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ISOLATION, MOLECULAR IDENTIFICATION AND ENZYME ACTIVITY OF AMYLASE PRODUCING THERMOPHILIC BACTERIA FROM HOT SPRINGS

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ABSTRACT

In this era, enzyme exploration in Indonesia continues to increase, and the amylase enzyme of about 30% of the total production is used worldwide. The great needs for amylase enzyme and its uses in several industrial and health fields lead to the search for potential natural resources in its production, including hot springs, which are very promising because of their thermophilic and thermostable properties. The aim of this study was to isolate, determine the activity and molecular identification of amylase-producing thermophilic bacteria from natural hot springs Dolok Tinggi Raja, Simalungun, North Sumatra. This study includes measurement of enzyme activity using the DNS method and molecular identification. The results were obtained from 20 isolated isolates, 6 isolates had amylase activity with the highest activity, namely the UTMTR VAR A10 isolate with an amylase enzyme activity of 0.2733 Unit/mL. Based on the molecular identification using 16SrRNA gene amplification based on the BLAST program and phylogenetic analysis with the MEGA X program, it was shown that the UTMTR VAR A10 isolate is a bacterium *Bacillus licheniformis* strain UTMTR VAR A10. Further research is needed to purify the enzyme in such a way that it can be applied to the industrial world.

I. INTRODUCTION

In this era, research on enzyme producing microbes in Indonesia continues to increase. Enzyme are widely used in various fields, such as diagnostic techniques, biotechnology industry, textile and paper industry, sewage and waste treatment, as well as food industry [1]. The application of enzymes in the field of biotechnology places increasing demand for thermophilic (active at high temperatures) and thermostable (stable at high temperatures) enzymes. Furthermore, this is due to high-temperature reactions, which can minimize the risk of contamination, increase mass transfer rates, and can shift the equilibrium towards product formation [2]. In addition, enzymes act as biocatalysts, namely accelerating the rate of chemical reactions without being involved in the reaction. Also, it convert substrate molecules into reaction (products), and its uses as

catalysts can reduce environmental pollution, which are non-toxic. The application of enzymes as catalysts is widely used in industry and health, one of which is the amylase enzyme [3].

The amylase enzyme is a type that can degrade starch, and its uses in the industrial sector is quite high, that is in the food, textile, paper, and detergent industries. Also, the use of amylase enzymes in the health sector such as pharmacy, medicine, and the environment. The amylase requirements used in the food and beverage industry are of great value, and it is one of the most important and widely used enzymes. Furthermore, this enzyme represents the second largest market group after proteases, and the amylase enzyme of about 30% of the total enzyme production is used in the world. The high need for amylase enzyme and its uses in several industrial and health fields lead to the search for potential natural resources in its production [4].

Enzymes derived from microorganisms, especially thermophilic microorganisms such as bacteria, are more demanding in the industry because they are stable at high temperatures, easy to produce in large quantities, can work on several substrates, have high reaction rates, do not require a bioreactor cooling system and the possibility of low contamination. Thermophilic bacteria can survive and thrive in high-temperature conditions because the content of enzymes, ribosomes, and proteins is more stable. The lipid membrane of thermophilic bacteria is also rich in saturated fatty acids and therefore forms a much stronger hydrophobic bond than mesophilic bacteria [5].

The natural habitat of thermophilic bacteria is widespread throughout the earth's surface, especially in geothermal areas such as hot springs, volcanic craters, and volcanic areas. Indonesia is one of the most active tectonic areas in the world with more than 70 active volcanoes and numerous geothermal areas. However, hot springs are the easiest places to sample bacterial isolates. This study aims to explore microorganisms from geothermal habitats, namely hot springs that have the potential to produce thermostable amylase enzymes to be developed as thermophilic microorganisms for industrial and health purposes. This exploration is carried out at the Dolok Tinggi Raja Simalungun geothermal hot spring, North Sumatra, which is a hot spring that has not been widely used for this purpose [6].

II. MATERIALS AND METHODS

II.1 STUDY SITE

This research was conducted in the Molecular Biology Laboratory of the Faculty of Medicine, Universitas Prima Indonesia, Medan, Indonesia. The samples were obtained from Dolok Tinggi Raja hot spring, Silau Kahean district, Simalungun Regency. Furthermore, geographically, Dolok Tinggi Raja is located between 3º 13' 30" North latitude and 98º 30' 49" East Longitude (Figure 1).

Figure 1: Sampling Location of Dolok Tinggi Raja, Simalungun, North Sumatera, Indonesia.
Source: Authors, (2020).

II.2 SAMPLING PROCEDURE

Hot water sampling at the Dolok Tinggi Raja Natural Hot Springs was performed at a depth of approximately 1-2 meters. Water samples of about ± 100 mL was taken and the sediment of about ± 10 grams was obtained from several different points in which the temperature and pH of the sampling point were previously measured. Then a water sample is placed in a sterile Winkler bottle and the sediment is placed in a sterile bottle that has been prepared and labeled. Furthermore, the samples were stored in cold boxes to be taken to the laboratory for further analysis [7].

II.3 MICROBIAL ISOLATION

Water samples of about 4 mL was taken from 3 different location and placed in a centrifuge tube then mixed until homogeneous. Furthermore, 2 grams of sediment samples were taken from 4 locations and place in a petridish then mixed until homogeneous. The two samples prepared were then isolated on the enrichment media by taking 1 mL or 1 gram of the sample, and then poured into a test tube containing 9 mL of median nutrient broth and homogenized, incubate at a temperature of ± 50ºC for 2x24 hours. The microbes isolated on the enrichment media are then inoculated on the nutrient agar media. The sample was diluted at a concentration of 10^4, and inoculated with the spread plate method. A sample of 0.1 mL (100 µL) was taken using micropipettes, and placed on the surface of the Nutrient Agar medium, and was spread using a spreader. The procedure was performed aseptically, and incubate at a temperature of ± 50ºC for 2x24 hours, and the isolates were ready for further testing [8], [9].

II.4 AMYLASE ENZYME SCREENING

The microbial isolates were inoculated in Nutrient Agar media containing 1% starch, and incubated for 24-48 hours at a temperature of ± 50ºC. Then, a drip of Lugol reagents [(w/ v) 1% iodine in 2% potassium iodide] was added on the surface of the media until it was distributed, and let it stand for ± 1 minute, then the remaining liquid on the surface of the media was discarded. The clear zones on the surface of the media was observed, and the presence of clear zones showed that microbes produced amylase [10].

II.5 PREPARATION OF GLUCOSE STANDARD CALIBRATION CURVES

A standard glucose solutions was prepared using glucose solutions at various concentrations ranging from 50-300 µg/mL. Furthermore, 1 mL of glucose solution was taken at each concentration and 1.5 mL of DNS solution was added, vortexed, then boiled for 5 minutes. The mixture was cooled under running water for 15 minutes and 20 mL of aquabidest was added, and vortexed. The mixture was then measured for its absorbance at a wavelength of 540 nm. Each absorbance result of each glucose solution with different concentrations was plotted on a regression line showing a linear relationship between absorbance and glucose level [11].

II.6 MEASUREMENT OF ENZYME ACTIVITY

The amylase enzyme activity was tested using the DNS method, and the test was carried out using 1 mL of bacterial sample inoculated on 25 mL of production media (2.55 g/L rice flour; 8.4 g/L yeast extract; 8.1 g/L NaCl) at a temperature of ± 50ºC for 48 hours. After incubation, the media is centrifuge at a speed of 10,000 rpm for 15 minutes. The results of the supernatant (crude enzymes) are used to determine the activity of the enzymes. Then a mixture of reactions containing 1 mL substrate (1% starch in 0.1 M buffer phosphate pH 7) and 1 mL crude enzyme (supernatant) was incubated at a temperature of 50ºC for 10 minutes. Furthermore, 1 mL substrate was added 1% starch in a phosphate buffer of 0.1 M pH 7) in an empty tube and then incubated at a temperature of 50ºC for 10 minutes. Each tube is filled by adding 2 mL of 3.5-dinitrosalicylic (DNS) acid reagents, then heated for 5 minutes, and cool under running water for 15 minutes, then 20 mL aquabidest was added. Each solution in the tube was tested for color intensity by absorbent at a wavelength of 540 nm using a Spectrophotometer UV-Vis. One unit of an enzyme (Unit/mL) is equivalent to the amount of enzyme required to release 1 µmol reduced sugar per minute in test conditions [10], [11], [12]. To see the size of one unit of enzyme activity, the following formula was used: Formula Activity Enzyme.

$$AE = \frac{Mg\ x\ 100}{BMg\ x\ MI} \quad (1)$$

Information:
AE: Enzyme Activity (Unit/mL Enzyme Filtrate)
Mg: Miligrams of Glucose resulting from the Hydrolysis of starch (mg)
BMg: Mr Glucose = 180
MI: Incubation period = 10 minutes

II.7 MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION

The components of the isolates were assigned to the macroscopic and microscopic morphological characteristics of the isolates and the biochemical components. For the macroscopic morphological characteristics, the colony shape, surface, color, margins, and edges were observed, while microscopic morphological characteristics were carried out with gram staining. The characteristics of the isolates were also determined biochemically by testing catalase, motility, gelatinase, TSIA, and Simon's citrate [13].

II.8 MOLECULAR IDENTIFICATION

Bacterial isolates planted or isolated overnight are transferred to a 1.5 ml microtube, and a 1 mL TE buffer was introduced and become homogeneous, and centrifuges at a speed of 11,000 rcf for 15 minutes. Furthermore, lysozyme was poured into the tube and incubated for 30 minutes at a temperature of 37ºC. After that, 125 µL ammonium acetate 7.5 M was added in the tube and then homogenized and placed on ice for 10 minutes. Then 500 µL chloroform was added to the tube and shaken firmly. The sample was crossed at 11,000 rcf for 10 minutes. The supernatant is taken and placed in a new tube, then isopropanol was added to the supernatant at ratio = 1/2:1. Also, the tubes were homogeneously reversed up and down several times, and centrifuge back at 18,000 crf for 10 minutes. The supernatant was removed and 500 µL ethanol 70% was added, and then centrifuged again at 18,000 crf for 10 minutes. The supernatant was discarded and the pellets was dried. Furthermore, 50 µL free water nuclease was added to the tube, and the solution was used as a DNA template for PCR. The PCR program used includes; pre-denaturation at 95ºC for 90 seconds, followed by 30 cycles consisting of denaturation at 95ºC for 30 seconds, annealing at 55ºC for 30 seconds, extension at 72ºC for 90 seconds, and a final extension at 72ºC for 3 minutes [14]. The primers used were primer 27 F: 5'AGA GTT TGA TCC TGG CTC AG-'3 and Primer 1492 R: 5' GGT TAC CTT GTT ACG ACT T-'3.

II.9 PHYLOGENETIC ANALYSIS

The sequencing results were trimmed and compiled using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/-bioedit.html). The sequential data assembled is then registered in BLAST along with the genomic data registered at the NCBI/National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST) to determine taxon or species with homology or a largest and closest molecular similarity. Then an alignment was carried out using the CLUSTAL W program and a phylogenetic tree was created using the MEGA X program [15].

III. RESULTS AND DISCUSSIONS

III.I AMYLASE ENZYME SCREENING

Based on the results of the research that has been carried out, from 20 isolates taken from hot springs Dolok Tinggi Raja, 6 isolates were able to produce amylase enzymes (Table 1). This is evidenced by the formation of a clear zone around the colony on nutrient agar containing 1% starch (Figure 2).

Table 1: Amylase Enzyme Screening Results.

No	Isolates	Amylase Enzyme
1	UTMTR VAR A1	+
2	UTMTR VAR A2	-
3	UTMTR VAR A3	-
4	UTMTR VAR A4	+
5	UTMTR VAR A5	+
6	UTMTR VAR A6	-
7	UTMTR VAR A7	-
8	UTMTR VAR A8	-
9	UTMTR VAR A9	-
10	UTMTR VAR A10	+
11	UTMTR VAR S1	-
12	UTMTR VAR S2	-
13	UTMTR VAR S3	-
14	UTMTR VAR S4	-
15	UTMTR VAR S5	+
16	UTMTR VAR S6	-
17	UTMTR VAR S7	-
18	UTMTR VAR S8	-
19	UTMTR VAR S9	+
20	UTMTR VAR S10	-

Source: Authors, (2020).

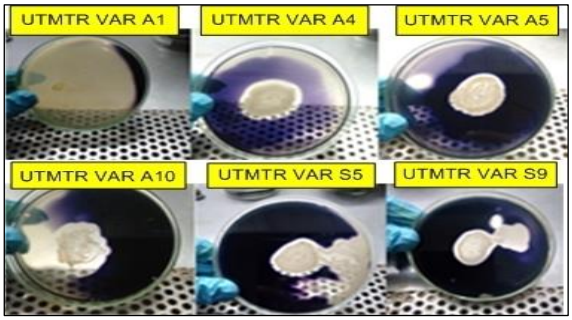

Figure 2: Isolates of UTMTR VAR A1, A4, A5, A10, S5 and S9 which can produce amylase enzymes are characterized by the formation of a clear zone around the colony.
Source: Authors, (2020).

In the amylase enzyme screening results, 6 isolates showed positive results with the formation of a clear zone on the addition of a lugol/iodine solution to starch [16].

III.2 ENZYME ACTIVITY

The results of the isolation of thermophilic bacteria from hot springs Dolok Tinggi Raja were discovered to be 6 isolates that produced amylase enzyme. Furthermore, the 6 isolates were subjected to further test in order to determine the enzyme activity. The results of enzyme activity can be seen in Table 2.

Table 2: Enzyme Activity Results based on Growth Time (Incubation Time).

Isolate	Incubation Time (Hours)	Glucose Concentration (mg/mL)	Enzyme Activity Unit (Unit/mL)
UTMTR VAR A1	0 Hours	1.397	0.0776
	4 Hours	2.8667	0.1593
	8 Hours	4.2347	0.2353
UTMTR VAR A4	0 Hours	1.22055	0.0678
	4 Hours	2.150	0.1194
	8 Hours	2.7528	0.1529
UTMTR VAR A5	0 Hours	1.4706	0.0817
	4 Hours	2.7478	0.1527
	8 Hours	4.6782	0.2599
UTMTR VAR A10	0 Hours	1.2911	0.0717
	4 Hours	3.1359	0.1742
	8 Hours	4.9202	0.2733
UTMTR VAR S5	0 Hours	1.53105	0.0851
	4 Hours	1.8012	0.1001
	8 Hours	3.81835	0.2121
UTMTR VAR S9	0 Hours	1.71655	0.0954
	4 Hours	2.4988	0.1388
	8 Hours	3.67725	0.2043

Source: Authors, (2020).

In Table 2, it can be seen that these 6 bacterial isolates have different enzymatic activities. The activity of the amylase enzyme is determined based on the concentration of reducing sugars produced by hydrolysis of starch by the amylase enzyme in bacterial isolates. Based on the research on measuring enzyme activity as a function of growth time, it can be seen that the time for amylase enzyme production is increasing. This is in line with previous studies showing that the production time increased from 0 hours until the maximum production time was 36 hours [17]. Also, other studies stated that the incubation time for enzyme production in *Bacillus licheniformis* bacteria at 0, 4, and 8 hours also increased [18].

Based on the results of the amylase enzyme activity test, the graph shows that the highest amylase enzyme activity was shown by the UTMTR VAR A10 isolate (Figure 3). The activity of the UTMTR VAR A10 isolate at the 0 hour was 0.0717 Unit/mL, the 4 hour was 0.1742 Unit/mL and the 8 hour was 0.2733 Unit/mL. The influence of incubation time on production of enzymes is very important. This is because in the field of biotechnology time-saving is needed [19]. This result is in line with the results of previous studies, which state that *Bacillus sp*. RSAII-1b begins to have an adaptation phase after 0 to 12 hours and the growth increases with increasing fermentation time. The amylase enzyme was secreted during the 15 hours resulting in an

enzyme activity of 0.0319 Unit/mL and increased at the 24 hours with the enzyme activity of 0.1323 Unit/mL. And the increase again resulted in the highest amylase enzyme activity of 0.0711 Units/mL at 33 hours [20]. Another study reported that the highest α-amylase enzyme activity was at the 36 hours of 3832.08 U/mg. When the incubation time changed from 36 to 72 hours, the enzyme production activity decreased by 49.17%. The activity of this enzyme decreases as the growth of microbes in the medium increases, but the nutrients become less numerous, which in addition to the secondary metabolites leads to a nutrient deficiency of the bacteria [21].

The growth of cells and amylase products *Bacillus sp*. reported depends on the strain, media composition and concentration, cultivation method, cell growth, nutrient requirements, pH temperature, incubation time, and thermostability. The production of amylase is influenced by the composition and concentration of the media, because the media a source of carbon. Furthermore, carbon is needed during fermentation because it increases the carbon used in the flour of rice and starch as a stimulator for the production of the enzyme. Starch and rice flour are slowly metabolized by bacteria as a source of complex carbohydrates and as an inductor for amylase secretion [22]. The enzyme activity decreases with increasing incubation time and after the maximum production time. This is because bacteria are no longer a source of carbon and therefore nutrients are reduced. Also, it is due to enzyme denaturation due to interactions with other compounds in the fermentation media [23].

According to Elmansy [22], the 72 hours observation was reported the optimal growth period for *Bacillus* strains and as the incubation time, which would result in more time for the number of colonies that cause the activity of the much weaker enzyme. The incubation period or the long growth period greatly reduced the α-amylase due to exhaustion of nutrients that causes the death of the microorganism, and the collection of the product side contained in the media products such as toxins, inhibitors, and proteolytic α-amylase by the enzyme protease, as well as the cells can reach the phase of decline and show the synthesis of amylase were reduced.

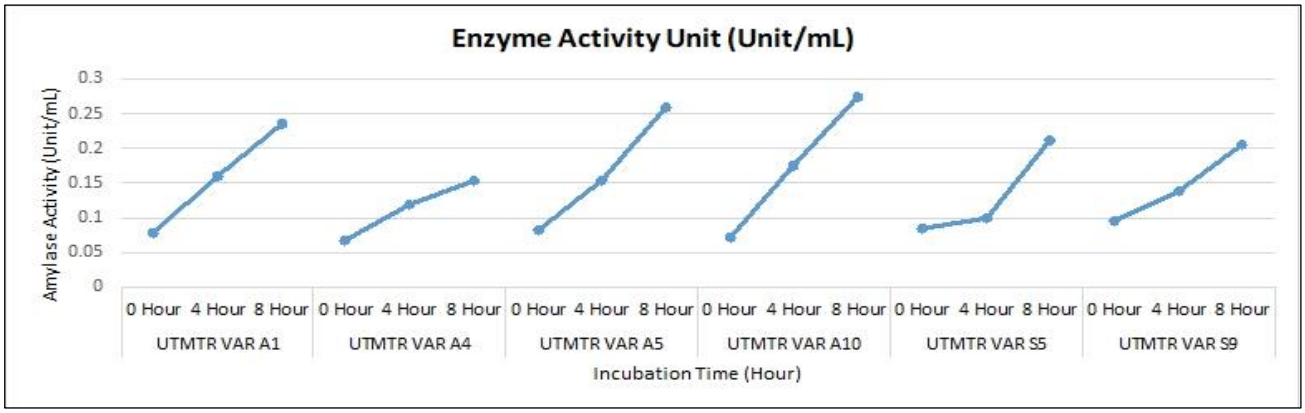

Figure 3: Amylase Enzyme Activity Test Results.
Source: Authors, (2020).

III.3 MORPHOLOGICAL AND BIOCHEMICAL CHARACTERERIZATION

The selected isolates with the highest amylase enzyme activity were subjected to macroscopic, microscopic, and biochemical characterization tests. The results are shown in Table 3.

Table 3: Enzyme Activity Results based on Growth Time (Incubation Time).

Characteristics	Result
Macroscopic	
• Shape	Circular
• Edge	Undulate
• Elevation	Flat
• Color	Cream
Microscopic	
• Gram stain	Gram (-) Bacil (+)
Biochemistry	
• Catalase	+
• Motility	-
• Gelatin	-
• TSIA	K/A, H2S:-, Gas:-
• Simon's citrate	-

Source: Authors, (2020).

The morphological characteristics of the UTMTR VAR A10 isolate were carried out macroscopically and microscopically. The colonies were reported to be cream colored and circular by macroscopic observation. Meanwhile, microscopically, it was observed that the colony of UTMTR VAR A10 isolates were gram-negative rod-shaped bacteria (Figure 4.A). Also, identification was carried out by biochemical characteristics as shown in Table 3. The results of the catalase test on this isolate were positive (Figure 4.B), as indicated by the presence of gas bubbles in the bacterial colony reaction to which 3% H2O2 reagent was added, the ability to convert H_2O_2 which was to oxygen and water decomposes [17], with the exception of the results of negative mobility tests, gelatin and citrate (Figure 4.C). The TSIA test shows that the Slant is red because the formation of the basic compound shows that *Bacillus* does not ferment lactose, maltose, and sucrose. Meanwhile, the color of the TSIA media on the butt is yellow, which means that *Bacillus* can ferment glucose. According to Cappucino [24] *Bacillus* can ferment glucose as an energy source, besides that TSIA shows that these bacteria are not able to produce sulfur and gas. Based on the results of the macroscopic, microscopic, and biochemical characteristic tests obtained by comparing the results in the Bergey's Manual of Systematic Bacteriology, it can be concluded that the UTMTR VAR A10 isolate was identified as *Bacillus sp*. Bacteria of the genus *Bacillus sp*. are the most widely reported as thermostable enzyme-producing bacteria [25]. Furthermore,

molecular identification was carried out to determine the species of these bacterial isolates.

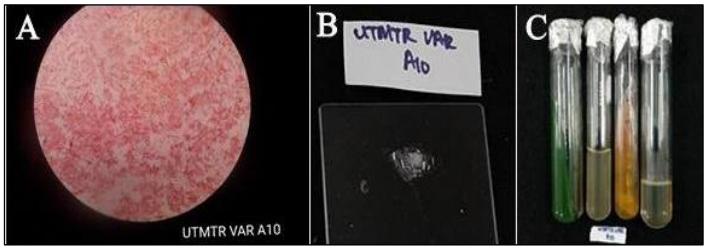

Figure 4: A. Microscopic Isolate UTMTR VAR A10; B. Catalase Test Results; C. Biochemical Characteristics Test Results: 1) Simon's Citrate; 2) Gelatin; 3) Triple Sugar Iron Agar (TSIA); 4) Motility
Source: Authors, (2020).

III.4 MOLECULAR IDENTIFICATION

Isolates UTMTR VAR A10 which has the highest amylase enzyme activity in further identification using 16SrRNA gene sequence analysis with primer 27 F: 5' --AGA GTT TGA TCC TGG CTC AG – 3' and Primer 1492 R: 5' – GGT TAC CTT GTT ACG ACT T – 3'. The results of the 16SrRNA sequencing analysis obtained were compared with the gene sequences in GenBank using BLAST search (Basic Local Alignment Search Tool) on NCBI. The 16SrRNA gene sequence alignment database showed that the UTMTR VAR A10 isolate had the highest 99.78% similarity to *Bacillus licheniformis* strain MPF 22 [26].

III.5 PHYLOGENETIC ANALYSIS

Based on the phylogenetic tree, the bacteria *Bacillus sp* isolates UTMTR VAR A10 has the closest homology/similarity to the *Bacillus licheniformis* strain MPF22 (Figure 5). These results are based on the alignment of the base pairs to determine the phylogenetic relationships between the sequences. The phylogenetic of this bacterial isolate used the neighbor-joining tree method with boostrap analysis of 1000 replications to test the phylogenetic tree branches. The higher the bootstrap repetition used, the better and more accurate the phylogenetic tree [17]. Therefore, based on the results obtained, it can be determined that the UTMTR VAR A10 isolate is a *Bacillus licheniformis* bacterium designated as strain UTMTR VAR A10.

The bacterium *Bacillus licheniformis* produces an amylase enzyme, a thermostable enzyme that is widely used in biotechnology. Amylase is an enzyme that can hydrolyze the statch. The enzyme can hydrolyze starch molecules to produce products that varies such as maltose, dextrin, and especially the molecules of glucose as the smallest unit. Furthermore, amylase is one of the enzymes widely used in industry, biotechnology, and health. The use of the amylase enzyme in the industrial sector is quite high, in the food industry (Food and Beverage), textiles. paper and detergents. Also, the use of amylase enzyme in the health sector such as pharmacy, medicine, and the environment is high [4].

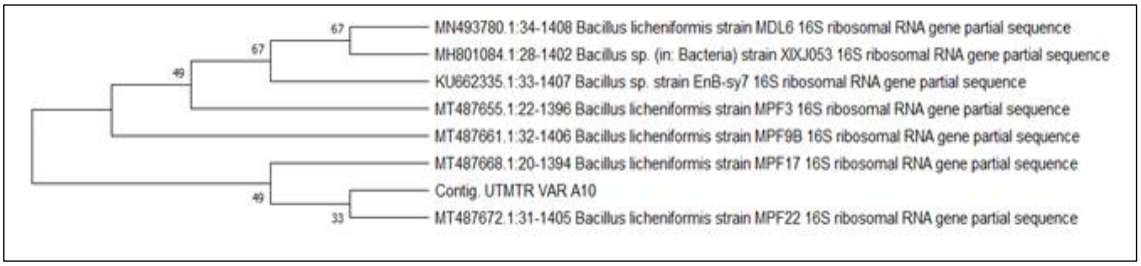

Figure 5: UTMTR VAR A10 Phylogenetic Chart / Tree with 7 related *Bacillus* species based on the 16SrRNA gene
Source: Authors, (2020).

IV. CONCLUSIONS

Based on the research results, it can be concluded this is the first report on the isolation of amylase-producing thermophilic bacteria from Hot Springs Dolok Tinggi Raja, Simalungun, North Sumatra. Molecular identification based on 16SrRNA gene amplification and sequencing stated that the UTMTR VAR A10 isolate was a bacterium *Bacillus licheniformis* strain UTMTR VAR A10. This thermophilic bacterium showed the amylase enzyme activity at the 8 hours of 0.2733 Unit/mL. Further research is needed to purify the enzyme so that it can be applied in the industrial world.

V. AUTHOR'S CONTRIBUTION

Conceptualization: Saryono, Titania T Nugroho and Edy Fachrial.
Methodology: Edy Fahrial, Saryono, and Harmileni.
Investigation: I Nyoman Ehrich Lister and Chrismis Novalinda Ginting.
Discussion of results: Edy Fachrial, Harmileni, and Vincentia Ade Rizky.
Writing – Original Draft: Edy Fachrial and Vincentia Ade Rizky.
Writing – Review and Editing: Harmileni and Vincentia Ade Rizky.
Resources: Chrismis Novalinda Ginting and Edy Fachrial.
Supervision: Saryono and I Nyoman Ehrich Lister.
Approval of the final text: Saryono and Titania T Nugroho.

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