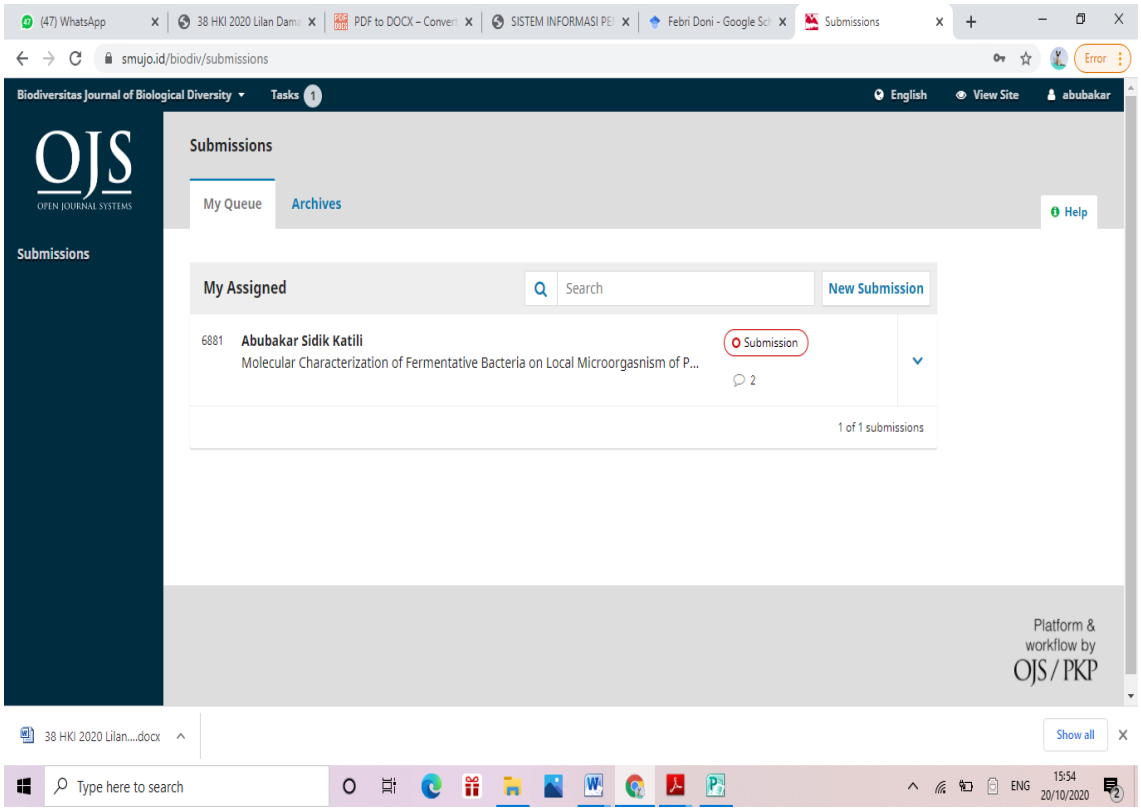

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Yuliana Retnowati

Molecular Characterization of Fermentative Bacteria on Local Microorganisms of *Pomacea canaliculata*

YULIANA RETNOWATI[1,♥], ABUBAKAR SIDIK KATILI[1,♥♥] (10 PT)

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Manuscript received: DD MM 2016 (Date of abstract/manuscript submission). Revision accepted: ..... 2016. (8 pt)

Abstract. Local Microorganisms (LMo) is a fermented liquid containing various microorganisms potentially as decomposer and bio-fertilizer. P.canaliculata is one of the rice weeds that potential as a basic ingredient of LMo because the high protein content. The objective of this study was to determine the the types of fermentative bacteria on Local Microorganisms of P.caniculata. The fermentation of LMo was conducted for 21 days and acidity changing was detected. Microbial population was determined at 7 day intervals based on the TPC method. The type of microbial was observed based on morphology characters Characterization and identification based on polyphasic taxonomy including macroscopic and microscopic morphological characters, and molecular characters based on 16S rRNA gene sequences. The results showed that P.canaliculata MOL 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. Isolation results obtained 3 types of bacteria based on morphological differences, namely isolates BFPc-01, BFPc-02, and BFPc-03, with each character being a milky white colony, Gram positive coccus; colony pink, coccus, Gram positive; and yellow, bacillus, Gram negative colonies. The results of the characterization of feremtti bacteria isolates based on the 16s rRNA gene sequence showed that the BFPc-01 isolate showed similarities to Klebsiella penumoniae MT604895.1 (99.04%), each of BFPc-02 and BFPc-03 isolates were closely related to Serratia sp (100%) and Microbacterium sp (100%). (9 pt)

Key words: Molecular characterization; fermentative; local microorgnisms, Pomacea cnaiculata. (9 pt)

Abbreviations (if any): All important abbreviations must be defined at their first mention there. Ensure consistency of abbreviations throughout the article.

Running title: a short title with five words

INTRODUCTION (10 PT)

Local Microorganisms (LOm) is a liquid containing various types of microorganisms that are involved in the process of overhauling various organic wastes. The degradative ability of microorganisms in LMo has been widely used for activation in the composting process so as to accelerate the compost ripening process and shorten the composting time. The microorganisms contained in LMo are also bio-fertilizing agents that have potential as commercial biological agents. The use of LMo has also been applied as a fertilizer to fertilize plant leaves, stimulate plant growth, and control pest and disease agents (Manullang et al., 2017; Sigit, 2018).

Local Microorganisms can be sourced from a variety of local materials, including cow urine, banana stalks, gamal leaves, fruits, stale rice, household waste, bamboo shoots, Cebreng leaves / legumes, banana weevils, vegetables, and elephant grass, so that it can play a role in the process of waste management, both solid and liquid waste (Manullang et al., 2017; Sigit, 2018). Another material that has the 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. The presence of this pest can damage thousands of hectares of rice seedlings or at a young age. These animals contain protein and high fat so that they have the potential to be a source of local microorganisms.

Local Microorganisms is 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 a 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 importantly of LMo role and its potential to be developed as a commercial product, the objective of this study was to reveal the fermentative bacteria in Local Microorganisms of *P.caniculata*. The results of this study are expected to be preliminary data for the development in the next analysis, including the ability to solubilizing phosphate and produce of IAA growth hormone.

MATERIALS AND METHODS (10 PT)

Procedures

Fermentation of P.canaliculata for generation of LMo

The P.canaliculata as much as 1 kg was crushed and to be added 200 grams of brown sugar and 5 liters of rice washing water, then stired evenly. The mixture was put into the fermenter and incubated on 37^{o}C for 21 days with 3 repetitions. The change in the degree of acidity was determined using a pH meter.

Isolation and purification fermentative bacteria

The suspension of P.canaliculata-fermentation product was collected by centrifugation technique. The suspension was mixed culture including fermentative microorganisms on P.canaliculata fermentation process. The fermentative bacteria were isolated using Nutrient Agar medium based on pour plate method and to be incubated on 37oC for 48 hours. The population of fermentative bacteria was determined based on the number of bacteria colony multiplied by the dilution factor. The population of fermentative bacteria was observed on 7 days interval times, i.e 0, 7, 14 and 21 days fermentation. The LMo suspension as much as 1 Ml was diluted on ringer solution, then carried out serial dilution up to 10^{-6} of level dilution. The each of dilution as much as 1 ml was patting on Nutrient Agar medium based on pour plate method, and incubated on 37^{o}C for 48 hours. The growth of bacteria colony was observed as the diversity of fermentative bacteria based on the morphology characters. The bacteria colony which showed differences morphology was isolated into the new Nutrient Agar medium based on streak plate method as a pure culture. Then the pure culture was characterized based on molecular characterization.

Molecular characterization of fermentative bacteria

Extraction of genomic DNA. The DNA genomic of fermentative bacteria was carried out follow the protocol of ZR Fungal/Bacterial DNA Kit™. The bacterial cells as much as 50-100 mg (wet weight) resuspended in up to 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.

Amplification of 16S rRNA gene. Amplification PCR by using MyTaq Red Mix (Bioline). PCR Master Mix 1x25µl: 9.5µl ddH_2O; 12.5µl MyTaq Red Mix, 2x; 1µl 20 µmol / µl 27F Primer (AGAGTTTGATCMTGGCTCAG); 1 µl 20 µmol / µl 1492R Primer (TACGGYTACCTTGTTACGACTT); and 1 µl DNA Template. PCR Condition (35 cycles) was follow an initial denaturation of 95^{o}C for 1 min; denaturation on 95^{o}C for 15 sec; annealing on 52^{o}C for 15 sec; extension on 68 ^{o}C for 45 sec; and hold on 4^{o}C for infinite of duration time. The PCR product was detected on agarosa-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 to determine the approximate phylogenetic position. Sequences were aligned by using ClustalW with representative bacteria 16S rRNA sequences, and a phylogenetic tree was constructed by using the Molecular Evolutionary Genetics Analysis (MEGA) software, version 6.06 (Retnowati et al 2017).

Data analysis

The Data of the population of fermentative bacteria served as a graphic log of $CFUMl^{-1}$ versus observation time. The characteristic of fermentative bacteria was analysis based on descriptive qualitative analysis to describe the morphological characters and to determine the similarity of 16 S rRNA sequence of fermentative bacteria isolate to 16S RNA sequence data on NCBI gene bank. The closely related of fermentative bacteria isolate to the type strain on NCBI was served as phylogenetic tree.

RESULTS AND DISCUSSION

Description of Local Microorganisms of Pomacea canaliculata

The fermentation of Pomacea canaliculata for generation of Local Microorganisms was carried out for 21 days. As a effect of fermentation process, there were formed the liquid as the mixed of microorganisms and metabolic product of microorganisms. The macromolecule of Pomacea canaliculata and brown sugar were used of microorganism as energy and carbon sources for their growth. Microorganism activities produced metabolic which changing chemical characters of suspension as a changing of acidity degree. The result of determination acidity

showed that the pH of liquid tend to decrease as long as fermentation process (Fig.1). The graphic showed that the acidity condition of fermentation was acid condition, that was predicted the microorganisms on fermentatation process as fermentative microorganisms.

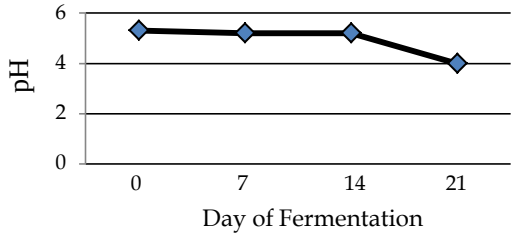

Figure 1. acidity degree of Pomacea canaliculata fermentation

Population of fermentative bacteria

The fermentation process of macromolecule on p. canaliculata was carried out by fermentative bacteria. They were able to grow on acid environment. The population was determined every 7 days interval time observation. The result showed that the population of fermentative bacteria was increase on 0 to 14 days fermentation, then decrease on 21 days fermentation. The highest population achieve on 14 days fermentation about more than 320.10^5 CFU/ml, and the lowest one on 21 days of fermentation about 20.10^5 CFU/ml.

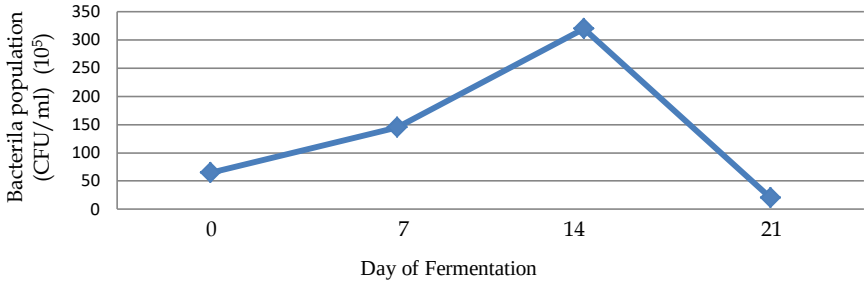

Figure2. The population of fermentative bacteria on Local Microorganisms of P.canaliculata

The fermentative bacteria on LMo of P.canaliculata consist of three types of bacteria. They were differences based on morphological characters, especially colour and shape of colony (Fig 3). They were white, pink, and yellow colony furthermore referred as BFPc-01, BFpc-02, and BFPc-03 isolate. The growth of the fermentative bacteria showed succession pattern as long as fermentation process. The white colony was growth on first to second weeks of colony, then followed by the growth of pink colony; and the last week the pink colony replaced by the yellow colony. The figure3 also showed the dominant isolate every times observation. The first up to third weeks of fermentation, the population of fermentative bacteria was dominated by BFPc-01 isolate, while the fourth week replaced by BFPc-03 isolate. The BFpc-02 isolate just found on third weeks fermentation belong to BFPc-01 isolate.

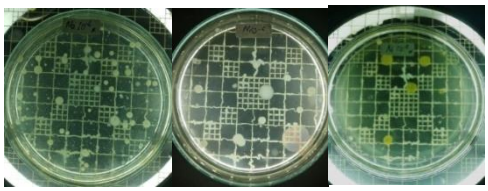

Figure3. The morphology of fermentative bacterioa on Local Microorganisms of P.canaliculata.

Diversity of fermentative bacteria

The fermentative bacteria on Local Microorganism of P.canaliculata carried out of molecular characterization based on sequence of 16S rRNA gene, then compared with the 16S rRNA sequence or genome sequence of type strain on NCBI gene bank. The result of molecular analysis was shown as phylogenetic tree that describe the evolutionary relationship between type strain and fermentative isolate bacteria (Fig 4). The phylogenetic tree showed that the BFPc-01 isolate was closely related to type strain of Klebsiella pneumonia MT604895.1 with 99.0% of sequence similarity. The BFpc-02 isolate was similar to Serratia marcescens MK414866.1, while the BFPc-03 isolate similar to Microbaterium sp. MT328062.1 with sequence similarity of 100% respectively.

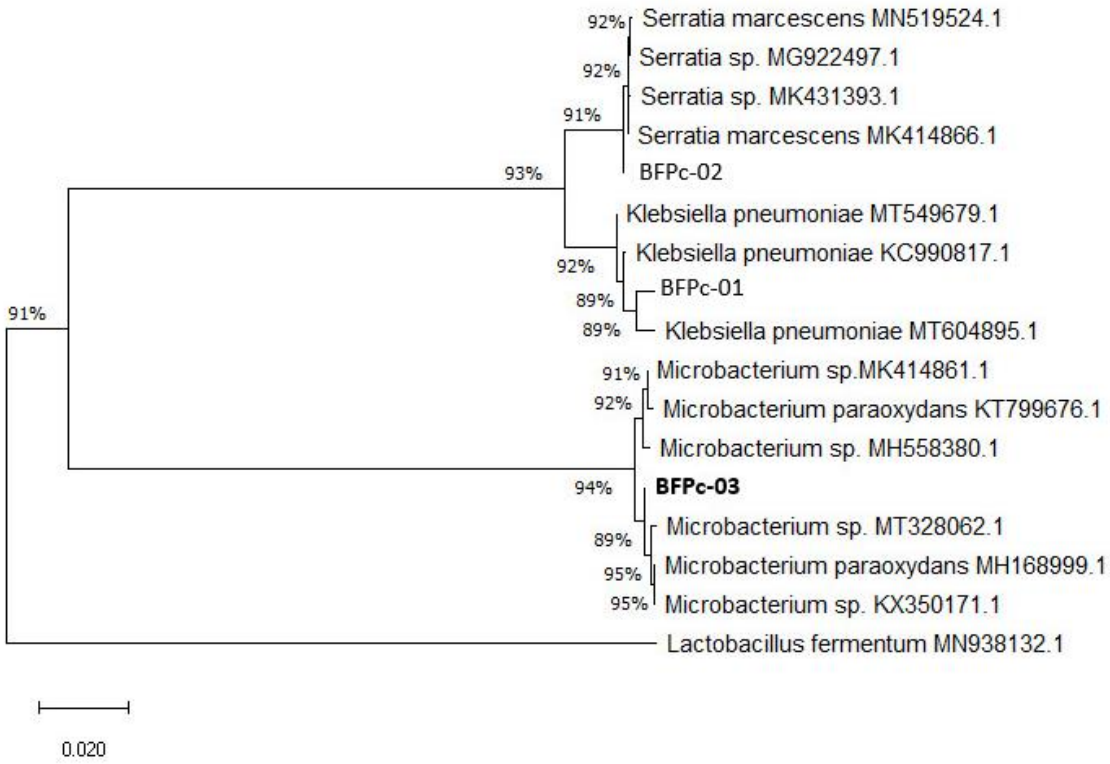

Figure4. Neighbour-joining phylogenetic tree inferred from 16S rRNA gene sequence. The phylogenetic tree shows the phylogenetic relationship of fermentative bacteria isolate with related type strain. Bootstrap values are expressed as percentages of 1000 replications. Bootstrap values, >50% are shown at branch points. Score bar represents 1 nucleotide substitution per 100 nucleotides.

Discussion

The fermentation process for generation Local Microorganisms (LMo) from Pomacea canaliculata was showed the changing of biological and chemical characters, including the bacterial population and acidity degree changing as long as fermentation process. The acidity degree of LMo was tent to decrease. This shows that the fermentation process is going well. Fermentation is the metabolisms on anaerobic conditions, whereas an aerobic respiration activity by aerobic microbes on the early step replaced by fermentation metabolism. The change in the degree of acidity / pH of the solution indicates that microbial activity changes basic macromolecules of substrate into organic acids. Organic acids such as lactic acid are classified as weak and can dissociate by releasing hydrogen ions. This release of hydrogen ions will be change the balance of the solution (Jay, 1992). Marsini et al, 2015 reported that the pH of the MOL tofu dregs tends to decrease after the third week of fermentation, then increases at the sixth week of fermentation. During under acidic conditions, there is a decomposition of

organic acids and dissolved nitrogen compounds to form ammonium, carbonic acid, and a small portion of CO2, N2, CH4, and H2 which induce increasing of pH (Seni 2013; Marsini dkk, 2015).

The fermentation process of LMo of *P.canaliculata* was carried out on 21 days. There were required of fermentative bacteria to decompose the macromolecule on the substrate into the simpler compounds perfectly. Purwasasmita, 2009; Suwastika et al, 2015; Manulang and Daryono, 2017 reported that LMo of Gliricidia leaf on 7 t0 21 days fermentation period have been applied as organic compos. Juanda et al, 2011 also reported that the effective time to generate LMo was varied depend on type of basic material. The LMo quality was influenced by number and type of microorganisms, pH and colour.

The activity of microbial on decomposition process of *P.canaliculata* macromolecule caused the changing of physical and chemical characters of LMo, including decreasing acidity level. The chemical character of LMo was influence the population and diversity of microbes. The microbes show a tendency to differ in response to environmental conditions. The result of observation about the type microbes on fermentation periods showed that there was a succession pattern of microbe growth. There was showed the ability of microbes to tolerance on environmental physicochemical conditions.

The population of fermentative bacterial in LMo of P.canaliculata tended to change during fermentation process. The population tends to decrease on 14 up to 21 days fermentation. It predicted that the bacteria have reached an intolerant phase to 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 of fermentation or 21 days of fermentation. The changes of bacterial population during fermentation assumed as a form of bacterial response to biotic and abiotic conditions, including oxygen levels, nutrient availability, competition, and pH. The bacterial population at the beginning of fermentation is an aerobic acid derived from the basic material. The bacteria use the macromolecules in the base material as nutrient sources which indicated by an increasing of bacterial population. Furthermore, the condition tends to change to be more anaerobic. In anaerobic conditions, aerobic bacteria suppressed and replaced by an anaerobic bacteria. The decreased acidity of MOL during the fermentation process is also a limiting factor for bacterial growth. The low acidity condition indicates that the fermentative bacteria were classified as acidophilic bacteria.

Fermentative bacteria on Local Microorganisms of *P.canaliculata* successfully identified based on molecular characters of 16S rRNA gene sequences. They were closely related with *Klebsiella pneumonia* MT604895.1, *Serratia marcescens* MK414866.1, and *Microbaterium* sp with similarity index about 99-100%. The fermentative bacteria on LMo of *P.canaliculata* were bacteria that play a role in the macromolecular decomposition process of *P.canaliculata*. The bacteria were presumed originate from the agricultural environment as a habitat of *P.canaliculata*. *Klebsiella pneumonia* was the kind of bacteria that naturally occurs in soil, and about 30% of strains can fix nitrogen under anaerobic conditions. As a free-living diazotroph, its nitrogen fixation system has been extensively studied, and is of agricultural interest, as *K. pneumoniae* has been shown to increase yields in agricultural conditions. As well as *Serratia marcescens* and *Microbaterium* sp were naturally found in soil, water and the surface of plant.

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