyas S, Witjoro A, Suarsini E, Aridowi D, Aini AN, Purnomo A, Permana YI, Aribah D. 2018. Vertical distribution of bacteria in various lakes of East Java, Indonesia. *J Phys: Conf Ser* 1093: 012017. DOI: 10.1088/1742-6596/1093/1/012017.
- Roper MM, Gupta VVSR. 2016. Enhancing non-symbiotic N₂ fixation in agriculture. *The Open Agric J* 10: 7-27. DOI: 10.2174/1874331501610010007.
- Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4: 406-425. DOI: 10.1093/oxfordjournals.molbev.a040454.
- Sembiring M, Sabrina T. 2021. Diversity of non-symbiotic nitrogen-fixing bacteria and their potential in andisols affected by the eruption of Mount Sinabung, North Sumatra, Indonesia. *Biodiversitas* 22: 3539-3544. DOI: 10.13057/biodiv/d220854.
- Stal LJ. 2017. The effect of oxygen concentration and temperature on nitrogenase activity in the heterocystous cyanobacterium *Fischerella* sp. *Sci Rep* 7: 5402. DOI: 10.1038/s41598-017-05715-0.
- Sumarno D, Muryanto T. 2015. Penentuan kandungan ammonia (N-NH₃) berdasarkan hasil analisa kandungan ammonium (N-NH₄) di daerah aliran sungai (DAS) Poso Kabupaten Poso Sulawesi Tengah. *Buletin Teknik Litkayasa Sumber Daya dan Penangkapan* 13 (2): 113-118. [Indonesian]
- Susilowati DN, Setyowati M. 2016. Analisis ktivitas nitrogenase dan gen NIFH isolat bakteri rhizosphere tanaman padi dari lahan sawah pesisir

- Jawa Barat. Al-Kaunyah J Biol 9: 125-138. DOI: 10.15408/kaunyah.v9i2.4036. [Indonesian]
- Yao S, Lyu S, An Y, Lu J, Gjermansen C, Schramm A. 2019. Microalgae-bacteria symbiosis in microalgal growth and biofuel production: a review. J Appl Microbiol 126: 359-368. DOI: 10.1111/jam.14095.
- Yusra Y, Azima F, Novelina N, Periadnadi P. 2014. Isolasi dan identifikasi mikroflora indigenous dalam Budu. Agritech 34: 316. DOI: 10.22146/agritech.9460. [Indonesian]
- Zulfarina Z, Rusmana I, Mubarik NR, Santosa DA. 2017. The abundance of nitrogen fixing, nitrifying, denitrifying and ammonifying bacteria in the soil of tropical rainforests and oil Palm plantations in Jambi. Makara J Sci 21: 187-194. DOI: 10.7454/mss.v21i4.8841.