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Isolation of Thermophilic Bacteria in the Hot Spring of Asin, Tuel, Tublay, Benguet

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ABSTRACT

Background and Objectives: Thermophilic bacteria are less studied but they are important group of microorganisms because of their ability to produce industrial enzymes.

Materials and Methods: In this study, thermophilic bacteria were isolated from hot spring of Asin, Tuba, Benguet. A bacterium
that could tolerate high temperatures was characterized by morphology, biochemistry and sequencing of its 16S rRNA gene.
The isolate was screened for protease and amylase activity. Phylogenetic affiliations of the isolate was studied.

Results: The bacterium with the ability to tolerate high temperatures was identified as *Bacillus* sp. both by morphology,
biochemistry and sequencing of its 16S rRNA gene. BLAST search analysis of the sequence showed maximum identity with
Bacillus sp (99% and 100% similarity). Phylogenetic analysis of the isolate revealed close affiliation with thermophilic *Bacillus* species.

Conclusion: The study confirmed that the isolated *Bacillus* sp. to be a true thermophile and could be a source of thermostable
amylase which can be exploited for food industries.

Introduction

Around the world, microorganism can be found. If plants and animals have millions of species, so as microorganism. These are living things that cannot be seen by our naked eye. Thus, a microscope is needed in order to view them. They can survive in different environment. Some can thrive in an extreme condition like steam vents, boiling mud pots and others. Microorganism would also prefer water, soil, air and inside other living things (Zion National Park). Bacteria is one of the microorganisms that can thrive in an environment with different condition. One condition considered is the temperature of the environment. Thus, bacteria can exhibit tolerance to a wide range of temperature. From the psychrophiles microbes that grow at cold temperatures, mesophiles microbes that grow at moderate temperatures and the thermophiles that grow at high temperatures (Pandey, A. et al. 2014; Trimulyono, G. et al. 2018).

This study focused on Thermophiles. They are bacteria that are abundant in extreme environment. They can survive in high temperature. They can be found in different heated geographic regions of Earth like hot springs, hydrothermal vents and others (NZQA). They have an optimum growth temperature of 50–55 °C, but can grow in the range of 40–60 °C (Gleeson et al. 2013). There were several studies of thermophiles that were conducted. Moreover, as stated by Reichle in his study that before studies of

biological organisms in hot springs began in the 1960s, scientists long thought that thermophilic bacteria could not survive in temperatures above 55°C (131°F). However, many bacteria were discovered that not only survived, but also thrived at higher temperatures. Brock and Freeze in 1969 reported a new species of thermophilic bacteria, which they named *Thermus aquaticus*, living in Mushroom Spring in the Lower Geyser Basin of Yellowstone National Park.Another is the study reflected the temperature optima of bacteria occurring at various temperatures along the thermal gradient (35–70°C) in a Yellowstone hot spring. The research was studied directly in nature by measuring the rate of incorporation in the dark of 14C-glucose or 14CO2 (Brock and Brock, 1968). Since then, different studies on thermophiles were conducted, usually from hot spring. The Taq polymerase in the Yellowstone became popular and it was extensively used in polymerase chain reaction (PCR) that was developed in the mid-1980s by K. Mullis, R. Saiki.

There are different species of thermophiles that were reported. Most of them are being utilized by industries. Some of these are the bacterial genera, *Thermus, Rhodothermus, Thermoanaerobacter Bacillus* and *Clostridium*. Archaeal of the genus of *Methanobacterium* and *Thermoplasma* are also used. Representative genera of hyperthermophiles archaea are *Methanococcus, Methanothermus, Archaeoglobus, Pyrococcus, Pyrodictium, Pyrolobus, Sulfolobus, Thermococcus,* and *Thermoproteus*. The thermophilic bacteria *Geobacillus kaustophillus, Aeribacillus pallidus, Geobacillus galactosidasus* and *Geobacillus toebii* isolated from Tunisia hot springs are reported to produce extremozymes such as amylases, proteases, xylanases, cellulases and mannases. Thermostable hydrolytic enzymes are also reported from Manikaran and Yumthang hot springs of Indian Himalayan regions (Angelin J. et al. 2022) Several studies have been conducted to identify and characterize thermophiles. Moreover, only few are recorded in the locale of Benguet. Thus, this study concentrates on the characterization and isolation of some thermophile found in Hot Spring of Asin, Tuel, Tublay, Benguet.

Methodology

Screening for Thermophilic bacteria

The study collected samples from water as well as from sediments. For the water samples, it was collected near the main source of the hot spring. The sediment samples were collected beneath the hot water. They were preserved and brought to the laboratory for further evaluation. In the laboratory, the different media and different materials to be used were already prepared.

Water samples were vortexed for 2-3 minutes and 20 µl of inoculum were transferred to a 10 ml nutrient broth. For the sediment samples, about 0.5 g of sediment was mixed with 5 ml distilled water. It was vortexed for 2-3 minutes and allowed to settle for 5 mins. 20 µl of the supernatant from the mixture was inoculated on 10 ml nutrient broth. Both water and sediment samples were incubated under 40^{o}C in 2 days. Broth cultures that exhibit turbidity were examined for the presence of putative thermophiles.

Morphological and Biochemical Characterization of thermophile

The broth cultures were then plated onto nutrient agar and cultured at 40°C in 18-24 hrs. Further, to isolate a pure culture, a single colony was picked and restreaked three times on nutrient agar. It was then incubated for 18-24 hrs. The pure culture was subjected to gram staining following the laboratory standards. They were also evaluated on there enzymatic activity for amylase and caseinase.

The pure cultures were sent to Philippine Genome Center, Diliman, Quezon City for the extraction and sequencing of 16S rRNA. Samples were extracted using Cetyltrimethyl ammonium bromide (CTAB) extraction method. The gel quality of genomic DNA (gDNA) were assessed by running it through gel

electrophoresis to ensure its purity, integrity, and size distribution. Gene amplification by PCR, components include: genomic DNA, 27F and 1492R primers, Taq Buffer, DNA polymerase, and dNTP Mix. Cycling parameters on thermal cycler: 98°C 3 min; 30 cycles of 94°C 30 sec, 54°C 30 seconds, 55°C 45 sec; 72°C 1 min; hold at 4°C. Capillary sequencing involves the incorporation of fluorescently labelled chain terminator ddNTPs; the reaction components include: amplicons, primers, and ABI BigDye® Terminator v3.1 Cycle Sequencing Kit. Cycling parameters on Bio-Rad T100 Thermal Cycler: pre-hold at 4°C; 96°C 1min; 25 cycles of 96°C 10 secs, 50°C 5 secs, 62°C 4 mins; hold at 4°C. Ethanol Precipitation was carried out to remove unincorporated ddNTPs, excess primers and primer dimers. Capillary electrophoresis on the ABI 3730xl DNA Analyzer using a 50cm 96-capillary array, POP7TM Polymer, and 3730xl Data Collection Software v3.1. Base calling on the Sequencing Analysis Software v5.4. Forward and reverse sequences were assembled to produce contigs. The assembled sequences were checked identified using FASTA search at NCBI database. The type and reference strains with the highest similarity to the sequences of the studied isolates were retrieved and used as data. The construction of phylogenetic tree was obtain using Mega 11 software.

Further, the evaluation of the physico-chemical properties of water and sediment were collected from sampling site. pH values were directly measured by immersing the electrode of pH meter in the water sample. A refractometer is used to measure the salinity of the water sample. The temperature was also obtain using a mercury thermometer right at the spot where water sample is taken. Color or samples were recorded through direct visual observation.

Results and discussion

Morphological characterization

Morphological characterization (Table 1) shows that the isolates exhibits white color. In terms of colony, round and smooth margins measuring ≤ 0.5-1.0 mm are observed. Under microscopic analysis, the thermophiles revealed a rod-shaped occurring singly, in pairs or in chains. Isolates were also identified to be gram positive.

Table 1. Morphological Characterization

Isolates	Cultural Characteristics			
	color	margin	form	Size of colony (mm)
Water	white	smooth	round	≤ 0.5-1.0
sediment	white	smooth	round	≤ 0.5-1.0

A gram-positive bacterium has a thick cell about 20-80nm. It is composed of multiple layer of peptidoglycan with as many as 30 layers of interconnected glycan chains. Thus, they have the ability to retain the purple stain. Peptidoglycan is also permeable to many substances including sugar, amino acids, and ions. Gram positive cell wall has teichoic acids and phosphate. The teichoic acids are chains of either ribitol-phosphate or glycerol-phosphate to which various sugars and D-alanine are usually attached (Nester et al., 2001; https://byjus.com/). Jordanian hot springs has isolates that shows the same characteristics. They were grey, creamy, and white; opaque or translucent; rough or smooth; with regular or irregular edges. Based on gram staining, the isolates were found mostly to be gram-positive (Mohammad B. et al., 2017). In addition, the cell morphology varied from short to long rods and cocci

with a dominance of Gram-positive bacteria in the isolates from Soldhar (Tapovan) hot spring in Central Himalayan Region, India (Arya M. et al., 2015).

Amylase and caseinase evaluation

Isolates for both water and sediment shows positive result upon employing starch hydrolysis. These represents positive amylase activity. The isolates degrade all the starch constituent of the medium.

An enzyme that is able to breakdown starch into smaller sugar is called amylase. This are being secreted by bacteria into the surrounding media, catalyzing the breakdown of starch into smaller sugars. These smaller sugars can then be absorbed by the cells and the cells can use the sugars as a source of energy and carbon.

Enzymes became widely important specially in the field of recombinant technology and protein engineering. Most of them are being used in different industries. Enzymes from microbes are preferred nowadays due to their economic feasibility, high yields, consistency, ease of product modification and optimization, regular supply due to absence of seasonal fluctuations, rapid growth of microbes on inexpensive media, stability, and greater catalytic activity. Microbial enzymes also has the most successful applications of solid-state fermentation. For the past years, researches on microbial enzymes production increased through solid-state fermentation. It was then used to produce large quantities for commercial and industrial purposes (Gurung et al., 2013; Heliyon. 2022).

In this study, the isolated bacteria were able to produce amylase and hydrolyze starch at 50^{o}C. The thermostability is one of the characteristics that is desirable in a major group of industrial enzymes. Researches nowadays are taking into the studying the thermostable amylotic enzymes to improve industrial method of starch degradation as well as production of other products like crystalline dextrose, glucose, maltose, dextrose syrup, and maltodextrins. Some bacteria like *Bacillus subtilis, Bacillus stearothermophilus, Bacillus licheniformis,* and *Bacillus amyloliquefaciens* are found to be good producers of thermostable α-amylase and are widely in use for commercial production of the enzyme for numerous applications. Today, thermostable amylases of Bacillus stearothermophilus or Bacillus licheniformis are used in starch processing industries (Gurung et al., 2013; I. Gomes et al., 2003). Moreover, the study shows that isolates belong to genus *Bacillus*. These species are referred to as “cell factories” which provides suitable volume of enzymes used as bulk chemicals (Bergquist et al., 2014). The bacterial strain used in the study of Carvalho R. et al., 2018 was Bacillus sp SMIA-2, a thermophilic strain isolated from a local soil sample. It shows a stable and high production of amylase enzymes. Isolates from sediment samples of Bora hot spring showed amylolytic activity. The amylolytic index of 0.8 mm and 0.5 mm was observed showing that these thermophilic bacteria are positive in amylase test (F M Gazali et al., 2018). In addition, these isolates are able to degrade starch completely implying the production of some amylase.

Caseinase is another enzyme being secreted so called exoenzyme. It catalyzes the breakdown of milk protein which called casein. It breaks down into small peptides and individual amino acids which are utilize by the organism for energy use or as building material. In the reaction process, hydrolysis causes the milk agar to clear around the growth area as it converts to its end products like small chains of amino acids, dipeptides, and polypeptides (bio.libretexts.org).

In this study, the isolates for both water and sediments shows a negative result for caseinase. Thus, the isolates cannot degrade milk. However, thermophiles are stated to have enzymes that can function at high temperatures on which, some of these enzymes are used in molecular biology and in washing agents. It may require a longer incubation period. As well as fastidious organisms may not grow in the medium and

are difficult to test (https://microbenotes.com/; https://bio.libretexts.org/). Moreover, thermostable proteases are usually produced using thermophilic strains belonging to genus *Bacillus* (Haki and Raksit, 2003; Lasa I, and Berenguer J.,1993). In the study of Nascimento et al., 2004, a thermophilic bacterium was evaluated on its protease activity. The protease production by thermophilic *Bacillus sp* strain SMIA-2 was cultivated in liquid cultures containing trisodium citrate and reached a maximum 9h, with levels of 1.93U/mg protein. It was observed that the microorganism utilized several carbon sources for the production of protease. Further their results showed that starch was the best substrate, followed by trisodium citrate, citric acid and sucrose. Another strain exhibited considerable protease activity in which its phylogenetic analysis of the isolate revealed close affiliation with thermophilic Bacillus species (Panda MK et al., 2013).

Analysis of 16S rDNA sequences

The small ribosomal subunit contains 16S rRNA in prokaryotes. They have been used for taxonomic and phylogenetic studies. A Single isolates of prokaryote microorganisms from different sources can be identified by amplification and sequencing using the same pair of primers and the same PCR and sequencing conditions. (Rivas R et al., 2004).

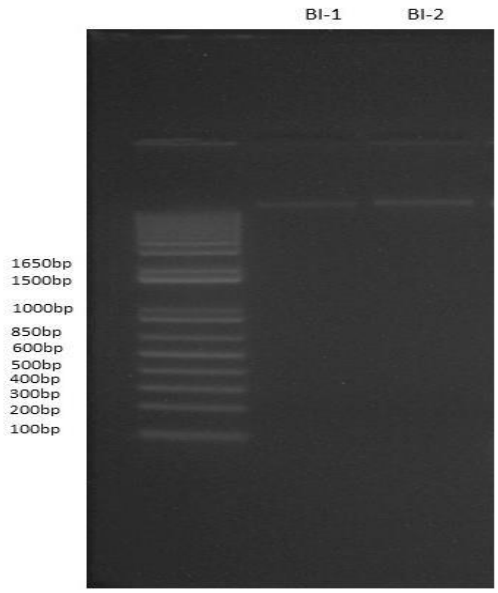

Figure 1. ***Agarose Gel Electrophoresis of genomic DNA with ~1500bp size. Genomic DNA was loaded to 1.2% agarose run in 100V for 45 minutes DNA marker 1kb plus ladder (Invitrogen).***

Based on the agarose gel electrophoresis (Figure 2), the size of 16S rRNA of all isolates is within the range of 1650bp to 100bp. This outcome was further corroborated by the number of nucleotides after the sequences were assembled. The length of a complete 16S rRNA contains ~1,500 base pairs composed of both variable and conserved regions with 1-10 copies present in Bacteria (Clarridge, 2004). From these results, the extracted 16S rRNA from the isolates in the study can be considered near full-length. The gel image shows that all bacterial samples were successfully amplified using 16s (27F & 1492R) primers (Figure 2).

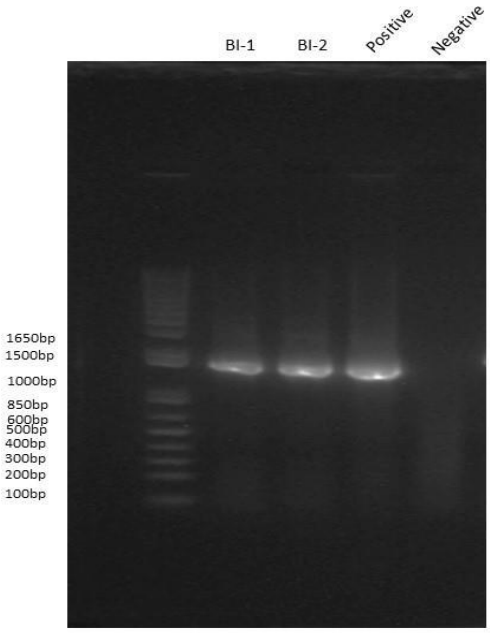

Figure 2. ***Agarose Gel Electrophoresis of amplified gene with ~1500bp size. Post-PCR product was loaded to 1.2% agarose run in 100V for 45 minutes DNA marker 1kb plus ladder (Invitrogen).***

Sequence analysis of 16S rDNA of each isolate was employed to confirm the identity of the two thermophiles. As a result the isolates were identified to be under *Bacillus sp.* For the water sample, the identified strain shows *Bacillus sp. hb27*. It has 100% identity with 100% query cover and a length of 1457. For sediment sample, a strain was identified to be *Bacillus sp. (in: firmicutes)*. It has 99.27% identity with 62% query cover and a length of 1359. Figure 3 shows the phylogenetic tree of the extracted isolates taken from both water and sediment samples. Some strains were accounted in order to construct the phylogenetic tree.

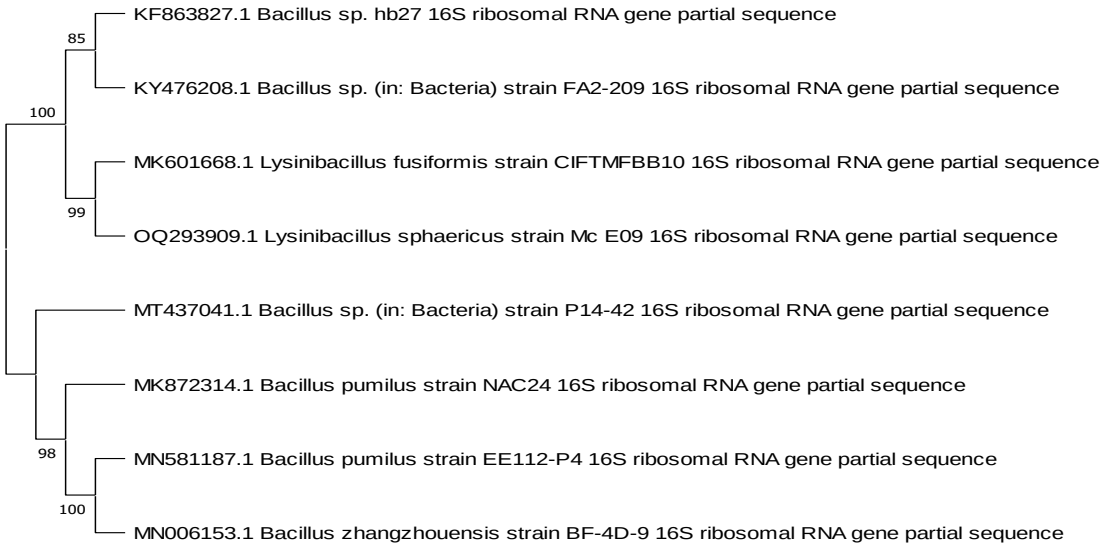

Figure 2. ***Phylogenetic tree. Lineage of Bacillus species from water and sediment samples.***

The study of Mohammad B. et al., 2017 in Jordanian hot springs shows isolates of thermophilic bacteria under *Bacillus sp*. Sequencing of the 16S rDNA of the isolates followed by BLAST search revealed that nine strains could be identified as *Bacillus licheniformis* and one isolate as *Thermomonas hydrothermalis*. Another study shows that the bacterium with the ability to tolerate high temperatures was identified as Bacillus sp. both by morphology, biochemistry and sequencing of its 16S rRNA gene. Its BLAST search analysis of the sequence showed maximum identity with Bacillus amyloliquefaciens (99% similarity) in which its phylogenetic analysis revealed close affiliation with thermophilic Bacillus species (Panda MK et al., 2013). Thermophilic bacteria were also isolated from various hot springs in Turkey. After evaluating several primer sets targeting the repetitive DNA elements of REP, ERIC, BOX and (GTG)5, the (GTG)5 and BOXA1R primers were found to be the most reliable technique for identification and taxonomic characterization of thermophilic bacteria in the genera of Geobacillus, Anoxybacillus and Bacillus spp (Adiguzel A. et al., 2009). Central Sulawesi has a hot spring named Bora hot spring with potential thermophiles. The isolated thermophilic bacteria from Bora Hot Spring namely TM022, TM023, TM024, TM026 were observed to bacillus and cuccus (Ifandi S. et al., 2018). Four thermophilic bacterial isolates viz., (Brevibacillus thermoruber, PS1), (Brevibacillus thermoruber, PS2), (Paenibacillus sp., PS3) and (Bacillus licheniformis, PS4) were isolated and characterized. These isolates was taken from natural hot water springs of Himachal Pradesh (india) (Verma A. et al., 2014). Also, the organism which was isolated from soil and capable of producing the caseinase enzyme was identified to be *Bacillus subtilis* based on the Biochemical tests and 16S rRNA sequencing result (Alhosien N. et al. 2020). In addition, *Bacillus subtilis* and *Bacillus pumilus* which are thermophilic bacteria are use to determine mutants for mesophilic bacteria (Droffner M. et al,.1985). This shows that the *Bacillus sp*. isolated in the study corroborates with these studies.

Physico-chemical properties

Sampling site shows that temperature is at 50ºC. Its pH is 7.57 and the salinity to be 0.46 mg/L. The color is observed to be clear. This result can be corroborated in some studies. The optimal temperature for growth of isolates of thermophilic bacteria was 65ºC and the optimal pH was 6-8 (Verma A. et al., 2014). Another, study showed that the isolates can grew over a wide range of temperatures (20–100 °C) and pHs (5–10) (Arya M. et al., 2015). Some thermophiles grew well at neutral to slight alkali pH (Takashi . et al. 1972). Some investigate the microbial diversity and community composition in several Costa Rican hot springs alongside the latitudinal axis of the country, with a range of temperatures (37–63°C), pH (6–7.5) and other geochemical conditions (Uribe-Lorío L. et al.,2019). Interestingly the study of Droffner M. et al,.1985 shows that thermophilic mutant strains were able to grow at temperatures between 50 degrees C and 70 degrees C at a frequency of less than 10(-10). Indeed, hot spring is an environment with high temperature and almost neutral wherein putative thermophiles may strive.

Conclusion

The 16S rRNA isolated from water and sediment of Asin Tuba Benguet are identified to be thermophilic bacteria. In, which they are under Bacillus sp. Its morphological and biochemical test coincide with the characteristics of a Bacillus bacteria. Further identification and additional test to ensure isolates identification is recommended.

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