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Identification and screening of enzymatic abilities of *Ktedonobacteria* from forest soil of Cisolok Geothermal Area, Indonesia

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Abstract. *Rachmania MK, Ningsih F, Sari DCAF, Eshananda Y, Sakai Y, Yabe S, Yokota A, Sjamsuridzal W. 2022. Identification and screening of enzymatic abilities of Ktedonobacteria from forest soil of Cisolok Geothermal Area, Indonesia. Biodiversitas 23: 4686-4695.* This study aimed to provide information on culturable *Ktedonobacteria* from forest soil in the Cisolok geothermal area and their potential as enzyme producers. Twelve ktedonobacterial isolates were obtained from this study and identified based on full-sequence of the 16S rRNA gene. Seventeen isolates (including five isolates from previous studies) were used for enzymatic screening and phylogenetic analyses. Screening of amylolytic (0.5% soluble starch) and cellulolytic (0.5% carboxymethylcellulose) (CMC) activities from ktedonobacterial isolates was performed on a ten-fold diluted R2A agar medium, and were incubated at 30 °C for 21 days. The EzBioCloud search revealed that all isolates showed low sequence similarities with *Dictyobacter aurantiacus* S-27^T (97.82 to 98.18%) as their closest related species. The phylogenetic tree showed that all isolates belong to the genus *Dictyobacter* (family Dictyobacteraceae of the class Ktedonobacteria). All isolates formed a monophyletic group and were placed as the sister clade to *D. aurantiacus*, as supported by a very strong bootstrap value (99%). Screening of amylolytic and cellulolytic abilities showed that most isolates (88.23%) were able to degrade both 0.5% starch and 0.5% CMC as substrates. This study revealed the presence of *Ktedonobacteria* with amylolytic and cellulolytic abilities in the forest soil of Cisolok geothermal area.

Keywords: 16S rRNA gene, amylase, cellulase, Cisolok geothermal area, *Ktedonobacteria*

INTRODUCTION

Actinomycete-like bacteria within the class *Ktedonobacteria* in the phylum Chloroflexi have recently attracted scientists' interest owing to their potency as promising new microbial resources in biotechnological and pharmaceutical fields (Zheng et al. 2019a). They are known for their potential ability to produce novel thermostable enzymes (Tomazini et al. 2019; Zheng et al. 2021) and bioactive compounds (Park et al. 2014; Igarashi et al. 2019). *Ktedonobacteria* were characterized for their filamentous morphology with branched mycelia and aerial non-motile spores (Yabe et al. 2010a), and relatively large genome size (5.54 to 13.66 Mbp; Zheng et al. 2019a). Their genomes harbor many secondary metabolite-related gene clusters, indicating that the class *Ktedonobacteria* possess broad potency as metabolite compounds producers with novel structures and metabolic pathways (Zheng et al. 2019a).

Studies based on the 16S rRNA gene sequences analysis from the environmental samples reveal that the class *Ktedonobacteria* inhabit diverse environments, including common terrestrial e.g., garden soil, sand, bark, (Yabe et al. 2017a), and rubber plantation soil (Effendi et al. 2020) to extreme environments e.g., uranium-contaminated soil (Brodie et al. 2006), compost (Yabe et al. 2017a), and rock in the Antarctic (Mezzasoma et al. 2021). This class was predominantly found in extreme acidic oligotrophic environments (Tebo et al. 2015), and recorded at low abundances in non-extreme environments, such as sandy soil (Acosta-Martinez et al. 2010). Current isolation and cultivation approach resulted in only a few *Ktedonobacteria* taxa which have been formally proposed since 2006, with 19 species within eight genera (Cavaletti et al. 2006; Yabe et al. 2021). Therefore, intensive efforts in developing isolation and cultivation methods are urgently required to increase the number of culturable *Ktedonobacteria* in an attempt to discover new natural products (Zheng et al. 2019a).

Carbohydrase enzymes play key roles in industrial and biotechnological applications, including in the food, textiles, and paper industries (Satyanarayana et al. 2013; Gopinath et al. 2017). Novel thermostable enzymes identified from *Ktedonobacteria* were as follows: β-

xylosidase from glycoside hydrolase family 5 (GH5) in the genome of *Thermogemmatispora* T81 (Tomazini et al. 2019), and β-1,4-glucanase from GH12 family (designated as ThazG12) which was cloned from *Thermosporothrix hazakensis* SK20-1^T (Zheng et al. 2021). These two strains, *Thermogemmatispora* T81 and *T. hazakensis* SK20-1^T, were isolated from high-temperature habitats, geothermal soil in the volcanic zone (Stott et al. 2008) and ripe compost (Yabe et al. 2010b), respectively. Therefore, the exploitation of bacteria isolated from extreme environments offers the opportunity to discover enzymes that are suited for industrial applications (Littlechild 2015).

Ktedonobacteria inhabiting Indonesia is still rarely explored, as currently only a few reports prior to this study (Yabe et al. 2017, presented in Universitas Indonesia Scholar Summit; Yabe et al. 2017b; Effendi et al. 2020). *Dictyobacter aurantiacus* was the only taxa at the species level which has been proposed and isolated from the soil of paddy fields in Gunung Salak, West Java (Yabe et al. 2017b). Metagenomic analysis based on the 16S rRNA gene sequences revealed that *Ktedonobacteria* was a major predominating class in the soil of plant agroforestry system rubber tree (*Hevea brasiliensis*) which intercropped with *Canna* sp. (Effendi et al. 2020). Therefore, study of the *Ktedonobacteria* diversity is needed in order to explore more diverse *Ktedonobacteria* in Indonesia.

Cisolok geothermal area is one of the prospecting geothermal areas in Indonesia (Herdianita and Mandradewi 2010) that provides a heat energy source from the volcanic activity below Mount Halimun, West Java (Sumartha et al. 2020). Thermal water from geysers along the Cisolok river discharges continuously and extends to the forest alongside the river, thus the forest near the Cisolok geysers was exposed to thermal water discharge (Figure 1). Our previous study successfully detected *Ktedonobacteria* predominated forest soil under the Bamboo tree of Cisolok geysers based on the 16S rRNA gene sequence analysis (Yabe et al. 2017, presented in Universitas Indonesia Scholar Summit). In addition, Rachmania et al. (2020) and Eshananda et al. (2020) obtained seven culturable *Ktedonobacteria* isolates from forest soils under the Bamboo tree and in the decayed Bamboo stem. After these works (Eshananda et al. 2020; Rachmania et al. 2020), we obtained twelve additional ktedonobacterial isolates from the same samples that have not yet been identified. These isolates need further characterization to clarify their taxonomic identities. The study focusing on the potency of these culturable *Ktedonobacteria* as carbohydrate-degrading enzymes (amylase and cellulase) producers from the Cisolok geothermal area is required for further investigation.

The aims of this study were to obtain information regarding the taxonomic identity of culturable *Ktedonobacteria* based on the 16S rRNA gene sequence data and their ability as enzyme producers. This study is the first report that described the enzymatic abilities of culturable *Ktedonobacteria* from the geothermal area in Indonesia. The exploration of *Ktedonobacteria* as new microbial resources is important for future potential applications.

MATERIALS AND METHODS

Microorganisms

Seventeen ktedonobacterial isolates were used in this study (Table 1). Three isolates (S3.2.1.5, S3.2.1.6, and S3.2.2.5) were obtained from our previous study (Rachmania et al. 2020). Three isolates (K17-1, K42, and K44) were obtained from Eshananda et al. (2020) and further purified since different colony morphologies were observed in the culture plate. As a result of purification, we obtained four (K17-1, K17-1A, K17-1B-1, K17-1B-2) and two (K44-1B, K44-2-1) isolates from K17-1 and K44, respectively. Additionally, we obtained seven other isolates from this study (K11A, K11A-2, K11B, K17-A, SL3-2-2-R3, SL3-2-2-R6-1, and SL3-2-2-R6-2). All isolates were obtained from forest soil around the Cisolok geothermal area (06º55.991S, 106º27.187E), West Java, Indonesia. Soil samples were obtained from topsoil under the Bamboo tree (S3.2.1) and soil in decayed Bamboo stem (S3.2.2).

Figure 1. Cisolok geothermal area, West Java, Indonesia. A. Forest area along of Cisolok river; B. One of spouting hot spring in Cisolok river.

Isolation of *Ktedonobacteria*

Culturable ktedonobacterial isolates were obtained according to Yabe et al. (2017b) using Reasoner's 2A (R2A) broth medium with 1:10 dilution and 2% (w/v) gellan gum was used as a solidifying agent. The pH of the isolation medium was adjusted to 5.5 and the medium was supplemented with 60 ppm sodium azide. Incubation was carried out at 30°C for three weeks. A firm pale orange colony which similar to the character of *Ktedonobacteria* colony (Yabe et al. 2017b) was selected and subsequently colony PCR was performed using the specific *Ktedonobacteria* primers KTED161F (5'-ATACCGGBGMGAAAKYGYCGAC-3') and GNSB941R (5'- AAACCACACGCTCCGCT-3') (Yabe et al. 2017a).

Culture maintenance and preservation

The pure culture was maintained on NBRC medium 231 with the addition of 2% gellan gum and the medium pH was adjusted to 7.0. Incubation was carried out at a temperature of 30°C. Mycelial suspensions of *Ktedonobacteria* isolates were preserved in 20% (v/v) glycerol solutions and the suspensions were stored at -80°C for long-term preservation. All *Ktedonobacteria* isolates were deposited at Universitas Indonesia Culture Collection (UICC), Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok, Indonesia.

Qualitative screening of hydrolytic enzymes

The screening of amylolytic and cellulolytic ktedonobacterial isolates was conducted according to the method of Yabe et al. (2017b) by using 0.5% (w/v) of soluble starch and 0.5% (w/v) carboxymethylcellulose (CMC), respectively. Each substrate was dissolved in Reasoner's 2A medium (1:10 dilution) with the addition of 2% of agar. Bacterial isolates were streaked on the plates, then the plates were incubated at 30°C for 21 days. The ability of isolates to degrade CMC was assessed by staining the plates with 0.2% (w/v) Congo red and washing with 1 M NaCl. Starch hydrolysis was observed using 1% (v/v) Lugol's iodine solution. Clear zones around colonies indicated positive results.

Amplification of 16S rRNA gene of isolated *Ktedonobacteria*

The full-length of 16S rRNA gene of two isolates (K17-1 and K42) from Eshananda et al. (2020) and twelve isolates obtained in this study (K17-1A, K17-1B-1, K17-1B-2, K44-1B, K44-2-1, K11A, K11A-2, K11B, K17-A, SL3-2-2-R3, SL3-2-2-R6-1, and SL3-2-2-R6-2) were determined in this study using eight (9F, 27F, 518F, 785F, 800R, 907R, 1492R, and 1510R) universal *Eubacteria* primers (Lane 1991). The genomic DNA of isolates was extracted according to the method of Zheng et al. (2019a) by using Puregene Yeast/Bact. Kit [Qiagen, Hilden, Germany]. The 16S rRNA gene sequences of isolates were amplified by polymerase chain reaction (PCR) with a primer set of 9F and 1510R (Lane 1991). The PCR amplification was performed using the following condition: initial denaturation at 95°C for 1 min, 30 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 30 s. The amplified PCR products were sequenced commercially by the 1st BASE DNA sequencing service (http://www.base-asia.com/dna-sequencing-services).

Phylogenetic tree construction

The 16S rRNA gene sequences of isolates were analyzed using ChromasPro software version 2.1.8. The similarity of 16S rRNA gene sequences of *Ktedonobacteria* isolates was compared to the 16S rRNA gene sequences of all known *Ktedonobacteria* type strains in the EzBioCloud database (https://www.ezbiocloud.net; Yoon et al. 2017). Pairwise sequence similarities of 16S rRNA gene between all isolates and type strains were estimated using the MEGA software version 11 software (Tamura et al. 2021).

All of the 16S rRNA gene sequences were aligned and phylogenetic trees were constructed using MEGA 11 software (Tamura et al. 2021). The phylogenetic inference methods used to construct the phylogenetic tree were neighbor-joining (NJ) (Saitou and Nei 1987), maximum parsimony (MP) (Swofford and Berlocher 1987), minimum evolution (ME) (Rzhetsky and Nei 1992), and maximum likelihood (ML) (Felsenstein 1981). The Kimura two-parameter model was applied to construct the tree (Kimura 1980) and the reliability of the tree was estimated by using 1000 bootstrap replications (Felsenstein 1985).

RESULTS AND DISCUSSION

Culturable ktedonobacterial isolates and phylogenetic analyses

Morphologically, all ktedonobacterial isolates showed a pale to bright orange color with a firm texture in the agar medium and formed small filamentous colonies after being incubated at a temperature of 30°C for 28 days (Figure 2). All *Ktedonobacteria* isolates obtained from forest soil in the Cisolok geothermal area are considered as mesophilic bacteria due to the growth temperature of isolation and cultivation occurring at 30°C. Member of *Ktedonobacteria* consists of mesophilic bacteria which grow optimally at 25 to 30 °C (Cavaletti et al. 2006; Wang et al. 2019; Yan et al. 2020; Yabe et al. 2021) and thermophilic bacteria which grow optimally at 50 to 65°C (Yabe et al. 2010b; King and King 2014; Zheng et al. 2019b). Mesophilic *Ktedonobacteria* comprises of families Dictyobacteraceae (Wang et al. 2019), Ktedonobacteraceae (Cavaletti et al. 2006), Ktedonosporobacteraceae (Yan et al. 2020), and Reticulibacteraceae (Yabe et al. 2021) within order Ktedonobacterales (Cavaletti et al. 2006).

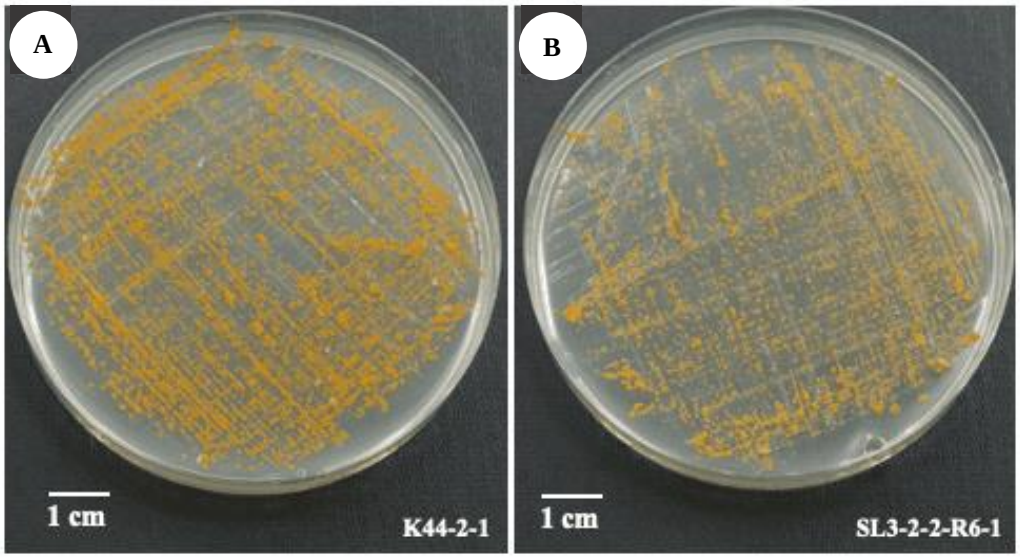

Figure 2. Ktedonobacterial isolates grown on NBRC 231 gellan gum medium at temperature of 30°C after 14 days. A. K44-2-1; B. SL3-2-2-R6-1

A total of 17 ktedonobacterial isolates from forest soil of the Cisolok geothermal area were used in phylogenetic analyses (Figure 3). The full sequence data of the 16S rRNA gene of 14 isolates were determined in this study (including isolates K17-1 and K42 whose 16S rRNA genes were previously partially sequenced in Eshananda et al. 2020). The 16S rRNA gene sequence data of another three isolates (S3.2.1.5, S3.2.1.6, and S3.2.2.5) were reported in our previous study (Rachmania et al. 2020). The 16S rRNA gene sequence similarity of all isolates was compared with all known species-type strains of the class *Ktedonobacteria* in the EzBioCloud database. EzBioCloud search results showed that all isolates belonged to the member of the class *Ktedonobacteria* and have low similarity values to their closest related taxon, *Dictyobacter aurantiacus* S-27^T (97.82 to 98.18%) (Table 1). The 16S rRNA gene sequences of isolates were then deposited in the GenBank/EMBL/DDBJ databases (LC715230-LC715246; Table 1).

Interestingly, all isolates from the soil of the Cisolok geothermal area are grouped with *D. aurantiacus*, which is also found in the geothermal area in West Java. *Dictyobacter aurantiacus* that was discovered by Yabe et al. (2017b) is the only taxon of the class *Ktedonobacteria* at the species level that has been found in Indonesia. It was isolated from the soil of paddy fields in Salak Mountain, in West Java. Mount Salak is an active volcano, another geothermal area in West Java. Other known species of *Dictyobacter* were obtained from the soil in geothermal areas e.g., *D. alpinus*, *D. kobayashii* (Wang et al. 2019), *D. vulcani* (Zheng et al. 2020), *D. fomicarum* (Yabe et al. 2021), and only one species was not obtained from geothermal sites e.g. *D. arantiisoli* (Yabe et al. 2021).

Dictyobacter aurantiacus S27^T showed a pale orange colony, growing optimally at a temperature of 25 to 30°C and at pH 6.0 (Yabe et al. 2017b). *Dictyobacter* isolates from this study were also showed similar morphology to *D. aurantiacus* by their pale to bright orange colony's color. These isolates also grow well at a temperature of 30°C and a pH of 7.0. All *Dictyobacter* species are mesophilic bacteria which able to grow at temperatures ranging from 11 to 37°C (optimal at 25 to 30°C) and pH ranging from 3.5 to 8.6, which is optimal at pH 6.0 to 7.0 (Yabe et al. 2017b; Wang et al. 2019; Zheng et al. 2020; Yabe et al. 2021).

Genus Dictyobacteraceae consists of six species with validly published names: *D. aurantiacus* (Yabe et al. 2017b), *D. alpinus*, *D. kobayashii* (Wang et al. 2019), *D. vulcani* (Zheng et al. 2020), and *D. arantiisoli*, *D. fomicarum* (Yabe et al. 2021). Species within the genus *Dictyobacter*: *D. aurantiacus* S27^T and *D. fomicarum* SOSP-9 form a clear sporangia structure on their mycelia (Cavaletti et al. 2006; Yabe et al. 2016; Yabe et al. 2021), while the other *Dictyobacter* species merely produce spores directly on aerial mycelia (Yabe et al. 2021). However, the microscopic observation, including spores characteristics, of all isolates is not conducted in this study.

The phylogenetic relationship between all *Dictyobacter* isolates from this study is shown in Figure 3. The NJ tree was supported by ML, ME, and MP trees. The tree showed that all isolates from the soil of the Cisolok geothermal area formed a monophyletic group and were placed as the sister clade to *D. aurantiacus* S27^T with very strong bootstrap support (99%). As shown in Figure 3, all isolates were separated from *D. aurantiacus.* The low similarity of their 16S rRNA gene to the type strain of *D. aurantiacus* S27^T and the phylogenetic analyses indicated that all isolates from Cisolok geothermal area are putative novel species of *Dictyobacter.* Further study is needed to provide a taxonomic description of this potential novel taxon.

Table 1. Sequence similarity search result of ktedonobacterial isolates used in this study and their closely related taxa

Isolate code	UICC strain number	Source	% Similarity to members of Dictyobacteraceae							Accession number
			S27^T	Uno11^T	SOSP1-9^T	Uno16^T	Uno17$^{T(*)}$	W12^T	Uno3^T	
K11A	B-129	S3.2.1	98.04	96.63	96.54	95.71	95.73	95.50	91.85	LC715236
K11A-2	B-130		98.18	96.63	96.54	95.79	95.73	95.57	91.85	LC715237
K11-B	B-131		98.04	96.55	96.44	95.61	95.55	95.68	91.71	LC715238
K17-1	B-132		98.04	96.76	96.73	95.75	95.76	95.68	91.99	LC715239
K17-1A	B-133		98.04	96.71	96.67	95.80	95.74	95.73	91.95	LC715240
K17-1B-1	B-134		98.18	96.56	96.54	95.93	95.80	95.72	91.77	LC715241
K17-1B-2	B-135		98.04	96.61	96.56	96.00	95.66	95.79	91.91	LC715242
K17-A	B-136		97.97	96.63	96.54	95.64	95.66	95.43	91.85	LC715243
K42	B-137		98.18	96.77	96.54	96.14	95.73	96.07	91.77	LC715244
K44-1B	B-138		97.82	96.46	96.43	95.88	95.61	95.96	91.76	LC715245
K44-2-1	B-139		98.08	96.83	96.50	96.24	95.88	96.32	91.80	LC715246
S3.2.1.5	B-126		97.41	96.21	96.13	95.42	95.51	95.13	91.26	LC715230
S3.2.1.6	B-127		97.41	95.99	95.92	95.13	95.58	95.13	91.11	LC715231
S3.2.2.5	B-128	S3.2.2	98.00	96.57	95.35	95.78	95.72	95.49	91.48	LC715232
SL3-2-2-R3	B-140		98.04	96.49	96.33	95.86	95.67	95.79	91.63	LC715233
SL3-2-2-R6-1	B-141		98.11	96.56	96.24	95.91	95.64	95.99	91.67	LC715234
SL3-2-2-R6-2	B-142		98.04	96.56	96.40	95.93	95.73	95.86	91.70	LC715235

Note: S27^T: *Dictyobacter aurantiacus* (LC210808); Uno11^T: *D. kobayashii* (LC278466); SOSP1-9^T: *D. fomicarum* (AM180155); Uno16^T: *D. alpinus* (LC278467); Uno17^T: *D. arantiisoli* (LC278468); W12^T: *D. vulcani* (LC422201); Uno3^T: *Tengunoibacter tsumagoiensis* (LC278465). (*): alignment data were retrieved from the NCBI database. The 16S rRNA sequence similarities data of S3.2.1.5, S3.2.1.6, and S3.2.2.5 were obtained from Rachmania et al. (2020).

Qualitative screening of hydrolytic enzymes

The ability of 17 ktedonobacterial isolates to degrade starch as tested on ten-fold diluted R2A agar supplemented with 0.5% (w/v) soluble starch at 30°C for 21 days is shown in Table 2. Observation of amylolytic activity of ktedonobacterial isolates was carried out using the qualitative starch-iodine method. The presence of a clear zone around ktedonobacterial colonies after the starch-containing plates were stained with 1% (v/v) iodine is shown in Figure 4. In this study, all of 17 ktedonobacterial isolates were positive for amylolytic activity at 30 °C as shown by the presence of clear zones around the colonies. The positive results of amylolytic activities in 17 isolates indicated that all isolates were able to produce amylolytic enzymes.

Screening for cellulase activity in 17 ktedonobacterial isolates was evaluated using ten-fold diluted R2A agar supplemented with 0.5% (w/v) carboxymethylcellulose (CMC) substrate for 21 days at a temperature of 30°C. The screening result of the cellulolytic activity for all ktedonobacterial isolates is shown in Table 2. The clear zone formation around all isolates on the CMC-containing medium after flooding the plates with 0.2% (w/v) Congo red is shown in Figure 5. Fifteen ktedonobacterial isolates formed clear zones in the tested medium, indicating that they were able to produce cellulolytic enzymes.

Observation of amylase- and cellulase-producing ktedonobacterial was carried out based on clear zone formation around the colonies. All 17 isolates showed amylolytic activity by producing extracellular amylase on 0.5% starch agar medium at 30°C. Meanwhile, 15 isolates produced extracellular cellulase due to their ability to degrade 0.5% CMC at 30°C. Therefore, 15 of 17 isolates (88.23%) were amylase- and cellulase-producing *Ktedonobacteria*, while 2 of 17 isolates (11.76%) were only amylase-producing *Ktedonobacteria*. These findings showed that *Ktedonobacteria* isolated from forest soil in the Cisolok geothermal area were able to produce extracellular carbohydrase to degrade the complex carbohydrate polymers into mono- and short oligomers. The possession of carbohydrate-degrading enzymes (amylase and cellulase) in *Ktedonobacteria* isolates may support their natural role as decomposers. By possessing these enzymes, the isolates may cooperate in decomposition processes that occur in soil or litter in the forest of the Cisolok geothermal area.

We cannot justify the variability level of clear zone intensity (Figures 4-5) as we only determined positive and negative activities based on the presence of clear zones around the colonies qualitatively. At this moment, we could not explain the level of enzymatic activities (as strong, medium, or weak). The intensity of the clear zone may be affected by the variabilities of the growth rate of the isolates and the variation of inoculum among the isolates. A further quantitative study using crude enzymes is needed to perform in order to classify the enzymatic activities (as strong, medium, or weak).

The comprehensive genome studies of *Ktedonobacteria* revealed that this class encodes 104 putative biosynthetic gene clusters (BGCs) with high unknown functions (Zheng et al. 2019a). Their genomes also comprised of 153 to 290 carbohydrate-active enzymes (CAZymes) which represented 2.53% to 3.77% of the total encoded proteins (Zheng et al. 2021). An unexpected findings revealed that the number of CAZymes in *Ktedonobacteria* genomes is higher compared to the prominent cellulolytic

actinomycetes. Interestingly, 4 to 28 CAZymes in *Ktedonobacteria* genomes were predicted to be putative extracellular enzymes, which exceeded the other Chloroflexi members (Zheng et al. 2021). Although the whole genome sequence of 17 *Ktedonobacteria* isolates used in this study was not yet determined, the phylogenetic analyses (Figure 3) indicated that all 17 isolates belong to *Ktedonobacteria* and *Dictyobacter aurantiacus* S27^T as their closest related species. According to Zheng et al. (2021), *Dictyobacter aurantiacus* S27^T has 265 CAZymes (3.55% of the total encoded proteins) and 21 extracellular CAZymes (0.28% of the total encoded proteins). Closely related species are most likely harbours similar biosynthetic gene clusters (BGCs). Additionally, the results of enzymatic screening performed in this study (Table 2) supported that they have CAZymes (amylolytic and cellulolytic) activities. All ktedonobacterial isolates in this study are likely to have similar CAZymes properties with *D. aurantiacus* S27^T.

Table 2. Enzymatic abilities of ktedonobacterial isolates from forest soil of Cisolok geothermal area

Isolate code	Degradative test	
	0.5% Starch	0.5% CMC
K11A	+	+
K11A-2	+	-
K11-B	+	+
K17-1	+	+
K17-1A	+	+
K17-1B-1	+	+
K17-1B-2	+	+
K17-A	+	+
K42	+	+
K44-1B	+	+
K44-2-1	+	+
S3.2.1.5	+	+
S3.2.1.6	+	+
S3.2.2.5	+	+
SL3-2-2-R3	+	+
SL3-2-2-R6-1	+	+
SL3-2-2-R6-2	+	-

Note: (+), positive for amylolytic/cellulolytic activities; (-), negative for amylolytic/cellulolytic activities

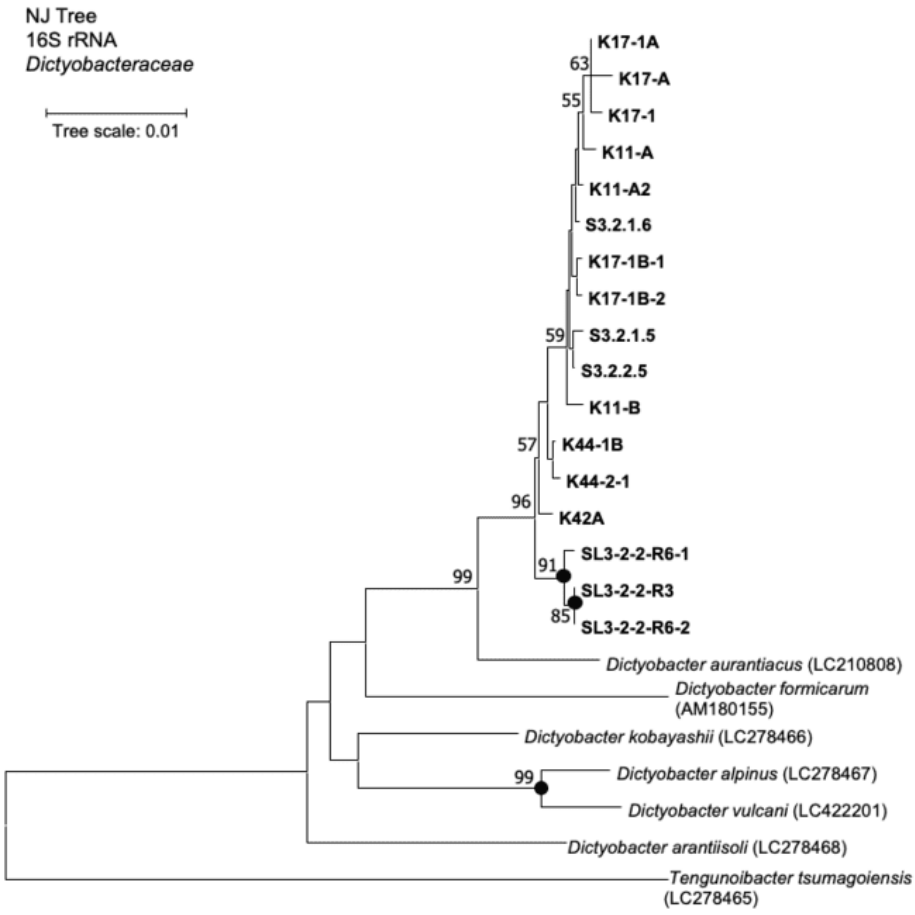

Figure 3. The neighbor-joining tree was constructed based on the 1323 aligned position of 16S rRNA gene sequences. *Tengunoibacter tsumagoiensis* (LC278465) was used as an outgroup. The closed circles indicated branches of the tree were found using maximum likelihood, minimum evolution, and maximum parsimony trees. Numbers at the branched nodes indicated the percentage of bootstrap values with 1000 replications and only more than 50% of bootstrap values were shown

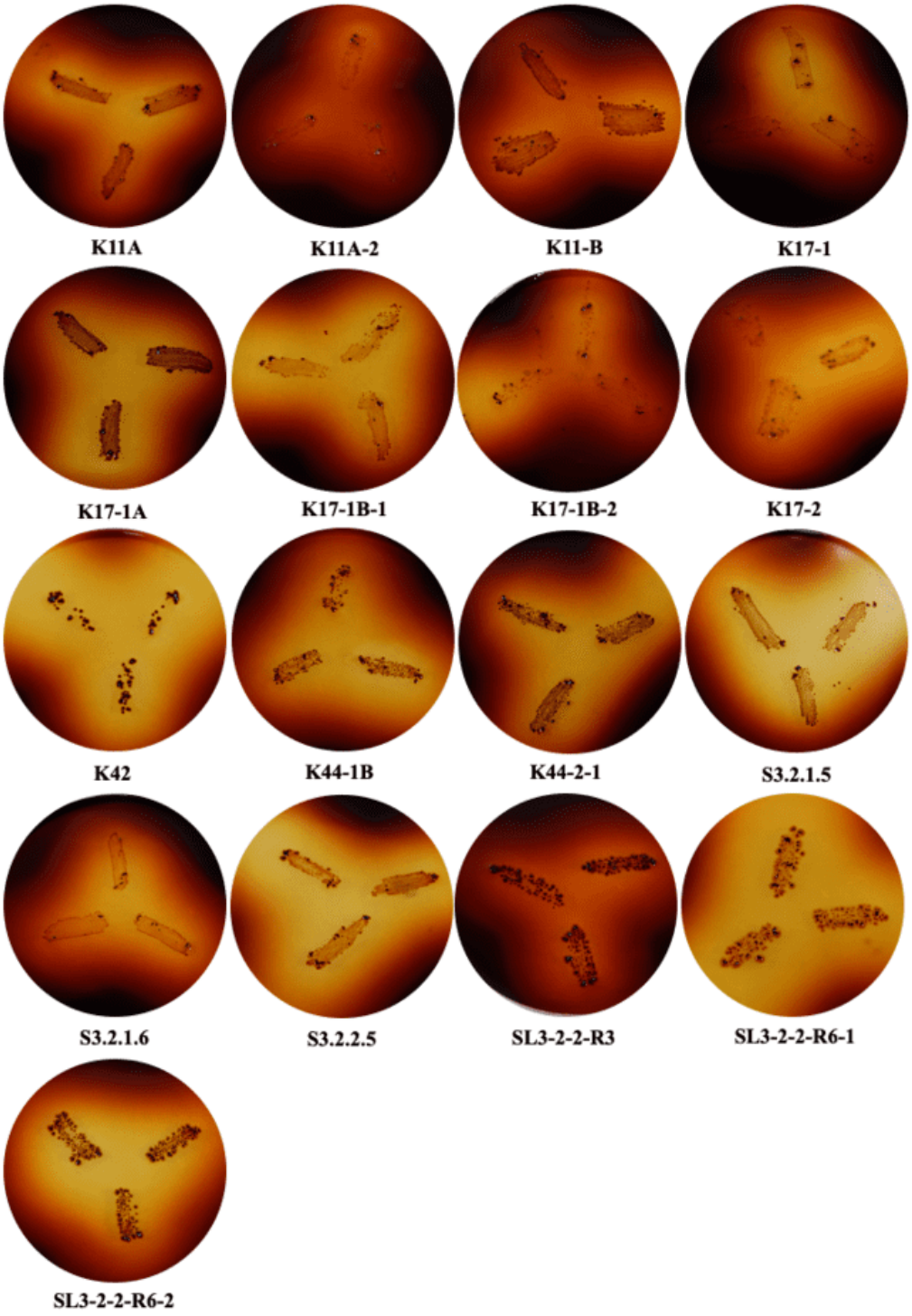

Figure 4. Formation of clear zone around colonies of ktedonobacterial isolates indicated positive for amylolytic activity

The great numbers of CAZymes in *Ktedonobacteria* genomes were supported by the ability of *Ktedonobacteria* to degrade various polysaccharides substrates e.g., starch, cellulose, xylan, and chitin (Cavaletti et al. 2006; Yabe et al. 2010b; Yabe et al. 2016; Yabe et al. 2017b; Wang et al. 2019; Yan et al. 2020; Yabe et al. 2021). The enzymatic abilities of *Dictyobacter* isolates from this study were supported by the previous scientists' reports that most Dictyobacteraceae members were able to degrade CMC, avicel, xylan, starch, and skim milk (Yabe et al. 2017b; Wang et al. 2019; Zheng et al. 2020), while *Tengunoibacter tsumagoiensis* Uno3^{T} was merely able to degrade xylan (Wang et al. 2019). Therefore, *Ktedonobacteria* could also be proposed as potential candidate for novel enzymes producers (Zheng et al. 2021).

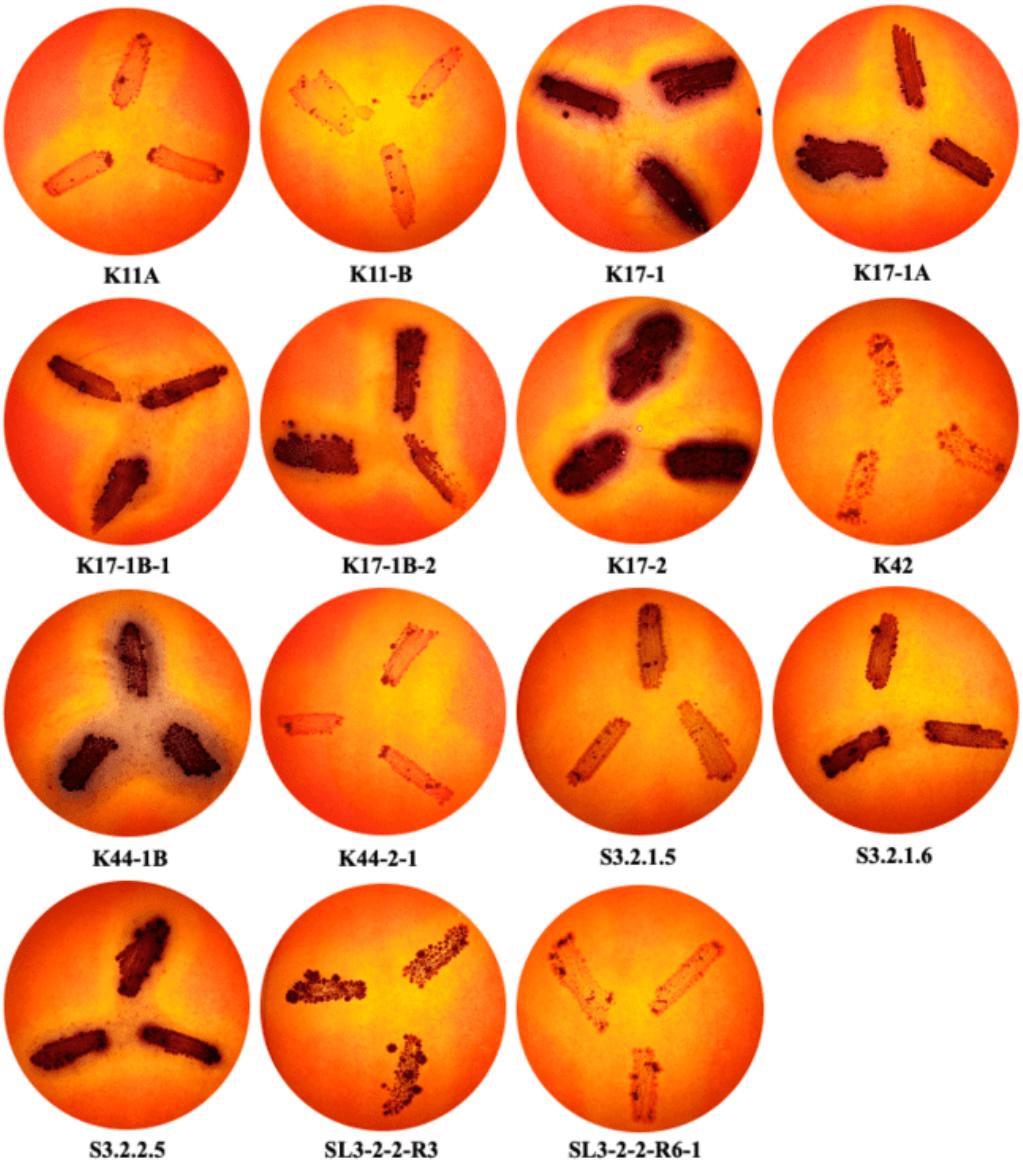

Figure 5. Formation of clear zone around colonies of ktedonobacterial isolates indicated positive for cellulolytic activity

In conclusion, the 16S rRNA gene sequence similarity search and phylogenetic analyses showed that all ktedonobacterial isolates belong to the genus *Dictyobacter* within the family Dictyobacteraceae of the class *Ktedonobacteria* and are represented as candidate novel species. Further taxonomic studies are needed for the description of this putative novel taxon. Most isolates (88.23%) showed enzymatic abilities on both 0.5% soluble starch and 0.5% CMC. This study revealed the presence of *Ktedonobacteria* with amylolytic and cellulolytic abilities in the forest soil of Cisolok geothermal area. These culturable *Ktedonobacteria* isolates serve as new genetic microbial resources for future prospecting applications as potential novel enzymes producers.

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