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Diversity and phylogenetic relationship of cellulolytic bacteria from the feces of Bali Cattle in South Central Timor, East Nusa Tenggara, Indonesia

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Abstract. *Missa H, Susilowati A, Setyaningsih R. 2016. Diversity and phylogenetic relationship of cellulolytic bacteria from the feces of Bali Cattle in South Central Timor, East Nusa Tenggara, Indonesia, Indonesia. Biodiversitas 17: 614-619.* There are three types of cattle farms with different kinds of feed in South Central Timor, that are maintained around Supul Lake, quarantined and left in the wild. This research aims to isolate, identify and determine the genetic relationship among of cellulolytic bacteria from the feces of Bali Cattle (*Bos javanicus javanicus* D´Alton, 1823, syn. *Bos javanicus sondaicus* Temminck, 1839/Blyth, 1842) in South Central Timor. The isolation of cellulolytic bacteria was done by using spread plate method on Carboxymethyl Cellulose (CMC) media. Cellulolytic activities were determined by the clear zone visibility using 0.1% congo red indicator. 16S rRNA encoding genes amplification was conducted using Polymerase Chain Reaction (PCR) using 63F and 1387r primers. Sequences of the 16S rRNA encoding genes were analyzed by bioinformatics using Nucleotide BLAST on NCBI website to determine the species of bacteria based on sequence similarity. The construction of the phylogenetic tree of cellulolytic bacteria was done using MEGA 7.0 software. The results were 48 isolates showing cellulase activity. There were 12 isolates from Supul Lake which have high cellulase activity namely: S1H6, S2H5 S2H7 S3H; in quarantine location: K1H6, K2H3, L2H7, K2H4, K1H2, K3H2; and in wild care system: L1H4 and L1H5. These activities were presented in clear zone about 7.08 to 1.47 cm. Based on the analysis of 16S rRNA encoding genes, there were 5 different genera found in 12 isolates with high cellulolytic activity. The isolates possessed similarity with *Pseudomonas* sp. 96%, *Acinetobacter* sp. 95%, *Bacillus* 97%, *Stenotrophomonas* 88%, and *Brachybacterium* sp. 97%. There were seven bacterial isolates having the potential to be declared as new bacterial species with <97% similarity percentage that are SIH6, S2H5, K2H3, K2H4, LIH4, LIH5, L2H7. Based on the phylogenetic tree cellulolytic bacteria showed the closest genetic relationship of 0.0% and farthest of 19.3% L2H7 isolate with Brachybacterium sp. S21F1

Keywords: 16S rRNA encoding gene, Bali Cattle, *Bos sondaicus*, cellulolytic bacteria, diversity, phylogenetic relationship

INTRODUCTION

Cellulolytic bacteria are bacteria that can hydrolyze cellulose complex into smaller oligosaccharides and ultimately into glucose (Lamid et al. 2011). Cellulolytic bacteria are naturally very common in agricultural soil, fertilizer or in the plant tissues, the rumen of ruminant animals and also on cattle feces. Cattle feces is the waste products of digestion secreted from the body in the form of solids (Hidayah et al. 2012). The availability of farm wastes is abundant, such as manure which is rarely used by the community of South Central Timor, therefore it becomes one of the causes of environmental pollution.

The problem of Bali Cattle (*Bos javanicus javanicus* D´Alton, 1823, syn. *Bos javanicus sondaicus* Temminck, 1839/Blyth, 1842) or livestock breeding in South Central Timor, East Nusa Tenggara is the shortage of the main fodder i.e. king grass, so as to maintain the potential of dairy farms that are part of Timor people's lives, the cattle breeders select one of three alternatives i.e. firstly, raising cattle extensively by means of cattle grazing in the field, orchard or yard, with the kind of food that are commonly consumed: agati leaves, banyan leaves, cottonwood leaves, banana stems and leguminous leaves; or secondly, breeding cattle in semi-intensive manner in the way that the cattle are kept in the shed at night, then grazed during the day around Lake Supul. The third is the fodder for cows is grazed from around Lake Supul, in the form of marshes grass and the water is from the lake. While producing Bali Cattle in South Central Timor, the breeders prefer an alternative technique by quarantining the cattle for fattening process since by being quarantined the feeding of the cattle is more regulated. The type of feed that is usually given is banana stems, as well as several grasses such as *Panicum maximum*, *Pennisetum purpureum*, and *Pennisetum purpureum*, and the drinking is water mixed with salt.

Alternative fodder has a quite high content of cellulose to affect the microbial enzymes in digesting the nutrients in the rumen which is ideally suited for a number of microorganisms. Cellulolytic bacterial isolates has a specific activity as a producer of cellulase enzymes, so it has the use of certain commercial functions such as garbage sewage treatment and is often used in the textile industrial field. Additionally, cellulase is also used in the pharmaceutical industry as an agent to help the digestive

system e.g. fiber material for diet purposes. Cellulase is also used in the fermentation process of biomass into biofuels, such as ethanol. Cellulase-producing cellulolytic bacteria are often found from the genus of *Pseudomonas*, *Cellulomonas*, *Bacillus*, *Micrococcus*, *Cellvibrio*, and *Cytophaga* (Lamid et al. 2011).

MATERIALS AND METHODS

Sampling

Sampling was from the feces of Bali Cattle in the South Central Timor, East Nusa Tenggara, Indonesia based on the predetermined sampling points, i.e., in semi intentions cattle raising around Lake Supul, in cattle quarantine and in extensive cattle care (wild) locations. Determination of sampling points used purposive sampling method. Sampling was determined by the difference in fodder. Feces of Bali Cattle were taken in wet condition for as much as 5-10 grams, and then they were inserted into the sample bottle and labeled with information of the place they were taken. Each sampling was feces of three different cattle which were taken from a nearby location.

Isolation of cellulolytic bacteria

Isolation of cellulolytic bacteria was carried on by diluting 1 g of sample into 9 mL of sterile distilled water aseptically. Then, serial dilution was made into 10^{-6}, and from serial dilution of 10^{-4} to 10^{-6}, it was taken 0.1 mL and it was distributed to CMC media using rod spreader. This technique was called spread plate technique. Finally, it was incubated at the temperature of 37°C for 4 x 24 hours (Syulasmi et al. 2009). Each colony of bacteria showing different morphology was taken and was considered as a pure culture.

Selection and cellulase activity test

Pure culture bacterial isolates on CMC slanted media were spotted on a petri dish containing CMC media, then incubated for 48 hours and at the end of incubation period, 0.1% congo red staining was conducted, being settled for 15 minutes and rinsed with 1M NaCl solution. If there were a clear zone around the colony, it indicated cellulose hydrolysis activity by cellulase enzymes (Jalgaonwala et al. 2011).

Genomic DNA extraction

The first stage to isolate the genomic DNA of bacteria was to grow the bacteria in CMC liquid medium and incubate it in the bacteria incubator shaker for 24 hours. A total of 1.5 mL of culture was centrifuged at 14.000 rpm speed. Then the DNA was extracted using Presto™ Mini gDNA bacterial kit (Geneaid).

16S rRNA encoding gene amplification by PCR

16S rRNA encoding genes were amplified using the polymerase chain reaction (Veriti Thermal Cycler). The reaction was conducted by mixing 12.5 µL of Kapa 2G Fast Ready Mix (Kapa Biosystem), 1.25 µL of 63 forward primers (63F: 5' CAGGCCTAACACATGCAAGTC 3'). 1.25 µL of 1387 reverse primer (1387r: 5' GGGCGGCGTGTACAAGGC 3') (Marchesi et al. 1998), 2 µL of DNA template and 8 µL of ddH_2O. PCR run with the following profiles: predenaturation at a temperature of 94°C for 2 min, followed by 30 cycles of denaturation stages at a temperature of 94°C for 30 seconds, annealing at a temperature of 55°C for 30 seconds, elongation at 72°C for 1 minute, and finalizing at a temperature of 72°C for 5 minutes. Then it was stored at 4°C for use at proper time and checked with electrophoresis (Marchesi et al. 1998). The DNA underwent sequencing in 1st Base Laboratory in Singapore. The DNA sequences were analyzed bioinformatically using Nucleotide BLAST on NCBI website (Waturangi et al. 2008) to determine similarity of the isolates compared to species existing in the database.

Phylogenetic relationship analysis

Genetic relationship among cellulolytic bacteria species from the feces of Bali Cattle around Lake Supul, in quarantine, and in wild care location that have been found in previous studies were analyzed using MEGA (Molecular Evolutionary Genetics Analysis) 7.0 software on a computer device (Kumar et al. 2015).

RESULTS AND DISCUSSION

Population of cellulolytic bacteria from Bali Cattle feces in South Central Timor

The population number of cellulolytic bacteria contained in the samples was determined randomly by multiplying the number of colonies being formed by liquidation factor on the concerned spread plate. The average populations of cellulolytic bacteria by plate count method on the feces of Bali Cattle in South Central Timor, especially in three cattle care sites around Lake Supul, cattle quarantine, and wild care areas, were eligible for the calculation of the bacterial population at dilution of $1x10^{-6}$. The highest cellulolytic bacteria population was on the feces of Bali Cattle in cattle quarantine which was equal to 1.68×10^8, while the lowest average population was on the feces of Bali Cattle around Lake Supul i.e. 1.05×10^8 (Figure 1).

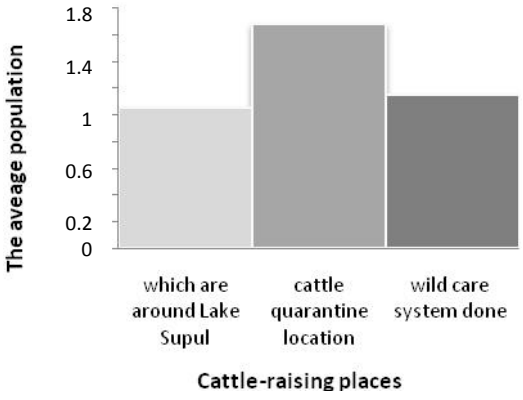

Figure 1. The average population number (10^6) of cellulolytic bacteria in the feces of Bali Cattle in South Central Timor based on the spread plate method on CMC media

The average population of cellulolytic bacteria per gram of Bali cattle feces in South Central Timor was considered high when compared to the population of cellulolytic bacteria per gram of rumen contents in the sheep rumen which was 2.98 x 10^7, was 5.12 x 10^7 in goat rumen, was 2.80 x 10^7 in deer rumen, was 4.76 x 10^7 in cattle rumen, was 7.58 x 10^7 in buffalo rumen (Thalib et al. 2000). Total population of cellulolytic bacteria varies depending on the feed consumed, the sampling time after feeding, different animal species, season and the availability of forage.

Cellulolytic activity of bacteria isolates

The isolation of cellulolytic bacteria from three sampling sites found 70 isolates. Each isolates was obtained from nine research samples. After being tested for its cellulase activity using 0.1% congo red staining, 48 isolates of positive cellulolytic bacteria were acquired. Therefore 12 bacterial isolates were selected to represent 48 isolates which were found for further research. The spread plate showing positive results of cellulolytic bacteria was presented in Figure 2.

Cellulose was hydrolyzed on CMC agar and if it was added with congo red staining, it would clear zone due to the reaction between congo red and bonding β-1,4-glycosidic contained in the cellulose polymer. Cellulose itself was hydrolyzed due to the activity of cellulase enzymes produced by the bacteria (Steensma 2001). With 1 mL of NaCl solution, it could be used to dilute the congo red dye around the colony, so that the clear zone was more visible (Sumardi 2004).

Cellulolytic bacterial isolates produced different clear zone diameter from one isolates to another. It can be caused by the size of the colonies that vary from one isolate to another. Cellulolytic activity index of cellulolytic bacteria from the feces of Bali Cattle in South Central Timor in three sites namely around Lake Supul, in cattle quarantine and in wild care were shown in the following diagram (Figure 3, 4, 5).

The highest index of clear zone on three cellulolytic bacterial populations in the feces of Bali Cattle in South Central Timor was produced by K1H6 isolates while the lowest ratio was produced by K1H3 isolates. When compared to the research conducted by Gusmailina et al. (2002) who obtained the highest index of clear zone around 5.8 and the research conducted by Hidayah et al. (2012) who obtained the highest index of clear zone around 3.7 then this K1H6 isolates was a cellulolytic bacteria that had high activity in degrading cellulose. Some microbes, mainly the bacteria types, had the ability to hydrolyze cellulose naturally through its cellulase activity. Although many microorganisms were able to degrade cellulose, only a few microorganisms could produce cellulase in a significant amount that was capable of hydrolyzing crystalline cellulose. Based on the graphic on figure 3, 4 and 5, it was selected 12 high activity cellulolytic bacterial isolates representing three sampling sites of Bali Cattle feces in South Central Timor for molecular identification. The selected isolates were SIH6, S2H5, S2H7, S3H1, K1H2, K1H6, K2H3, K2H4, K3H2, L1H4, L1H5, L2H7.

Amplification of 16S rRNA genes encoding

Isolation of DNA generally consists of four stages: cell lysis process, DNA binding, washing, and precipitating. After DNA isolation stage was completed, DNA concentration measurement was done to obtain DNA purity so as to qualify for the molecular analysis. DNA isolation results show that the DNA of 12 high activity cellulolytic bacteria was isolated properly. Isolation can be said to be pure and qualified to proceed to the molecular analysis if the value ratios of A260/280 were from 1.8 to 2.0 (Sambrook et al. 1989).

The results of the amplification of 16S rRNA genes by PCR was analyzed using 0.5% (w/v) agarose gel electrophoresis for 45 minutes at a voltage of 85 volts and a current of 300 mA to see the size of the DNA as the amplification product. Profile amplicons of 16S rRNA gene amplification by PCR can be seen in Figure 6.

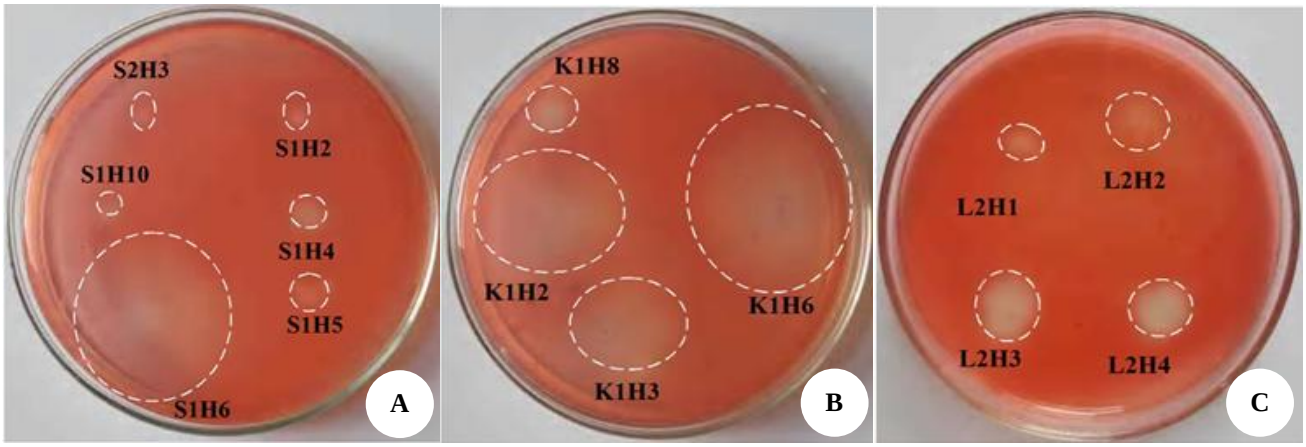

Figure 2. Clear zone showed cellulase activity of bacteria isolates in CMC media with congo red staining from Bali Cattle feces in South Central Timor. A. Around Lake Supul, B. Cattle quarantine, C. Wild care area

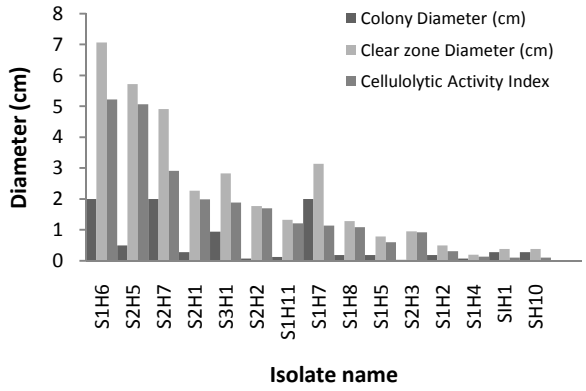

Figure 3. Cellulolytic activity index of the bacteria from Bali feces around Lake Supul represented by clear zone diameter reduced by diameter of the bacteria colony

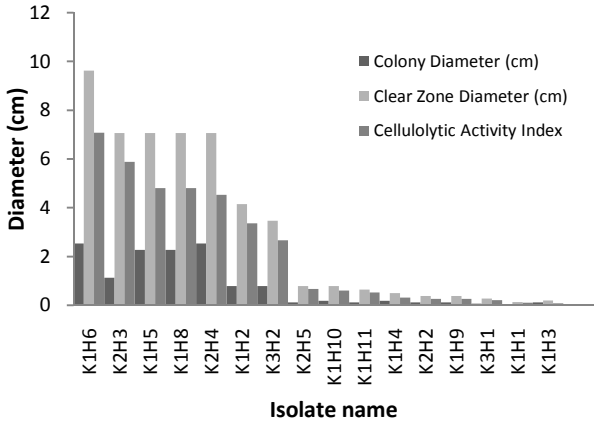

Figure 4. Cellulolytic activity index of the bacteria from Bali Cattle feces in quarantine represented by clear zone diameter reduced by diameter of the bacteria colony

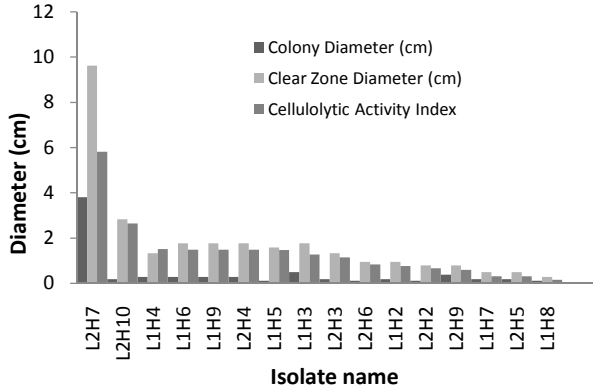

Figure 5. Cellulolytic activity index of the bacteria from Bali Cattle feces in wild care represented by clear zone diameter reduced by diameter of the bacteria colony

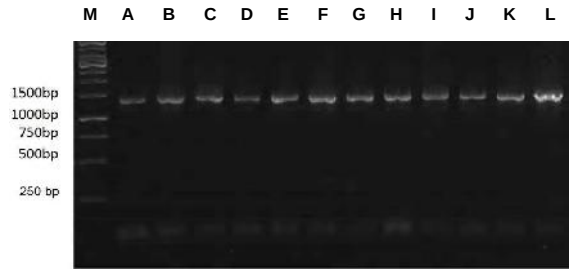

Figure 6. Electropherogram amplicon of 16S rRNA encoding genes cellulolytic bacteria isolated from Bali Cattle feces; M. Marker DNA, A. S1H6, B. S2H5, C. S2H7, D. S3H1, E. K1H2, F. K1H6, G. K2H3, H. K2H4, I. K3H2, J. L1H4, K. L1H5, L. L2H9

Table 1. Cellulolytic bacteria similarities by 16S rRNA encoding genes using the BLAST program

Isolate codes	Most similar species	Access number	Similarity (%)
S1H6	*Pseudomonas* sp. 53 (2015)	KU321296.1	96%
S2H5	*Acinetobacter* sp. XT-40	KR063566.1	95%
S2H7	*Pseudomonas* sp. C25	KT361090.1	97%
S3H1	*Bacillus cereus* ASDS9	KF256128.1	98%
K1H2	*Uncultured Bacterium* Clone QXJ-18	KJ957714.1	97%
K1H6	*Brachybacterium* sp. MCCC IA09822	KU560279.1	97%
K2H3	*Bacillus Cereus* MER 35	KT719615.1	95%
K2H4	*Pseudomonas* sp. DGM MH46	JF923454.1	96%
K3H2	*Bacillus Cereus* YBNY-1	KU363977.1	97%
L1H4	*Pseudomonas* sp. UIWRF 1386	KR189244.1	96%
L1H5	*Pseudomonas* sp. HJX22	KP979553.1	88%
L2H7	*Stenotrophomonas* BAB 5314	KT254651.1	80%

It is shown in the picture that the gene coding for 16S rRNA bacteria is amplified properly. This was shown by the existence of bands which were bright and bold as well as parallel to one another. This indicates that the primers being used are attached to the DNA template on the optimum temperature for primer annealing (Utami et al. 2012). The emergence of single band shows that the primers pair being used are specific and only stick to the expected position (Ratnayani et al. 2009).

Identity of cellulolytic bacteria based on 16S rRNA encoding genes sequences

The identification of bacteria using a gene encoding for 16S rRNA sequencing involves a comparison between the results and the reference sequences stored in the GenBank database. The results of the analysis of 16S rRNA gene using BLAST can be seen in Table 1.

The similarity percentage of ≥ 99% indicated that the species being compared were the same species, whereas by 97-99% of similarity percentage, it can be stated that the isolates being compared were in the same genus and by <97% of similarity percentage, it can be stated that the isolates had the potential to be declared as a new species of bacteria (Drancourt et al. 2000). Based on this research, the

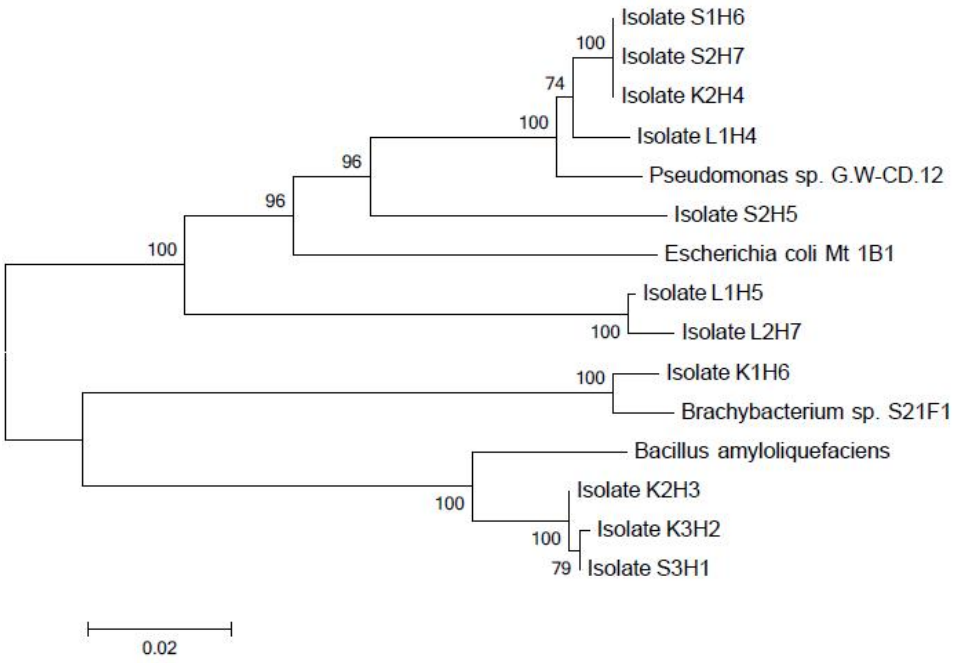

Figure 7. Phylogenetic relationships on the basis of 16S rRNA encoding gene. Based on the distance matrix, a neighbor-joining tree was reconstructed using MEGA software version 7.0. The bootstrap was performed with 1000 replicates. The bar indicated a 25-nucleotide difference. Genetic relationship of each of these bacteria isolates from each location of raising cattle i.e. around Supul Lake (S), in quarantine (K) and wild care system (L) was not in the same group. The cellulolytic bacterial isolates clustered and had a close relationship with the bacteria in the same genus with reference genus (*Pseudomonas, Bacillus, Brachybacterium*)

bacterial isolates that showed genus similarity were S2H7, S3H1, K1H2, K1H6, and K3H2 isolates, and there were seven bacterial isolates that have the potential to be declared as new bacterial species that are S1H6, S2H5, K2H3, K2H4, L1H4, L1H5, and L2H7 isolates.

Phylogenetic relationship of high activity cellulolytic bacteria

Phylogenetic relationship of cellulolytic bacteria among the isolates and some bacteria that have been found in this research was discoverable using a phylogenetic tree by looking at the genetic distance (Figure 7). Based on the phylogenetic tree, each of these species which were found in each location formed a group together, but they spread across all groups. This suggests that the bacteria isolated from each location have non-adjacent phylogenetic relationship. They formed groups based on the proximity of the genus. Despite being on the different location and different kind of food, the isolates of high activity bacteria joined the groups that has similar bacterial genus and those that show close relationship.

The results of the analysis of genetic distance between cellulolytic bacteria isolates were as follows: the closest genetic distance was 0.0% on S2H7 isolates with K2H4 isolates, S1H6 isolates with K2H4 isolates, and S2H7 isolates with S1H6 isolates. While the farthest genetic distance was 19.3%, i.e. L2H7 isolates with *Brachybacterium* sp. S21F1. The scale of 0.02 referred to the evolutionary distance on the length of the branch.

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