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Submission date: 25-Aug-2019 03:05AM (UTC-0700)
Submission ID: 1163132817
File name: MSF.967.83.pdf (392.48K)
Word count: 4417
Character count: 24034

Materials Science Forum
ISSN: 1662-9752, Vol. 967, pp 83-92
doi:10.4028/www.scientific.net/MSF.967.83

Submitted: 2018-10-18
Revised: 2019-03-31
Accepted: 2019-05-01
Online: 2019-08-16

Molecular Barcoding Based 16S rRNA Gene of Thermophilic Bacteria from Vulcanic Sites, Linow Lake, Tomohon

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Keywords: thermophilic bacteria, 16S rRNA gene, Linow Lake.

Abstract. Thermophilic bacteria live at temperatures above 45^0 C. Many investigations focused on their potential as sources of highly active enzymes 'termostable enzyme' and other products such as antibiotics and compatible solutes. Lake Linow is an active volcanic lake located in Tomohon City, North Sulawesi, Indonesia. Lake Linow becomes the habitat of thermophilic bacteria. A study has been conducted to obtain isolates of thermophilic bacteria and to identifikasi berdasarkan gen 16 s RNA. Bacterial DNA extraction procedure using the Presto TM Mini gDNA Bacteria Kit Geneaid protocol, with modifications. Amplification of 16s RNA gene using PCR method. Visualization of 16 s RNA amplicon genes with automatic electrophoresis capiler Qiaxel, Qiagen. Sequencing was carried out using Singapore's First BASE Sequencing service. The results showed that IL2 isolates and IL3 isolates could live up to 70^{0}C. Alignment analysis results using NCAST BLBI IL2 isolates showed 99% similarity with Bacillus thuringiensis strain H2682 (accession number CP009720.1). While isolate of IL3 thermophilic bacteria showed 94% similarity with Bacillus licheniformis strain 14DA11 (accession number CP023168.1). The results of phylogeny reconstruction with neighbor joining method, gene sequence 16S rRNA isolate IL2 showed the closest relation with Bacillus thuringiensis strain HD1011 (accession number CP009335.1). While IL3 isolate showed the closest relation with Bacillus licheniformis strain 14DA11 (accession number CP023168.1).

Introduction

The largest geothermal resource in the world is in Indonesia, which spreads to 252 locations in 26 provinces. This is because Indonesia is included in one of the ring of fire zones namely areas that often experience earthquakes and volcanic eruptions that surround the Pacific Ocean basin [13]. North Sulawesi Province especially Tomohon City is one of the cities in Indonesia that has geothermal resources because of the geographical location of Tomohon City which is surrounded by Mount Lokon and Mount Mahawu and the mountains of Masarang. Lake Linow is part of Mount Lokon volcanic area with high sulfur activity and hot mud. Lake Linow is located in Lahendong-Tomohon Selatan Subdistrict, Tomohon City and is adjacent to the geothermal power development area. In addition to being developed as a tourist attraction because of the beauty of the lake with the phenomenon of water color change, Lake Linow also lives endemic biota including komo and sayok (local names) which are members of the order odonata, kabos (Channa striata) and other biota [12].

One characteristic of the active volcano area is high temperatures. This condition is a barrier for living beings to be able to adapt and survive. In the western Linow lake area, there is still activity of hot mud with soil surface temperatures of 42^{0}C -> 60^{0}C and at a depth of about one meter the soil temperature varies between 45^{0}C -> 70^{0}C so that the area is not covered with grass and trees [11]. In this condition only microorganisms that can withstand high temperatures can survive and grow

optimally. This heat-resistant microorganism is called thermophilic bacteria. Thermophilic bacteria are bacteria that can live at high temperatures (above 35^0C) [4]. The term thermophilic was first used by Miquel in 1879, which is to describe organisms capable of living at high temperatures [10]. Thermophilic bacteria are known to grow optimally in a temperature range of 55^0C-80^0C [8]. Such high temperatures are generally fatal to organisms, but thermophilic bacteria are still able to live and even grow optimally. This bacterium carries out a thermostability mechanism through the interaction between DNA and protein and the efficiency of repairing damaged DNA by using certain enzymes [7]. Thermophilic bacteria are one of the microorganisms that currently have commercial value because they produce thermostable enzymes that are useful in industry. Its ability to live in a high temperature environment causes these microbes to excel from other microbes. The genus of Cyanobacteria, purple bacteria, green bacteria, Bacillus, Clostridium, Thiobacillus and Spirochaeta [2] are groups of thermophilic microbes.

Thermophilic bacteria in the Lake Linow volcanic pathway is one of the interesting biological studies because of the potential to find new isolates. The identification of thermophilic bacteria can be carried out conventionally through the characterization of biochemical and microscopic properties of bacterial cells, to molecular based. Generally, in identifying bacteria in the laboratory still using conventional identification methods. The problem arises when many conventional bacterial identification errors are found, which is that most bacteria are difficult to cultivate due to inappropriate nutrition and growth conditions, cells that cannot be cultured and intrinsic properties of many organisms [5]. Therefore, molecular methods are needed which are relatively faster and more precise, one of them is by using the 16S rRNA gene. Identification using the molecular method of the 16S rRNA gene is one of the methods used to identify bacteria. The advantages of the molecular method of the 16S rRNA gene because this gene has a unique and different sequence for each bacterial species, are relatively constant, and have a small mutation rate. The use of 16S rRNA gene sequences along with bioinformatics analysis allows the identification of thermophilic bacterial isolates to the species level as well as being a reinforcement (verification) of the results of conventional identification.

Materials and Methods

Research methods

This study uses descriptive research methods. The results of the research data were obtained through laboratory experiments. The research stages were bacterial isolation and thermotolerant test, total DNA extraction and purification, 16S rRNA gene amplification by PCR method, visualization of 16S rRNA gene amplicons by electrophoresis method, sequencing and sequence analysis. The stages of this study can be simply described in the research flow chart (Figure 1).

Figure 1. Location Map of Lake Linow

Sampling of Lake Linow hot Mud

Samples were taken aseptically from Lake Linow hot mud at 3 different points. Each sampling location is measured in situ using a digital thermometer. The sludge sample was taken as much as 100 ml and then put into a sample thermos of 0.7 L to maintain the temperature. The sample was

then taken to the FMIPA-UNIMA Molecular Biology Laboratory and immediately microbial isolation and selection was carried out. The samples were measured again by physical and chemical parameters before being used in the laboratory.

Thermo-tolerance test

Each pure bacterial isolate was tested again to ensure that bacterial isolates obtained were actually included in thermophilic bacteria. The thermo-tolerance test is intended to see the optimum growth temperature of bacteria. The same pure bacterial isolate, first rejuvenated on the new NA growth medium, incubated at 45^{0}C for 24 hours, growing isolates were tested again at 55^{0}C for 24 hours then tested further at higher temperatures of 65^{0}C and 70^{0}C.

Molecular Identification of Bacteria Using the 16 S rRNA gene

Before identification using the 16S rRNA gene, purified bacterial isolates were previously inoculated into general fertilizing media (Nutrient Agar) and incubated for 2 x 24 hours with the best growing temperature of 55^{0}C.

a. DNA extraction and purification

Bacterial suspension from pure culture is prepared aseptically. DNA of bacterial isolates was extracted with Presto TM Mini gDNA Bacteria Kit Geneaid with procedures according to the Kit protocol. The stages of DNA extraction and purification are: Lysis, DNA Binding, Washing and Elution

b. Analysis of DNA concentration and purity

The results of total DNA extraction were then analyzed for concentration and purity by using UV / VIS 35 Spectrometer Lambda Perkin Elmer. DNA purity can be seen with a A260 / A280 ratio between 1.8 - 2.0 nm. If <1.8 means that it is contaminated with proteins and or components of protein derivative contaminants that affect DNA molecules, and if> 2.0 means contaminated with RNA (Kit Protocol), with the following DNA Concentration and Purity formula:

Concentration of DNA= $Absorbansi\ 260\ x\ 50$ [1]

$$Purity\ of\ DNA = \frac{Absorbance\ 260}{bsorbance\ 280}$$

c. Amplification of 16S rRNA gene, by PCR method

The extracted DNA was amplified using the PCR method using Eppendorf's Master cycles pro PCR machine. The PCR process using 2x MyTaq HS Red Mix Bioline and Primer 16S rRNA used in the amplification process by PCR is 16S (App. 425 bp) [3] : 16sA (5′CGC CTG TTT AAC AAA AAC AT 3′) (Foward), 16sB2 (5′TTT AAT CCA ACA TCG AGG 3′) (Reverse). The conditions and components of the PCR are:

Table 1. The Components of PCR

PCR Component	Volume (µL)
2x MyTaq HS Red Mix Bioline	25
Primer Forward	1
Primer Reverse	1
DNA isolat bakteri	4
DdH_2O	21
Total	**50**

Table 2. The Condition of PCR (Modification)

Cyclus	Duration (second)	Temperature (°C)	Fase
	60	94	Denaturasi
	30	50	Annealing
35 x	30	72	Ekstension
	60	72	Final Ekstension

The amplicon visualization has been done using the automated electrophoresis method of Qiaxcel, Qiagen.

e. Bioinformatics Sequencing and Analysis

Sequencing uses ABI PRISM 3730xl Genetic Analyzer Develop by Applied Biosystems, USA, through Singapore FIRST BASE sequencing services. The sequencing output from Singapore's FIRST BASE is in the form of .seq files analyzed using Geneious Bioinformatics software 10.1.3. The reading result with Geneious 10.1.3 is sequence chromatogram, the base sequence of the thermophilic 16S rRNA gene gene or called sequence and sequence characteristics. The bacterial 16S rRNA gene sequence obtained is then used for alignment analysis using the online BLAST (Basics Local Alignment Searching Tools) method on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Selected sequences of BLAST results are then used to reconstruct phylogeny trees. Reconstruction of phylogeny trees using MEGA 7.0 software. The phylogeny tree model used was determined by analysis of suitable models in MEGA 7.0 software. Construction of phylogeny trees using the Neighbor Joining method with 1000x boostrap. Phylogeny tree construction was also carried out online on the NCBI website.

Results and Discussion

Thermo-tolerant test of thermophilic bacteria

Thermotolerant test was carried out on pure culture of bacterial isolates obtained to see the optimum temperature of bacterial growth. The thermotolerant test is carried out according to the method used by Ambrasari et al. 2005, with a scratch technique on Agar Nutrient media. Incubation is carried out for 2 x 24 hours at a constant temperature. Thermotolerant test results at a constant temperature of 45^{0}C obtained six isolates, each isolate St.1.1 and St.1.3 from station I, isolates St.2 and isolates St.2.2 from station II, and isolates St.3.1, isolates 3.2 from station III . Thermo-tolerant test results at 55^{0}C obtained four isolates, each isolate St.1.1 from station I, isolate St.2 from station II, and isolates St.3.1, isolate St.3.2 from station III. Thermo-tolerant test results of 65^{0}C obtained three isolates namely isolate St.1.1 from station I, and isolates St.3.1, isolate St.3.2 from station III. Whereas from the results of the thermo-tolerant test at a temperature of 70^{0}C obtained two isolates, namely isolates St.3.2 and isolates St.3.3, each from station III. From the results of the thermo-tolerant test, it was found that the growth temperature of thermophilic bacteria which was successfully cultivated from Lake Linow volcanic site ranged from 45^{0}C - 70^{0}C, with optimum growth temperature of 55^{0}C - 65^{0}C. The growth of colonies at temperatures of 45^{0}C - 70^{0}C is different, seen from the morphology of the colony which includes the shape of colonies, edges of colonies and elevations (Table 5).

Total DNA extraction and purification.

The isolate samples used in DNA extraction and purification were obtained from the isolation and thermo-tolerance test, IL2 and IL3 isolates (Figure 5). DNA of bacterial isolates was extracted with Presto TM Mini gDNA Bacteria Kit Geneaid with gram positive bacterial research procedure according to the Kit protocol. After getting the total DNA from the extraction results, DNA concentration and purity analysis was carried out with UV / VIS 35 Spectrometer Lambda Perkin Elmer. DNA purity can be seen with a A260 / A280 ratio between 1.8 - 2.0 nm. If <1.8 means

contaminated with proteins and / or components of protein derivative contaminants that affect DNA molecules, and if> 2.0 means contaminated with RNA (Kit Protocol). Concentration and purity test results (A260 / A280) DNA resulting from extraction of IL2 isolates showed a concentration value of 8.25 µg / ml and purity value (A260 / A280) 0.29 while IL3 isolates showed a concentration value of 13.3 µg / ml and purity (A260 / 280) 1.33. The total DNA volume extracted from the two isolates were 100 µl each (Table 7). These results were obtained after several modifications to the Kit protocol at the time of proteinase-K immersion.

Amplification and Visualization of Amplicon 16S rRNA gene.

The process of 16S rRNA gene amplification on chromosomal DNA from bacterial isolates (Figure 6) was performed using 16S rRNA primers (App. 425 bp) :[3] with Foward 16sA (5´CGC CTG TTT AAC AAA AAC AT 3´) and Reverse 16sB2 (5´TTT AAT CCA ACA TCG AGG 3´) via the PCR method with Eppendorf's Master cycles pro PCR machine. Amplification is carried out on PCR conditions according to the protocol. Modification of PCR conditions was carried out after several times no visualization of the results of amplification was obtained through automatic electrophoresis. The next process after PCR is the electrophoresis process, using KIT DNA screening Qiagen (Qiaxel) with work procedures according to the KIT protocol. Visualization of amplicons using automatic electrophoresis obtained different band thickness of IL2 and IL3 isolates. The most amplitude based on band thickness is obtained by IL2 (Figure 7).

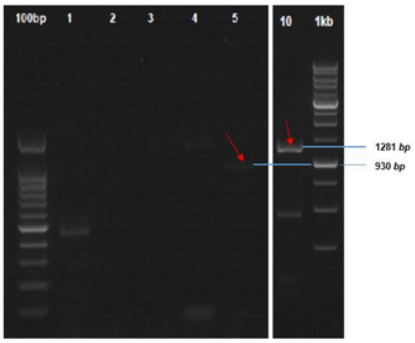
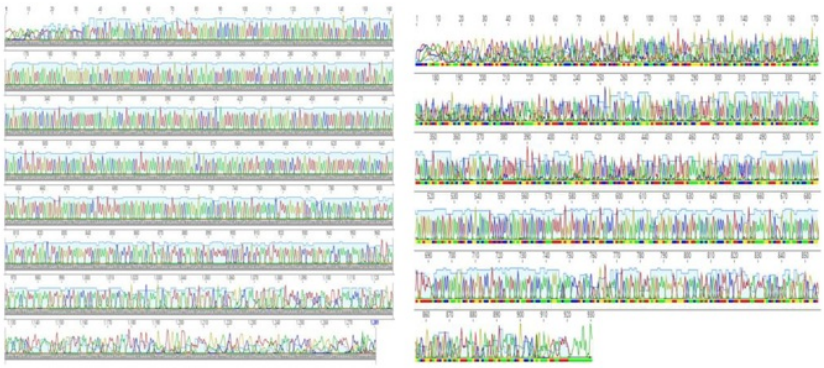


Figure 7. Visualization of amplicons, thermophilic bacterial 16s rRNA gene.

Sequencing

Sequencing results in the form of file seq. from Firt BASE Singapore analyzed using the Geneious 10.1.3 program (Drummond et al. 2012), to obtain the nucleotide sequence of the 16S rRNA gene of thermophi bacteria from the Lake Linow volcanic site in Tomohon. The nucleotide sequencing obtained by the 16S rRNA gene length of IL2 isolate was 1281 bp while IL3 930 bp. Sequencing works well as evidenced by sequencing chromatogram bands which show the basic type separation takes place well (Figure 8).



(a) (b)

Figure 8. Sequence chromatogram of 16S rRNA gene isolate IL2 (a) isolate IL3 (b)

The following is a sequence of nucleotide bases of 16S rRNA gene of Lake thermophilic bacteria Linow isolates IL2 and IL3.

Characteristics of 16S rRNA Gene, Thermophilic Bacteria.

The characteristics of thermophilic bacterial sequences of IL2 and IL3 isolates have different base and frequency compositions (Table 5). Comparison of Guanine and Cytosine IL2 isolates was 37.9% while IL3 isolates were 44.0%. IL2 and IL3 sequences after being analyzed in contig to get consensus sequences, analyzed the alignment on the NCBI site. The Basic Local Aligment Searching Test (BLAST) method was used to obtain the similarity of 16S rRNA sequences of thermophilic Bacteria from Lake Linow volcanic site from those reported by other researchers from various parts of the world and recorded in the NCBI bank gene. BLAST results of IL2 isolates showed the highest similarity sequence (99%) with Bacillus thuringiensis strain H2682 (accession number CP009720.1), while IL3 showed the highest similarity sequence (94%) with Bacillus licheniformis strain 14DA11 (accession number CP023168.1) (Table 6).

Table 5. Characteristics of two 16S rRNA gene sequences, thermophilic bacteria isolates IL2 and IL3.

Characteristics	IL2		IL3	
	Freq	%	Freq	%
A	435	34,0	261	28,1
C	201	15,7	196	21,1
G	285	22,2	213	22,9
T	360	28,1	260	28,0
GC	486	44,2	409	44,0
Leght of sekuens	1281	100,0 %	930	100,0 %

Table 6. Results of BLAST.

	IL2					
Description	*Max score*	*Total score*	*Query cover*	*E value*	*Ident*	*Accession*
Bacillus thuringiensis strain H2682, *complete genome*	2213	2213	96%	0.0	99%	CP009720.1
Bacillus cereus strain S2-8, *complete genom*	2207	2207	96%	0.0	99%	CP009605.1
Bacillus cereus strain 3a, *complete genom*	2207	2207	96%	0.0	99%	CP009596.1
Bacillus thuringiensis strain HD1011, *complete genome*	2207	2207	96%	0.0	99%	CP009335.1
Bacillus cereus strain AH820, *complete genom*	2207	2207	96%	0.0	99%	CP001283.1

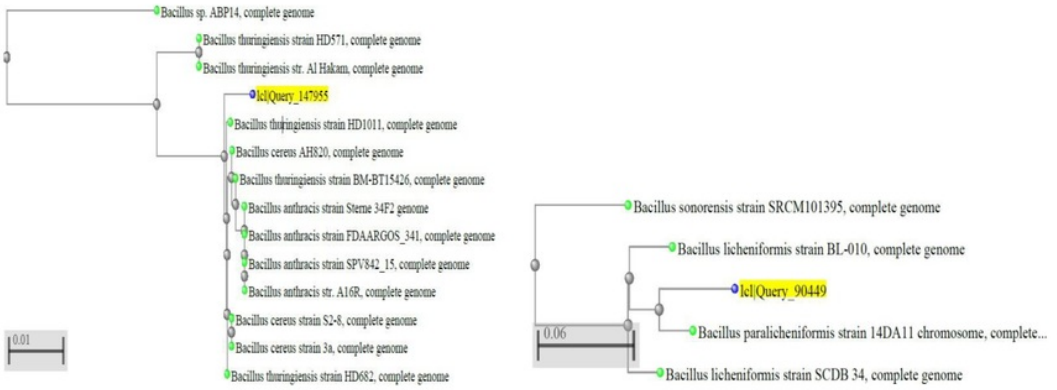
Bacillus thuringiensis strain BM-BT15426, *complete genome*	2202	2202	96%	0.0	99%	CP020723.1
Bacillus anthracis strain 14RA5914, *complete genom*	2191	2191	96%	0.0	99%	CP02300 1.1
Bacillus anthracis strain FDAARGOS 341, *complete genom*	2191	2191	96%	0.0	99%	CP022044.1
Bacillus anthracis strain Sterne 34F2 *genom*	2191	2191	96%	0.0	99%	CP019726.1
Bacillus anthracis strain SPV842, *complete genom*	2191	2191	96%	0.0	99%	CP019588.1

IL3

Description	*Max score*	*Total score*	*Query cover*	*E value*	*Ident*	*Accession*
Bacillus paralicheniformis strain 14DA11 *choromosome complete genome*	1376	1376	95%	0.0	94%	CP023168.1
Bacillus licheniformis strain SCDB 34, *complete genome*	1301	1301	95%	0.0	93%	CP014793.1
Bacillus licheniformis strain BL-010, *complete genome*	1266	1266	95%	0.0	92%	CP022477.1
Bacillus sonorensis strain SRCM101395, *complete genome*	878	878	95%	0.0	84%	CP021920.1

Reconstruction of phylogen based on 16s rRNA gene.

Phylogeny reconstruction based on 16S rRNA IL2 sequences and 16S rRNA IL3 sequences was carried out in two approaches. Online reconstruction was carried out on the NCBI website (www.blast.ncbi.nllm.niv.gov/Blast.cgi) and reconstruction using the MEGA Program 7.0. Two approaches were used to obtain the comparison of phylogeny trees formed and comparison of the position of IL2 isolates and IL3 isolates. Reconstruction of phylogeny on the NCBI site was carried out with the Neighbor Joining method using ten of the highest sequences of BLAST results for IL2 Isolates and four of the highest sequences of BLAST results for IL3 (Figure 14). The results of the reconstruction of phylogeny on the NCBI site placed IL2 isolates on the closest evolutionist position or relationship with Bacillus thuringiensis strain HD1011 (accession number CP009335.1), whereas IL3 isolates had the closest evolutionary relationship with Bacilus licheniformis strain 14DA11 (accession number CP023168.1).



a. IL2 b. IL3

Figure 13. Phylogeny tree of thermophilic bacteria isolates IL2 and IL3, compared with 16s rRNA gene sequences similar to the NCBI gene bank.

Discussion

The isolates obtained from the Linow Lake volcanic site were identified and characterization based on morphology, microscopic and molecular methods using the molecular method of the 16S rRNA gene. 16S rRNA gene has been widely used as a molecular code for bacterial identification [9]. 16S rRNA encoding gene can be used as a molecular marker because this gene has a unique and different sequence for each bacterial species, is relatively constant, and has a small mutation rate. DNA isolation is the initial stage of DNA-based molecular analysis. Samples of thermophilic bacterial DNA were obtained from extraction and purification using Preto TM Mini gDNA Barteria Kit Geneaid with Gram positive bacterial research procedures according to the Kit protocol. The results of the analysis of DNA concentration and purity using Perkin Elmer's UV / VIS 35 Lambda Spectrophotometer obtained DNA isolates concentration of IL2 bacteria which was 8.25 with a purity of 0.29 and the results of DNA isolation analysis of IL3 bacterial isolates were 13.3 with a purity of 1.33 (table 4.4) Samples of IL3 bacterial isolates had higher DNA concentrations compared to DNA samples of IL2 isolates, and also the purity value was 1.33. These results were obtained after modification of the proteinase-K immersion time according to the Kit protocol for 10 minutes at 600C to 12 minutes of immersion time.

DNA isolation results are said to be pure when the 260/280 absorbance ratio ranges between 1.8 - 2.0 nm. If the absorbance ratio is 260/280 <1.8, this indicates that the DNA isolate produced is contaminated with protein and / or protein derivative contaminants that affect DNA molecules, whereas if the absorbance ratio is 260/280> 2.0 then the DNA isolate is produced contaminated with RNA (Kit Protocol) (Barbosa et al. 2007). The total DNA extraction results were amplified by PCR machine using 2x MyTaq HS Red Mix Bioline and 16S rRNA primers (App. 425 bp) :[3] with Foward 16sA (5′CGC CTG TTT AAC AAA AAC AT 3′) and Reverse 16sB2 (5′TTT AAT CCA ACA TCG AGG 3′). Amplification was carried out with modified PCR conditions, namely denaturation phase 94^0C for 60 seconds, an annealing phase of 50^0C for 30 seconds, a phase of 72^0C extension for 30 seconds and the final extension of 72^0C for 60 seconds. The amplicon produced on the PC2 isolate of IL2 looks thicker, while the IL3 amplicon looks thinner (figure 4.5). Thin tape means that the PCR process runs but is not optimal so that the process of multiplying DNA molecules does not work perfectly. The less optimistic PCR process can occur due to a lack of annealing temperature, the DNA concentration resulting from extraction is too little.

Sequence analysis results through the BLAST program for sequences of IL2 bacterial isolates showed similarity (99%) with Bacillus thuringiensis H2682 strain of the 10 highest sequences in NCBI with a sequence length of 1281 bp. Sequences of IL3 bacterial isolates showed genetic similarity (94%) with Bacillus licheniformis strain 14DA11 from the 4 highest sequences in NCBI with a length of 930 bp sequences. Although the value of the similarity level of the two isolates is high, 99% and 94% of the same sequences at NCBI Gene Bank, but this cannot be used as a reference that IL2 isolates are Bacillus licheniformis H2682 strain and IL3 isolate is Bacillus licheniformis strain 14DA11 because none BLAST results showed 100% similarity index with the second sequence of isolates obtained.

Phylogeny analysis indicated that IL2 isolates had a kinship relationship with Bacillus thuringiensis strain HD1011 (accession number CP009335.1), while IL3 isolates showed a kinship with Bacillus licheniformis strain 14DA11 (accession number CP023168.1). Data of the base sequence of the encoding gene 16S rRNA allows it to be used to construct phylogenetic trees that can show ancestors and kinship relationships of organisms, but organisms that are closely related or identical based on these parameters do not necessarily have physiological similarities. This is because the gene encoding 16S rRNA is not a functional gene for survival and prokaryota adaptation in certain environments. Research on thermophilic bacteria, especially the Bacillus group through 16S rRNA gene amplification has also been carried out. Habbie, et al (2014) who successfully identified thermophilic bacteria Bacillus licheniformis strain DSM13 ATCC 14580 and Bacillus aerus 24K 16S strain from Lapindo hot mud through 16S rRNA gene amplification. Helin et al [6] succeeded in isolating and identifying thermophilic bacteria from the Gedong Songo water

source with 16S rRNA gene analysis method. The results showed that there were similarities shown by the Geobacillus thermoleovourans bacteria which could grow in the temperature range between 65^0C to 75^0C.

Conclusion

Six isolates of thermophilic bacteria that were successfully isolated from 3 sampling points on the volcanic site of Lake Linow in Tomohon City. Characteristics of thermophilic bacterial isolates of IL2 and IL3 isolates have different base and frequency compositions. Comparison of Guanine and Cytosine IL2 isolates was 44.2% while IL3 isolates were 44.0%. Sequences of IL2 bacterial isolates showed similarities (99%) with Bacillus thuringiensis H2682 strains of the 10 highest sequences in NCBI with a length of 1281 bp sequences and sequences of IL3 bacterial isolates showing similarity (94%) with Bacillus licheniformis strain 14DA11 of the 4 highest sequences at NCBI with a length of 930 bp.

Acknowledgment

Thanks to the Directorate of Research and Community Service, Ministry of Research, Technology and Higher Education, the Republic of Indonesia, which has funded this research through the Penelitian Unggulan Perguruan Tinggi Scheme, 2017-2018 year.

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