JOURNAL OF TROPICAL LIFE SCIENCE
2022, Vol. 12, No. 2, 173 – 182
http://dx.doi.org/10.11594/jtls.12.02.03

Research Article

Bacterial Community Structure in Sago Pith and Sago Waste Water and Its Potential Uses as Organic Acids Producer

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Article history:
Submission September 2021
Revised November 2021
Accepted March 2022

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ABSTRACT

Sago is one of the commodities in South Sulawesi. The extraction process of sago flour produces wastewater that still contains organic matter and smells sour. The sour odor produced allows acid-producing bacteria that can be used for waste treatment. This research aims to explore the bacterial community structure in sago pith and sago wastewater through high-throughput sequencing technology and its potential uses as organic acids producer. Samples were obtained from a traditional sago factory in Palopo City, South Sulawesi, Indonesia. The acidity degree, total dissolved solids, and temperature were measured in the sago pool at the sampling area, while the nutrient contents were analyzed by titration method in Testing Laboratory of Food Quality and Food Safety, Brawijaya University. Bacterial cells in the sago wastewater were acquired through a multilevel filtering process on filter paper with pore nitrocellulose membrane sizes of 0.45 and 0.20 µm. Total DNA trapped in the nitrocellulose membrane with a pore size of 0.20 µm was isolated using FastDNA Spin Kit (MPBIO), and the V3-V4 regions of 16S rDNA (341f-806r) were amplified. Amplicons were analyzed by Miseq of Illumina and further analyzed by Muscle v.3.8.31, QIIME v.1.7.0, and R v.2.15.3. The result shows that nutrient content in sago pith is higher than in sago wastewater. Both samples are dominated by Phylum Proteobacteria and share 189 common bacterial species. The dominant bacteria that can produce organic acid in sago pith are *Dysgonomonas* sp., *Propionispira* sp., and *Lactobacillus pentosus*. While, *Lactobacillus mali* and *Gluconobacter frateurii* are the dominant organic acid-producer bacteria in sago wastewater.

Keywords: Bacterial community structure, Next generating sequencing, Sago pith, Sago wastewater

Introduction

Sago (*Metroxylon sago* Rottb.) is one of the Indonesian plantations and identified as one of the priorities national plant researches in 2020-2024 (2020-2024 PRN). Sago is widespread across Sumatera, Kalimantan, Sulawesi, Maluku, and Papua. South Sulawesi is one of the sago-producing areas in Indonesia, which produced 2.560 tons in 2015; 3.069 tons in 2016; 3.073 tons in 2017; 3.136 tons in 2018; 2.964 tons in 2019; and an estimated 3.026 tons in 2020. The highest sago production in South Sulawesi was reported in North Luwu and Luwu regencies with dry sago flour production of 2.070 tons and 888 tons in 2018 with an area of 1.805 ha and 1.335 ha, respectively[1]. The plantation area is certain to increase, in line with the government's plan to build a sago techno-park in Palopo City.

Sago flour is extracted from the sago pith using a large volume of water and produces sago

How to cite:
Tenriawaru EP, Suharjono, Ardyati T, Zubaidah E (2022) Bacterial Community Structure in Sago Pith and Sago Waste Water and Its Potential Uses as Organic Acids Producer. Journal of Tropical Life Science 12 (2): 173 – 182. doi: 10.11594/jtls.12.02.03.

wastewater of 94-97% from the total weight of sago stems [2, 3]. Based on observation at the factory site, the sago wastewater has not been utilized by the community and is disposed of into the river. A total of 10-22 tons of sago wastewater is disposed of into the river per day, thus it has the potential to be a pollutant [2]. The sago wastewater contains carbon and nitrogen with a ratio of 105:0.12 [2] and has a sour smell. The carbon and nitrogen content and the sour odour allow acid-producing bacteria in sago wastewater. Therefore, research on the community of acid-producing bacteria in sago wastewater is needed as an initial step for a solution to utilize sago wastewater. However, research on bacterial communities in sago wastewater is still limited.

Several studies on bacteria in sago plants that have been reported are nitrogen-fixing bacteria associated with sago plants and contaminant bacteria in sago flour, which originated from the water. Sago-associated bacteria are *Klebsiella pneumonia, Klebsiella oxytoca*, *Enterobacter oryzae, Raoultella ornithinolytica*, *Burkholderia tropica*, *Cronobacter sakazakii* or *Cronobacter turicensis*, *Pantoea cypripedii*, *Erwinia aphidicola* [4], and *Stenotrophomonas* sp. [5]. Contaminant bacteria are *Salmonella, Bacillus cereus, Staphylococcus* spp., and *Clostridium perferingens* [6]. In addition, some amylolytic bacteria, namely *Bacillus* sp.PSA10 and *Bacillus* sp. PPK5 found in wet sago flour from sago processing area in Kendari, South Sulawesi [7], *Bacillus mycoides, Bacillus cereus, Bacillus licheniformis, Bacillus alvei*, and *Serratia liquefaciens* in sago waste product from Susupu, North Maoluccas [9]. Some cellulolytic bacteria, i.e., *Burkholderia cepacia* A1E (have a 99,37% similarity of 16S rDNA sequence with *Burkholderia cepacia* JCM 2799) found in sago pith waste in traditional sago industry, Palopo, South Sulawesi [10], *Serratia liquefaciens, Acinetobacter iwofii, Bacillus licheniformis*, and *Bacillus cereus* in sago waste product from Susupu, North Maoluccas [9].

Research on the bacterial community in plants is still limited because some bacteria cannot be cultured [8]. The possible method for studying the bacterial community is via high-throughput sequencing (HTS) technology, which is also known as next-generation sequencing (NGS). This method uses metagenome-based approaches, namely isolating total DNA from the environment without growing bacteria first [8, 11]. Research on bacterial communities in plants using metagenome-based approaches has been carried out on peels of banana, guava, mango, papaya, and passion fruit. [12], tomato [13, 14], apple [15], watermelon [8], grapes, lettuce, mushrooms, spinach, sprouts, strawberries, peaches, and peppers [16]. Research on the bacterial community in sago plants using metagenome-based approaches has not been done.

This study attempts to compare the nutritional contents between sago pith and sago wastewater, analyze the diversity and community structure of bacteria, and its potential use of dominant bacteria as organic acid-producer. The diversity and bacterial community structure were analyzed by using metagenomic-based approaches.

Material and Methods

A sampling of sago wastewater and sago pith

Samples of sago wastewater and sago pith were obtained in three replications from a traditional sago factory in Telluwanua District, Palopo City, South Sulawesi Province, Indonesia. The shredded sago pith sample was placed into a sterile plastic bag, while the sago wastewater was placed in a sterilized bottle. The samples were stored in a cool box containing ice gel for transport to the Microbiology Laboratory, Faculty of Mathematics and Natural Science, Brawijaya University to be prepared for further analysis.

Measurement of abiotic factors

Degree of acidity (pH), Total Dissolve Solid (TDS), and temperature of the pool of samples were measured at the time of sampling, while the nutrient contents of sago wastewater and sago pith were determined at the Testing Laboratory of Food Quality and Food Safety, Department of Agricultural Product Technology, Faculty of Agricultural Technology, Brawijaya University. The analysis of the nutrients followed the recommended method by Sudamiaji et al. [17] and included the total sugar, reducing sugar, starch, amylose, and vitamin C.

Bacterial chromosomal DNA extraction

All replicate samples of sago wastewater and sago pith was mixed into one sample. The mixed sago wastewater was filtered using a vacuum pump membrane filter (Merck) with a series of pore diameters 11.0 µm, 0.45 µm, and 0.20 µm, respectively. The 0.20 µm filter membrane that

contained bacterial cells was cut into small pieces and inserted in the Lysing Matrix E Tube. The mixed sago pith weighed as much as 3 g and was put into Lysing Matrix E Tube. The bacterial DNA was extracted according to FastDNA Spin Kit (MPBIO) Germany protocol with modifications: 1) increased homogenize time for samples-sodium phosphate buffer from 5 to 10 seconds using vortex; 2) suspension was incubated for ten minutes at room temperature (18 ±1 ºC) after which the supernatant-protein precipitate solution was inverted. The increasing homogenization time aimed to mix the sample and sodium phosphate buffer properly, whereas as the incubation of the supernatant-precipitate solution in order to completely precipitate of protein-supernatant.

Amplification

The extracted DNA was analyzed qualitatively with 1% agarose gel electrophoresis. The DNA concentration and purity were measured using NanoDrop Spectrophotometer. The DNA was diluted into 1.0 ng.µL^{-1} using sterile distilled water. Next-generation sequencing was carried out in Novogen, Singapura, using the illumine platform based on pair-end algorithms. The 16S rDNA sequences were amplified using primers 341f (3'-CCTAYGGGRBGCASCAG-5') and 806r (3'-GGACTACNNGGGTATCTAAT-5'). These specific primers were designed for the V3-V4 region of 16S rDNA with a length of 466 bp. The PCR reaction was carried out with Phusion® High Fidelity PCR Master Mix (New England Biolabs, United Kingdom). The quality of the 16S rDNA amplicon was analyzed using 2% agarose gel electrophoresis. Amplicon with a size 400-450 bp was purified using the Qiagen Gel Extraction Kit (Qiagen, Germany) and analyzed using the Illumina platform.

Data Analysis

Paired-end reads were connected using the FLASH v.1.2.7 program, filtered using the QIIME v.1.7.0 program, and the chimera was detected and removed using the UCHIME algorithm to obtain effective tags. All the effective tags were grouped by 97% DNA similarity into the Operational Taxonomic Unit (OTU). The number of effective tags included the total tags and annotated tags as taxon tags. The tags that were found once and only in one sample is shown as a unique tag.

Sequence analyses were performed by UPARSE software v7.0.1001 [18] using all effective tags. Representative sequences for each OTU were screened for annotation by the MOTHUR Program [19, 20] and references from the SILVA database. The taxonomy was constructed using the MUSCLE Program. The alpha bacteria diversity was analyzed using the QIIME Program v.1.7.0 and displayed with the Program R v.2.15.3 [21].

Table 1. Abiotic factors of sago wastewater and sago pith

Parameters	Sago wastewater (LS1)	Sago pith (ES)
pH	6.40	6.00
TDS (ppm)	111.00	-
Temperature (°C)	33.00	30.00
Total sugar (%)	0.01	0.44
Reducing sugar (%)	>0.01	0.16
Starch (%)	>0.01	18.29
Amylose (%)	>0.01	6.47
Vitamin C (mg.100 g^{-1})	10.07	96.32

Results and Discussion

Abiotic factors

Abiotic factors included the pH, TDS, temperature, total sugar, reducing sugar, starch, amylose, and vitamin C (Table 1). pH and temperature were higher in sago wastewater than in sago pith, while nutrient contents in sago pith were higher than in sago wastewater.

The sago pith contains about 65% starch and the remaining is fiber [22]. Sago starch is the glucose polymer chain consisting of two glucans, namely amylose and amylopectin [23]. Sago pith contains sugar, reducing sugar, starch, amylase, and vitamin C (Table 1). Sago flour is extracted from sago pith by using the hand-washing method for shredded sago pith and precipitate in water. The extraction process separates sago flour, fiber, and sago wastewater as the residue [2]. The nutrient contents of sago pith are mainly precipitate in flour, some trapped in pith fibers, and a small portion dissolves in sago wastewater [22]. Sago flour nutrient contents are also affected by variety and leaf area [24].

Bacterial diversity

The number of tags, taxon tags, and OTUs number in sago pith was also higher than sago

wastewater, but the unique tags in sago wastewater were larger than in sago pith (Table 2). It showed that the bacterial composition of sago pith was higher than in sago wastewater. However, some tags on sago wastewater were only found once, which was indicated by a unique tag.

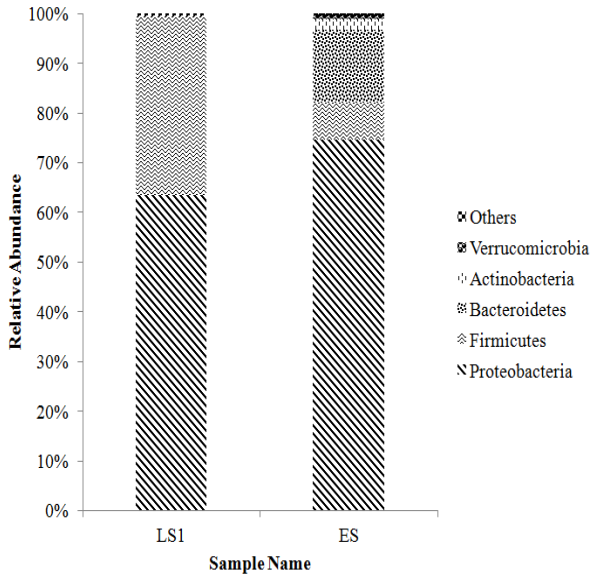

Figure 1. Bacterial communities' structure at the phylum level. X axis represent the sample name and Y axis represent relative abundance (%) of phyla. LS1 means sago wastewater and ES means sago pith. The color in each diagram represents the relative abundance of each phylum.

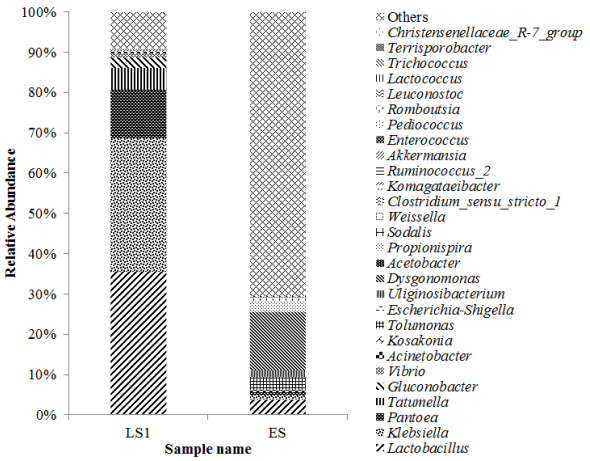

Figure 2. Bacterial communities structure at the genus level. X axis represent the sample name and Y axis represent relative abundance (%) of genera. LS1 means sago wastewater and ES means sago pith. The color in each diagram represents the relative abundance of each genus.

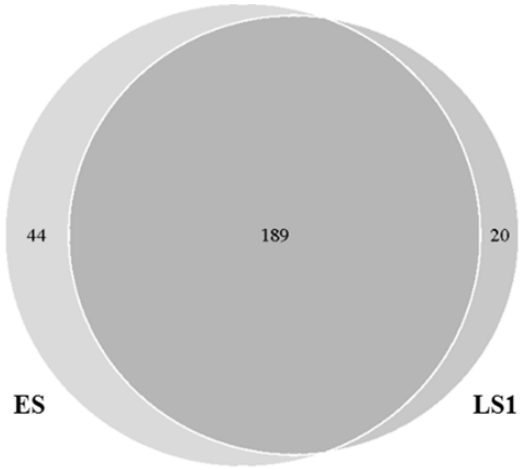

Figure 3. Venn diagram of OTU number. Each circle and color represent one sample/group, the value represents the OTUs number, value in left circle represent the number of specific OTUs in sago pith (ES), value in right circle was the number of specific OTUs in sago wastewater (LS1), value in overlapping part represent the shared OTUs of both samples.

Even though sago pith and sago wastewater come from the same sago stem, the bacterial composition of sago pith and sago wastewater was different (Figure 1 and 2). Overall, there were 44 species that were only found in sago pith but not found in sago wastewater (Figure 3). This indicated that the production process affected the composition and diversity of bacteria in the sample. The extraction process of sago starch allowed the presence of bacterial species from the sago pith dissolved in sago wastewater and was shown as overlapping species on the Venn diagram (Figure 3). The overlapping circle means that both samples shared 189 species. Meanwhile, several other species that were previously found in the sago pith were trapped in the pith fibers or deposited in the sago starch so that they were not found in sago wastewater. On the other hand, there were also 20 species that were only found in sago wastewater but did not originate from sago pith (Figure 3). The species is thought to have originated from the water used to extract sago starch. The water sources used varies and are not sterile or filtered. The extraction method is not sterile, which could result in microbial contamination from water and the environment [6].

In this study, the microbial diversity was determined by Shannon and Simpson indices. Both bacterial diversity indices provided the commu-

Table 2. Bacterial diversity, bacterial richness, tags, and OTUs number

Parameters	Sago wastewater (LS1)	Sago pith (ES)
Total tags	118,049.00	119,020.00
Taxon tags	113,000.00	115,610.00
Unique tags	5,049.00	3,410.00
OTUs number	220.00	236.00
Shannon diversity index	3.05	2.92
Simpson diversity index	0.81	0.70
Chao1 richness index	210.13	237.33
ACE richness index	211.75	237.86

Remarks: Total tags represent the number of effective tags, taxon tags represent the number of annotated tags, unique tags represent the number of rare tags that only appeared once and only in one sample, OTUs represent the number of genera or species which were grouped based on the ≥ 97% similarity of sequences

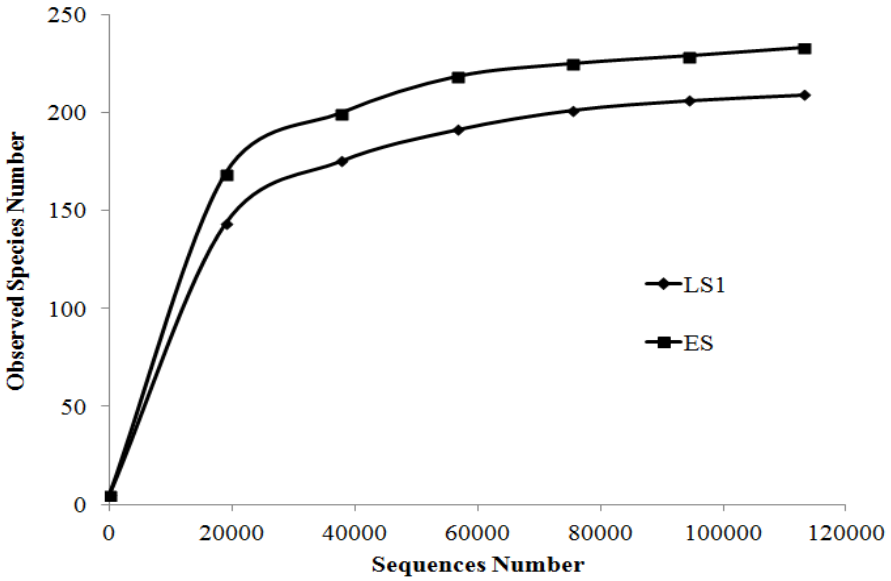

Figure 4. Rarefaction curve. Each curve represented a single sample and shaped by each sample name supplied in the mapping file, value on the X-axis represent sequences number and value on the Y-axis represent observed species number, LS1 line represent sago wastewater and ES line represent sago pith.

nity composition of the samples by estimating evenness (Simpson index) and richness (Shannon index) [25]. The richness was also measured by using two types of non-parametric indices: 1) Chao1 to estimate the number of species in the community, and 2) ACE to estimate the species coverage [25]. The parametric index of richness was determined by the rarefaction curve. It showed that the number of OTUs in sago pith was higher than sago wastewater. The bacterial diversity and richness of each sample showed that bacterial diversity in sago wastewater was higher than in sago pith but lower in richness (Table 2 and Figure 4).

Kim *et al*. [25] suggested that the number of species present, its numerical composition, and bacterial diversity was important factors to characterize the bacterial community in a niche. Bacterial diversity, number of tags, taxon tags, OTUs number of sago pith were higher than sago wastewater. It is influenced by the nutrient content in sago wastewater and sago pith. Bacteria need nutrients such as carbon, nitrogen [26, 27], and vitamin for their growth. The results showed that sago pith contained higher nutrition than in sago wastewater. The high nutrient content of sago pith allows more bacteria to grow in the sample, compared to sago wastewater which has limited nutritional content.

Likewise, in the richness index, sago pith was higher than sago wastewater. Richness index was inversely proportional to diversity index, in which

bacterial diversity at sago wastewater was higher than at sago pith (Table 2). Richness and diversity calculation of bacterial diversity of both samples were based on the number of OTUs (sequences with similarity ≥ 97%). Kim *et al.* [25] argued that the richness index measured the number of species (OTUs) in the community, while the diversity index measured the richness and evenness of species (OTUs). The dominance of certain species (OTUs) will reduce the diversity index. Sago wastewater showed that there was no dominant phylum or species. Meanwhile, the sago pith was dominated by Phylum Proteobacteria (Figure 1) and Genus *Dysgonomonas* (Figure 2).

Richness, OTU number, and bacterial diversity were also affected by pH and nutrient content in the sample. Richness and OTU numbers decreased at pH 6.40 and temperature 33ºC (in sago wastewater) and increased at pH 6.00 and temperature 33ºC. Meanwhile, total sugar and vitamin C increased the species richness and OTU number but decreased the bacterial diversity. This was probably because vitamin C can stimulate the growth of certain bacteria and inhibit other bacteria.

Bacterial community structure and its potential uses

The bacterial community in sago wastewater consists of Phylum Proteobacteria, Firmicutes, Bacteriodetes, Actinobacteria, Verrumicrobia, Acidobacteria, Nitrospirae, Chloroflexi, and Cyanobacteria. Whereas, the sago pith consisted of the Phyla Proteobacteria, Firmicutes, Bacteriodetes, Actinobacteria, Verrumicrobia, Acidobacteria, Nitrospirae, Cyanobacteria, Chloroflexi, and Gemmatimonadetes (Figure 1). Phylum Proteobacteria has the highest number of phylum for both samples, with relative abundance in sago wastewater and sago pith were 63% and 75%, respectively. In sago wastewater, Phylum Firmicutes with the relative abundance of 36% had the second-highest rank. Phylum Bacteriodetes (15%) and Firmicutes (7%) had the second and third rank of relative abundance in sago pith. The lowest relative abundance of phylum in sago wastewater was Verrumicrobia (0.01%) and at sago pith was Cyanobacteria (0.004%).

For the genus level, sago wastewater and sago pith consist of 76 genera and 79 genera, respectively (Figure 2). The dominant genera were shown as a taxonomic tree in Figure 5. There are no dominant species in sago wastewater, but in sago pith, the Phylum Proteobacteria was the most dominant, as much as 75% (Figure 1) and Genus *Dysgonomonas* as much as 14%, while the percentage of their genera was a maximum 4% (Figure 2).

It is interesting that the sago pith is dominated by Genus *Dysgonomonas*. This was different from previous studies which reported that species of this genus were found from clinical sources, associated with insects [28, 29], sugarcane bagasse [30], food waste and its fermentation [31–33], but has never been reported to be found in sago pith.

Genus *Dysgonomonas* is known as lignocellulosic and polysaccharide-degrading bacteria into biofuels [29]. The abundance of the Genus *Dysgonomonas* in sago pith was due to the presence of sufficient amounts of starch and amylose (Table 1). *Dysgonomonas* is able to produce ferulic and vanillic acid from lignin [34]. This ability was thought to cause smell sour and lower pH of sago pith than sago wastewater.

Besides being caused by Genus *Dysgonomonas*, the sour odor and low pH in sago wastewater are also thought to be caused by several genera known as acid producers, namely *Lactobacillus*, which is capable of producing lactic acid [35, 36], and *Gluconobacter frateurii*, which was known as the acetic acid producer and vitamin C [37]. The high content of vitamin C in sago pith (Table 1) was thought to be related to the presence of *Gluconobacter frateurii*.

In contrast to sago pith, sago wastewater is dominated by the Genus *Lactobacillus*, *Klebsiella*, *Pantoea*, *Tatumella*, and *Gluconobacter* (Figure 2). Although the results of this study showed that the content of sugar, reducing sugar, starch, and amylose were limited in sago wastewater (Table 1), the nutrient content was still able to support microbial growth. In addition, sago wastewater from traditional sago factory in Telluwanua District, Palopo City, South Sulawesi Province, Indonesia, contains vitamin and micro-nutrients needed by microbes, namely nitrogen (0,01%), phosphorus (0.01-0.12 ppm), potassium (10.46-50.71 ppm), magnesium (5.84-7.94 ppm), and calcium (0.62-2.62 ppm) [38].

Among the five genera in sago wastewater, the genus *Lactobacillus* and *Gluconobacter* are acid-producing genera [35, 36]. These bacteria were suspected to be the cause of the sour smell and pH decrease in sago wastewater after being storage for

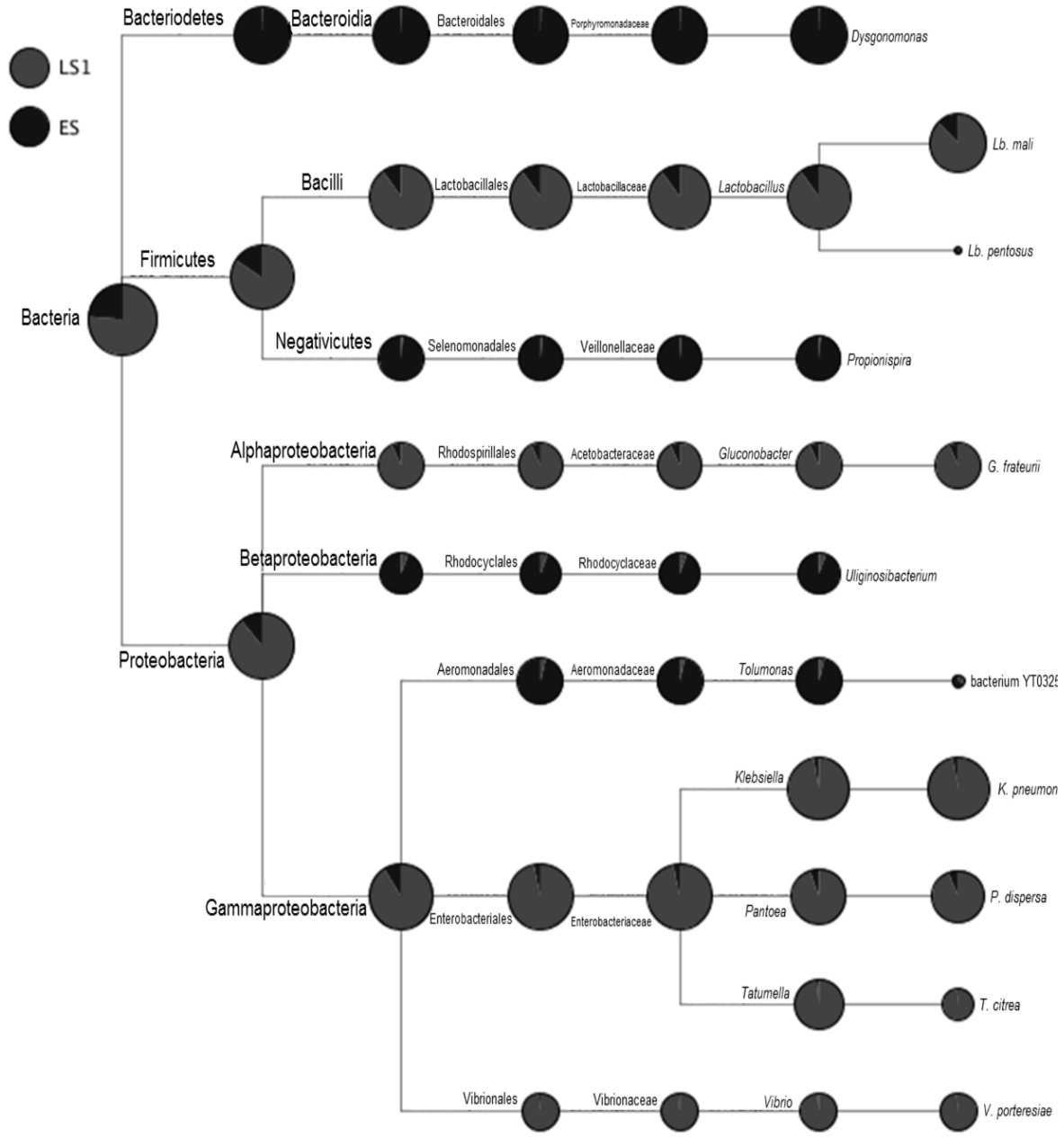

Figure 5. Taxonomic tree of top ten abundance genera in both samples. The column represents different taxonomic rank, the size and color of slice area represents relative abundance of species on each sample

a long time. These acid-producing bacteria can be used commercially as starter cultures to produce organic acids, fermented food, and bioactive compounds [39, 40]. However, the potential uses of these bacteria for acid production need further elucidation. Furthermore, the potential for industrial application of the bacteria presents in sago pith and sago wastewater is for further study.

In addition to acid-producing bacteria, there are also several other bacteria that can be explored for their ability as lignin-degrading agents in the waste products of sago flour production, namely Genus *Dysgonomonas* and *Tolumonas* [34, 41, 42]. The presence of lignin-degrading bacteria is associated with the presence of lignin content in sago pith. Linggang et al. [43] suggested that waste sago pith fiber (sago hampas) produced from the sago flour extraction process contains 3.9% lignin.

Likewise, the genus *Pantoea*, *Vibrio,* and *Klebsiella* can also be tested for their ability as N-fixing bacteria or as phosphorus-solubilizing bac-

teria [44, 45]. The presence of these bacteria in the sample provides an opportunity for processing waste from the sago flour production, as a starter culture for industry, and as plant growth-promoting rhizobacteria.

Among the species found in sago pith and sago wastewater, *Klebsiella pneumonia* and Genus *Pantoea* and *Vibrio* have been reported to be isolated from sago palm [4, 46], while *Lactobacillus mali* was reported to be isolated from sago wastewater in sago home industry west Malangke, North Luwu, South Sulawesi, Indonesia [47]. Meanwhile, other genera have only been reported to be found from plants or plant rhizosphere [35, 36, 41, 42], while in sago is still unknown. Thus, these results could provide new knowledge to this research of bacterial community in sago pith and sago wastewater.

Conclusion

Sago wastewater has lower nutritional content than sago pith. The bacterial community of sago wastewater and sago pith shared 189 common species, 20 specific species for sago wastewater, and 44 for sago pith. Sago wastewater and sago pith were dominated by Phylum Proteobacteria. Sago wastewater is mainly composed of *Lactobacillus mali*, *Gluconobacter frateurii, Klebsiella pneumonia, Pantoea dispersa, Tatumella citrea,* and *Vibrio porteresiae*. Sago pith is mainly composed of *Dysgonomonas* sp., *Propionispira* sp., *Uliginosibacterium* sp., *Tolumonas* bacterium YT0325, and *Lactobacillus pentosus.* The bacteria that could be a potential source for organic acid-producer were *Dysgonomonas* sp., *Lactobacillus mali*, *Lactobacillus pentosus*, *Gluconobacter frateurii*, and *Propionispira* sp.

Acknowledgement

Acknowledgements are addressed to the Ministry of Education and Culture of the Republic of Indonesia as the funder of this research through the 2017 BPPDN scholarship, the colleagues at Faculty of Science UNCP who have facilitated the sampling process, Dr. Hasria Alang, M.Kes. who has assisted in DNA extraction, reviewers, and all who have helped this research.

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