

# Molecular identification of amylase-producing thermophilic bacteria isolated from Bukit Gadang Hot Spring, West Sumatra, Indonesia

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**Abstract.** Ardhi A, Sidauruk AN, Suraya N, Pratiwi NW, Pato U, Saryono. 2020. Molecular identification of amylase-producing thermophilic bacteria isolated from Bukit Gadang Hot Spring, West Sumatra, Indonesia. *Biodiversitas* 21: 994-1000. Amylase is one of the hydrolytic enzymes that is widely used in a wide number of industrial processes such as food, fermentation, textile, paper, detergent, and pharmaceutical industries. Amylase produced by thermophilic bacteria may be thermostable, which is very beneficial in several applications requiring high temperature, for example, the process of gelatinization, liquefaction, and saccharification are performed in high temperature involved in the starch processing. In this study, the amylase-producing ability of thermophilic bacteria isolated from Bukit Gadang hot spring, West Sumatra, Indonesia, was checked and followed by molecular identification. Thirteen isolates that were successfully isolated from the hot springs were microscopically and macroscopically characterized, biochemically tested, and determined their amylase enzyme activity both qualitatively and quantitatively. The isolate that performed the best amylase activity was identified using the molecular technique. The DNA sequencing was carried out in 16S rRNA and continued with BLAST search for species identification. The result of molecular identification showed that the isolate with the best amylase activity was identified as *Bacillus licheniformis*. The optimum amylase production (231.33 U/ml) and the best enzyme-specific activity (101.79 U/mg) were obtained at the incubation time of 36 hours.

**Keywords:** Amylase, bacteria, identification, thermophilic, West Sumatra

## INTRODUCTION

Amylase is an extracellular enzyme consisting of  $\alpha$ -,  $\beta$ -, and glucoamylase, that hydrolyzes starch or oligosaccharides randomly into glucose units, including dextrans and small polymers (Dash et al. 2015). This enzyme is essential in biotechnology, cosmetics, nutrition, and pharmacy (Kumar et al. 2014). The amylase is widely used in industry because it can increase production and requires less time and space for production (Deljou and Arezi 2016). Amylase is also used in the manufacture of high-fructose syrup, as it can break down starch, ferment starch to ethanol, and treat wastewater in starch processing (Baltaci et al. 2017).

Amylase can be produced from plants, animals, or microbes (Gazali and Suwastika 2018; Saryono et al. 2018; Ardhi et al. 2019). However, amylase production using bacteria is generally preferred over fungi, because it generates various characteristics and advantages as bacterial cells are quickly grown, and cell production scales are easily increased (Deljou and Arezi, 2016).

Amylase produced by thermophilic bacteria may be thermostable, which is very beneficial in several applications requiring high temperatures. One of the most

widespread applications of thermostable  $\alpha$ -amylase is in the starch industry that involves the process of gelatinization, liquefaction, and saccharification that are performed in high temperature (Mehta and Satyanarayana 2016). A thermostable amylase can reduce the risk of contamination, the cost of external cooling, and increasing the rate of diffusion (Fossi et al. 2014).

Thermophilic microorganisms live in extreme environments and grow optimally at temperatures between 45-80°C (Alrumman et al. 2018). Thermophilic microorganisms are stable to many solvents, detergents, and acidic and basic pH so that these microorganisms have attracted many researchers' attention (Mohammad et al. 2017). Adiguzel et al. (2009) isolated and identified thermophilic bacteria from hot springs in Turkey using phenotypic, genotypic, methyl ester fatty acid methods, rep-PCR, and sequencing at 16S rRNA to obtain isolates with the best characteristics. Alrumman et al. (2018) succeeded in isolating 84 isolates from three hot springs in Saudi Arabia, and the optimum enzyme production was obtained after 72 hours of incubation at the beginning of the stationary phase. Panda et al. (2013) stated that the thermophilic *Bacillus* sp. isolated from India might be a thermostable source of protease in the pharmaceutical and

industrial fields. Fachrial et al. (2019) succeeded in isolating and molecularly identifying thermophilic bacteria from the Panen hot spring, Sumatera Utara, Indonesia, which was identified as *Bacillus subtilis* JCM 1465 and able to produce carbohydrases and proteases.

Indonesia is rich in hot springs, but it is still a lack of reports on the production of thermostable enzymes. In this study, isolation and amylase enzyme activity tests were carried out from thermophilic bacteria isolated from Bukit Gadang, West Sumatra, Indonesia. Bacteria with the ability to produce the best thermostable amylase enzymes were then molecularly identified.

## MATERIALS AND METHODS

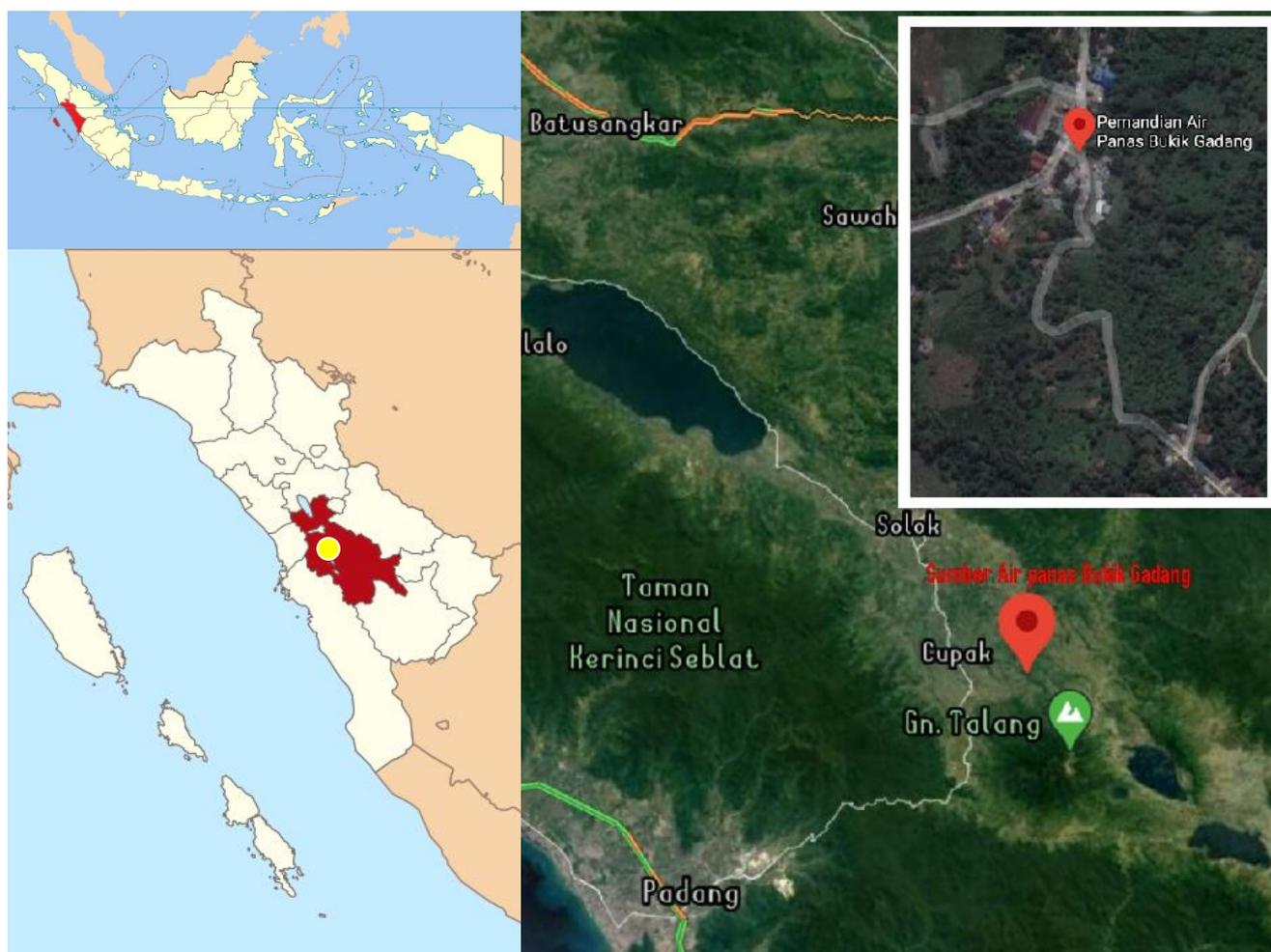
### Isolation of Thermophilic Bacteria

Samples were taken from the Bukit Gadang Hot Springs, Solok Regency, Province of West Sumatra, Indonesia (Figure 1) using random sampling techniques. The hot water sample was put into a sterile flask, accompanied by a measurement of the temperature and pH of the water. The sample was inoculated using the pour

plate method (Goto, 2007). In this method, a total of 1 ml of homogeneous sample was placed in the center of a sterile petri dish using a sterile pipette. Nutrient Agar (NA) medium was poured into the petri dish containing the inoculum and then mixed well. After the solidification of the agar, the plate is inverted incubated according to the original temperature (45°C, 50°C, and 55°C) for 24-48 hours. Purification was carried out several times until a pure colony was obtained.

### Identification of Thermophilic Bacteria

Bacterial culture was inoculated into NA and Nutrients Broth (NB) media and incubated at 45, 50, and 55°C. Bacterial growth was observed after 24-48 hours through its optical density measurements using the spectrophotometric method (Spectrophotometer Thermo Scientific 10S UV-Vis) against its turbidity at a wavelength of 660 nm. The microscopic test was carried out by morphological observation and gram staining. The shape of the cell could be either *coccus*, *basil*, or *spiral*. Subsequently, the samples were tested for their resistance to acids using carbol fuchsin solutions and their ability to produce catalase.



**Figure 1.** Location of Bukit Gadang Hot Springs, Solok, West Sumatra, Indonesia

### Screening of amylase-producing ability

The ability of bacteria to produce amylase was observed based on their ability to degrade starch. Each bacterial isolate was inoculated on NA media containing starch for 48 hours at the temperature of their original habitat and then tested by dripping iodine solution around the bacterial colony. The ability to produce amylase was demonstrated by the formation of clear zones around bacterial colonies. The isolate producing the largest clear zone ratio was chosen for the next subsequent step. Statistical analysis of the clear zone ratio used SPSS Duncan's multiple range test with a level of 5%.

### Amylase production

The thermophilic isolate was inoculated in a medium containing 1% starch, 0.5% yeast extract, 0.5% peptone, and 0.05% MgSO<sub>4</sub>.7H<sub>2</sub>O and incubated at 50°C for 48 hours at pH 7. Enzyme measurement was performed every 12 hours based on the Nelson-Somogyi method (Nelson, 1944) and expressed in unit (one unit is the amount of enzyme which releases 1 μmole glucose). The determination of protein content of crude enzyme was carried out using the Lowry (1951) method.

### Molecular identification

Bacterial isolates were grown on NB for 24 hours, then centrifuged for 10 minutes at 4000 rpm so that DNA pellets were obtained. The pellets obtained were added buffer and lysozyme, then incubated at 37 °C for 30 minutes, then added protein K, and re-incubated at 60 °C for 10 minutes. After incubation, the GB buffer was added and incubated at 70 °C for 10 minutes and continued with centrifuge for 10 minutes at 4000 rpm. The supernatant obtained was added with 200 μL absolute ethanol, then centrifuged and added with 400 μL WI buffer. After centrifugation, DNA pellets were added with 600 μL wash buffer, re-centrifuged, and continued with the addition of warm eluent buffers. The

mixture was allowed to stand for 3 minutes at room temperature, re-centrifuged, and stored in the refrigerator.

Molecular identification was carried out based on the sequence of 16S rRNA. PCR amplification of 16S rRNA genes used forward primer: 24F (5'AGA TGA TGA TCC TGG CT-'3) and reverse primer: 1541R (5'AAG GAG GTG CAG CCG CA-'3). This gene could be used as a standard for new bacterial species for phylogenetic trees (Lau et al. 2002). The success of PCR product amplification was known through 1.2% agarose gel electrophoresis. The existence of a single band of DNA indicated that the DNA was successfully amplified.

A total of 50 μL PCR amplification reaction mixture were made from 25 μL (1x) Master mix, 1 μL primer 24F (0.2 mM), 1 μL primer 1541R (0.2 mM), 2.5 μL bacterial DNA, and 20.5μL dH<sub>2</sub>O. The stages of the PCR program conducted in this study were pre-denaturation performed at 95 °C for 3 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 50 °C for 30 seconds, extension at 72 °C for 1 minute, and the final extraction at 72 °C for 10 minutes. The amplified PCR products were visualized using an electrophoretic gel. The emergence of PCR DNA bands marked the success of PCR. Furthermore, the PCR products were sequenced. The similarity of sequences was determined using BLAST (<http://www.ncbi.nlm.nih.gov/Blast>). Multiple sequences alignment used the Neighbour-Joining (N-J) method to determine the closeness of the isolates utilizing the Clustal X version 2.1 program, and the alignment results were viewed using the Mega version 6 program.

## RESULTS AND DISCUSSION

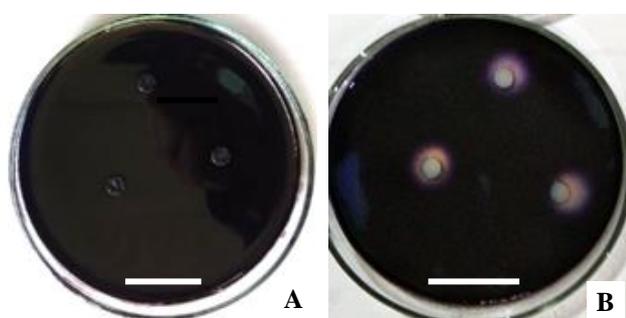
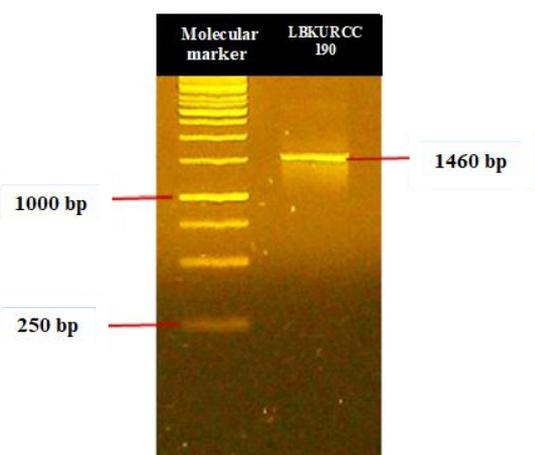
As many as thirteen thermophilic bacteria were isolated and further identified by morphological observation and biochemical reactions (Table 1).

**Table 1.** Morphological identification and biochemical tests of thermophilic isolates from Bukit Gadang Hot Springs, West Sumatra

| Temp. | Isolates code (LBKURCC) | Slant NA-media | Stab NA-Media | NB-Media           | Cell shape/<br>Gram staining | Genus (sp.)        | Acid resistance | Catalase | Starch hydrolysis |
|-------|-------------------------|----------------|---------------|--------------------|------------------------------|--------------------|-----------------|----------|-------------------|
| 45°C  | 235                     | Beaded         | Echinulate    | Sediment           | Basil/ (-)                   | <i>Thermus</i>     | -               | +        | -                 |
|       | 236                     | Filiform       | Echinulate    | Uniform turbidity  | Basil/ (-)                   | <i>Thermus</i>     | -               | +        | -                 |
|       | 237                     | Filiform       | Beaded        | Uniform turbidity  | Basil/ (-)                   | <i>Thermus</i>     | -               | +        | +                 |
|       | 238                     | Spreading      | Echinulate    | Uniform turbidity  | Basil/ (-)                   | <i>Bacillus</i>    | -               | +        | -                 |
|       | 239                     | Beaded         | Papillilate   | Sediment           | Basil/ (-)                   | <i>Thermus</i>     | -               | +        | +                 |
|       | 240                     | Beaded         | Echinulate    | Sediment           | Basil/ (-)                   | <i>Thermus</i>     | -               | +        | -                 |
| 50°C  | 185                     | Echinulate     | Villose       | Pellicle (aerobic) | Basil/ (-)                   | <i>Thermus</i>     | -               | -        | +                 |
|       | 186                     | Spreading      | Papillilate   | Sediment           | Coccus/ (-)                  | <i>Pseudomonas</i> | -               | +        | +                 |
|       | 187                     | Filiform       | Papillilate   | Pellicle (aerob)   | Coccus/ (-)                  | <i>Pseudomonas</i> | -               | +        | +                 |
|       | 188                     | Echinulate     | Echinulate    | Sediment           | Basil/ (-)                   | <i>Thermus</i>     | -               | +        | -                 |
|       | 189                     | Spreading      | Echinulate    | Pellicle (aerob)   | Coccus/ (-)                  | <i>Pseudomonas</i> | -               | -        | +                 |
|       | 190                     | Spreading      | Echinulate    | Pellicle (aerobic) | Basil/ (-)                   | <i>Bacillus</i>    | -               | +        | +                 |
| 55°C  | 260                     | Beaded         | Papillilate   | Sediment           | Basil/ (+)                   | <i>Bacillus</i>    | -               | +        | -                 |

**Table 2.** The ratio of the clear zone produced by thermophilic bacteria

| Temp. | Isolates code (LBKURCC) | Ratio                   | Genus (sp.)        |
|-------|-------------------------|-------------------------|--------------------|
| 45°C  | 235                     | 0.00±0.00 <sup>d</sup>  | <i>Thermus</i>     |
|       | 236                     | 0.00±0.00 <sup>d</sup>  | <i>Thermus</i>     |
|       | 237                     | 1.79±0.28 <sup>bc</sup> | <i>Thermus</i>     |
|       | 238                     | 0.00±0.00 <sup>d</sup>  | <i>Bacillus</i>    |
|       | 239                     | 2.00±0.26 <sup>bc</sup> | <i>Thermus</i>     |
|       | 240                     | 0.00±0.00 <sup>d</sup>  | <i>Thermus</i>     |
| 50°C  | 185                     | 2.01±0.89 <sup>bc</sup> | <i>Thermus</i>     |
|       | 186                     | 2.28±0.05 <sup>ab</sup> | <i>Pseudomonas</i> |
|       | 187                     | 1.66±0.18 <sup>b</sup>  | <i>Pseudomonas</i> |
|       | 188                     | 0.00±0.00 <sup>d</sup>  | <i>Thermus</i>     |
|       | 189                     | 2.26±0.02 <sup>ab</sup> | <i>Pseudomonas</i> |
| 55°C  | 190                     | 2.78±0.38 <sup>a</sup>  | <i>Bacillus</i>    |
|       | 260                     | 0.00±0.00 <sup>d</sup>  | <i>Bacillus</i>    |

**Figure 2.** Clear zone on starch media: A. Control, B. Amylase-positive. Bar = 2 cm**Figure 3.** PCR product of thermophilic bacteria LBKURCC190

There were three genera of bacteria obtained, namely *Thermus* sp., *Bacillus* sp., and *Pseudomonas* sp (Table 1). The genus of *Bacillus* appeared at all isolated temperatures because they were able to survive in environments with high temperatures (Mohammad et al. 2017). Thermophilic isolates were found to be mostly gram-negative bacteria in the form of basil. The biochemical tests indicated that all

isolates were negative to the acid-resistant test or unable to live in environments with high acid concentrations. Eleven of the 13 isolates were positive for the catalase test marked by the emergence of gas bubbles from hydrogen peroxide ( $H_2O_2$ ), which was decomposed into oxygen and water. Hydrogen peroxide was formed when aerobic metabolism so that microorganisms that grew in the aerobic environment could decompose the substance (Luhova et al. 2003). Whereas in the starch hydrolysis test, seven isolates had the ability to hydrolyze the starch. Starch hydrolysis results could be in the form of smaller molecules such as glucose, maltose, and dextrin (Reddy et al. 2003).

Only 7 of the 13 isolates could produce amylase, which was marked by the appearance of a clear zone around the bacterial colony (Table 2). This clear zone showed that the starch had been hydrolyzed by the amylase produced by bacteria. *Bacillus* sp. (LBKURCC190) isolated at 50°C produced the largest clear zone ( $2.78 \pm 0.38$ ) compared to the other two genera, namely *Thermus* sp. and *Pseudomonas* sp. Some previous studies also reported that *Bacillus* sp was the genus most widely used to produce amylase. Previous research from Fatoni and Zufahair (2012) and Dash et al. (2015) stated that *Bacillus* species, primarily *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, and *B. stearothermophilus*, produced about 60% of commercial enzymes. Almost all genera of *Bacillus* synthesize the  $\alpha$ -amylase (Deljou and Arezi, 2016). Figures of clear zone resulted from *Bacillus* are shown in Figure 2.

Morphological identification and biochemical testing, however, could only identify up to the genus level and performed a low level of accuracy. Further molecular identification was carried out on isolates with the highest amylase activity, which was LBKURCC 190. The amplified PCR products on the 16S rRNA of the LBKURCC190 was 1460 bp (Figure 3). This result was consistent with the statement of Clarridge (2014) that the target of 16S rRNA region amplification was around 1500 bp.

The results of 16S rRNA sequencing analysis showed that LBKURCC190 isolates had the highest similarity (>98%) with *Bacillus licheniformis* found in GenBank. Based on the alignment (Figure 4), it showed that LBKURCC190 isolate had a close relationship with *Bacillus licheniformis* strain ATCC 14580. These results were obtained based on changes in base arrangement from the alignment results. Changes in the base arrangement that occurred in alignment between base pairs and between sequence pairs were used to determine the phylogenetic relationship between sequences.

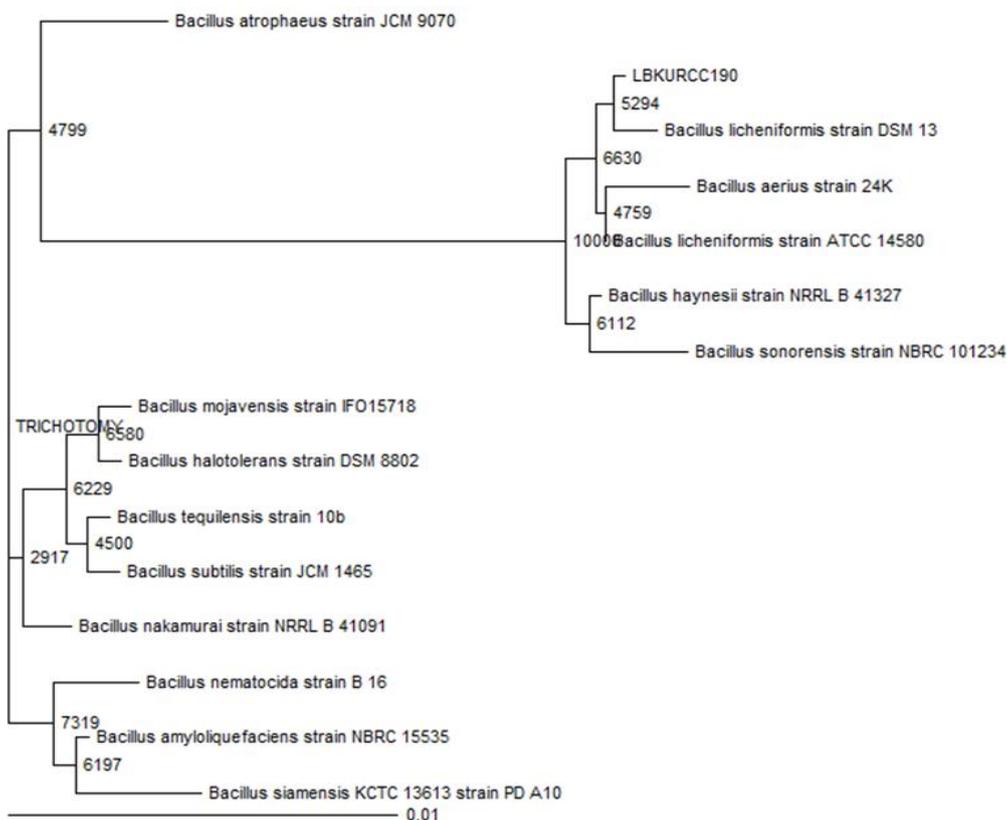
A phylogram of LBKURCC190 thermophilic bacteria was obtained using the N-J Tree method with 10,000 bootstrap replicates. The bootstrap analysis is carried out to test the branches of a reliable phylogenetic tree and how well the phylogenetic tree model data sets are used. The higher the bootstrap repetition rate is used, the accuracy of the phylogenetic tree obtained will be better and more accurate. Several studies had also used bootstrap analysis with 10,000 repetitions in fungal and bacterial testing (Gordillo-Fuenzalida et al. 2019; Anwar et al. 2016;

Krimitzas et al. 2013). Based on a phylogram with 15 strains and four subclusters, thermophilic bacteria *Bacillus* sp. LBKURCC190 had the closest kinship with *Bacillus licheniformis* DSM13 strain with a branching rate of 5294. These results were following macroscopic and microscopic data, where LBKURCC190 isolate showed the basil form and was a gram-positive bacterium. Similar results were also obtained by Mohammad et al. (2017), which isolated thermophilic bacteria from hot springs in Jordan and identified the bacteria as *Bacillus licheniformis* species.

The production of  $\alpha$ -amylase using thermophilic bacteria *B. licheniformis* had been successfully carried out by Abdel-Fattah et al. (2012) with the optimum combination in media composition. Amylase is an extracellular enzyme, so that the enzyme is in the fermented supernatant. Amylase activity was influenced by fermentation time. It could be seen from the growth of bacteria based on optical density values, which the higher the enzyme activity, the higher the optical density value. This was because when bacteria grew, bacterial cells would increase and the turbidity level would be even higher. Figure 5 illustrates the change in turbidity of the production media after 48 hours of fermentation. The bacterial growth curve was analyzed by measuring optical density every 12 hours to observe the phase of bacterial growth and to determine the age of the culture (Figure 6).

*Bacillus licheniformis* LBKURCC190 showed a significantly increased growth at 24 hours, with an OD value of 1.119 and enzyme activity 173 U/ml, and did not

change at 36 hours with an OD value of 1.101 and an increasing enzyme activity reaching 231.33 U/ml. In this phase, the bacterial growth was swift; however, after reaching the optimum point until 48 hours, it started to decrease. This could be the nutrients were still inadequate numbers until 48 hours, so that bacterial growth continued, and it decreased after the nutrients began to run out until the death phase. In this phase of death, the number of cells that died was more than living cells. In Figure 6 (a), this phase was shown by decreasing curve plots. Figures 6b and 6c showed the optimum amylase production was obtained at 36-hour fermentation time (231.33 U/ml), and the optimum amylase specific activity was 101.79 U/mg protein at the same fermentation time. The specific activity of this enzyme meant the amount of enzyme activity per amount of protein present in the enzyme mixture tested. Different results were found by Gurudeeban et al. (2011), in which the maximum amylase activity produced by *Bacillus megaterium* was obtained under an incubation period of 72 hours, the temperature of 35°C, and pH 6.5. The enzyme activity obtained was relatively smaller when compared to other studies (Abdel-Fattah et al. 2012), where the optimal amylase produced by *Bacillus licheniformis* isolate AI20 obtained reached 384 U/ml. Production of amylose was influenced by media compositions, which were the type of carbon and nitrogen sources, as well as the concentration of yeast extract and peptone (Gurudeeban et al. 2011; Alamanaa et al. 2019).



**Figure 4.** Phylogenetic tree of LBKURCC190 isolate with 15 bacterial strains using the Neighbour-joining analysis

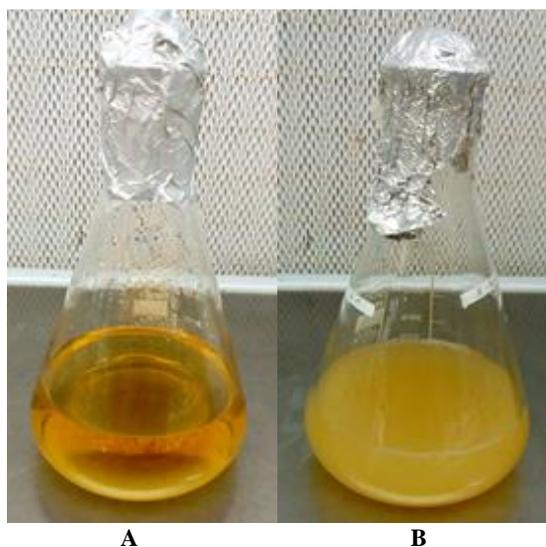


Figure 5. Production media for amylase (A) 0 hour (B) 48 hours

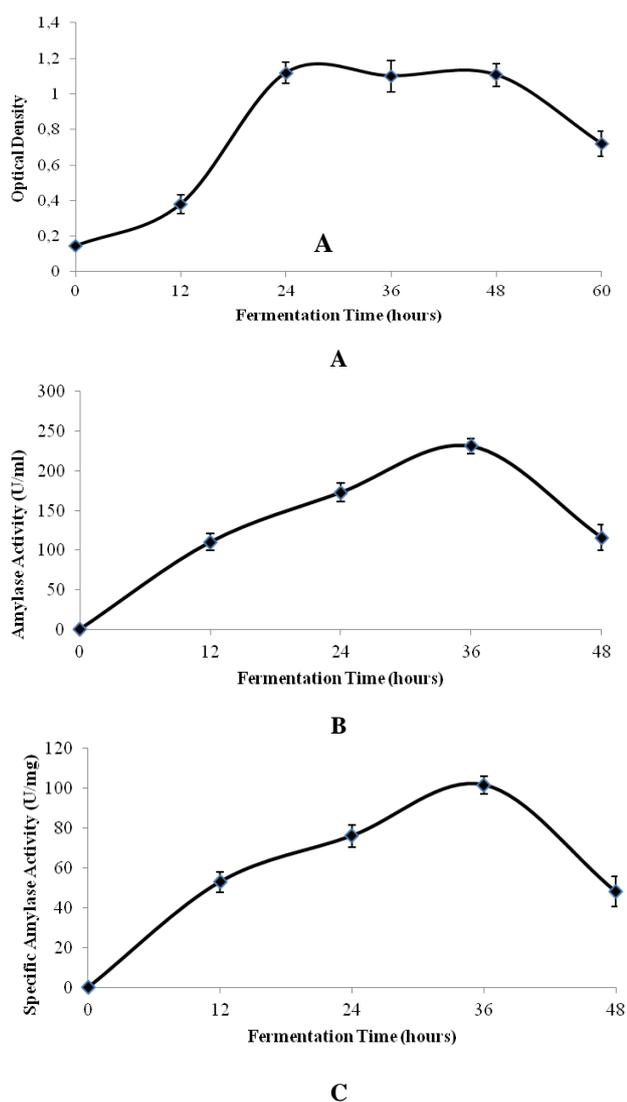


Figure 6. A. Optical density, B. Amylase activity, C. Specific amylase activity of *Bacillus licheniformis* LBKURCC190

To conclude, there were 13 isolates obtained from Bukit Gadang hot spring, West Sumatra, Indonesia, identified as *Bacillus* sp., *Thermus* sp., and *Pseudomonas* sp. The isolate of LBKURCC190 performed the highest amylase activity and was molecularly identified as *Bacillus licheniformis*. The optimum amylase production was obtained at 36-hour fermentation time, with the enzyme activity of 231.33 U/ml, and the enzyme-specific activity of 101.79 U/mg protein.

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