

Cellulolytic enzyme-producing thermophilic *Actinobacteria* isolated from the soil of Cisolok Geysers, West Java, Indonesia

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Abstract. *Setyaningsih PP, Ningsih F, Rachmania MK, Syafitri WA, Sari DCAF, Yabe S, Yokota A, Oetari A, Sjamsuridzal W. 2019. Cellulolytic enzyme-producing thermophilic Actinobacteria isolated from the soil of Cisolok Geysers, West Java, Indonesia. Biodiversitas 20: 3134-3141.* This study investigated 17 thermophilic *Actinobacteria* isolated from the soil of geysers in the Cisolok geothermal area, West Java, as potential producers of cellulase. Screening for cellulase was performed on minimal (Mm) agar medium with and without the addition of 1% (w/v) carboxymethylcellulose (CMC) and microcrystalline cellulose (MCC), then incubated at 45, 50, 55 and 60°C for up to 7 days. Formation of clear zones around colonies indicated cellulose hydrolysis. The results showed that 15, 14, 4, and 3 isolates showed cellulolytic activity on CMC agar medium at 45, 50, 55, and 60°C, respectively, after 7 days of incubation. Three potential isolates showed cellulolytic activity on MCC agar medium after being incubated for 7 days at 45°C. Molecular identification based on the 16S rRNA gene was performed for three isolates with positive cellulolytic activity at 60°C. The results showed that the three isolates are closely related to *Actinomadura keratinilytica* WCC-2265^T with 99.93-100% sequence similarities. A phylogenetic tree based on 16S rRNA gene sequences confirmed that the three isolates were clustered together with *Actinomadura keratinilytica* WCC-2265^T with 100% bootstrap value. The tree also showed that cellulase producers and non-cellulase producers in *Thermomonosporaceae* are grouped into different clades.

Keywords: Cellulase, Cisolok geothermal area, thermophilic *Actinobacteria*, soil

INTRODUCTION

Actinobacteria is one of the largest phyla within the domain *Bacteria* which comprises Gram-positive bacteria with high G+C content in their DNA. They are frequently found in soil due to their abilities in carbon cycling (Edwards 1993), and are mostly mesophilic (Satyanarayana et al. 2013). *Actinobacteria* can also be found in high-temperature habitats such as geothermal areas, deserts, and deep-sea hydrothermal vents (Satyanarayana et al. 2013), with growth temperatures, range from 40 to 80°C (Shivlata and Satyanarayana 2015). These thermophilic *Actinobacteria* has gained interest and are largely studied for their metabolites production, such as various antibiotics and industrial important thermostable enzyme (Hamedi and Wink 2017; Lewin et al. 2017).

Thermophilic *Actinobacteria* were reported to produced many important enzymes, such as amylases, cellulases, lipases, proteases, and xylanases. Studies on extracellular enzymes produced by thermophilic *Actinobacteria* have garnered interest for application in various industries due to their thermostability (Satyanarayana et al. 2013). The

advantages of thermostable enzymes from thermophiles include great stability, and enhanced activity in the presence of common protein denaturants (Matsui et al. 2000). They also reduce the risk of contamination (Haki and Rakshit 2003). Cellulases, as one of important enzymes, are widely used in several industries, such as textiles, pulps, and biofuels (Satyanarayana et al. 2013). Cellulases act upon cellulose by breaking the polysaccharide down into oligosaccharide and glucose (Rani et al. 2016).

Cellulose is one of the most abundant polysaccharides and is a major component of plants, composed of glucose units linked by β -1,4-glycosidic bonds (Sharma and Yazdani 2016). Thermostable cellulases are required to break the crystalline celluloses, such as biopolishing of cotton fabric in the textile industry because it is a more efficient process at high temperature (Ando et al. 2002). Cellulase-producing bacteria have more advantages than fungi e.g. better stability, shorter generation time, easy to cultivate using inexpensive carbon and nitrogen sources, and larger amount of enzymes secreted (Li et al. 2008). Additionally, several thermophilic *Actinobacteria* have been reported as cellulase producers, for example,

Thermomonospora cellulolytica YIM 77510^T and *T. amylolytica* YIM 77502^T (Jiao et al. 2015), *Actinomadura keratinilytica* T16-1 (Sukkhum et al. 2011), and *A. miaoliensis* TF1 (Sriyapai et al. 2018).

The studies on thermophilic *Actinobacteria* from extreme environments e.g. volcanic areas, hydrothermal areas, and geysers, are still either unexplored or less-explored which showed promising researches for exploring their potential as thermostable enzyme producers (Mehta and Satyanarayana 2013). The Cisolok geothermal area, as one of less-explored extreme environments in West Java, is one of the potential habitats which has high microbial diversity with *Proteobacteria* and *Cyanobacteria* as major abundant phyla (Seo et al. 2012). Previous studies by Yokota et al. (2016) and Mawarid et al. (2016) reported that thermophilic bacteria identified as *Paenibacillus cisolokensis* and *Brevibacillus* sp., respectively, isolated from the litter of Cisolok geothermal area, which showed positive results for cellulase enzyme activity at temperature of 50°C.

Previous study by Sjamsuridzal et al. (unpublished data), successfully obtained 17 thermophilic *Actinobacteria* isolates from soil in Cisolok geothermal area. Syafitri et al. (2019) reported that all of these *Actinobacteria* isolates could grow at 45°C, and 15 of the 17 isolates showed amylolytic activity at 45°C. According to Shivilata and Satyanarayana (2015), thermophilic *Actinobacteria* are able to grow in temperatures ranging from 40 to 80°C. A similar observation was reported by Kumar et al. (2014), where thermophilic bacteria isolated from the Manikaran hot springs in India had growth in a temperature range of 45 to 70°C. However, until recently, the reports regarding cellulose-degrading thermophilic *Actinobacteria* from soil in Cisolok geothermal area, has not been described yet. The aims of this study were to investigate the cellulolytic activity of thermophilic *Actinobacteria* isolated from soil in the Cisolok geothermal area and to identify potential thermophilic *Actinobacteria* that show cellulolytic activity at high temperatures.

MATERIALS AND METHODS

Source of isolates

The 17 isolates used in this study were obtained from five soil samples taken from the Cisolok geysers at three sampling locations. Seven isolates (SL1-1-R-2, SL1-1-R-4, SL1-1-R-7, SL1-1-R-8, SL1-2-R-2, SL1-2-R-3, and SL1-2-R-4) were obtained from soil around a big geyser (6°57'221"S, 106°27'507"E), three isolates (SL2-2-R-1, SL2-2-R-12, and SL2-2-R-15) were obtained from soil around a small geyser (6°57'189"S, 106°27'365"E), and seven isolates (SL3-1-R-14, SL3-1-R-16, SL3-2-R-5, SL3-2-R-17, SL3-2-R-18, SL3-2-R-33, and SL3-2-R-37) were obtained from forest soil near a geyser (6°57'482"S, 106°28'655"E). Thermophilic *Actinobacteria* were isolated according to Yabe et al. (2017) and Ningsih et al. (2019) with some modifications. The isolation was performed using 1% International *Streptomyces* Project (ISP) 1 agar medium incubated at 45°C for 3 to 4 weeks.

Pure isolates were grown on ISP 1 agar medium at

45°C for 7 days. All of the isolates were preserved as agar block in 20% (v/v) glycerol solution at - 80°C and as lyophilized cells using the L-drying method for long-term preservation (Ningsih et al. 2019). All isolates are deposited in the Universitas Indonesia Culture Collection (UICC), Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, in Depok, Indonesia.

Cellulolytic activity assay

Screening of cellulolytic activity of 17 thermophilic *Actinobacteria* isolates was performed on minimal (Mm) agar with the addition of 1% (w/v) carboxymethyl cellulose (CMC) as a substrate and incubated at 45, 50, 55, and 60°C for up to 7 days. The cellulolytic activity was observed after 3 and 7 days of incubation by staining the plates with 0.2% (w/v) Congo red and then rinsing twice with 1 M NaCl solution. The formation of a clear zone around a bacterial colony indicated the hydrolysis of cellulose (Meddeb-Mouelhi et al. 2014). The experiment was carried out in triplicate. The potential isolates with positive cellulolytic activity up to 60°C were subjected to cellulolytic activity assay on Mm agar with the addition of 1% (w/v) microcrystalline cellulose (MCC) as a substrate and incubated at 45°C for 7 days. The enzyme activity was observed by staining the plates with 0.2% (w/v) Congo red and washing twice with 1 M NaCl solution. A clear zone formation around the bacterial colony would indicate the enzyme activity.

Molecular identification based on 16S rRNA gene sequence

The isolates were grown in ISP 1 broth medium for 7 days at 45°C and mycelium was harvested by centrifugation. Genomic DNA was extracted using a Genomic DNA Mini Kit (Blood/Cultured Cell) (Geneaid, New Taipei City, Taipei) according to the manufacturer's protocol. The 16S rRNA gene was amplified using MyTaq Red Mix (Bioline, London) with two universal primers: 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1510R (5'-GGCTACCTTGTTACGA-3') (Weisburg et al. 1991). The PCR reactions were carried out as follows: initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 15 sec, annealing at 56°C for 15 sec and extension at 72°C for 10 sec. The PCR products were sequenced using four universal eubacterial primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3'; *Escherichia coli* positions 8-27), 785F (5'-GGATTAGATACCCTGGTA-3'), 800R (5'-TACCAGGGTATCTAATCC-3'), and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'; *E. coli* positions 1492-1510) (Weisburg et al. 1991; Jin et al. 2015) by 1st BASE Sequencing Service, Malaysia (<https://base-asia.com/dna-sequencing-services.com>). Sequences obtained in this study were analyzed using the software program ChromasPro V.2.1.8. (Technelysium, Tewantin, Australia) and then compared with sequence on the EzBioCloud Database (<https://www.ezbiocloud.net/>) (Yoon et al. 2017). A phylogenetic tree was constructed according to the neighbor-joining method used by Saitou and Nei (1987) with the MEGA 7.0 program (Kumar et al. 2016).

Distance matrices for the aligned sequences were calculated by the two-parameter method used by Kimura (1980). The robustness of individual branches was estimated by the bootstrapping of 1,000 replications (Felsenstein 1985).

RESULTS AND DISCUSSION

Cellulolytic activity assay

Screening for cellulolytic activity was performed by incubating the isolates at 45, 50, 55, and 60°C on Mm agar medium with CMC as substrate. The results of the enzyme screening are shown in Table 1. In total, 15 isolates were able to hydrolyze CMC after being incubated for 3 days at 45°C. Fourteen out of 17 isolates showed cellulolytic activity on a CMC agar medium after being incubated for 3 to 7 days at 50°C. Only four isolates showed CMC hydrolysis after being incubated for 3 to 7 days at 55°C. A cellulase assay was conducted for four isolates with positive cellulolytic activity at 55°C and then at 60°C. After being incubated for up to 7 days, only three isolates (SL1-2-R-2, SL1-2-R-3, and SL1-2-R-4) showed cellulolytic activity on CMC agar medium at 60°C. Cellulolytic activity on the CMC agar medium was detected by the formation of a clear zone on the plates, as seen in Figure 1.

Isolates that showed cellulolytic activity on the CMC agar medium were able to hydrolyze β -1,4-glycosidic linkage in cellulose to a monomeric unit of glucose. Cellulase belongs to the glycoside hydrolases that are able to hydrolyze glycoside bonds (Satyanarayana et al. 2013). The cellulase system has three major groups based on their catalytic action: endoglucanase, exoglucanase, and β -glucosidase. Therefore, using a different substrate might give different cellulolytic activity results (Lynd et al. 2002). Endoglucanase cleaves at random internal

amorphous regions of the cellulose polysaccharide chain and releases oligosaccharides and new chain ends (Lynd et al. 2002). Carboxymethyl cellulose (CMC) is soluble cellulose derivate that is used as a standard substrate for endoglucanase production because of its amorphous cellulose form (Tang et al. 2011). Amorphous noncrystalline regions are attacked in the initial stages of cellulose breakdown because of their accessibility, which makes them more easily hydrolyzed (Sharma and Yazdani 2016). The ability to hydrolyze CMC showed that the isolates were able to produce endoglucanase. Detection of endoglucanase activity was performed by staining on agar plate with dyes. Residual long chains of polysaccharide on an agar plate will adsorb the dyes, therefore, the part of the plate with hydrolyzed polysaccharides will show a clear zone as the activity of endoglucanase (Tang et al. 2011).

Bacterial isolate SL2-2-R-15 showed growth on the CMC agar medium at 45 to 55°C while SL3-1-R-16 showed growth at 45 to 50°C. However, these isolates did not show cellulolytic activity after being incubated on the CMC agar medium for up to 7 days. The result showed that these two isolates (SL2-2-R-15 and SL3-1-R-16) were able to grow on the CMC agar medium but not able to hydrolyze CMC as a substrate. Carboxymethyl cellulose (CMC) is generally used for endoglucanase production, and some organisms cannot hydrolyze CMC (Lynd et al. 2002). Although the isolates were not able to use CMC as a substrate, they were still able to grow on CMC agar medium because the assay medium consisted of yeast extract and agar. Yeast extract contains amino acids, peptides, water-soluble vitamins, and carbohydrates that can be used as nutrient sources for microorganisms (Costa et al. 2002). Agar used as a bacteriological media consists of two polysaccharides, agarose, and agaropectin (Suzuki et al. 2003). Some microorganisms are able to hydrolyzed agar by producing agarase and metabolize it as carbon and energy source (Chi et al. 2012).

Table 1. Cellulolytic activity of 17 *Actinobacteria* isolates after 3 and 7 days of incubation at 45, 50, 55, and 60°C

Isolate code	CMC						MCC		
	45°C		50°C		55°C		60°C		45°C
	3d	7d	3d	7d	3d	7d	3d	7d	7d
SL1-1-R-2	+	+	+	+	*	*	n/a	n/a	n/a
SL1-1-R-4	+	+	+	+	*	*	n/a	n/a	n/a
SL1-1-R-7	+	+	+	+	*	*	n/a	n/a	n/a
SL1-1-R-8	+	+	+	+	*	*	n/a	n/a	n/a
SL1-2-R-2	+	+	+	+	+	+	n/a	+	+
SL1-2-R-3	+	+	+	+	+	+	n/a	+	+
SL1-2-R-4	+	+	+	+	+	+	n/a	+	+
SL2-2-R-1	+	+	+	+	+	+	*	*	n/a
SL2-2-R-12	+	+	+	+	*	*	n/a	n/a	n/a
SL2-2-R-15	-	-	-	-	-	-	n/a	n/a	n/a
SL3-1-R-14	+	+	*	+	*	*	n/a	n/a	n/a
SL3-1-R-16	-	-	-	-	*	*	n/a	n/a	n/a
SL3-2-R-5	+	+	+	+	*	*	n/a	n/a	n/a
SL3-2-R-17	+	+	+	+	*	*	n/a	n/a	n/a
SL3-2-R-18	+	+	*	*	*	*	n/a	n/a	n/a
SL3-2-R-33	+	+	+	+	*	*	n/a	n/a	n/a
SL3-2-R-37	+	+	+	+	*	*	n/a	n/a	n/a
Total positive cellulolytic activity	15	15	13	14	4	4	n/a	3	3

Note: (+): positive cellulolytic activity; (-): negative cellulolytic activity; (*): no growth; (n/a): data not available

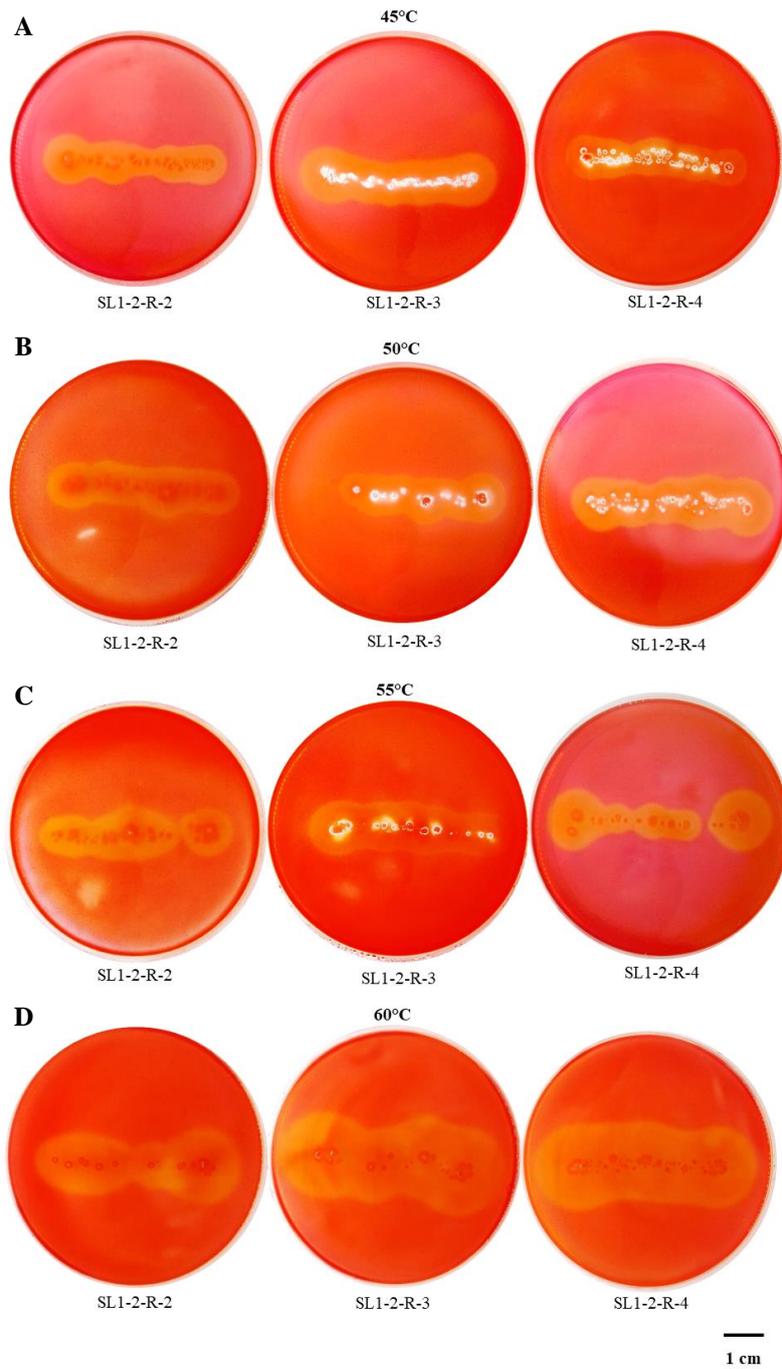


Figure 1. Isolates with positive cellulolytic activity on CMC agar medium after 3 days of incubation at: A. 45°C, B. 50°C, C. 55°C, D. After 7 days of incubation at 60°C

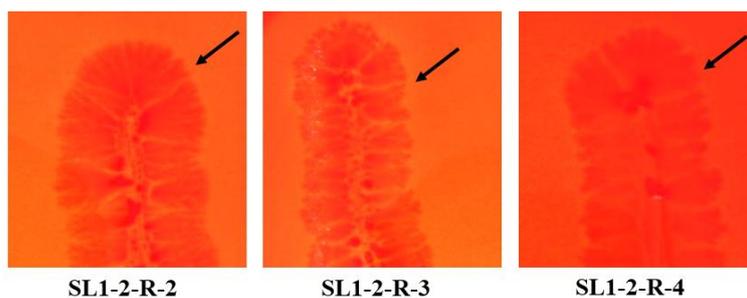


Figure 2. Positive result of cellulolytic activity on MCC medium after 7 days of incubation at 45°C

Screening for cellulolytic activity on MCC agar medium was performed for the three potential isolates (SL1-2-R-2, SL1-2-R-3, and SL1-2-R-4) with positive cellulolytic activity on CMC agar medium at 60°C. The results showed that the three isolates were able to grow and show cellulolytic activity on MCC agar medium at a temperature of 45°C after being incubated for 7 days. Clear zones were weakly detected on MCC agar medium as seen in Figure 2. The result of this study showed that three isolates were also able to produce exoglucanase. Microcrystalline cellulose (MCC) was used to detect the ability of isolates to produce exoglucanase (Lynd et al. 2002). Exoglucanase acts on the reducing or nonreducing ends of the cellulose polysaccharide chain and releases cellobiose as a product. Exoglucanase presumably acts by peeling cellulose chain from the microcrystalline structure. Cellulose has two regions, amorphous and crystalline, where amorphous regions are more accessible to enzyme degradation, while crystalline cellulose molecules have constituent molecules of individual microfibrils that are packed tightly. Thus, they are recalcitrant to be hydrolyzed by enzymes (Sharma and Yazdani 2016). The hydrolysis of MCC needs the synergism of endoglucanase and exoglucanase, which is called exo-endo synergy. Exo-endo synergy leads endoglucanase to release cellulose chains with new ends and thus becomes available to be attacked by exoglucanase (Bettache et al. 2018).

This study showed that three thermophilic *Actinobacteria* isolates (SL1-2-R-2, SL1-2-R-3, and SL1-2-R-4) were able to show cellulolytic activity on CMC and MCC agar media which means these isolates have the ability to produce endoglucanase and exoglucanase (Tang et al. 2011). Previous study reported that two *Streptomyces* strains, M7a and M23, isolated from forest soil were able to produce complete cellulase systems, including endoglucanases, exoglucanases, and β -glucosidases, to degrade cellulose to monomer glucose (Semêdo et al. 2000). Several factors that affect the growth and enzyme production of thermophilic *Actinobacteria* are temperature, pH, and nutrients. According to Al-Tai et al. (1989) and Manivasagan et al. (2010), *Actinobacteria* have the ability to show cellulolytic activity in a pH range from 4 to 9. In another study, Jiao et al. (2015) isolated and identified *Thermomonospora cellulositytica* YIM 77510^T and *T. amylolytica* YIM 77502^T from geothermally heated soil in Tengchong county, China, that were able to produce cellulase and grow at 25 to 55°C with the optimum growth temperature at 45°C. Several studies reported that growth and cellulases production of *Actinobacteria* such as *Thermomonospora curvata* are promoted by the addition of yeast extract in the medium as a nitrogen source (Semêdo et al. 2000).

Molecular identification based on 16S rRNA gene sequences

Molecular identification based on 16S rRNA gene sequences was performed for three isolates with cellulolytic activity at 60°C. All three isolates (SL1-2-R-2, SL1-2-R-3, and SL1-2-R-4) are closely related to *Actinomadura keratinilytica* WCC-2665^T, with 100% (SL1-2-R-2 and SL1-2-R-4) and 99.93% (SL1-2-R-3) similarities. *Actinomadura keratinilytica* WCC-2665^T is a thermophilic *Actinobacteria* in the family *Thermomonosporaceae* with growth temperature ranges from 30 to 55°C; however, there is still no information about the cellulolytic activity of *A. keratinilytica* from type strain WCC-2665^T (Puhl et al. 2009). Sukkhum et al. (2011) identified *A. keratinilytica* strain T16-1 isolated from soil in Huai Kha Khaeng Wildlife Sanctuary, Uthaitani province, Thailand which was able to degrade cellulose and grow at temperatures of 30 to 60°C.

A phylogenetic tree analysis of the three isolates along with other species from the genus *Actinomadura* and other genera in the family *Thermomonosporaceae*, showed that most of the species are thermophilic *Actinobacteria* (see Table 2). The members of the family *Thermomonosporaceae* are mainly found in soil, and some of them have been isolated from compost. Some genera of *Thermomonosporaceae* are also known as enzyme producers such as *Actinomadura gamaensis* NEAU-Gz5^T (cellulase; Abagana et al. 2016), *Actinomadura kijaniata* NBRC 14229^T (amylase, xylanase, urease; Horan and Brodsky 1982), *Thermomonospora curvata* DSM 43183^T (CMCase, xylanase; Goodfellow and Trujillo 2012), and *Actinocorallia herbida* ATCC 51528^T (amylase; Inuma et al. 1994). The phylogenetic tree showed that the genera *Actinomadura* are paraphyletic in the family *Thermomonosporaceae*. *Actinomadura* appears in the same clade as the genera *Spirillospora* and *Actinocorallia* (Figure 3). This finding is similar to a previous report of Nouioui et al. (2018), showing that the type genus of *Thermomonosporaceae* appears to be paraphyletic and that some *Actinomadura* species showed an uncertain position within *Thermomonosporaceae*. The phylogenetic tree also showed that cellulase producers and non-cellulase producers in *Thermomonosporaceae* are grouped into different clades. Based on an alignment comparison of sequence position in the 16S rRNA gene, there are two sites that appear to determine the difference between the cellulase and non-cellulase producers. The differences were located in *A. keratinilytica* WCC-2265^T at positions 976 and 1011, which were determined to be cytosine and guanine, respectively, in the 16S rRNA gene analysis of the cellulase producers clade. In the 16S rRNA gene of the non-cellulase producer's clade, sites 976 and 1011 were determined to be guanine and cytosine, respectively.

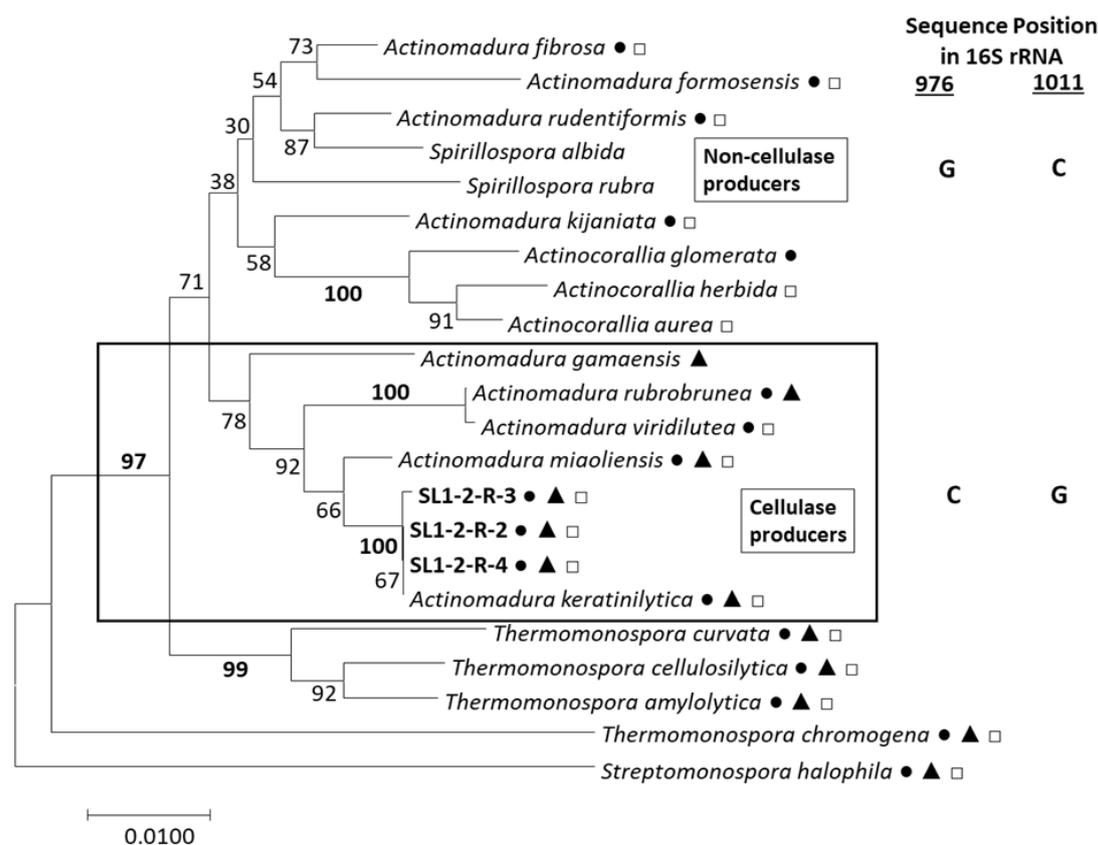


Figure 3. Bootstrapped neighbor-joining tree, inferred from 1272 aligned sites of 16S rRNA gene sequence, showing phylogenetic relationships among three isolates used in this study and closely-related taxa in the family *Thermomonosporaceae*. *Streptomonospora halophila* YIM 9155^T was used as an outgroup. Bootstrap values at branching points are expressed as percentages from 1000 replications. The scale bar indicates 1 base change per 100 nucleotide positions. Bracketed group assigned to the thermophilic cellulase producers lineage. Note: (●): thermophilic; (▲): cellulase producer; (□): other enzymes producer

Table 2. List of Operational Taxonomic Units (OTUs) used in the phylogenetic analysis

Species	Strain	DNA database accession no.	Growth range temperature	Source	Reference
<i>Actinomadura keratinilytica</i>	SL1-2-R-2	LC484202	25-60°C	Soil	This study
<i>Actinomadura keratinilytica</i>	SL1-2-R-3	LC484203	25-60°C	Soil	This study
<i>Actinomadura keratinilytica</i>	SL1-2-R-4	LC484204	25-60°C	Soil	This study
<i>Actinomadura keratinilytica</i>	WCC-2265 ^T	EU637009	30-55°C	Compost	Puhl et al. (2009)
	T16-1	-	30-60°C	Soil	Sukkhum et al. (2011)
<i>Actinomadura miaoliensis</i>	BC 44T-5 ^T	EF116925	25-55°C	Compost	Tseng et al. (2009)
	TF1	-	40-60°C	soil	Sriyapai et al. (2018)
<i>Actinomadura rubrobrunea</i>	NBRC 15275 ^T	BCQU01 000204	37-65°C	Soil	Goodfellow and Trujillo (2012)
<i>Actinomadura viridilutea</i>	IFO14480 ^T	D86943	37-65°C	Desert soil	Goodfellow and Trujillo (2012)
<i>Actinomadura gamaensis</i>	NEAU-Gz5 ^T	KT989505	15-40°C	Soil	Abagana et al. (2016)
<i>Actinomadura formosensis</i>	NBRC 14204 ^T	AJ293703	23-41°C	Soil	Goodfellow and Trujillo (2012)
<i>Actinomadura kijaniata</i>	NBRC 14229 ^T	BCQR01 000335	28-50°C	Soil	Horan and Brodsky (1982)
<i>Actinomadura fibrosa</i>	ATCC 49459 ^T	AF163114	20-45°C	Soil	Goodfellow and Trujillo (2012)
<i>Actinomadura rudentiformis</i>	HMC1 ^T	DQ285420	30-45°C	Soil	Goodfellow and Trujillo (2012)
<i>Thermomonospora cellulosilytica</i>	YIM 77510 ^T	AB859254	25-55°C	Soil	Jiao et al. (2015)
<i>Thermomonospora amylolytica</i>	YIM 77502 ^T	AB859253	25-55°C	Soil	Jiao et al. (2015)
<i>Thermomonospora curvata</i>	DSM 43183 ^T	AF002262	30-53°C	Compost	Goodfellow and Trujillo (2012)
<i>Thermomonospora chromogena</i>	ATCC 43196 ^T	AF116558	40-53°C	Compost	Goodfellow and Trujillo (2012)
<i>Actinocorallia herbida</i>	ATCC 51528 ^T	D85473	12-38°C	Soil	Iinuma et al. (1994)
<i>Actinocorallia aurea</i>	DSM 44434 ^T	AB006177	20-30; 37°C	Soil	Tamura et al. (2007)
<i>Actinocorallia glomerata</i>	NBRC 15960 ^T	AJ293704	7-41°C	Compost	Goodfellow and Trujillo (2012)
<i>Spirillospora albida</i>	ATCC 15331 ^T	D85498	18-35°C	Soil	Goodfellow and Trujillo (2012)
<i>Spirillospora rubra</i>	JCM 6875 ^T	AF163123	20-37°C	Soil	Goodfellow and Trujillo (2012)

In conclusion, *Actinobacteria* isolated from soils in the Cisolok geothermal areas are thermophiles. All of the isolates were able to grow at temperatures ranging from 45 to 60°C. The thermophilic *Actinobacteria* isolates are also potential producers of the cellulase enzyme as demonstrated by CMC hydrolysis at temperatures of 45°C by 15 isolates, 50°C by 14 isolates, 55°C by four isolates, and 60°C by three isolates, and MCC hydrolysis at temperature of 45°C by three isolates. The three isolates (SL1-2-R-2, SL1-2-R-3, and SL1-2-R-4) that showed positive cellulolytic activity were able to hydrolyze both CMC and MCC as substrates. This result indicates that isolates from soil of geothermal area could produce cellulase at high temperatures. The molecular identification based on 16S rRNA gene sequences showed that three isolates with positive cellulolytic activity at 60°C are closely related to *Actinomadura keratinilytica* from family *Thermomonosporaceae* that is known as thermophiles and enzymes producer. Further studies are needed, especially for confirmation of thermostable cellulase production from these isolates.

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REFERENCES

- Abagana AY, Sun P, Liu C, Cao T, Zheng W, Zhao S, Xiang W, Wang X. 2016. *Actinomadura gamaensis* sp. nov., a novel actinomycete isolated from soil in Gama, Chad. *Antonie Leeuwenhoek* 109 (6): 833-839.
- Al-Tai AM, Abdul-Razzak S, Al-Attayah SS, Abdul-Nour BA. 1989. Cellulase production from Actinomycetes isolated from Iraqi soils: II. Cell growth and cellulase activity of *Streptomyces* sp. strain AT7 different temperatures. *J Islamic Acad Sci* 2 (3): 185-188.
- Ando S, Ishida H, Kosugi Y, Ishikawa K. 2002. Hyperthermostable endoglucanase from *Pyrococcus horikoshii*. *Appl Environ Microbiol* 68 (1): 430-433.
- Bettache A, Azzouz Z, Boucherba N, Bouiche C, Hamma S. 2018. Lignocellulosic biomass and cellulolytic enzymes of *Actinobacteria*. *SAJ Biotechnol* 5: 1-9.
- Chi WJ, Chang YK, Hong SK. 2012. Agar degradation by microorganisms and agar-degrading enzymes. *Appl Microbiol Biotechnol* 94 (4): 917-930.
- Costa E, Teixidó N, Usall J, Atarés E, Viñas I. 2002. The effect of nitrogen and carbon sources on growth of the biocontrol agent *Pantoea agglomerans* strain CPA-2. *Lett Appl Microbiol* 35 (2): 117-120.
- Edwards C. 1993. Isolation properties and potential applications of thermophilic actinomycetes. *Appl Biochem Biotechnol* 42: 161-179.
- Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39 (4): 783-791.
- Goodfellow M, Trujillo ME. 2012. Family III. Thermomonosporaceae Rainey, Ward-Rainey and Stackebrandt 1997, 486 emend. Zhang, Wang and Ruan 2001, 381 emend. Zhi, Li and Stackebrandt 2009, 600. In: Goodfellow M, Kämpfer P, Busse H-J, Trujillo ME, Suzuki K, Ludwig W, Whitman WB. (eds) *Bergey's Manual of Systematic Bacteriology*, 2nd Edition. Springer, New York.
- Haki, GD, Rakshit SK. 2003. Developments in industrially important thermostable enzymes: a review. *Biores Technol* 89: 17-34.
- Hamed J, Wink J. 2017. Introduction. In: Wink J, Mohammadpanah, Hamed J (eds) *Biology and Biotechnology of Actinobacteria*. Springer, Gewerbestrasse.
- Horan AC, Brodsky BC. 1982. A novel antibiotic-producing *Actinomadura*, *Actinomadura kijaniata* sp. nov. *Intl J Syst Evol Microbiol* 32 (2): 195-200.
- Iinuma S, Yokota A, Hasegawa T, Kanamaru T. 1994. *Actinocorallia* gen. nov., a new genus of the order *Actinomycetales*. *Intl J Syst Bacteriol* 44 (2): 230-234.
- Jiao J-Y, Liu L, Zhou E-M, Wei D-Q, Ming H, Xian W-D, Yuan C-G, Zhong J-M, Li W-J. 2015. *Actinomadura amyolytica* sp. nov. and *Actinomadura cellulositytica* sp. nov., isolated from geothermally heated soil. *Antonie Leeuwenhoek* 108: 75-83.
- Jin L, Lee HG, Ko SR, Ahn CY, Oh HM. 2015. *Jatrophilhabitans fulvus* sp. nov., an actinobacterium isolated from grass soil. *Intl J Syst Evol Microbiol* 65 (10): 3476-3480.
- Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 16 (2): 111-120.
- Kumar M, Yadav AN, Tiwari R, Prasanna R, Saxena AK. 2014. Deciphering the diversity of culturable thermotolerant bacteria from Manikaran hot springs. *Ann Microbiol* 64: 741-751
- Kumar S, Stecher G, Tamura T. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol Biol Evol* 33 (7): 1870-1874.
- Lewin GR, Carlos C, Chevrette MG, Horn HA, McDonald BR, Stankey RJ, Fox BG, Currie CR. 2017. Evolution and ecology of *Actinobacteria* and their bioenergy applications. *Annu Rev Microbiol* 70: 235-254.
- Li W, Zhang WW, Yang MM, Chen YL. 2008. Cloning of the thermostable cellulase gene from newly isolated *Bacillus subtilis* and its expression in *Escherichia coli*. *Mol Biotechnol* 40: 195-201.
- Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS. 2002. Microbial cellulose utilization: fundamentals and biotechnology. *Microbiol Mol Biol Rev* 66 (3): 506-577.
- Manivasagan P, Gnanam S, Sivakumar K, Thangaradjou T, Vijayalakshmi S, Balasubramanian T. 2010. Isolation, identification, and characterization of multiple enzyme-producing *Actinobacteria* from sediment samples in Kodyakarakai coast, the Bay of Bengal. *Afr J Microbiol Res* 4 (14): 1550-1559.
- Matsui I, Sakai Y, Matsui E, Kikuchi H, Kawarabayasi Y, Honda K. 2000. Novel substrate specificity of a membrane-bound β -glycosidase from the hyperthermophilic archaeon *Pyrococcus horikoshii*. *FEBS Lett* 467: 195-200.
- Mawarid AT, Sjamsuridzal W, Yabe S, Santoso I, Ningsih F, Nurlaili DG, Oetari A, Yokota A. 2016. Polyphasic identification of a thermophilic bacterium from geyser of Cisolok, Indonesia. *AIP Conf Proc* 1729: 020067. DOI: 10.1063/1.4946970.
- Meddeb-Mouelhi F, Moisan JK, Beauregard M. 2014. A comparison of plate assay methods for detecting extracellular cellulase and xylanase activity. *Enzyme Microb Technol* 66: 16-19.
- Mehta D, Satyanarayana T. 2013. Diversity of hot environments and thermophilic microbes. In: Satyanarayana T, Littlechild J, Kawarabayasi Y (eds.). *Thermophilic Microbes in Environmental and Industrial Biotechnology*. Springer, Berlin.
- Ningsih F, Yokota A, Sakai Y, Nanatani K, Yabe S, Oetari A, Sjamsuridzal W. 2019. *Gandjariella thermophila* gen. nov., sp. nov., a new member of the family *Pseudonocardiaceae*, isolated from forest soil in a geothermal area. *Intl J Syst Evol Microbiol*. DOI 10.1099/ijsem.0.003594
- Nouioui I, Carro L, García-López M, Meier-Kolthoff J, Woyke T, Kyrpidis NC, Pukall R, Klenk H-P, Goodfellow M, Göker M. 2018. Genome-based taxonomic classification of the phylum *Actinobacteria*. *Front Microbiol* 9.
- Puhl AA, Selinger LB, McAllister TA, Inglis GD. 2009. *Actinomadura keratinilytica* sp. nov., a keratin-degrading actinobacterium isolated from bovine manure compost. *Intl J Syst Evol Microbiol* 59 (4): 828-834.
- Rani ME, Kumar NV, Gunaseeli R, Kannan ND. 2016. Comparative biochemistry and kinetics of microbial cellulase. In: Gupta VK (ed.) *New and Future Developments in Microbial Biotechnology and Bioengineering: Microbial Cellulase System Properties and Applications*. Elsevier, Amsterdam.

- Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4 (4): 406-425.
- Satyanarayana T, Littlechild J, Kawarabayasi Y. 2013. *Thermophilic Microbes in Environmental and Industrial Biotechnology: Biotechnology of Thermophiles*, 2nd ed. Springer, New York.
- Semêdo LTAS, Gomes RC, Bon EPS, Soares RMA, Linhares LF, Coelho RRR. 2000. Endocellulase and exocellulase activities of two *Streptomyces* strains isolated from a forest soil. *Appl Biochem Biotechnol* 84-86: 267-276.
- Seo MJ, Kim JN, Pyun YR. 2012. 16S rRNA gene sequence-based microbial diversity analyses of the geothermal areas of Cisolok, Kamojang, and Likupang in Indonesia. *Korean J Microbiol Biotechnol* 40: 268-273.
- Sharma S, Yazdani SS. 2016. Diversity of microbial cellulase system. In: Gupta VK (ed.) *New and Future Developments in Microbial Biotechnology and Bioengineering: Microbial Cellulase System Properties and Applications*. Elsevier, Amsterdam.
- Shivlata L, Satyanarayana T. 2015. Thermophilic and alkaliphilic *Actinobacteria*: biology and potential applications. *Front Microbiol* 6: 1014.
- Sriyapai P, Chansiri K, Sriyapai T. 2018. Isolation and characterization of polyester-based plastics-degrading bacteria from compost soils. *Microbiology* 87 (2): 290-300.
- Sukkhum S, Tokuyama S, Kongsaree, Tamura T, Ishida Y, Kitpreechavanich V. 2011. A novel poly (L-lactide) degrading thermophilic actinomycetes, *Actinomadura keratinilytica* strain T16-1 and pla sequencing. *Afr J Microbiol Res* 5 (18): 2575-2582.
- Suzuki H, Sawai Y, Suzuki T, Kawai K. 2003. Purification and characterization of an extracellular β -agarase from *Bacillus* sp. MK03. *J Biosci Bioeng* 95 (4): 328-334.
- Syafitri WA, Ningsih, F, Setyaningsih PP, Rachmania MK, Sari DCAF, Yabe S, Yokota A, Oetari A, Sjamsuridzal W. 2019. Screening for amylolytic activity and characterization of thermophilic *Actinobacteria* isolated from a geothermal area in West Java, Indonesia. *Biodiversitas* 20 (7): 1929-1938.
- Tamura T, Hatano K, Suzuki K. 2007. Classification of '*Sarracenospora aurea*' Furihata et al. 1989 as *Actinocorallia aurea* sp. nov. *Intl J Syst Evol Microbiol* 57: 2052-2055.
- Tang W, Chen X, Zhang H, Chen F, Li X. 2011. Limitation of the development on cellulose hydrolysis by cellulase assay and search for the true cellulase degrading crystalline cellulose. In: Golan AE (ed.). *Cellulase: Types and Action, Mechanism, and Uses*. Nova Science Publishers, Inc. New York.
- Tseng M, Yang SF, Hoang KC, Liao HC, Yuan GF, Liao CC. 2009. *Actinomadura miaoliensis* sp. nov., a thermotolerant polyester-degrading actinomycete. *Intl J Syst Evol Microbiol* 59 (3): 517-520.
- Weisburg, WG, Barns SM, Pelletier DA, Lane DJ. 1991. 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* 173 (2): 697-703.
- Yabe S, Sakai Y, Abe K, Yokota A, Také A, Matsumoto A, Sugiharto A, Susilowati D, Hamada D, Nara K, Sudiana IM, Otsuka S. 2017. *Dictyobacter aurantiacus* gen. nov., sp. nov., a member of the family *Ktedonobacteraceae*, isolated from soil, and emended description of the genus *Thermosporothrix*. *Intl J Syst Evol Microbiol* 67: 2615-2621.
- Yokota A, Ningsih F, Nurlaili DG, Sakai Y, Yabe S, Oetari A, Santoso I, Sjamsuridzal W. 2016. *Paenibacillus cisolokensis* sp. nov., isolated from litter of a geyser. *Intl J Syst Evol Microbiol* 66: 3088-3094.
- Yoon, SH, Ha SM, Kwon S, Lim J, Kim Y, Seo H, Chun J. 2017. Introducing EzBioCloud: A taxonomically united database of 16S rRNA and whole-genome assemblies. *Intl J Syst Evol Microbiol* 67: 1613-1617.