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Research Article

Bioprospecting and Molecular Identification of Amylase and Cellulase Producing Thermophilic Bacteria from Sediment of Nglimut Hot Springs, Kendal Regency

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ABSTRACT

The utilisation of enzymes in the industry has brought numerous benefits and advantages to production processes. Enzymes serve as biocatalysts, efficiently catalyzing reactions and hydrolysis in biochemical processes. However, there are challenges in applying enzymes in the industry, particularly concerning enzyme stability. The obstacle encountered in the production processes involving industrial enzyme applications is the low stability of enzymes when used at high temperatures. Heat-sensitive enzymes undergo damage or denaturation. Thermophilic microorganisms are chosen because they hold the potential to produce thermophilic enzymes. The thermophilic enzymes exhibit better heat stability compared to other enzymes, making them an effective alternative for future industrial production processes. This study aims to isolate thermotolerant bacteria from Nglimut Hot Spring sediment, screen for cellulase- and amylase-producing isolates, and molecularly identify the best isolate using *16S rRNA* barcode. The results show that 22 bacterial isolates were found in the sediment of a hot spring; TS-14 was the best isolate in producing amylase, with the highest average amylolytic index of 2.38, whereas TS-15 had the highest cellulolytic index of 2.11. Based on *16S rRNA* identification, TS-14 showed an homological identity of 79% with *Bacillus amyloliquefaciens*, while TS-15 had a 100% homological identity with *Bacillus licheniformis*. These results were important as the first step of screening bacterial potential to produce thermophilic enzymes that could be applied in the downstream processing in future industrial and biotechnology companies.

INTRODUCTION

Thermophilic enzymes have significant potential across various industrial processes due to their exceptional stability, functional capability under high temperatures, and ability to withstand structural alterations. These enzyme varieties find widespread application in sectors such as the chemical industry, food production, pharmaceuticals, paper manufacturing, and textiles. Currently, enzymes derived from microorganisms are more fre-

quently utilised within industrial contexts. An enzyme earns the "thermostable" label when it demonstrates a high transition or denaturation temperature while maintaining extended functionality at this elevated temperature. In this context, the temperature considered high exceeds the boundary of thermophilic growth (>55°C). Typically, extracellular enzymes exhibit heightened stability as they remain unaffected by intracellular factors like compatible solutes. Furthermore, enzymes can be influenced by additional factors, including pH, water content, and temperature. As a result, thermophilic enzymes present an alternative for industrial applications that require robust enzymes capable of enduring extreme environmental conditions, owing to their reliability under such circumstances (Turner 2007).

Nglimut Hot Spring in Gonoharjo, Kendal Regency, Central Java, Indonesia, was chosen because it harbors a diverse range of microorganisms that thrive in high-temperature environments. The presence of plantations and other biodiversity in the area surrounding Mount Ungaran further contributes to the potential of the microorganisms living in the hot spring to produce thermophilic enzymes. The coordinates of the Nglimut Hot Spring are within 110°19'47.3"E to 110°20'12.3"E longitude and 7°08'56.9"S to 7°09'42.1"S latitude, at an elevation of 700 meters above sea level. Research conducted by Emianto (2011) indicated that the reservoir temperature in Gonoharjo is approximately 207.53°C, but the actual reservoir temperature might be higher or lower than the calculated value. This difference is attributed to the fact that many dissolved elements in the geothermal fluid near the surface precipitate, especially Na-K-Ca elements, causing differences in chemical content between the fluid sample and the fluid in the reservoir (Emianto 2011).

Bacteria originating from hot springs are microorganisms capable of thriving in elevated temperatures, from 45°C to temperatures exceeding 100°C. Groups of bacteria derived from these hot spring environments can generate enzymes characterized by inherent stability at high temperatures and resilience against changes in physical and chemical conditions. An illustrative instance of such enzymes is cellulase, as pointed out by Khalil (2011). The attention directed towards these bacteria from hot springs is primarily due to their potential as a source of robust enzymes that retain their functionality in high-temperature environments. Worth mentioning is the cellulase enzyme, which holds substantial commercial importance. Cellulase enzymes exhibit considerable promise in the conversion of agricultural cellulosic materials into glucose feedstocks and their role in bioethanol production (Mohammad et al. 2017).

Cellulase enzymes are primarily synthesized by fungi, bacteria, and protozoa, which facilitate the hydrolysis of cellulose in a process called cellulolysis. The significance of cellulase enzymes lies in their diverse range of applications. Industries such as textiles, food production, detergents, leather, and paper manufacturing require stable and functional enzymes under extreme pH and temperature conditions. Additionally, cellulase enzymes play a crucial role in biomass fermentation for biofuels, altering fibers, and even in pharmaceutical applications. Bacterial organisms tend to outpace fungi in cellulase production due to their faster growth rate. Noteworthy bacterial genera that exhibit cellulolytic properties encompass *Cellulomonas* sp., *Pseudomonas* sp., *Bacillus* sp., and *Micrococcus* sp. (Shanmugapriya et al. 2012).

Amylase is a commercial enzyme that makes up 25% of the world's enzyme market needs (Reddy et al. 2003). Amylase can hydrolyze amylum and produce glucose. Amylase originating from thermophilic bacteria can have high thermostability, it may be stable in the presence of sub-

stances that can denature enzymes and stable in an acidic environment, so it has high commercial value for its use in industrial processes and biotechnology. Thermostable amylase is increasingly used in industrial processes and biotechnology (Sianturi 2008). Various industrial processes that use amylase include the food industry, fermentation, textiles, paper, detergents, and pharmaceuticals (de Souza & de Oliveira Magalhães 2010).

The production of amylase is affected by temperature, pH, enzyme concentration, substrate concentration, and inhibitor effects (Poedjiadi et al. 2006; Soeka et al. 2015). This study aims (i) to screen for potentially thermophilic enzymes, especially amylase and cellulase, from sediment of Nglimut hot springs, Kendal Regency, (ii) to determine the optimum condition for enzyme production by temperature variation via qualitative assay, and (iii) to identify the best cellulase and amylase-producing isolate through 16S rRNA barcode. The data provided significant information as fundamental research of the potential thermophilic enzyme from sediment of hot springs, which was previously never disclosed. This study improves our understanding of the basic qualitative assay and is important as the first step of screening the potential thermophilic enzymes, which could later be optimized using the quantitative assay and applied in downstream processing in future industrial and biotechnology companies.

MATERIAL AND METHOD

Sampling site

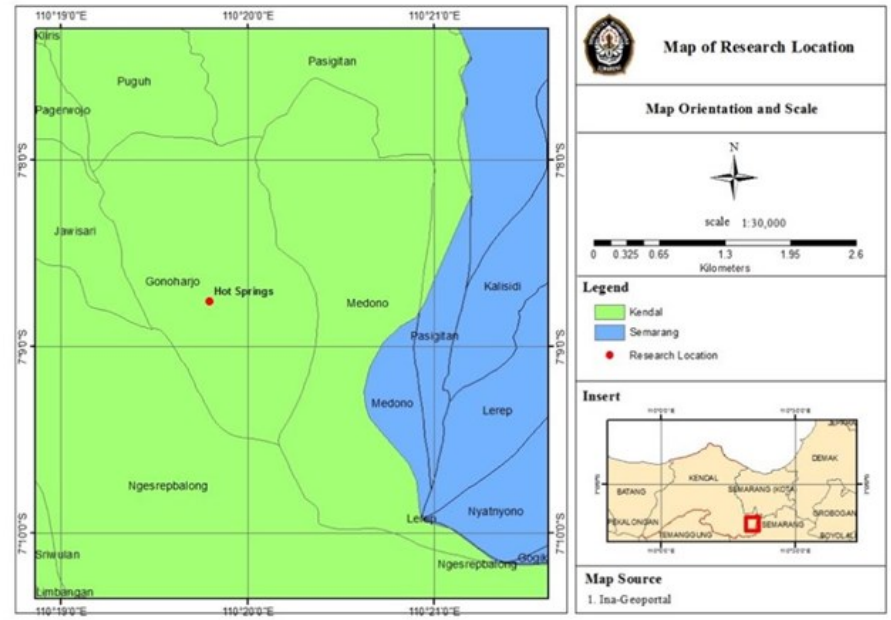
The Nglimut, Gonoharjo, Kendal Regency had the longitude coordinates 110°19'47.3"E to 110°20'12.3"E and latitude coordinates 7°08'56.9"S to 7°09'42.1"S with the temperature approximately of 45-50°C. Temperature differences occur during summer and rainy seasons. The research was carried out by collecting sediment samples from the Nglimut hot springs in Gonoharjo, Kendal Regency (Figure 1). Samples were taken and placed in a hot water flask so that the temperature is maintained until reaching the laboratory for further testing. The bacterial isolation, cellulase and amylase enzyme screening, temperature and pH variation assay, as well as molecular identification using *16S rRNA* gene markers were done in this study.

Bacterial Isolation

Isolation of thermophilic bacteria was done on Nutrient Agar (NA) (Hi Media, India) and Thermus Agar (TA) (Hi Media, India) media. The screening process of isolating thermophilic bacteria was taking a 1-gram sample of the hot spring sediment, placing it into 9 milliliters of sterile distilled water, followed by employing a serial dilution method to create dilutions ranging from 10^{-1} to 10^{-7}. Each dilution (1 mL) was introduced onto NA and TA media using the spread plate technique. Subsequently, incubating was done at a temperature of 45°C for 48 hours. The colonies grown in the media were then observed for colony morphology. The gram staining was performed for microscopic observation (Khalila et al. 2020).

Cellulase Screening

Cellulase activity screening was done on CMC Agar media, made by mixing 1.36 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 2 g NaCl, 1 g $(NH_4)_2SO_4$, 0.01 g $FeSO_4.7H_2O$, 3 g CMC, 1 g yeast extract, and 15 g agar powder into 1 L of distilled water in an Erlenmeyer flask (Naresh et al. 2019). All thermophilic bacterial isolates were tested for cellulase activity at 45°C, 50°C,



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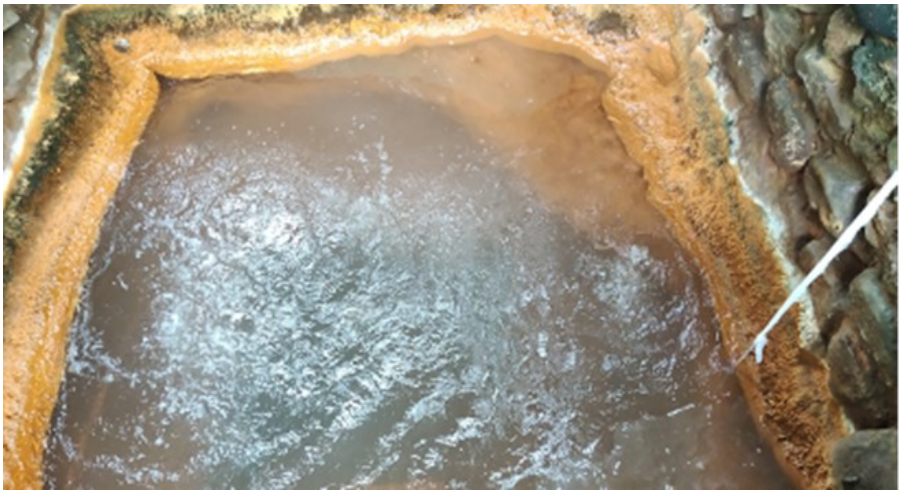

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Figure 1. Sampling Map of research location in Nglimut Hot Spring (**A**) , Nglimut Hot Spring, Gonoharjo (**B**).

and 55ºC in duplicates. The cellulase enzyme test followed the procedure from Naresh et al. (2019). A sterilized paper disc was placed on top of the enzyme test media. Each liquid culture of bacterial isolates was inoculated onto the paper disc and then incubated for 48 hours. The test results were then calculated using the cellulolytic index equation (Naresh et al. 2019):

$$\text{Cellulolytic index} = \frac{(\text{Clear zone diameter} - \text{bacterial colony diameter})}{\text{bacterial colony diameter}}$$

The isolates of thermophilic bacteria that produce the highest cellulase activity were then identified molecularly to determine their identity.

Amylase Screening

Amylase activity screening was done by making an amylase selective medium with 2 g of yeast extract, peptone 5 g, $MgSO_4.7H_2O$ 0.5 g, NaCl 0.5 g, $CaCl_2.2H_2O$ 0.15 g, starch 10 g, and agar 20 g. The ingredients were put into a glass beaker, and sterile distilled water was added to a volume of 1 L. Afterwards, it was incubated for 48 hours at 45ºC, 50ºC, and 55ºC in duplicates. Sterilized paper discs were placed on the enzyme test media. Each liquid culture of bacterial isolates was inoculated onto the paper discs and incubated for 48 hours. After 48 hours of incubation, the media around the colonies were covered with an iodine solution. Positive results

are indicated by the presence of a clear zone around the colony (Zuraidah 2020). The amylolytic index is measured using the following formula:

$$\text{Amylase index} = \frac{(\text{Clear zone diameter} - \text{bacterial colony diameter})}{\text{bacterial colony diameter}}$$

Enzyme Production Characterisation

Characterization of amylase and cellulase production was carried out by incubation at various temperatures of 40°C, 45°C, 50°C, and 55°C for 48 hours. This characterisation aims to determine the optimal temperature of bacteria in producing thermophilic enzymes. Enzyme characterization was performed by preparing cellulase enzyme screening media, CMC Agar media, as described above. The bacterial isolates used in the experiment were selected based on the largest clear zone index values from each previous enzyme screening. Bacterial isolates TS-14 and TS-15 were inoculated from liquid media, with 5 µL each, onto paper discs placed on the enzyme characterization test media. The results were indicated by the presence of clear zones around the bacterial colonies.

DNA Extraction and *16S rRNA* Molecular Identification

The DNA extraction was performed following the Insta-Gene Protocol using Bio-Rad InstaGene Matrix kit (USA), for bacterial DNA extraction (Gray et al. 2014). The DNA concentration was checked using a nanodrop spectrophotometer (Thermo Fisher Scientific Nanodrop 2000 Spectrophotometer, USA). DNA was amplified using a PCR thermocycler (Labnet MultiGene OptiMax Thermal Cycler, USA). The amplification of genomic DNA was done using primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and primers 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') (Gislin et al. 2018). The PCR mastermix was 50 µL consisting of 25 µL MyTaq PCR Kit, 2 µL Forward primer, 2 µL Reverse primer, 19 µL ddH_2O, and 2 µL sample. The result of PCR was then electrophoresed (Mupid-EXu Electrophoresis, Japan) using a 1% Agarose Gel stained with FloroSafe Stain (1st BASE, Singapore) in 1X TAE Buffer for 30 min with a strength of 100 volts. The band of the DNA target was compared with the 1 KB DNA Marker (Promega, United States). The visualisation of the DNA bands uses Gel Documentation tool (UVITEC UVIDOC HD2, United Kingdom). The amplicon was then purified and continued with the sequencing process (Genetika Science, Jakarta). The sequencing outcomes were compared with the information available on GenBank using the Basic Local Alignment Search Tool (BLAST) program on the NCBI website (www.ncbi.nlm.nih.gov). This was done to acquire similar results for the sequences. After aligning the sequences, the phylogenetic tree was analyzed using the software Molecular Evolutionary Genetics Analysis 11 (MEGA 11). The phylogenetic tree was constructed using the Neighbor-Joining Tree and the Kimura-2 model parameters. To establish the connection between thermophilic bacteria and other bacterial species, this process was repeated 1000 times with bootstrap replication.

RESULTS AND DISCUSSION

Twenty-two thermophilic bacterial isolates of sediment samples from Nglimut Hot Spring were obtained on TA medium. Different macroscopic and microscopic characteristics are shown in Figure 2.

The positive results of the screening test conducted on the thermophilic bacterial isolates TS 1 - TS 22 were evidenced by the appearance of a clear zone around the colonies after iodine exposure. The clear zone indicated the presence of amylase activity (Table 1).

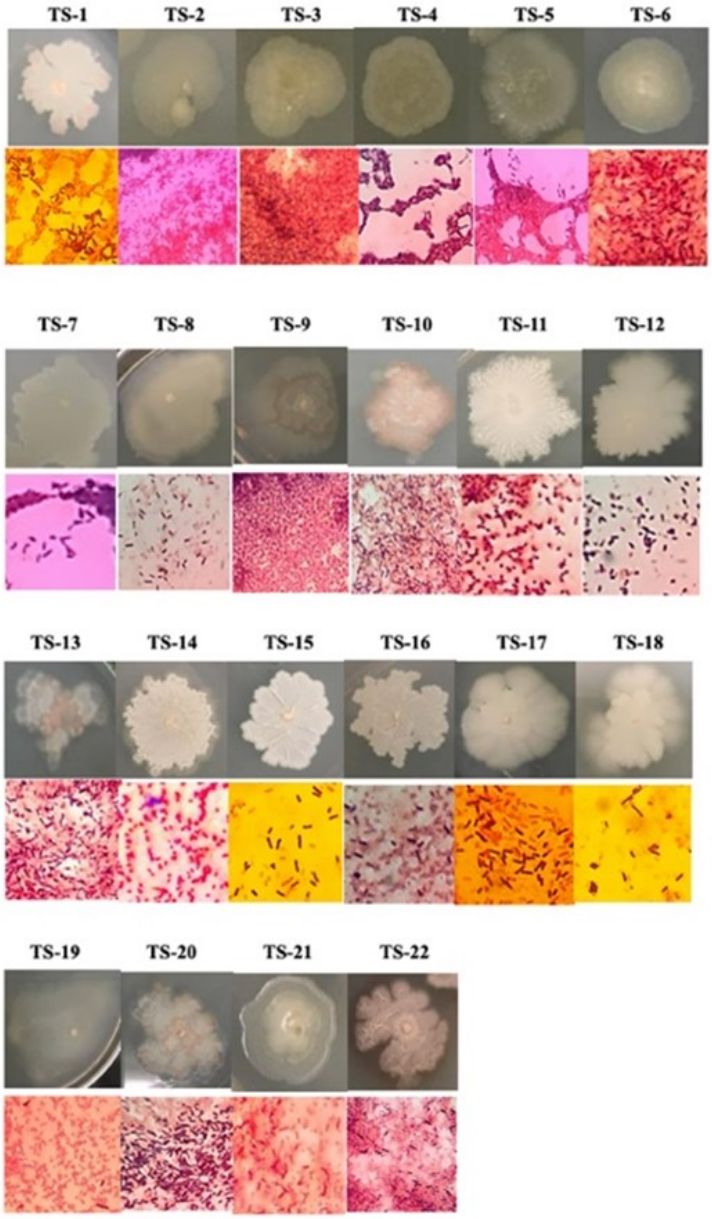


Figure 2. Macroscopic and Microscopic characterization of thermophilic bacterial isolates TS1-TS22 with 100X magnification.

Table 1 shows a compilation of 22 bacterial isolates sourced from hot spring deposits. Furthermore, 15 isolates were positive in producing amylase, while 7 isolates were positive for cellulase production. The clear zone formed indicates amylase activity in dismantling starch molecules in the growth medium. The appearance of a clear zone around the bacterial colony can be attributed to the disintegration of starch mediated by amylase, thus preventing the formation of complexes between starch and iodine (Octarya et al. 2011).

The amylolytic index can be calculated based on the diameter of the clear zone. This is in line with Zuraidah et al. (2020) and Mawati et al. (2021), stating that after incubation, the clear zone formed on each paper disc was observed and measured with a vernier caliper. TS 14 isolate had the highest average amylolytic index of 2.38. This indicates that the TS 14 isolate could produce higher amylase than the other isolates. The TS 14 isolate was further tested for its amylase activity by treating it with variations in temperature to determine the optimal cultivation temperature for the production of amylase. In addition, molecular identification was carried out on the TS 14 isolate to determine the species of the isolate.

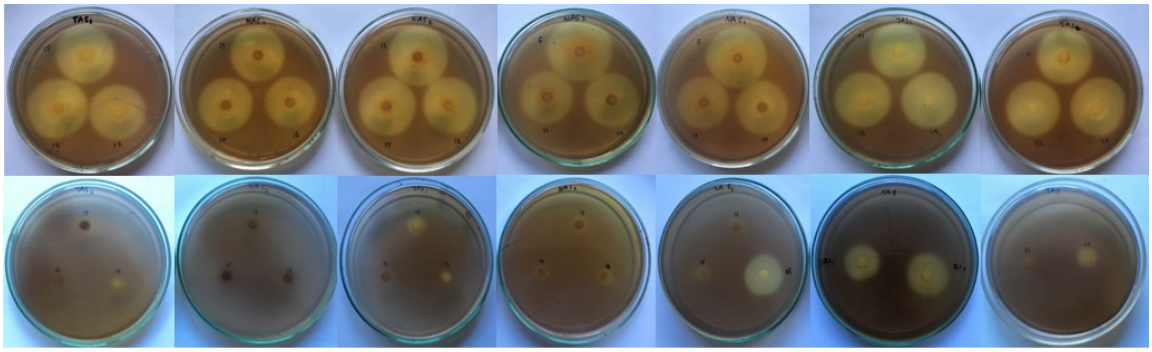
Table 1. Screening of Potential Amylase and Cellulase Production from TS1-TS22.

Code	Macroscopic and Microscopic Characterization							Enzyme Production	
	Colony Forming	Margin	Elevation	Color	Texture	Cell Forming	Gram	Amylase	Cellulase
TS-1	Irregular	Lobate	Flat	Pinkish white	Dry	Rod	+	+	-
TS-2	Round	Smooth	Flat	Yellowish white	Moist	Rod	-	-	-
TS-3	Irregular	Lobate	Flat	Yellowish white	Mucoid	Rod	+	-	-
TS-4	Irregular	Irregular	Flat	Yellowish white	Mucoid	Rod	+	-	-
TS-5	Irregular	Irregular	Flat	Clear white	Moist	Rod	+	-	-
TS-6	Round	Smooth	Umbonate	White	Mucoid	Rod	+	-	-
TS-7	Irregular	Irregular	Flat	White	Mucoid	Rod	+	-	-
TS-8	Irregular	Irregular	Flat	White	Dry	Rod	+	+	-
TS-9	Irregular	Irregular	Flat	Pinkish white	Mucoid	Rod	+	+	-
TS-10	Irregular	Irregular	Raised spreading edge	Pink	Mucoid	Rod	+	+	-
TS-11	Irregular	Irregular	Flat	Milky white	Dry	Coccus	+	+	+
TS-12	Irregular	Irregular	Flat	Milky white	Dry	Rod	+	+	+
TS-13	Irregular	Irregular	Raised spreading edge	Pinkish white	Mucoid	Rod	+	+	
TS-14	Irregular	Irregular	Raised spreading edge	Milky white	Dry	Coccus	-	+	+
TS-15	Irregular	Lobate	Raised spreading edge	Milky white	Dry	Rod	+	+	+
TS-16	Irregular	Irregular	Raised spreading edge	Milky white	Dry	Rod	+	-	+
TS-17	Irregular	Irregular	Flat	Milky white	Dry	Rod	+	+	+
TS-18	Irregular	Irregular	Flat	Milky white	Dry	Rod	+	+	-
TS-19	Irregular	Lobate	Flat	Milky white	Dry	Rod	+	+	-
TS-20	Irregular	Irregular	Flat	Milky white	Dry	Rod	+	+	-
TS-21	Irregular	Irregular	Flat	White	Dry	Rod	+	+	+
TS-22	Irregular	Irregular	Flat	Pinkish white	Mucoid	Rod	+	+	-

Figure 3 shows that a temperature of 40ºC was the best condition for treatment interaction. This is in line with Konsula (2004), and Fitriani (2013), stating that thermophilic *Bacillus* spp. produce extracellular thermostable *alpha*–amylase with an optimum growth temperature of 40ºC.

In the screening of thermophilic amylase, TS-14 obtained the highest index value of 2.37 at a temperature of 40°C, while temperatures of 45°C, 50°C, and 55°C were tested. Figure 3 illustrates the results of the screening of thermophilic amylase, with an index value of 1.62 at 45°C, 0.73 at 50°C, and no activity observed at 55°C. This is due to the nature of thermophilic amylases produced by microorganisms with a maximum temperature tolerance of 50°C (Mohammad et al. 2017).

All thermophilic bacteria isolates were tested for cellulase activity at 40ºC, 45ºC, 50ºC, and 55ºC. The results show that 7 isolates were capable of producing cellulase (Figure 3). Qualitative assessment of cellulase activity in thermophilic bacterial isolates is seen from the clear zone



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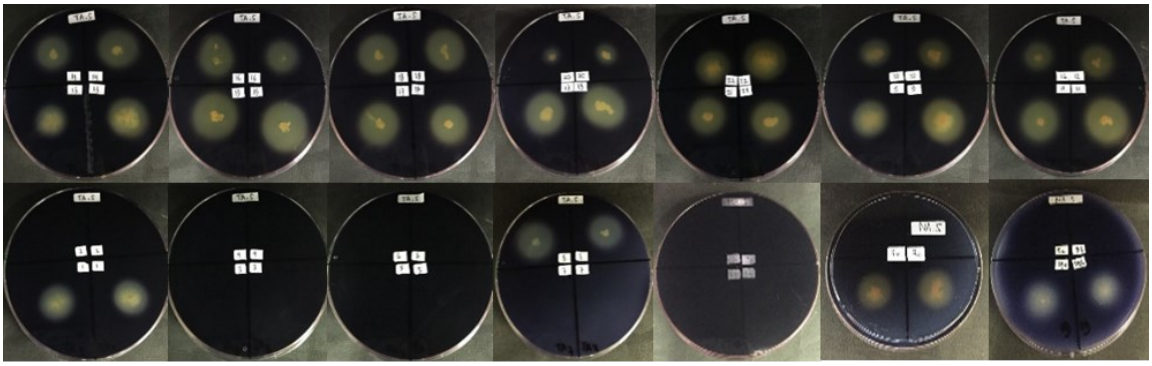

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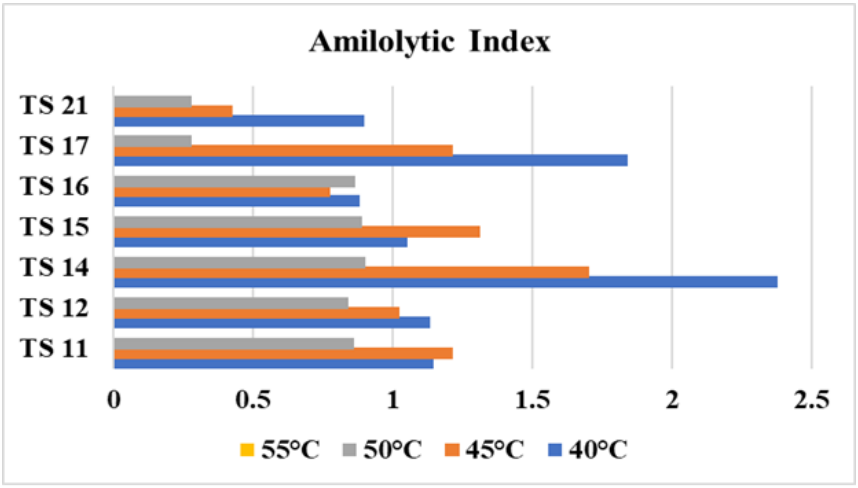

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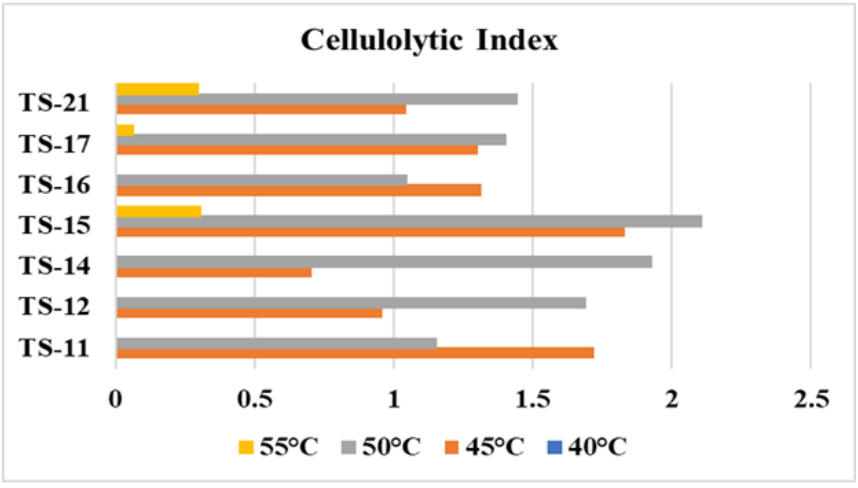

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Figure 3. Characterisation of cellulase production (**A**). Characterisation of amylase production (**B**). Amylolytic index based on difference temperature (**C**). Cellulolytic index based on difference temperature (**D**)

produced around bacterial colonies. The appearance of a clear zone around the colony on CMC media is the result of cellulose breakdown by bacteria that have cellulolytic abilities (Khalila et al. 2020).

The clear zone was then calculated with the cellulolytic index, and the results were averaged. The clear zone around the colony was measured to select the highest cellulase producer (Figure 3D) (Shaikh et al. 2013). The highest cellulolytic index of each temperature was isolate TS 15 with an index value of 1.83 at 45°C, 2.11 at 50°C, and 0.31 at 55°C. At 40°C, no enzyme activity is observed because this temperature is not within the optimal range for thermophilic microorganisms to generate cellulase. According to Gilter's classification, thermophilic microorganisms exhibit enzyme production at a minimum temperature of 45°C and a maximum temperature of 70°C (Akour 2019). This indicates that the TS 15 isolate was the best producer of cellulase enzymes and would be further identified molecularly.

Identification of the 16S rRNA gene was carried out using an amplification of the genomic DNA with primers 27F and 1492R primers. The 16S rRNA gene is part of the prokaryote genome, which has conserved parts and a hypervariable region that makes it valuable for the identification of bacterial species. The electrophoretic PCR product of the TS 14 and TS 15 isolate (Figure 2) shows an amplicon with a size of 1500 bp.

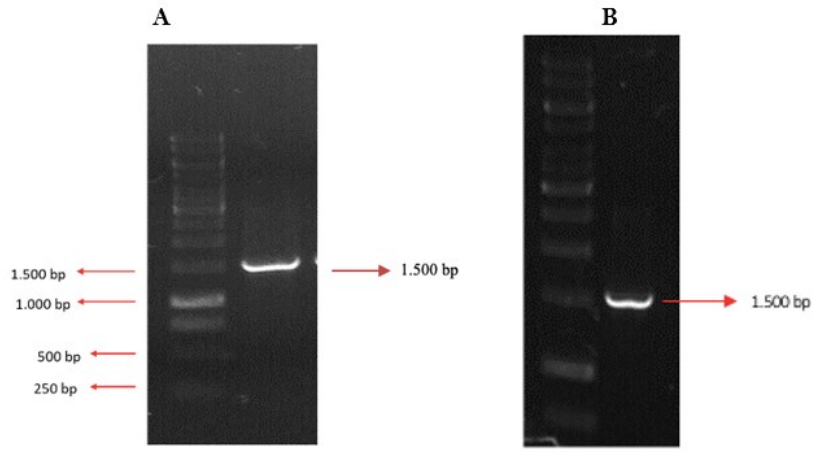

Figure 4. The gel electrophoresis result of the PCR-amplified product of the *16S rRNA* gene obtained from TS 14. (**A**), The gel electrophoresis findings for the PCR-generated product of the *16S rRNA* gene obtained from TS 15. (**B**)

The visualization of PCR amplification using Gel Electrophoresis showed white bands with a length of approximately 1500 bp depicted in Figure 4. The visible band on the doc gel indicates successful amplification. This is in line with Noer (2021), stating that the 16S rRNA gene sequence length is about 1500 bp and consists of conserved regions, relatively large genes, with interspecific polymorphisms to exhibit statistically valid measurement differences. The PCR products of both TS Sequencing results in the form of forward and reverse sequences were then edited using Bioedit software to become consensus sequences.

The phylogenetic tree of TS-14 and TS-15 isolates was made using MEGA X software, constructed using the Neighbor-joining tree method, and tested using the Bootstrap method with a value of 1,000 replications. According to Telle et al. (2011), bootstrap analysis is a method to test how well the model data set is. The bootstrap value is indicated by numbers next to the branches of the phylogenetic tree. A neighbor-joining tree is an approach used to construct a tree illustrating kinship relationships, relying on the nearness of evolutionary distances.

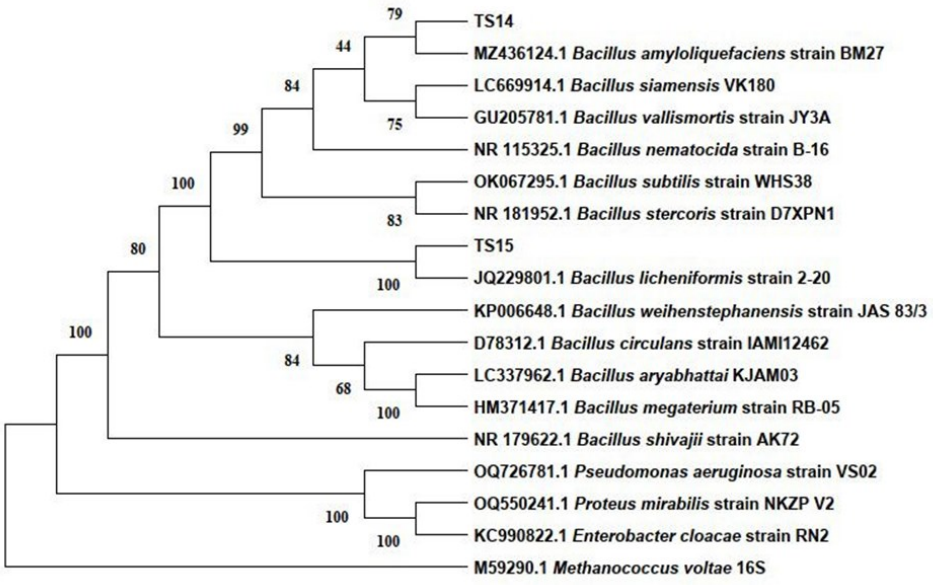

Figure 5. TS-14 and TS-15 isolate sequence phylogenetic tree.

The phylogenetic tree construction is shown in Figure 5. It shows that TS-14 is related to *Bacillus amyloliquefaciens* with a bootstrap value of 79%. The formation of amylase activity of TS-14 was influenced by the temperature, with an optimal value of 40°C. This was in accordance with the results of Ningsih et al. (2012), stating that the amylase enzyme produced by *B. amyloliquefaciens* has an optimum temperature of 30-60°C.

The TS-15 consensus sequences were matched against data in the Genbank on the BLAST program within the NCBI site. The results show that TS-15 isolates had a 100% similarity of its bootstrap value with *B. licheniformis*. A similarity percentage of 99% indicates that the query sequence with the database sequence is the same sequence and has similarities at the species level (Shofa et al. 2019). The bootstrap value shows close kinship if it has a high value, which is more than 70% (Widyadnyana et al. 2015). It has been commonly reported that cellulases can be produced by *B. licheniformis* at temperatures ranging from 30 to 60°C (Karim 2015). The genetic relationship is described by the value of the genetic distance, where the lower the genetic distance, the closer the genetic relationship (Butet el al. 2019).

CONCLUSION

The screening of thermophilic enzyme-producing bacteria from sediment of hot springs in Nglimut, Gonoharjo, Central Java resulted in two promising isolates. TS14, which has the highest potential of amylase formation, was molecularly identified as *B. amyloliquifaciens* and has an amylase index of 2.38 at 40°C. TS 15 exhibits the highest potential for manufacturing thermophilic cellulase enzyme with a cellulase index of 2.11 at 50°C and is molecularly identified as *B. licheniformis* species. This finding is the first attempt to screen and optimize the amylase and cellulase enzymes via qualitative assay. In the future, it will be exciting to test the potential isolates with other enzyme activity and also the quantitative assay to determine the specific activity of the enzyme.

AUTHORS CONTRIBUTION

A.B., N., and W.W. designed the study; J.S., R.S.M. and A.R.M. carried out the laboratory work; D.W., L.H., and L. analysed data and wrote the manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests that could have appeared to influence the work reported in this paper.

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