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by Dr Abubakar Sidik Katili

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Identification of fermentative bacteria on local microorganisms of golden snail (*Pomacea canaliculata* Lamarck, 1822)

YULIANA RETNOWATI♥, ABUBAKAR SIDIK KATILI♥♥

Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Gorontalo. Jl. Prof. Dr. Ing. BJ. Habibie, Bone Bolango 96119, Gorontalo Province, Indonesia. Tel.: +62-435-827213, Fax.: +62-435-827213, ♥email: yuliana_ri@yahoo.com, ♥♥abubakarsidik@ung.ac.id

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Abstract. *Retnowati Y, Katili AS. 2021. Identification of fermentative bacteria on local microorganisms of golden snail (*Pomacea canaliculate *Lamarck, 1822)). Biodiversitas 22: 778-784.* Local Microorganisms (LMo) is a fermented liquid containing various microorganisms that potentially act as decomposers and bio-fertilizer. *P. canaliculata* is one of the rice pests that is a basic ingredient of LMo because of its high protein content. The objective of this study was to determine the types of fermentative bacteria on Local Microorganisms of *P. canaliculata*. The fermentation of LMo was conducted for 0, 7, 14, and 21 days. Microbial population was determined at 7-day intervals based on the TPC method. Characterization and identification based on polyphasic taxonomy including macroscopic and microscopic morphological characters., Molecular identification was based on 16S rRNA gene sequences. The results showed that LMo of *P. canaliculata* had a low degree of acidity and tended to decrease during the incubation period, from pH 5.3 to 4.0. Bacterial population tends to increase at 0-14 fermentation days and decreases after 21 days. The isolation results showed that the 3 bacterial isolates namely BFPc-01, BFPc-02, and BFPc-03 were isolated based on morphological differences. The morphological characters of BFPc-01 was milky white color colony, Gram-negative, coccus; BFPc-02 isolate was pink, colony color, Gram-negative, coccus; and BFPc-03 isolate was yellow color colony, bacillus, Gram-positive. The results of molecular characterization based on the 16s rRNA gene sequence showed that BFPc-01 isolate similar to *Klebsiella pneumoniae* MT604895.1 (99.04%), BFPc-02 isolate closely related to *Serratia sp*. (100%), and BFPc-03 isolate similar to *Microbacterium* sp. (100%).

Keywords: Fermentative, local microorganisms, molecular characterization, *Pomacea canaliculata*

INTRODUCTION

The Local Microorganisms (LMo) is a liquid containing various microorganisms involved in the overhauling of various organic wastes (Neli and Suliasih 2015). The ability of microorganisms in LMo to decomposition has been widely used for activation in the composting process (Astuti et al. 2014; Manullang et al. 2018). Gong et al. (2017) reveal that intestinal tract of *P. canaliculata* contains cellulase-producing bacteria, while Li et al. (2019) found *that Ochrobactrum* sp., *Sediminibacterium* sp., *Desulfovibrio* sp., and *Cloacibacterium* sp. on the gut of *P. canaliculata*. The microorganisms contained in LMo are also bio-fertilizer agents, which have the potential of commercial biological agents. LMo is also used as a fertilizer to fertilize plant leaves, stimulate plant growth, and control pest and disease agents (Ranamukhaarachchi and Wickramasinghe 2006; Setiawan et al. 2016; Siregar et al. 2017; Widjajanto 2017; Manullang et al. 2018; Pane and Marwazi 2020).

Local Microorganisms can be obtained from a variety of local materials, including cow urine, banana stalks, Gamal leaf, fruits, stale rice, household waste, bamboo shoots, Cebreng leaves/legumes, banana weevils, vegetables, and elephant grass, so that it can play a role in solid and liquid waste management (Neli and Suliasih 2015; Thakur et al. 2016; Widjajanto 2017; Manullang et al. 2018; Roeswitawati 2018). Another material that has potential as a basic material for LMo is the golden snail (*P. canaliculata*). *P. canaliculata* is a member of the Ampullaridae tribe which is known as a pest on rice plants (Halwart 2008; Brito and Joshi 2016; Siregar et al. 2017; Rao et al. 2018). The presence of this pest can damage thousands of hectares of rice seedlings in the early age (Rao et al. 2018; Wagiman et al. 2019). This pest contains protein and high fat so that they have the potential to be a source of local microorganisms (Chimsung and Tantikitti 2014; Visca Jr and Palla 2018).

Local Microorganisms are composed of various types of microbes that play a role in the process of breaking down macromolecules in basic materials. Some of the microorganisms in LMo act as bio-fertilizers are *Rhizobium* sp., *Azospirillum* sp., *Azotobacter* sp., *Pseudomonas* sp., and *Bacillus* sp. Suhastyo et al. (2013) reported that the LMo of banana weevil contains a number of bio-fertilizer bacteria, including *Bacillus* sp., *Aeromonas* sp., and *Aspergillus* sp. Some of these bio-fertilizer microbes show activity as solubilizing phosphate and produce indole acetic acid (IAA) growth hormone. Rani et al. (2017) reported that the LMo of Bintaro fruit contains a number of solubilizing phosphate and IAA-producing bacteria. The objective of this study was to determine the identity of fermentative bacteria in Local Microorganisms of *P. canaliculata*.

MATERIALS AND METHODS

Fermentation of *Pomacea canaliculata*

Fermentation process was initiated by sample preparation. The fresh 1 kg meat of *P. canaliculata* was crushed with 200 grams of sucrose and 2 liters of coconut water. The mixture was incubated at 37°C for 21 days for fermentation process with 3 replicates (modified of Astuti et al. 2016). The change in acidity was determined using a pH meter at 7-day intervals. The fluid of mixture was used as source of fermentative bacteria through isolation and purification.

Isolation, purification, and determine population of fermentative bacteria

The activities were conducted on interval time of 7 days, i.e. 0, 7, 14, and 21 days of fermentation process. The fermentative bacteria were isolated from pour plate method (Okolie et al. 2013). The fluid from fermentation product (1 mL) was serially diluted to make 10^{-6} dilution. 1 mL Sample of each level dilution was poured into Nutrient Agar medium and incubated at 37°C for 48 hours. The growth of bacterial colonies was observed based on the morphological characters. The pure culture of various bacterial colonies was maintained on Nutrient Agar medium by streak plate method (modified of Martí-Quijal et al. 2020). Then, the pure culture was further characterized based on molecular characterization. The population of fermentative bacteria was determined based on the number of bacteria colonies multiplied by the dilution factor.

Molecular characterization of fermentative bacteria

Extraction of genomic DNA

The DNA genomic of fermentative bacteria was carried out by following the protocol of ZR Fungal/Bacterial DNA Kit™ (Dangre-Mudey and Tankhiwale 2016). The bacterial cells as much as 50-100 mg (wet weight) were resuspended in 200 µL of PBS isotonic buffer to a ZR BashingBead™ Lysis Tube. Secure in a bead beater fitted with a 2.0 mL tube holder assembly and process at maximum speed for 5 minutes. Centrifuge the ZR BashingBead™ Lysis Tube in a microcentrifuge at ≥10,000 x *g* for 1 minute. Transfer up to 400 µL supernatant to a Zymo-Spin™ IV Spin Filter (orange top) in a Collection Tube and centrifuge at 7,000 rpm (~7,000 x *g*) for 1 minute. Add 1,200 µL of fungal/bacterial DNA binding buffer to the filtrate in the collection Tube from Step 4. Transfer 800 µL of the mixture from Step 5 to a Zymo-Spin™ IIC Column in a Collection Tube and centrifuge at 10,000 x *g* for 1 minute. Discard the flow-through from the Collection Tube and repeat Step 6. Add 200 µL DNA Pre-Wash Buffer to the Zymo-Spin™ IIC Column in a new Collection Tube and centrifuge at 10,000 x *g* for 1 minute. Add 200 µL DNA Pre-Wash Buffer to the Zymo-Spin™ IIC Column in a new Collection Tube and centrifuge at 10,000 x *g* for 1 minute. Add 500 µL Fungal/Bacterial DNA Wash Buffer to the Zymo-Spin™ IIC Column and centrifuge at 10,000 x *g* for 1 minute. Transfer the Zymo-Spin™ IIC Column to a clean 1.5 mL microcentrifuge tube and add 100 µL DNA Elution Buffer directly to the column matrix. Centrifuge at 10,000 x *g* for 30 seconds to elute the DNA. Characterization of DNA extraction product for quality (purity) and quantity (concentration and extraction efficiency) was determined using a 1 µL extract analyzed via spectrophotometer. Ratio of absorbance of light at 260 nm and 280 nm (A260/A280) between 1.7 to 2 was taken as indicator for pure DNA (Dangre-Mudey and Tankhiwale 2016).

Amplification of 16S rRNA gene

Amplification PCR by using MyTaq Red Mix (Bioline). PCR Master Mix 1x25µL: 9.5µL ddH_2O; 12.5 µL MyTaq Red Mix, 2x; 1 µL 20 µmol/µL 27F Primer (AGAGTTTGATCMTGGCTCAG); 1 µL 20 µmol/ µL 1492R Primer (TACGGYTACCTTGTTACGACTT), and 1 µL DNA Template (Okolie et al. 2013). The forward and reverse 16S rRNA gene universal primers generate a 1.5 kb fragment. PCR Condition (35 cycles) followed an initial denaturation of 95°C for 1 min; denaturation on 95°C for 15 sec; annealing on 52°C for 15 sec; extension on 68 °C for 45 sec; and hold on 4°C for 48 hours. The PCR product was detected on agarose-gel electrophoresis using 1 Kb DNA ladder as marker.

16S rRNA gene sequencing and Phylogenetic analysis

The PCR products of fermentative bacteria were purified by using ZymocleanÔ Gel DNA Recovery Kit (Zymo Research) and sequenced based on bi-directional sequencing method. All the sequences obtained from sequencing phase were analyzed and edited by using BioEdit soft-ware (Retnowati et al. 2017). Initially, all the 16S rRNA gene sequences were compared to sequences in GenBank by using the online service of Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the approximate phylogenetic position. Sequences were aligned using ClustalW with representative bacteria 16S rRNA sequences, and a phylogenetic tree was constructed using the Molecular Evolutionary Genetics Analysis (MEGA) software 6.06. A rooted neighbor-joining tree was constructed using the sequence of the 16S rRNA gene *Lactobacillus fermentum* MN938192.1, obtained from GenBank as outgroup species (Retnowati et al. 2017).

Data analysis

The population of fermentative bacteria was analyzed based on descriptive quantitative analysis. The data of acidity degree changing was represented as graphic ph versus time of observation, while the data of fermentative bacteria population was represented as a graphic log of cfu mL^{-1} versus observation time. Molecular characterization data were compared with the NCBI GenBank.

RESULTS AND DISCUSSION

Description of fermentation process of *Pomacea canaliculata*

The fermentation of *P. canaliculata* was carried out for 21 days. As long as fermentation process, there were occur decomposition of macromolecule performed by microorganisms, especially fermentative bacteria. The fermentative bacteria were used macromolecule from *P. canaliculata* and sucrose as nutrient sources that support their growth. Fermentation process was showed by the changing acidity degree for 21 days (Figure 1). The pH of fluid tends to decrease from 5.5 to 4.0 for 21 days of incubation. Decreasing pH showed that the fermentation process presumed produced organic acid.

Population of fermentative bacteria

Fermentative bacteria carried out the fermentation process of macromolecule on *P. canaliculata*. Bacterial population was observed every 7 days of interval. The result showed that the population of bacteria was found to increase from 0 to 14 days of fermentation, and then decrease to 21 days. The highest 320 × 10^5 CFU/mL population achieved on 14 days of fermentation, and the lowest 20 × 10^5 CFU/mL at 21 days of fermentation (Figure 2).

Morphological characters of fermentative bacteria

Three bacterial isolates namely BFPc-01, BFPc-02 and BFPc-03 were isolated based on morphological characters. The isolates of fermentative bacteria were characterized based on morphological character including shape and color of colony, and shape of cell, based on microscopic observation, and Gram staining (Table 1).

Identification of fermentative bacteria based on molecular character

The fermentative bacteria on Local Microorganisms of *P. canaliculata* carried out molecular characterization based on the 16S rRNA gene sequence. The sequence of 16S rRNA gene of three types of fermentative bacteria was detected at about 1500 bp (Figure 3). The figure showed that the 27F and 1427R universal primer compatible with amplification of 16S rRNA gene of fermentative bacteria. The long 16S rRNA gene sequence of BFPc-01, BFPc-02, and BFPc-03 isolates was detected on 1359, 1399, and 1355 bp. The 16S rRNA gene sequence of fermentative bacteria isolates showed in Table 2. The sequence of each isolate was compared with the 16S rRNA sequence or genome sequence of type strain on NCBI gene bank through BLAST analysis. The result of molecular analysis was shown as phylogenetic tree that describes the evolutionary relationship between type strain and fermentative isolate bacteria (Figure 4). The phylogenetic tree showed that the BFPc-01 isolate was closely related to *Klebsiella pneumonia* strain 2483^T with 99.0% of sequence similarity. The BFPc-02 isolate was similar to *Serratia marcescens* strain IAE.169^T, while the BFPc-03 isolate similar to *Microbaterium* sp. strain YPS-003 with sequence similarity of 100% respectively.

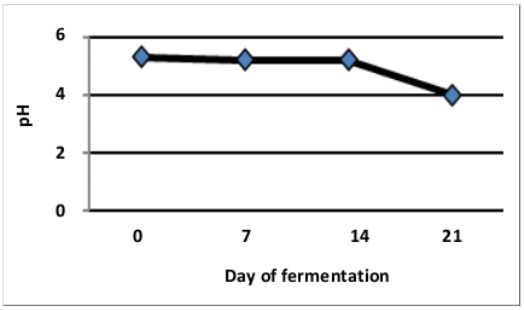

Figure 1. The decreasing of pH on fermentation process of *Pomacea canaliculata*

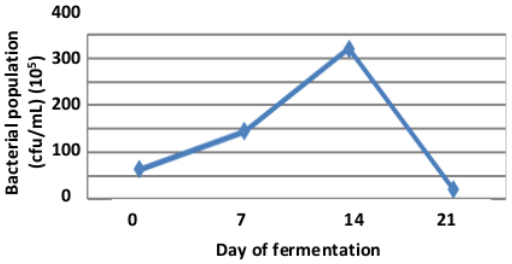

Figure 2. The population of fermentative bacteria on Local Microorganisms of *Pomacea canaliculata*

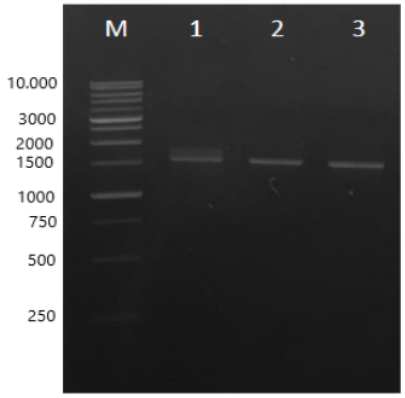

Figure 3. Chromatogram of 16S rRNA gene sequence of bacterial isolates

Table1. Morphological characters of bacterial isolates

Isolate bacteria	Morphological characters			
	Shape of colony	Color of colony	Shape of cell	Response to Gram staining
BFPc-01	Circular	White	Coccus	Gram-negative
BFPc-02	Circular	Pink	Bacillus	Gram-negative
BFPc-03	Circular	Yellow	Coccus	Gram-positive

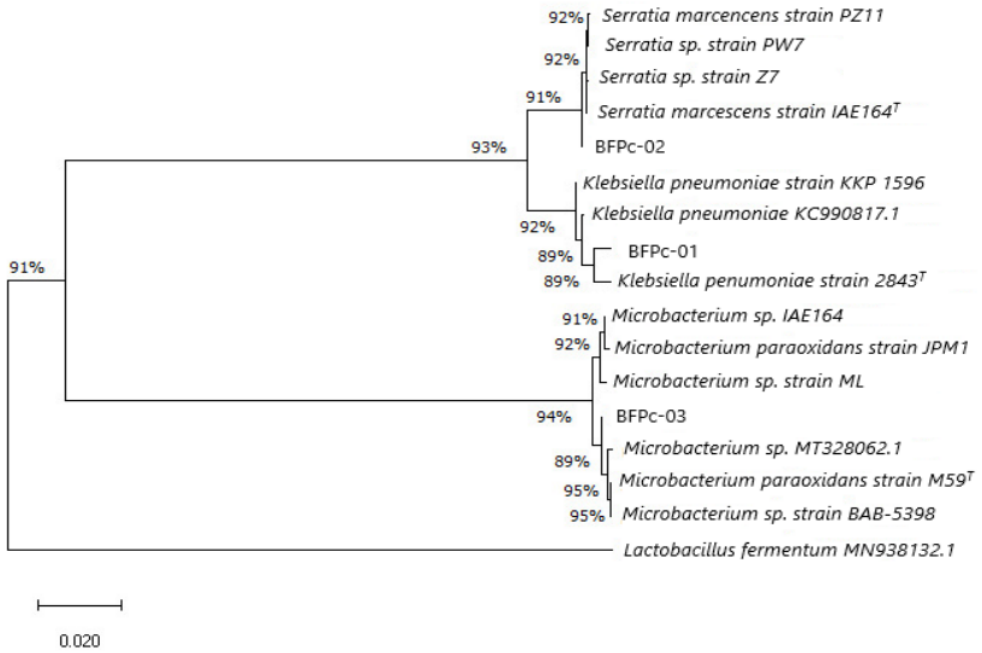

Figure 4. Neighbor-joining phylogenetic tree inferred from 16S rRNA gene sequence. The phylogenetic tree shows the phylogenetic relationship of BFPc-01 isolate to *Klebsiella pneumoniae* strain 2483^T (MT604895.1), BFPc-02 to *Serratia marcescens* strain IAE.169^T (MK414866.1), and BFPc-03 closely related with *Microbacterium* sp. strain YPS-003 (MT328062.1). Bootstrap values are expressed as percentages of 1000 replications.

Discussion

Fermentation of *Pomacea canaliculata* presumed that the decomposition of macromolecule produced organic acids. This is indicated by decreasing pH of fluid as long as fermentation process. Organic acids, such as lactic acid, are classified as weak and can dissociate by releasing hydrogen ions. The release of hydrogen ions can change the balance of the solution (Jay 1992). Marsini et al. (2015) reported that the pH of the LMo of tofu decreases after the third week of fermentation, then increases at the sixth week of fermentation. Under acidic conditions, decomposition of organic acids and dissolved nitrogen compounds to form ammonium, carbonic acid, and a small portion of CO_2, N_2, CH_4, and H_2 induce increasing in pH (Seni 2013; Marsini et al. 2015).

The fermentation process of LMo of *P. canaliculata* was carried out on 21 days. The fermentative bacteria were required to decompose macromolecule on the substrate into the simpler compounds completely. Suwastika et al. (2015); Manullang et al. (2018), reported that LMo of *Gliricidia* leaf had been applied as organic manure for 7 to 21 days. Juanda et al. (2011) also reported that the effective time to generate LMo was depended on type of basic material. The LMo quality was influenced by number and type of microorganisms, pH, and color.

Table 2. 16s rRNA gene sequence of bacterial isolates

No	Sample Name	Sequences
1	BFPc-01	**Sequence Assembly 1359bp** 1 CTCGGGTGAC GAGCGGCGGA CGGGTGATTA ATGTCTGGGA GACTGCCTGA TGGAGGGGGA 61 TAACTACTGG AAACGGTAGC TAATACCGCA TAACGTCGCA AGACCAAAGT GGGGGACCTT 121 CGGGCCTCAT GCCATCAAAT GTGCCCAGAT GGGATTAGCT AGTAGGTGGG GTAACGGCTC 181 ACCTAAGCGA CGATCCCTAG CTGGTCTGAG AGGATGACCA GCCACACTGG AACTGAGACA 241 CGGTCCAGAC TCCTACGGGA GGCAGCAGTG GGGAATATTG CACAATGGGC GCAAGCCTGA 301 TGCAGCCATG CCGCGTGTGT GAAGAAGGCC TTCGGGTTGT AAAGCACTTT CAGCGGGGAG 361 GAAGGCGATA AGGTTAATAA CCTTGTCGAT TGACGTTACC CGCAGAAGAA GCACCGGCTA 421 ACTCCGTGCC AGCAGCCGCG AGTAATACGG AGGGTGCAAG CGTTAATCGG AATTACTGGG 481 CGTAAAGCGC ACGCAGGCGG TCTGTCAAGT CGGATGTGAA ATCCCCGGGC TCAACCTGGG 541 AACTGCATTC TAAACTGGCA GGCTAGAGTC TTGTAGAGGG GGGTAGAATT CCAGGTGTAG 601 CGGTGAAATG CGTAGAGATC TGGAAGAATA CCGGTGGCGA AGGCGGCCCC CTGGACAAAG 661 ACTGACTCTC AGGTGCGAGA GCGTGGGGAG CACACAGGAT TAGATACCCT GGTAGTCCAC 721 GCCGTAAACG ATGTCGATTT GGAGGTTGTG CCCTTGAGGC GTGGCTTCCG GAGCTAACGC 781 ATTAAATCGA CCGCCTGGGG AGTACGGCCG CAAGGTTAAA ACTCAAATGA ATTGACGGGG 841 GCCCGCACAA GCGGTGGAGC ATGTGGTTTA ATTCAATGCA ACGCGAAGAA CCTTACCTGG 901 TCTTGACATC CACAGAACTT TCCAGAGATG GATTGGTGCC TTCGGGAACT GTGAGACAGG 961 TGCTGCATGG CTGTCGTCAG CTCGTGTTGT GAAATGTTGG GTTAAGTCCC GCAACGAGCG 1021 CAACCCTTAT CCTTTGTTGC CAGCGGTTCG GCCGGGAACT CAAAGGAGAC TGCCAGTGAT 1081 AAACTGGAGG AAGGTGGGGA TGACGTCAAG TCATCATGGC CCTTACGACC AGGGCTACAC 1141 ACGTGCTACA ATGGCATATA CAAAGAGAAG CGACCTCGCG AGAGCAAGCG GACCTCATAA 1201 AGTATGTCGT AGTCCGGATT GGAGTCTGCA ACTCGACTCC ATGAAGTCGG AATCGCTAGT 1261 AATCGTAGAT CAGAATGCTA CGGTGAATAC GTTCCCGGGC CTTGTACACA CCGCCCGTCA 1321 CACCATGGGA GTGGGTGGCA AAAGAAGTAG GTAGCTTAA
2	BFPc-02	**Sequence Assembly 1399bp** 1 TCGAGCGGTA GCACAGGGGA GCTTGCTCCC TGGGTGACGA GCGGCGGACG GGTGAGTAAT 61 GTCTGGGAAA CTGCCTGATG GAGGGGGATA ACTACTGGAA ACGGTAGCTA ATACCGCATA 121 ACGTCGCAAG ACCAAAGAGG GGGACCTTCG GGCCTCTTGC CATCAGATGT GCCCAGATGG 181 GATTAGCTAG TAGGTGGGGT AATGGCTCAC CTAGGCGACG ATCCCTAGCT GGTCTGAGAG 241 GATGACCAGC CACACTGGAA CTGAGACACG GTCCAGACTC CTACGGGAGG CAGCAGTGGG 301 GAATATTGCA CAATGGGCGC AAGCCTGATG CAGCCATGCC GCGTGTGTGA AGAAGGCCTT 361 CGGGTTGTAA AGCACTTTCA GCGAGGAGGA AGGTGGTGAA CTTAATACGT TCATCAATTG 421 ACGTTACTCG CAGAAGAAGC ACCGGCTAAC TCCGTGCCAG CAGCCGCGGT AATACGGAGG 481 GTGCAAGCGT TAATCGGAAT TACTGGGCGT AAAGCGCACG CAGGCGGTTT GTTAAGTCAG 541 ATGTGAAATC CCCGGGCTCA ACCTGGGAAC TGCATTTGAA ACTGGCAAGC TAGAGTCTCG 601 TAGAGGGGGG TAGAATTCCA GGTGTAGCGG TGAAATGCGT AGAGATCTGG AGGAATACCG 661 GTGGCGAAGG CGGCCCCCTG GACGAAGACT GACGCTCAGG TGCGAAAGCG TGGGGAGCAA 721 ACAGGATTAG ATACCCTGGT AGTCCACGCT GTAAACGATG TCGATTTGGA GGTTGTGCCC 781 TTGAGGCGTG GCTTCCGGAG CTAACGCGTT AAATCGACCG CCTGGGGAGT ACGGCCGCAA 841 GGTTAAAACT CAAATGAATT GACGGGGGCC CGCACAAGCG GTGGAGCATG TGGTTTAATT 901 CGATGCAACG CGAAGAACCT TACCTACTCT TGACATCCAG AGAACTTTCC AGAGATGGAT 961 TGGTGCCTTC GGGAACTCTG AGACAGGTGC TGCATGGCTG TCGTCAGCTC GTGTTGTGAA 1021 ATGTTGGGTT AAGTCCCGCA ACGAGCGCAA CCCTTATCCT TTGTTGCCAG CGGTTCGGCC 1081 GGGAACTCAA AGGAGACTGC CAGTGATAAA CTGGAGGAAG GTGGGGATGA CGTCAAGTCA 1141 TCATGGCCCT TACGAGTAGG GCTACACACG TGCTACAATG GCGTATACAA AGAGAAGCGA 1201 CCTCGCGAGA GCAAGCGGAC CTCATAAAGT ACGTCGTAGT CCGGATTGGA GTCTGCAACT 1261 CGACTCCATG AAGTCGGAAT CGCTAGTAAT CGTAGATCAG AATGCTACGG TGAATACGTT 1321 CCCGGGCCTT GTACACACCG CCCGTCACAC CATGGGAGTG GGTTGCAAAA GAAGTAGGTA 1381 GCTTAACCTT CGGGAGGGC
3	BFPc-03	**Sequence Assembly 1355bp** 1 GTGGATCAGT GGCGAACGGG TGAGTAACAC GTGAGCAACC TGCCCCTGAC TCTGGGATAA 61 GCGCTGGAAA CGGCGTCTAA TACTGGATAT GTGACGTGAC CGCATGGTCT GCGTTTGGAA 121 AGATTTTTCG GTTGGGGATG GGCTCGCGGC CTATCAGCTT GTTGGTGAGG TAATGGCTCA 181 CCAAGGCGTC GACGGGTAGC CGGCCTGAGA GGGTGACCGG CCACACTGGG ACTGAGACAC 241 GGCCCAGACT CCTACGGGAG GCAGCAGTGG GGAATATTGC ACAATGGGCG AAAGCCTGAT 301 GCAGCAACGC CGCGTGAGGG ATGACGGCCT TCGGGTTGTA AACCTCTTTT AGCAGGGAAG 361 AAGCGAAAGT GACGGTACCT GCAGAAAAAG CGCCGGCTAA CTACGTGCCA GCAGCCGCGG 421 TAATACGTAG GGCGCAAGCG TTATCCGGAA TTATTGGGCG TAAAGAGCTC GTAGGCGGTT 481 TGTCGCGTCT GCTGTGAAAT CCCGAGGCTC AACCTCGGGC CTGCAGTGGG TACGGGCAGA 541 CTAGAGTGCG GTAGGGGAGA TTGGAATTCC TGGTGTAGCG GTGGAATGCG CAGATATCAG 601 GAGGAACACC GATGGCGAAG GCAGATCTCT GGGCCGTAAC TGACGCTGAG GAGCGAAAGG 661 GTGGGGAGCA AACAGGCTTA GATACCCTGG TAGTCCACCC CGTAAACGTT GGGAACTAGT 721 TGTGGGGTCC ATTCCACGGA TTCCGTGACG CAGCTAACGC ATTAAGTTCC CCGCCTGGGG 781 AGTACGGCCG CAAGGCTAAA ACTCAAAGGA ATTGACGGGG ACCCGCACAA GCGGCGGAGC 841 ATGCGGATTA ATTCGATGCA ACGCGAAGAA CCTTACCAAG GCTTGACATA TACGAGAACG 901 GGCCAGAAAT GGTCAACTCT TTGGACACTC GTAAACAGGT GGTGCATGGT TGTCGTCAGC 961 TCGTGTCGTG AGATGTTGGG TTAAGTCCCG CAACGAGCGC AACCCTCGTT CTATGTTGCC 1021 AGCACGTAAT GGTGGGAACT CATGGGATAC TGCCGGGGTC AACTCGGAGG AAGGTGGGGA 1081 TGACGTCAAA TCATCATGCC CCTTATGTCT TGGGCTTCAC GCATGCTACA ATGGCCGGTA 1141 CAAAGGGCTG CAATACCGTG AGGTGGAGCG AATCCCAAAA AGCCGGTCCC AGTTCGGATT 1201 GAGGTCTGCA ACTCGACCTC ATGAAGTCGG AGTCGCTAGT AATCGCAGAT CAGCAACGCT 1261 GCGGTGAATA CGTTCCCGGG TCTTGTACAC ACCGCCCGTC AAGTCATGAA AGTCGGTAAC 1321 ACCTGAAGCC GGTGGCCTAA CCCTTGTGGA GGGAG

Fermentative microorganisms performed the fermentation process of *P. canaliculata*. The fermentative bacteria population in LMo of *P. canaliculata* changes during fermentation process. The population decreases from 14 to 21 days of fermentation. This showed that the bacteria reached an intolerant phase of environmental conditions. Marsiningsih et al. (2015) and Budiyani et al. (2016) reveal that the bacterial population in LMo of tofu and banana weevils decreased after the third week or 21 days of fermentation. Changes in bacterial population during fermentation are caused by biotic and abiotic conditions such as oxygen levels, nutrient availability, competition, and acidity level. At the beginning of fermentation, the bacterial population is an aerobic acid derived from the basic material. The bacteria use macromolecules as nutrient sources that indicate increased bacterial population.

The decreased acidity of LMo during the fermentation process is also a limiting factor for bacterial growth. The low acidity indicated that the fermentative bacteria were classified as acidophilic bacteria (Salvatore et al. 1992). Three fermentative bacteria isolated from fermentation of *P. canaliculata* were successfully identified based on molecular characters of 16S rRNA gene sequences. BFPc-01 isolate was closely related with *Klebsiella pneumonia* strain 2483^T (MT604895.1), BFPc-02 isolate was similar with *Serratia marcescens* strain IAE.169^T (MK414866.1), and BFPc-03 isolate was closely related with *Microbaterium* sp. strain YPS-003 (MT328062.1). Gong et al. 2013 reported that bacteria identified as *Bacillus subtilis* was found in intestinal tract of *P. canaliculata*. *Bacillus subtilis* was a type of cellulose-producing bacteria isolated from intestinal tract of *P. canaliculata*. *B. subtilis* was not found in Local Microorganism of *P. canaliculata* because it may not be adapted to fermentation condition.

Furthermore, fermentation process of *P. canaliculata* was focused on macromolecule decomposition of *P. canaliculata* as nutrient sources for their growth. There was no cellulose component in fermentation process of *P. canaliculata*. Some pathogenic bacteria are also found in *P. canaliculata* including *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* (Chobchuenchom and Bhumiratana 2003). All three fermentative bacteria isolate from Local Microorganisms of *P. canaliculata* were pathogenic bacteria. *Klebsiella pneumonia, Serratia marcescens,* and *Microbaterium* sp. were pathogenic bacteria naturally occurs in soil, water, and the surface of plant (Gundogan 2014; Vankova et al. 2015; Eduardo and Nora 2019). The three types of bacteria presumed originated locally habitat on agricultural land.

In summary, Local Microorganisms of *P. canaliculata* can infect farmers and did not recommended to be applied as decomposers or liquid organic fertilizer.

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