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File size: 323.49K  
Page count: 7  
Word count: 3,825  
Character count: 22,006  
Submission date: 04-Jan-2022 09:58AM (UTC+0800)  
Submission ID: 1737267872

BIODIVERSITAS  
Volume 22, Number 2, February 2021  
Pages: 776-784  
ISSN: 1412-033X  
E-ISSN: 2085-4722  
DOI: 10.13057/biodiv0220231

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

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Manuscript received: 2 October 2020; Revision accepted: 15 January 2021.

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**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 Sulisih 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 *Oribacterium* sp., *Scotinibacterium* sp., *Delffontia* sp., and *Clostridium* 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 (Rasumikharshechi and Wickramasinghe 2006; Setiawan et al. 2016; Siregar et al. 2017; Widjanto 2017; Manullang et al. 2018; Pane and Marwat 2020).

Local Microorganisms can be obtained from a variety of local materials, including cow urine, banana stalks, Gatal leaf, fruits, waste rice, household waste, bamboo shoots, Cebeng leaves/legumes, banana weevils, vegetables, and elephant grass, so that it can play a role in solid and liquid waste management (Neli and Sulisih 2015; Thakur et al. 2016; Widjanto 2017; Manullang et al. 2018; Roceswatiwati 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 Ampullariidae tribe which is known as a pest on rice plants (Halwart 2008; Retno 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 (Chamsung and Tanikitti 2014; Visesa 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. Sahatyo 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 in solubilizing phosphate and produce indole acetic acid (IAA) growth hormone. Rani et al. (2017) reported that the LMO of banana 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*.

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

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**Submission date:** 04-Jan-2022 09:58AM (UTC+0800)

**Submission ID:** 1737267872

**File name:** 6881-Article\_Text-36395-1-10-20210119\_1.docx (323.49K)

**Word count:** 3825

**Character count:** 22006

by Yuliana Retnowati, Abubakar Sidik Katili

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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 shed 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<sup>-6</sup> 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 DNase-free 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 1x25 µL: 9.5 µL ddH<sub>2</sub>O; 12.5 µL MyTaq Red Mix, 2x; 1 µL 20 µmol/µL 27F Primer (AGAGTTTGATCMTGGCTCAG); 1 µL 20 µmol/µL 1492R Primer (TACGGYTACCTTGTTA-3'), and 1 µL DNA Template (Okolie et al. 2013). The forward and reverse 16S rRNA gene universal primers generate a 7 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 Zymoclean® 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 software (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<sup>-1</sup> 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  $320 \times 10^5$  CFU/mL population achieved on 14 days of fermentation, and the lowest  $20 \times 10^5$  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 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<sup>T</sup> with 99.0% of sequence similarity. The BFPc-02 isolate was similar to *Serratia marcescens* strain IAE.169<sup>T</sup>, while the BFPc-03 isolate

similar to *Microbaterium* sp. strain YPS-003 with sequence similarity of 100% respectively.

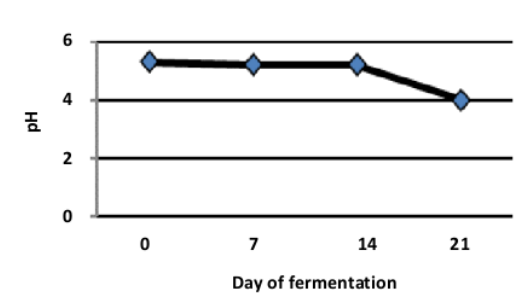


Figure 1. The decreasing of pH on fermentation process of *Pomacea canaliculata*

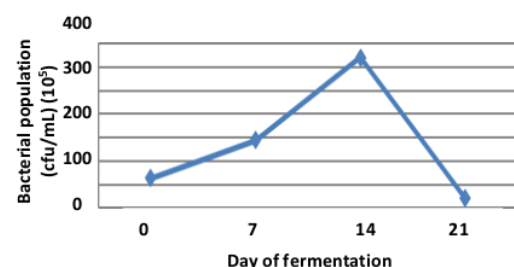


Figure 2. The population of fermentative bacteria on Local Microorganisms of *Pomacea canaliculata*

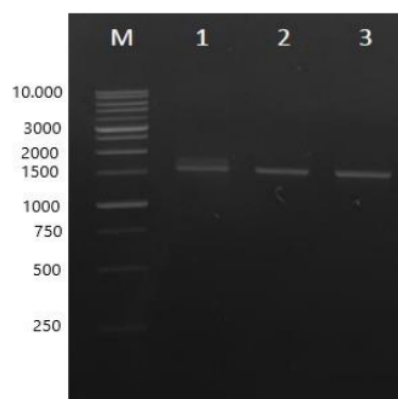
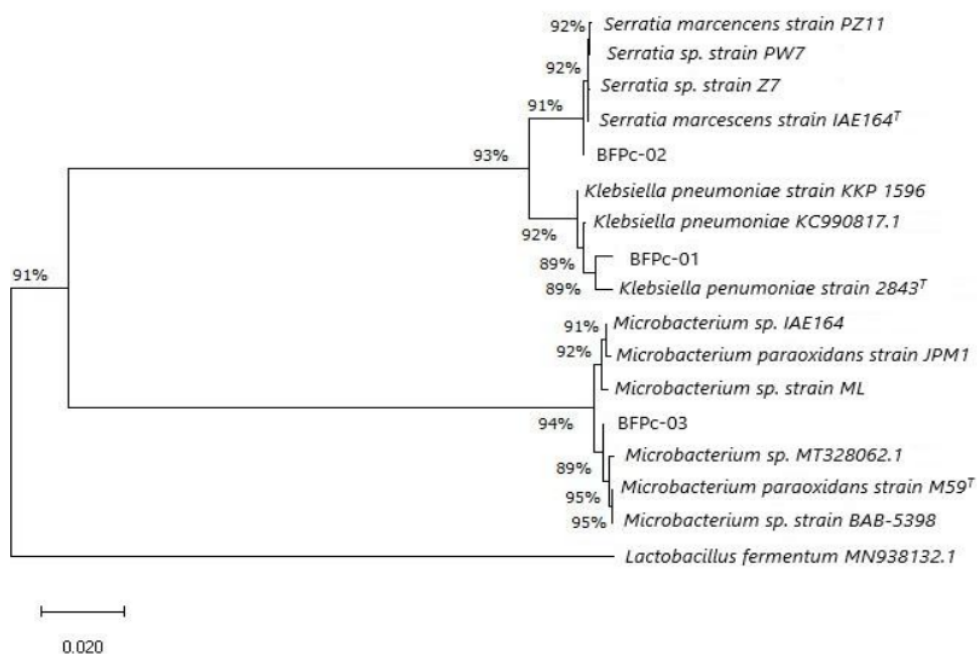


Figure 3. Chromatogram of 16S rRNA gene sequence of bacterial isolates



**Table 1.** 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<sup>T</sup> (MT604895.1), BFPc-02 to *Serratia marcescens* strain IAE.169<sup>T</sup> (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<sub>2</sub>, N<sub>2</sub>,

CH<sub>4</sub>, and H<sub>2</sub> 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	<b>Sequence Assembly 1359bp</b>
		1 CTCGGGTGAC GAGCGGCGGA CGGGTGATTA ATGTCTGGGA GACTGCCTGA TGGAGGGGGA
		61 TAACACTGCG AACCGGTAGC TAATACCGCA TAACGTGCGA AGACCAAGT GGGGGACCTT
		121 CGGGCCCTCAT GCCATCAAAAT GTGCCCAGAT GGGATTAGCT AGTAGGTGGG GTAACGGCTC
		181 ACCTAAGCGA CGATCCCTAG CTGGTCTGAG AGGATGACCA GCCACACTGG AACTGAGACA
		241 CGGTCCAGAC TCCTACGGGA GGCAGCAGTG GGAATATTG CACAATGGGC GCAAGCCTGA
		301 TGCAGCCATG CCGCGTGTGT GAAGAAGGCC TTCGGTTGT AAAGCACTTT CAGCGGGGAG
		361 GAAGGCGATA AGGTTAATAA CCTGTGCGAT TGACGTTACC CGCAGAAGAA GCACCGGCTA
		421 ACTCCGTGCC AGCAGCGCGG AGTAATACGG AGGGTGCAAG CGTTAATCGG AATTACTGGG
		481 CGTAAAGCGC ACGCAGGCGG TCTGTCAAGT CGGATGTGAA ATCCCCGGGC TCACCTCTGG
		541 AACTGCATTC TAAACTGGCA GGCTAGAGTC TTGTAGAGGG GGGTAGAATT CCAGGTGTAG
		601 CGGTGAAATG CGTAGAGATC TGAAGAATA CCGGTGGCGA AGGCGGCCCC CTGGCAAAAG
		661 ACTGACTCTC AGGTGCGAGA GCGTGGGGAG CACACAGGAT TAGATACCCCT GGTAGTCCAC
		721 CCGCTAAACG ATGTGCAATT GGAGGTTGTG CCCTTGAGGC GTGGCTTCCG GAGCTAACGC
		781 ATTAAATCGA CCGCCTGGGG AGTACGGCGG CAAAGTTAAA ACTCAAATGA ATTGACGGGG
		841 GCGCGCACAA GCGGTGGAGC ATGTGGTTTA ATTCAATGCA ACGCGAAGAA CCTTACCTGG
		901 TCTTGACATC CACAGAACTT TCCAGAGATG GATTGGTGCC TTCGGGAATC GTGAGACAGG
		961 TGCTGCATGG CTGTGCTCAG CTCGTGTTGT GAAATGTTGG GTTAAGTCCC GCAACGAGCG
		1021 CAACCCCTTAT CCTTTGTTGC CAGCGGTTTC GCCGGGAAT CAAAGGAGAC TGCCAGTGAT
		1081 AAATCGGAGG AAGGTGGGGA TGACGTCAAG TCATCATGGC CCTTACGACC AGGGCTACAC
		1141 ACGTGCTACA ATGGCATATA CAAAGAGAAG CGACCTCGCG AGAGCAAGCG GACCTCATAA
		1201 AGTATGTGCT AGTCCGATT GAGTCTGCA ACTCGACTCC ATGAAGTCGG AATCGCTAGT
		1261 AATCGTAGAT CAGAATGCTA CCGTGAATAC GTTCCCGGGC CTTGTACACA CCGCCCGTCA
		1321 CACCATGGGA GTGGGTGGCA AAAGAAGTAG GTAGCTTAA
2	BFPc-02	<b>Sequence Assembly 1399bp</b>
		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 CGGTGTGTGA AGAAGGCCTT
		361 CGGGTTGTAA AGCACTTTCA GCGAGGAGGA AGGTGGTGAA CTTAATACGT TCATCAATTG
		421 ACGTTACTCG CAGAAGAAGC ACCGGCTAAC TCCGTGCCAG CAGCCGCGGT AATACGGAGG
		481 GTGCAAGCGT TAATCGGAAT TACTGGGCGT AAAGCGCACG CAGGCGGTTT GTTAAGTCAG
		541 ATGTGAAATC CCGGGGCTCA ACCTGGGAAC TGCAATTGAA ACTGGCAAGC TAGAGTCTCG
		601 TAGAGGGGGG TAGAATTCCA GGTGTAGCGG TGAAATGCGT AGAGATCTGG AGGAATACCG
		661 GTGGCGAAGG CGGCCCCCTG GACGAAGACT GACGCTCAGG TGGGAAAGCG TGGGGAGCAA
		721 ACAGGATTAG ATACCCCTGGT AGTCCACGCT GTAAACGATG TCGATTTGGA GGTGTGTGCC
		781 TTGAGGCGTG GCTTCCGGAG CTAACGCGTT AAATCGACCG CCTGGGGAGT ACGGCCGACA
		841 GGTTAATACT CAAATGAATT GACGGGGGCC CGCACAAGCG GTGGAGCATG TGGTTTAATT
		901 CGATGCAACG CGAAGAACCT TACCTACTCT TGACATCCAG AGAACTTTCC AGAGATGGAT
		961 TGGTGCCCTC GGGAACTCTG AGACAGGTGC TGCATGGCTG TCGTCAGCTC GTGTTGTGAA
		1021 ATGTTGGGTT AAGTCCCAGC ACGAGCGCAA CCCTTATCCT TTGTTGCCAG CGGTTCCGGC
		1081 GGGAACTCAA AGGAGACTGC CAGTGATAAA CTGGAGGAAG GTGGGGATGA CGTCAAGTCA
		1141 TCATGGCCCT TACGAGTAGG GCTACACACG TGCTACAATG CGGTATACAA AGAGAAGCGA
		1201 CCTCGCGAGA GCAAGCGGAC CTCATAAAGT ACGTCGTAGT CCGGATTGGA GTCTGCAACT
		1261 CGACTCCATG AAGTCGGAAT CGCTAGTAAT CGTAGATCAG AATGCTACGG TGAATACGTT
		1321 CCGGGGCCCT GTACACACCG CCCGTCACAC CATGGGAGTG GGTGCAAAA GAAGTAGGTA
		1381 GCTTAACCTT CGGGAGGGC
3	BFPc-03	<b>Sequence Assembly 1355bp</b>
		1 GTGGATCAGT GCGCAACGGG TGAGTAACAC GTGAGCAACC TGCCCCTGAC TCTGGGATAA
		61 GCGCTGGAAA CGGCGTCTAA TACTGGATAT GTGACGTGAC CGCATGGTCT GCGTTTGGA
		121 AGATTTTTCG GTTGGGGATG GGCTCGCGGC CTATCAGCTT GTTGGTGAGG TAATGGCTCA
		181 CCAAGGCGTC GACGGGTAGC CGGCTGAGA GGTGACCGG CCACACTGGG ACTGAGACAC
		241 GGGCCAGACT CCTACGGGAG CGACGAGTGG GGAATATTGC ACAATGGGCG AAAGCCTGAT
		301 CGAGCAACCG CGCGTGAGGG ATGACGGCCT TCGGGTTGTA AACCTCTTTT AGCAGGGAAG
		361 AAGCGAAAGT GACGGTACCT GCAGAAAAAG CGCCGGCTAA CTACGTGCCA GCAGCCGCGG
		421 TAATACGTAG GGCACAAGCG 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 GATACCCCTGG TAGTCCACCC CGTAAACGTT GGGAACTAGT
		721 TGTGGGGTCC ATTCACGGGA TTCCGTGACG CAGCTAACGC ATTAAGTTCC CCGCCTGGGG
		781 AGTACGGCCG CAAGGCTAAA ACTCAAAGGA ATTGACGGGG ACCCGCACAA GCGGCGGAGC
		841 ATGCGGATTA ATTCGATGCA ACGCGAAGAA CCTTACCAAG GCTTGACATA TACGAGAACG
		901 GGGCAGAAAT GGTCAACTCT TTGGACACTC GTAAACAGGT GGTGCATGGT TGTCTCAGC
		961 TCGTGTCTGT AGATGTTGGG TTAAGTCCCG CAACGAGCGC AACCTCGTCT CTATGTTGCC
		1021 AGCACGTAAT GGTGGGAAC CTGCGGATAC TGCCGGGGTC AACTCGGAGG AAGGTGGGGA
		1081 TGACGTCAAA TCATCATGCC CCTTATGTCT TGGGCTTCAC GCATGCTACA ATGGCCGGTA
		1141 CAAAGGGCTG CAATACCGTG AGGTGGAGCG AATCCCAAAA AGCCGGTCCC AGTTCCGGATT
		1201 GAGGTCTGCA ACTCGACCTC ATGAAGTCGG AGTCGCTAGT AATCGCAGAT CAGCAACGCT
		1261 GCGGTGAATA CGTTCCCGGG TCTTGTACAC ACCGCGGCTC AAGTCATGAA AGTCGGTAAC
		1321 ACCTGAAGCC GGTGGCTTAA 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 Budiyan 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<sup>T</sup> (MT604895.1), BFPc-02 isolate was similar with *Serratia marcescens* strain IAE.169<sup>T</sup> (MK414866.1), and BFPc-03 isolate was closely related with *Microbacterium* 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 *Microbacterium* 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.

25

## ACKNOWLEDGEMENTS

The authors are grateful for the non-tax revenue of Universitas Negeri Gorontalo years 2020 and to the

Chancellor of Universitas Negeri Gorontalo, who has allocated PNPB research funds in 2020. We are also thanks Adam Suduri for his help with data collecting.

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