BIODIVERSITAS
Volume 20, Number 7, July 2019
Pages: 1929-1938

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d200720

Screening for amylolytic activity and characterization of thermophilic *Actinobacteria* isolated from a geothermal area in West Java, Indonesia

WINDA AYU SYAFITRI[1,*], FITRIA NINGSIH[1,2,*], PUTRI PRATIWI SETYANINGSIH[1], MAZYTHA KINANTI RACHMANIA[1], DHIAN CHITRA AYU FITRIA SARI[1,2], SHUHEI YABE[3,4], AKIRA YOKOTA[3], ARIYANTI OETARI[1,2], WELLYZAR SJAMSURIDZAL[1,2,♥]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Kampus UI Depok, Depok 16424, Indonesia.
[2]Center of Excellence for Indigenous Biological Resources-Genome Studies, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Kampus UI Depok, Depok 16424, Indonesia. ♥email: sjwelly@hotmail.com; sjwelly@sci.ui.ac.id.
[3]Department of Microbial Resources, Graduate School of Agricultural Science, Faculty of Agriculture, Tohoku University, 468-1 Aoba, Aramaki, Aoba-ku, Sendai, Miyagi 980-0845, Japan.
[4]Hazaka Plant Research Center, Kennan Eisei Kogyo Co., Ltd., 44 Aza-Inariyama, Oaza-Ashitate, Murata-cho, Shibata-gun, Miyagi 989-1311, Japan.

Manuscript received: 11 June 2019. Revision accepted: 24 June 2019.

Abstract. *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: 1929-1938.* In this study, we describe the screening for amylolytic activity of 17 thermophilic *Actinobacteria* isolates obtained from the soil of Cisolok geysers, a geothermal area in West Java, Indonesia. All isolates were screened for amylolytic activity by the starch-agar plate method at various temperatures. The results showed that all of isolates were able to grow at 45ºC. The growth abilities of the isolates grown in ISP 1 medium varied at temperatures from 45 to 60ºC. Fifteen of the 17 isolates showed amylolytic activity at 45ºC, 13 showed such activity at 50ºC, and four showed activity at 55ºC. Only three isolates, designated SL1-2-R-2, SL1-2-R-3, and SL1-2-R-4, showed growth and amylolytic activity at 60ºC. These three isolates were selected for molecular identification. The nearly full-length of 16S rRNA gene sequences data showed that these three isolates have a similarity of 99.93-100% with *Actinomadura keratinilytica* WCC-2265^T and of 98.74-98.91% with *A. miaoliensis* BC 44T-5^T. Phylogenetic tree shows that all three isolates are clustered together in a monophyletic group with the type strain of *A. keratinilytica* WCC-2265^T as their most closely related species, with 100% bootstrap support. Based on sequencing of the 16S rRNA gene, phylogenetic comparison, and phenotypic characterization, the three isolates were identified as *A. keratinilytica*.

Keywords: Amylolytic activity, geothermal area, soil, thermophilic *Actinobacteria*

INTRODUCTION

Actinobacteria are Gram-positive filamentous bacteria, which grow by a combination of tip extension and branching of the hyphae. Most *Actinobacteria* are saprophytic and soil-dwelling organisms, thus they are abundant in soil (Barka et al. 2016). *Actinobacteria* occur in various environments, including extreme environments defined by high temperatures, such as geothermal and volcanic areas, terrestrial hot springs, and geysers (Mehta and Satyanarayana 2013; Shivlata and Satyanarayana 2015). Thermophilic *Actinobacteria* can grow at relatively high temperatures ranging from 40 to 80 ºC (Shivlata and Satyanarayana 2015). In order to survive under these extreme environmental conditions, thermophilic *Actinobacteria* use adaptive strategies such as production of specific enzymes (Shivlata and Satyanarayana 2015).

Actinobacteria are also known to play an important role as producers of several enzymes (Liu et al. 2016), and are able to use a wide variety of nutritional sources, including various complex polysaccharides. Various enzymes of industrial use have been derived from various genera of *Actinobacteria* (Salwan and Sharma 2018).

Starch is a plant polysaccharide of considerable significance for humans that can be processed enzymatically into a variety of different products (Wang and Copeland 2013). Starch is a biopolymer composed of two polymers, amylose and amylopectin. Amylose is an essentially linear molecule in which the glucose units are linked through α-1,4 bonds. Amylopectin is a highly branched structure with α-1,6 bonds at the branch point (Sundarram et al. 2014). The starch industry demands large amount of amylolytic enzymes for hydrolysis and modification, which requires a combination of enzymes, including α-amylases, glucoamylases or β-amylases and isoamylases or pullulanases (Kikani et al. 2010).

Amylases degrade starch by the hydrolysis of α-1,4 and α-1,6-glycosidic linkages to smaller carbohydrates consisting of glucose units (Sundarram et al. 2014). Amylases play an important role in the food, fermentation, textile, and paper industries (Gopinanth et al. 2017). Starch conversion requires α-amylases to be active at high temperatures during gelatinization (100-110°C) and liquefaction (80-90°C) for low-cost processes (Kikani et al. 2010). A previous study reported that mesophilic enzymes are often not well suited for the harsh reaction conditions

*These authors contributed equally to the work

required in industrial processes because of the lack of enzyme stability (Demirjian et al. 2001). Thermostable amylase enzymes that deliver stable performance at high temperatures hold real potential for pharmaceuticals and agrochemicals, and the search for novel thermostable amylase enzymes continues to stimulate the search for microorganisms in extreme environments (Lamilla et al. 2017).

The discovery of a thermostable enzyme produced by *Actinobacteria* has attracted the interest of researchers and prompted them to explore geothermal regions (e.g. volcanic areas, hydrothermal areas, and geysers) (Mehta and Satyanarayana 2013). Cisolok is one of the geothermal areas in West Java, Indonesia (Purnomo and Pichler 2014), that is still less exploited, and is thus a high-potential area for isolation of novel thermophilic *Actinobacteria*. In our previous study, we identified a new genus and species of *Actinobacteria* from soil in the geothermal area of Cisolok, namely *Gandjariella thermophila* gen. nov., sp. nov., which has the ability to hydrolyze 1% (w/v) starch at temperature of 45 ºC (Ningsih et al. 2019, pers. comm.). Sjamsuridzal et al. (unpublished data) found two isolates of thermophilic *Actinobacteria* (designated as LC2-6A and LC2-6B, identified as *Actinomadura keratinilytica*) in a litter sample from the Cisolok geysers that produced various extracellular enzymes, such as amylase, cellulase, and xylanase at 50ºC. The same isolates also showed antibiotic activity at 50ºC to Gram-positive bacteria, *Kocuria rhizophila* NBRC 12078^T (Yokota et al. unpublished data).

In the framework of taxonomic study and bioprospecting of culturable rare-thermophilic *Actinobacteria* in geothermal areas in Indonesia, Sjamsuridzal et al. isolated 17 thermophilic *Actinobacteria* from the soil of Cisolok geysers in 2017. However, so far no information has been reported about their amylolytic activities at high temperatures and their identity based on 16S rRNA gene sequence, phylogenetic analyses, and phenotypic characterization.

Until recently, there have been no reports of thermophilic *Actinobacteria* from Indonesia producing thermostable amylase. Therefore, thermophilic *Actinobacteria* isolated from a geothermal area of Cisolok could be an important resource for the discovery of new thermostable α-amylase, which could be utilized in various industries. The aims of this study were to screen for amylolytic activity of the 17 thermophilic *Actinobacteria* isolates from a geothermal area of Cisolok and characterize the isolates with greatest potential.

MATERIALS AND METHODS

Source of the Actinobacterial isolates

A total of 17 *Actinobacteria* isolates were obtained during a study on the diversity of thermophilic *Actinobacteria* from the soil of Cisolok geysers, a geothermal area in West Java, Indonesia. The isolates were selected from three different sampling locations: (A) soil around big geyser (6º57'221"S, 106º27'507"E); (B) soil around small geyser (6º57'189"S, 106º27'365"E); (C.1) soil under *Gmelina* tree (6º57'482"S, 106º28'655"E); and (C.2) soil under Bamboo tree (6º57'482"S, 106º28'655"E) (Table 1). Sampling locations in the geothermal area of Cisolok are shown in Figure 1. The isolates were grown on International *Streptomyces* Project (ISP) 1 agar medium and incubated at 45 ºC for seven days. The isolates were preserved as agar block in 20% (v/v) glycerol at -80 ºC, and as lyophilized cells for long-term preservation (Ningsih et al. 2019, pers. comm.). All isolates were deposited at Universitas Indonesia Culture Collection (UICC), Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok, Indonesia.

Screening for amylolytic activity

Screening was conducted on Minimal (Mm) agar medium according to Meddeb-Mouelhi et al. (2014) with the addition of 1% (w/v) soluble starch and incubated at 45, 50, 55, and 60ºC for up to seven days. The Mm agar without the addition of soluble starch served as a control. The degradation of starch was detected by flooding the plates with 1% (v/v) Lugol's iodine solution. Clear zones around the colonies indicated positive results for amylase activity (Nithya et al. 2017). Growth ability at various high temperatures was also tested on ISP 1 agar medium at 45, 50, 55, and 60 ºC for up to seven days. The experiment was carried out in triplicate.

Identification of selected isolates by sequencing of the 16S rRNA gene

DNA extraction for 16S rRNA gene sequencing was conducted from mycelial suspensions of selected isolates grown in ISP1 broth medium at 45ºC for seven days. DNA was extracted using the genomic DNA mini kit [Geneaid] following the instructions of the kit protocol. PCR amplification for the 16S rRNA gene was carried out using the conditions specified in the MyTaq^{TM} Red Mix [Bioline] protocol, with universal primers for the 16S rRNA gene: 9F (5′-GAGTTTGATCCTGGCTCAG-3′), and 1510R (5′-GGCTACCTTGTTACGA-3′) as described by Weisburg et al. (1991). The PCR conditions specified initial denaturation at 95°C for three minutes, 35 cycles of denaturation at 95°C for 15 seconds, annealing at 56°C for 15 seconds, and extension at 72°C for one minute. The amplified 16S rRNA gene were sequenced using the 1st BASE DNA sequencing service (http://www.base-asia.com/dna-sequencing-services), using the following universal eubacterial primers for 16S rRNA gene: 785F (5′-GGATTAGATACCCTGGTA-3′), 800R (5′-TACCAGGGTATCTAATCC-3′), 27F (5′-AGAGTTTGATCMTGGCTCAG-3′), and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) (Weisburg et al. 1991; Jin et al. 2015).

The nearly full-length 16S rRNA gene sequences of selected thermophilic *Actinobacteria* isolates were analyzed using ChromasPro v.2.1.8 software and through a sequence-similarity search on EzTaxon-e (https://www.ezbiocloud.net/) (Yoon et al. 2017). The sequences of selected isolates were aligned with the sequences of type strains retrieved from the

DDBJ/EMBL/GenBank databases. Phylogenetic analysis was conducted in the Molecular Evolutionary Genetics Analysis version 7.0.26 (MEGA v7.0.26) software package (Kumar et al. 2016) using the neighbor-joining method (Saitou and Nei 1987) with bootstrap values based on 1000 replications (Felsenstein 1985). Evolutionary distances of the phylogenetic tree were calculated according to the Kimura two-parameter method (Kimura 1980).

Morphological, physiological, and biochemical characterization of selected isolates

Morphological characteristics of selected isolates were examined on ISP 1 agar medium incubated at 45 ºC for seven to 14 days (Puhl et al. 2009). The characteristic of the colony, the production of soluble pigment, and the color of the substrate hyphae were determined according to Shirling and Gottlieb (1966). Substrate and aerial mycelia were observed on 50% of Reasoner's 2A (R2A; Reasoner and Geldreich 1985) gellan medium incubated at 45 ºC for seven days. The formation of aerial hyphae and the morphology of the substrate mycelia were observed using a digital microscope (Hirox; KH-8700).

All physiological tests were conducted on ISP 1 and ISP 2 medium incubated at 45 ºC for seven to 14 days (Puhl et al. 2009). Growth at temperatures of 25, 30, 35, 40, 45, 50, 55, 60, and 65 ºC; and at various NaCl concentrations (1, 2, 3, 4, 5, 6, 7, 8, and 9% w/v) were tested on ISP 2 medium. The ability to grow at pH 4.0-10.0 was determined on ISP 1 medium using buffer systems according to Xu et al. (2005): pH 4.0-5.0, 0.1 M citric acid/0.1 M sodium citrate; pH 6.0-8.0, 0.1 KH_2PO_4/0.1 NaOH; pH 9.0-10.0, 0.1 M $NaHCO_3$/0.1 M Na_2CO_3; pH 11.0, 0.05 M Na_2HPO_4/0.1 M NaOH; pH 12.0-13.0, 0.2 M KCl/0.2 M NaOH. All physiological tests were performed in triplicate.

The ability of selected isolates to use various carbon sources: *D*-cellobiose, *D*-fructose, *D*-galactose, *D*-glucose, *D*-mannitol, *D*-mannose, *D*-melibiose, *D*-raffinose, *D*-ribose, *D*-xylose, *L*-arabinose, *L*-rhamnose, *L*-sorbose, lactose, maltose, *myo*-inositol, ribitol, sucrose, trehalose, and xylitol, was examined on ISP 9 agar medium according to Shirling and Gottlieb (1966). Carbon sources were added at 1% (w/v) final concentration and the medium was incubated at 45 ºC for seven to 14 days. The degradation of adenine (0.5% w/v), casein (1% w/v skimmed milk), gelatin (0.4% w/v), guanine (0.05% w/v), hypoxanthine (0.4% w/v), *L*-tyrosine (0.5% w/v), and xanthine (0.4% w/v) were detected in modified Bennett's agar (MBA) medium (Williams et al. 1983). Tween 80 (1% v/v) utilization was examined for opacity on Sierra's (1957) medium. Catalase production was observed by the addition of 3% (v/v) hydrogen peroxide to colonies grown on MBA medium, after seven days incubation at 45 ºC (Williams et al. 1983). All biochemical tests were performed in triplicate.

Table 1. The list of *Actinobacterial* isolates from three sampling locations in geothermal area of Cisolok, Indonesia.

No.	Sampling location 1 Big geyser	Sampling location 2 Small geyser	Sampling location 3 Soil forest near Cisolok geysers
1.	SL1-1-R-2	SL2-2-R-1	SL3-1-R-14
2.	SL1-1-R-4	SL2-2-R-12	SL3-1-R-16
3.	SL1-1-R-7	SL2-2-R-15	SL3-2-R-5
4.	SL1-1-R-8		SL3-2-R-17
5.	SL1-2-R-2		SL3-2-R-18
6.	SL1-2-R-3		SL3-2-R-33
7.	SL1-2-R-4		SL3-2-R-37
Total	7	3	7

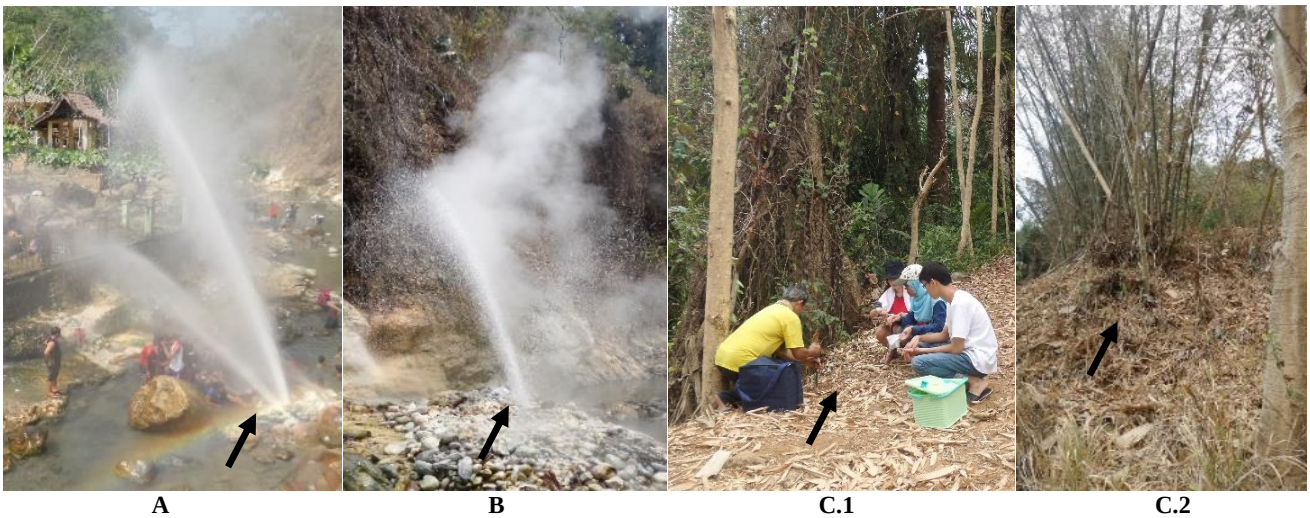

Figure 1. Sampling locations in geothermal area of Cisolok, Indonesia: A. Soil around big geyser; B. Soil around small geyser; C.1. Soil under *Gmelina* tree, and C.2. Soil under Bamboo tree

RESULTS AND DISCUSSION

Amylolytic activity of thermophilic *Actinobacteria*

The ability of the 17 isolates of *Actinobacteria* to grow at various temperatures was tested on ISP 1 agar medium incubated at 45, 50, 55, and 60ºC for three to seven days (Table 2). The results showed that all isolates grew at 45 ºC. Sixteen isolates were able to grow at 50ºC, and six isolates at 55ºC. The ability to grow at 60ºC was only shown in five isolates, designated SL1-2-R-2, SL1-2-R-3, SL1-2-R-4, SL2-2-R-15, and SL3-1-R-16. These result confirmed that the 17 isolates of thermophilic *Actinobacteria* isolated from the soil of Cisolok geysers are thermophiles. According to Shivlata and Satyanarayana (2015), thermophilic *Actinobacteria* grow well at temperatures ranging from 40 to 80ºC.

The starch degradation ability of the 17 isolates as tested on Mm agar medium supplemented with 1% (w/v) soluble starch is shown in Table 3. Amylolytic activity from the isolates was observed using the starch-iodine method based on the qualitative observation of the decrease in the iodine color intensity. In this study, 15, 13, and four out of 17 isolates were positive for amylolytic activity at 45, 50, and 55ºC, respectively, while only three isolates showed growth and amylolytic activity at 60ºC. Meanwhile, two isolates, designated SL2-2-R-15 and SL3-1-R-16, showed negative results for amylolytic activity at 45, 50, and 55ºC, even after seven days of incubation (Table 3). Figure 2 shows the presence of amylolytic activity on three representative isolates, designated SL1-2-R-2, SL1-2-R-3, and SL1-2-R-4, at temperatures of 45-60 ºC, indicated by the formation of clear zones around the colonies. Fifteen isolates showed clear zones on 1% soluble starch, indicating that they produce α-amylase. Polysaccharides, with the addition of iodine (I_2) will bind and form a dark purple color. Amylose plays a role in the formation of the dark purple color in the reaction with iodine. The clear zone formed indicates that starch has been hydrolyzed by amylase to oligosaccharides or monosaccharides (Immel and Lichtenthaler 2000; Lalitha et al. 2012).

The clear zones formed around the colonies have different diameters at each incubation temperature up to 60 ºC. The clear zones of the isolates incubated at 45, 50, and 55 ºC were bigger than the clear zones of the isolates incubated at 60ºC (Figure 2). These results indicated that the higher the incubation temperature, the lower the colonies' ability to produce amylase. According to Haritha et al. (2010), there are many factors which affect *Actinobacteria* growth and enzyme production e.g. temperature, pH, and substrate including the chemical and biological environment. In the present study, only three isolates grew and showed amylolytic activity at temperatures from 45 to 60ºC after seven days of incubation. The three selected isolates, selected based on their amylolytic activity at 60ºC, were further identified by molecular analysis based on 16S rRNA gene sequences data.

Phylogenetic analysis of selected isolates

The 16S rRNA gene sequences of three selected isolates (SL1-2-R-2, SL1-2-R-3, and SL1-2-R-4) were sequenced using four universal eubacterial primers (27F, 785F, 800R, and 1492R). The nearly full-length 16S rRNA gene sequences of SL1-2-R-2 (1492 bp), SL1-2-R-3 (1497 bp), and SL1-2-R-4 (1456 bp) were obtained. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence of three isolates (SL1-2-R-2, SL1-2-R-3, and SL1-2-R-4) are LC484202-LC484204.

Table 2. Growth of 17 thermophilic *Actinobacteria* on ISP 1 agar medium for seven days.

Isolate code	Temperatures of incubation (ºC)			
	45	50	55	60
SL1-1-R-2	+	+	-	n/a
SL1-1-R-4	+	+	-	n/a
SL1-1-R-7	+	+	-	n/a
SL1-1-R-8	+	+	-	n/a
SL1-2-R-2	+	+	+	+
SL1-2-R-3	+	+	+	+
SL1-2-R-4	+	+	+	+
SL2-2-R-1	+	+	+	-
SL2-2-R-12	+	+	-	n/a
SL2-2-R-15	+	+	+	+
SL3-1-R-14	+	+	-	n/a
SL3-1-R-16	+	+	+	+
SL3-2-R-5	+	+	-	n/a
SL3-2-R-17	+	+	-	n/a
SL3-2-R-18	+	-	-	n/a
SL3-2-R-33	+	+	-	n/a
SL3-2-R-37	+	+	-	n/a
Total	17	16	6	5

Note: (+): growth; (-): no growth; (n/a): not available, the test was not conducted

Table 3. Amylolytic activities of 17 thermophilic *Actinobacteria* on Mm agar supplemented with 1% (w/v) starch for seven days.

Isolate codes	Temperatures of incubation (ºC)			
	45	50	55	60
SL1-1-R-2	+	+	*	n/a
SL1-1-R-4	+	+	*	n/a
SL1-1-R-7	+	+	*	n/a
SL1-1-R-8	+	+	*	n/a
SL1-2-R-2	+	+	+	+
SL1-2-R-3	+	+	+	+
SL1-2-R-4	+	+	+	+
SL2-2-R-1	+	+	+	*
SL2-2-R-12	+	+	*	n/a
SL2-2-R-15	-	-	-	n/a
SL3-1-R-14	+	*	*	n/a
SL3-1-R-16	-	-	-	n/a
SL3-2-R-5	+	+	*	n/a
SL3-2-R-17	+	+	*	n/a
SL3-2-R-18	+	*	*	n/a
SL3-2-R-33	+	+	*	n/a
SL3-2-R-37	+	+	*	n/a
Total	15	13	4	3

Note: (+): amylase produced; (-): not produced; (*): no growth; (n/a): not available, the test was not conducted

A sequence-similarity search through EzTaxon-e (https://www.ezbiocloud.net/) showed that these three isolates (SL1-2-R-2, SL1-2-R-3, and SL1-2-R-4) have the highest similarities to members of the genus *Actinomadura*, and are closely related to *A. keratinilytica* WCC-2265^T (100%, 99.93%, and 100%, respectively) and *A. miaoliensis* BC 44T-5^T (98.81%, 98.74%, and 98.91%, respectively). The genus *Actinomadura* is a member of family *Thermomonosporaceae* of phylum *Actinobacteria* (Trujillo and Goodfellow 2015). The phylogenetic tree of the three isolates is shown in Figure 3. Based on a phylogenetic comparison, these three isolates were considered as members of the genus *Actinomadura*, and were determined to be closely related to *A. keratinilytica*, *A. miaoliensis*, *A. rubrobrunea*, and *A. viridilutea*, with strong bootstrap supports (94%). All of three isolates were grouped together in a monophyletic group with the type strain of *A. keratinilytica* WCC-2265^T as their most closely related species, with 100% bootstrap support. Based on the percentage of sequence similarity and the phylogenetic placement, all three isolates were identified as *A. keratinilytica.*

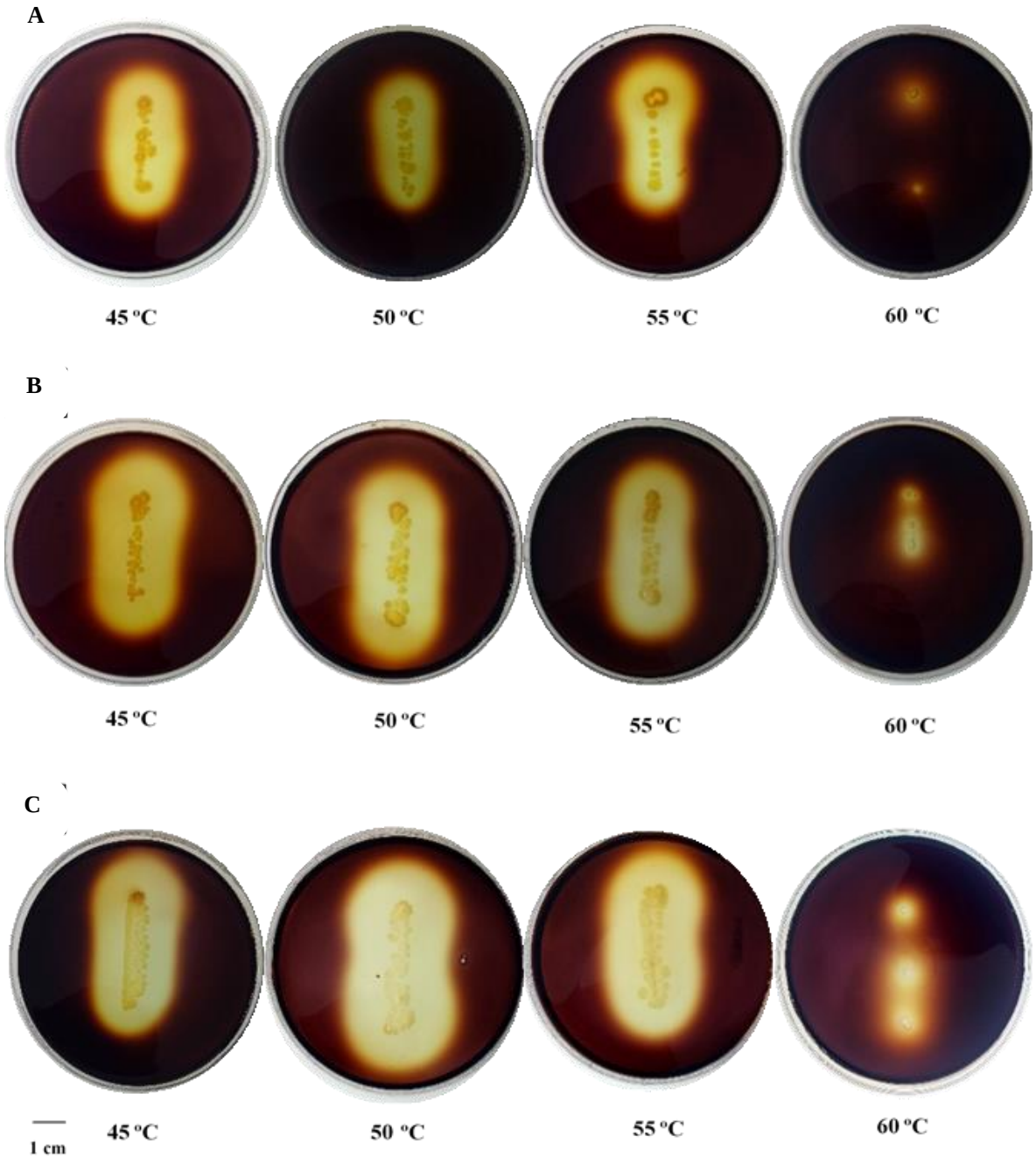

Figure 2. Amylolytic activity of thermophilic *Actinobacteria* isolates on Mm agar medium supplemented with 1% (w/v) starch, incubated at 45 to 60 °C for three to seven days; A. SL1-2-R-2, B. SL1-2-R-3, C. SL1-2-R-4. The clear zone formed indicates that starch has been hydrolyzed

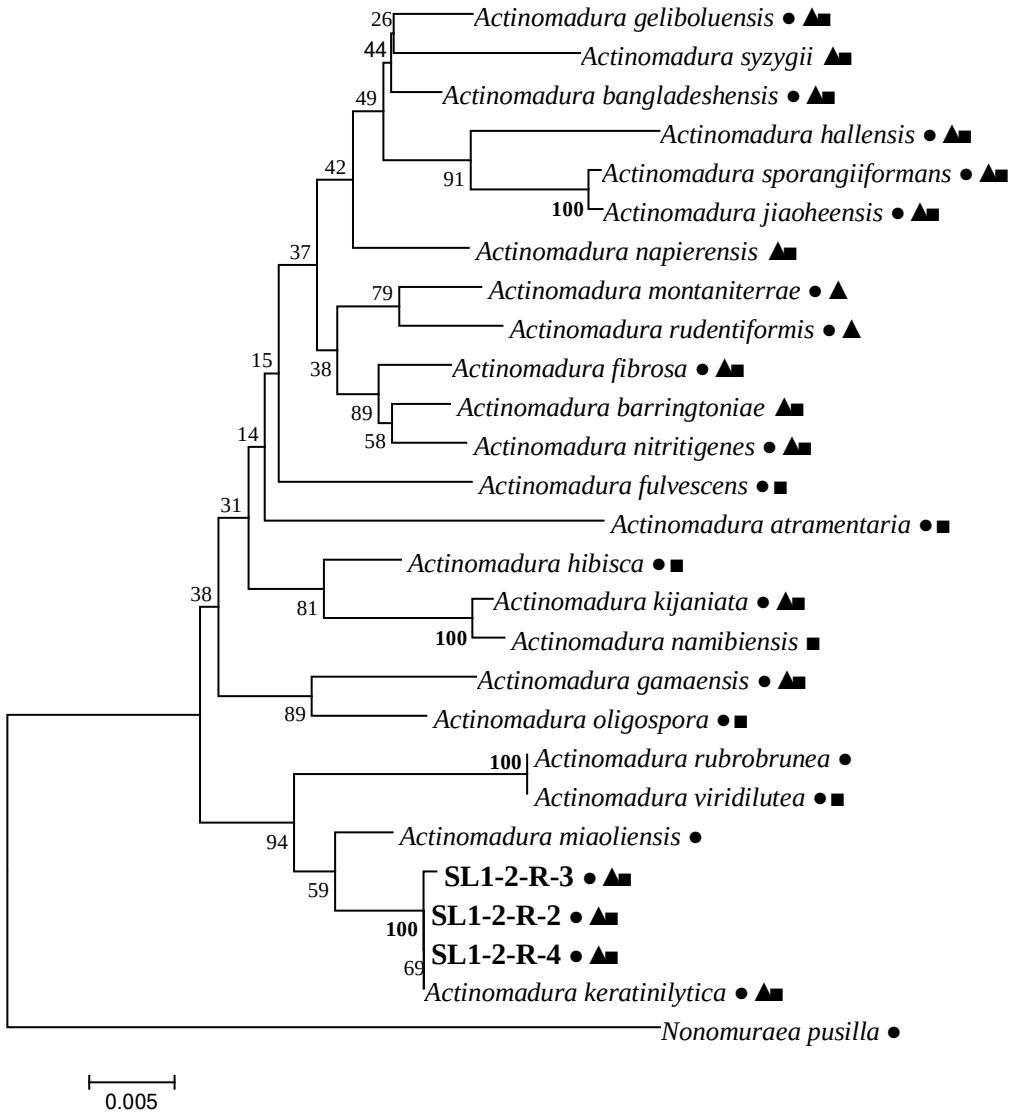

Figure 3. Phylogenetic relationships between three isolates (SL1-2-R-2; SL1-2-R-3; SL1-2-R-4) and type strains of related species of the genus *Actinomadura* based on nearly full-length 16S rRNA gene sequences. The phylogenetic tree was reconstructed by the neighbor-joining tree (Saitou and Nei 1987) using the MEGA software package (Kumar et al. 2016). *Nonomuraea pusilla* IFO 14684^T was used as an outgroup. Numbers at branch points indicate bootstrap percentages based on 1000 replications (Felsenstein 1985); Bar 0.005, represents substitutions per nucleotide position. ●: thermophilic, temperature range from 40 to 80^{o}C (Shivlata and Satyanarayana 2015); ▲: produces amylase; ■: produces other enzymes

The phylogenetic tree in Figure 3 shows that the three isolates (SL1-2-R-2, SL1-2-R-3, and SL1-2-R-4) and the type strain of *A. keratinilytica* WCC-2265^T were grouped together with thermophilic species of *Actinomadura*, e.g. *A. rubrobrunea*, *A. viridilutea*, and *A. miaoliensis.* According to Trujillo and Goodfellow (2012), *A. rubrobrunea* and *A. viridilutea* are thermophilic, with growth range between 37 and 65 ^{o}C. Tseng et al. (2009) reported that *A. miaoliensis* has a growth range between 25 and 55 ^{o}C, and *A. keratinilytica* has growth range between 30 and 60 ^{o}C (Sukkhum et al. 2011) with optimal growth is at 45 ^{o}C (Puhl et al. 2009). As shown in Figure 3, the three isolates and all closely related species are thermophilic, except for three species: *A. barringtoniae*, *A. syzygii*, and *A. napierensis.* These three species have growth temperature lower than 40^{o}C (Cook et al. 2005; Rachniyom et al. 2015; Rachniyom et al. 2018) (Table 4).

As seen in Table 4, most closely related species of the three isolates in the genus *Actinomadura* are thermophilic and are able to produce amylase, other enzymes, or both. The type strain of *A. keratinilytica* WCC-2265^T displayed the ability to degrade keratin and utilize it as a sole carbon and nitrogen source (Puhl et al. 2009). Sukkhum et al. (2011) reported that *A. keratinilytica* strain T16-1, which was isolated from a soil sample, produces several enzymes, e.g. amylase, cellulose, gelatinase, and protease.

Table 4. List of three selected isolates and the type strains from the genus *Actinomadura* used in the phylogenetic analyses and their salient characteristics

Species	Strain no.	Accession no.	Growth range (°C)	Amylase	Other enzymes	Sources	Reference
A. keratinilytica	SL1-2-R-2	LC484202	25-60	+	+	Soil	This study
A. keratinilytica	SL1-2-R-3	LC484203	25-60	+	+	Soil	This study
A. keratinilytica	SL1-2-R-4	LC484204	25-60	+	+	Soil	This study
A. keratinilytica	WCC-2265	EU637009	30-60	+	+	Compost	Puhl et al. (2009) Sukkhum et al. (2011)
A. miaoliensis	BC 44T-5	EF116925	25-55	-	nd	Soil	Tseng et al. (2009) Hoang et al. (2013)
A. rubrobrunea	NBRC 15275	BCQU01000204	37-65	nd	nd	Soil	Trujillo and Goodfellow (2012)
A. viridilutea	NBRC 14480	D86943	37-65	nd	+	Desert soil	Trujillo and Goodfellow (2012)
A. hibisca	NBRC 15177	BCRO01000158	15-40	nd	+	Soil	Tomita et al. (1990)
A. gamaensis	NEAU-Gz5	KT989505	15-40	+	+	Soil	Abagana et al. (2016)
A. montaniterrae	CYP1-1B	LC126428	20-45	+	nd	Soil	Songsumanus et al. (2016)
A. barringtoniae	GKU 128	KF667497	14-38	+	+	Roots	Rachniyom et al. (2018)
A. oligospora	ATCC 43269	AF163118	15-42	-	+	Soil	Mertz and Yao (1986)
A. nitritigenes	DSM 44137	AY035999	45	+	+	Experimental biofilters	Trujillo and Goodfellow (2012)
A. fibrosa	ATCC 49459	AF163114	20-45	+	+	Soil	Trujillo and Goodfellow (2012)
A. kijaniata	NBRC 14229	BCQR01000335	28-50	+	+	Soil	Horan and Brodsky (1982)
A. namibiensis	DSM 44197	AJ420134	28	nd	+	Soil	Wink et al. (2003)
A. geliboluensis	A8036	HQ157187	20-45	+	+	Soil	Sazak et al. (2012)
A. syzygii	GKU 157	KF667496	20-34	+	+	Roots	Rachniyom et al. (2015)
A. fulvescens	IFO 14347	U49005	10-45	nd	+	nd	Terekhova et al. (1982) Wink et al. (2003)
A. hallensis	H647-1	DQ076484	20-45	+	+	Soil	Lee and Jeong (2006)
A. bangladeshensis	3-46-b3	AB331652	20-45	+	+	Soil	Lee (2012)
A. jiaoheensis	NEAU-Jh1-3	KM000835	15-42	+	+	Soil	Zhao et al. (2015)
A. sporangiiformans	NEAU-Jh2-5	KM000834	20-42	+	+	Soil	Zhao et al. (2015)
A. rudentiformis	HMC1	DQ285420	30-45	+	nd	Soil	Trujillo and Goodfellow (2012)
A. atramentaria	IFO 14695	U49000	15-42	-	+	Soil	Miyadoh et al. (1987) Qin et al. (2009)
A. napierensis	B60	AY568292	28	+	+	Soil	Cook et al. (2005)

Note: +: thermophilic, able to produce amylase/other enzymes; -: mesophilic, not able to produce amylase/other enzymes; nd: no data

Morphological, physiological, and biochemical characterization of selected isolates

The characteristics of the three selected isolates (SL1-2-R-2, SL1-2-R-3, and SL1-2-R-4) as the genus *Actinomadura* were also confirmed by conducting morphological, physiological, and biochemical analyses. The characteristics comparison between all three isolates with closely related species from the genus *Actinomadura* is shown in Table 5. All three isolates are aerobic, Gram-stained positive, and catalase positive. They grow at temperature of 25 to 60°C (optimum, 45 to 50°C), at pH 6.0-9.0 (optimum, pH 7.0-8.0), and concentrations of 0-5% (optimum, 1%) NaCl in the medium (Table 5). This data is well supported by Puhl et al. (2009) and Sukkhum et al. (2011). According to Puhl et al. (2009), the type strain of *A. keratinilytica* WCC-2265^T has a temperature growth range between 30 and 55 °C, with optimal growth at 45°C. Meanwhile, Sukkhum et al. (2011) reported that the growth range of *A. keratinilytica* strain T16-1 isolated from a soil sample was 30 to 60°C.

The isolates showed optimum growth on ISP 1, ISP 2, ISP 3, and modified Bennett's media, solidified with 2% agar or gellan. The colonies were ivory, with wrinkled surfaces on ISP 2 medium, incubated at 45°C for seven days (Figure 4). Substrate and aerial mycelia of all isolates, observed on 50% of R2A gellan medium, are non-fragmented, branched, with ivory color, and showed flexuous spore arrangement (data not shown). These morphological characteristics are consistent with the description of species *A. keratinilytica* (Puhl et al. 2009).

The SL1-2-R-2 isolate was able to utilize 18 out of 20 sole carbon sources tested, all except for *D*-galactose and *D*-mannitol. The SL1-2-R-3 and SL1-2-R-4 isolates were not able to utilize *D*-fructose, *D*-galactose, and *D*-mannitol. The type strain of *A. keratinilytica*, WCC-2265^T (Puhl et al. 2009), was able to utilize *D*-fructose and *D*-mannitol as sole carbon sources. Degradative tests showed that all isolates hydrolyzed all tested compounds, excluding adenine for isolates SL1-2-R-3 and SL1-2-R-4, while currently, no data is available for *A. keratinilytica* WCC-2265^T (Table 5).

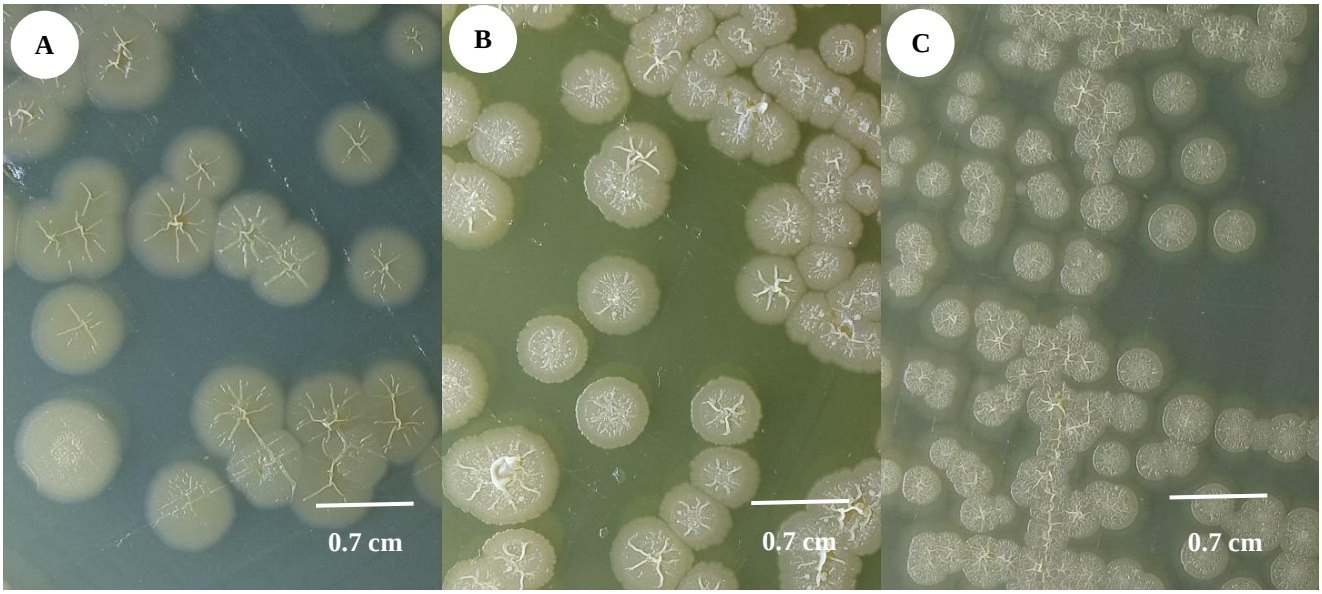

Figure 4. Colony morphology of three selected thermophilic *Actinobacteria* isolates on ISP 2 agar incubated at 45 ºC for seven days: A. Isolate SL1-2-R-2; B. Isolate SL1-2-R-3; C. Isolate SL1-2-R-4

Table 5. Morphological, physiological, and biochemical characteristics of isolates SL1-2-R-2, SL1-2-R-3, SL1-2-R-4, and the most closely related species from the genus *Actinomadura*. Strains: 1, SL1-2-R-2 (this study); 2, SL1-2-R-3 (this study); 3, SL1-2-R-4 (this study); 4, *A. keratinilytica* WCC-2265^T (Puhl et al. 2009); 5, *A. miaoliensis* BC 44T-5^T (Horan and Brodsky 1982); 6, *A. rubrobrunea* NBRC 15275^T (Tseng et al. 2009)

Characteristics	1	2	3	4	5	6
Spore morphology						
Arrangement	Flexuous	Flexuous	Flexuous	Flexuous-straight	nd	Curved
Colony characteristics on ISP 2						
Substrate hyphae	Ivory	Ivory	Ivory	nd	nd	nd
Aerial hyphae	nd	nd	nd	nd		
Soluble pigment	-	-	-	Brown	nd	nd
Growth at/in						
25ºC	+	+	+	+	+	-
60ºC	+	+	+	-	-	+
pH 6.0-9.0	+	+	+	+	nd	nd
0-5 % NaCl	+	+	+	+	nd	+
Catalase activity	+	+	+	nd	nd	nd
Growth on sole carbon source:						
D-Cellobiose	+	+	+	nd	nd	nd
D-Fructose	+	-	-	+	+	+/-
D-Galactose	-	-	-	nd	-	nd
D-Glucose	+	+	+	+	+	+
D-Mannitol	-	-	-	+	nd	+
D-Mannose	+	+	+	nd	nd	nd
D-Melibiose	+	+	+	nd	nd	nd
D-Raffinose	+	+	+	+	nd	+/-
D-Ribose	+	+	+	nd	nd	nd
D-Xylose	+	+	+	+	+	+/-
L-Arabinose	+	+	+	+/-	-	-
L-Rhamnose	+	+	+	+	nd	+
L-Sorbose	+	+	+	nd	nd	nd
myo-Inositol	+	+	+	+	-	+/-
Lactose	+	+	+	nd	nd	nd
Maltose	+	+	+	nd	nd	nd
Xylitol	+	+	+	nd	nd	nd
Ribitol	+	+	+	nd	nd	nd
Sucrose	+	+	+	+	nd	+
Trehalose	+	+	+	nd	+	nd
Hydrolysis of:						
Adenine	+	-	-	nd	nd	nd
Casein	+	+	+	nd	+	nd
Guanine	+	+	+	nd	nd	nd
Hypoxanthine	+	+	+	nd	nd	nd
L-Tyrosine	+	+	+	nd	+	nd
Starch	+	+	+	nd	nd	nd
Tween 80	+	+	+	nd	nd	nd
Xanthine	+	+	+	nd	nd	nd

Note: (+): positive; (-): negative; (nd): no data

As shown in Table 4, many species of *Actinomadura* have been isolated from soil, including the three isolates used in this study and all the other isolates named except for *A. barringtoniae* and *A. syzygii* (roots); *A. keratinilytica* (compost); and *A. nitrigenes* (experimental biofilters). According to Trujillo and Goodfellow (2015), the genus *Actinomadura* is widely distributed in soil and probably has a role in organic matter turnover. Therefore, it can be suggested that soil is promising habitat to explore the diversity of *Actinomadura*. Actinomadurae are slower growing than streptomycetes (Trujillo and Goodfellow (2015), and are therefore considered rare *Actinobacteria*, which are often very difficult to isolate and cultivate. This study is the first report for *Actinomadura* found in the soil of geysers in Indonesia.

In summary, the findings presented in this study show that soil around the geysers of Cisolok supports a

thermophilic community of *Actinobacteria*, and almost all isolates are positive for amylolytic activities (at temperatures of 45 to 60ºC). Fifteen out of 17 isolates tested showed clear zones on 1% soluble starch, indicating that they produced α-amylase. It can, therefore, be concluded that *Actinobacteria* in the soil of the Cisolok geysers are a promising source of amylase producers. Based on percentage of sequence similarity, the phylogenetic placement, and phenotypic characterization, the three selected isolates (SL1-2-R-2; SL1-2-R-3; SL1-2-R-4) were identified as *A. keratinilytica*. Further study about their amylase activity and its potential as a thermostable enzyme is needed for further applications.

ACKNOWLEDGEMENTS

This research was funded by *Hibah Publikasi Internasional Terindeks untuk Tugas Akhir Mahasiswa* (PITTA) UI 2018 no. 2331/UN2.R3.1/HKP.05.00/2018 to W.S. We would like to acknowledge the financial assistance provided by the Department of Microbial Resources, Graduate School of Agricultural Science, Faculty of Agriculture, Tohoku University, Japan.

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