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Lipolytic bacteria isolated from Indonesian sticky rice cake *wajik* and *jenang* experiencing with rancidity

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Abstract. *Susilowati A, Sari SLA, Setyaningsih R, Muthmainna HN, Handarwati H, Pangastuti A, Purwoko T. 2018. Lipolytic bacteria isolated from Indonesian sticky rice cake* wajik *and* jenang *experiencing with rancidity. Biodiversitas 19: 351-356.* Lipolytic bacteria are lipase producing bacteria. These bacteria can be isolated from various sources such as vegetable oil wastes, industrial dairy products, oil-contaminated soil, seeds, and foods containing fat. The lipolytic bacteria were isolated from *wajik*, *jenang manten* and *jenang alot* that have been cultured for 4-35 days using minimum media containing MgSO4.7H2O 0.03%, K2HPO4 0.005%, (NH4) SO40.5% (w/v) supplemented with olive oil as a carbon source. Lipolytic activity was marked by orange luminescence when it was exposed to UV light at 350 nm on colonies grown on media containing olive oil-rhodamine B. Lipolytic bacteria were identified based on colony morphology, cell morphology and sequences of 16S rRNA gene. The 16S rRNA gene sequences were analyzed to determine the identity of bacterial isolates based on the percentage of sequence identity using BLAST Nucleotide software on NCBI website. Results showed there were 7 lipolytic bacteria of 52 isolates. Those bacteria were Gram-negative with spherical and rods shape. The identity of four isolates obtained in *wajik* indicated 97-98% of similarity to *Acinetobacter* and one isolate showed 96% of similarity to *Pseudomonas*. The last two isolates obtained from *jenang manten* had 99% similarity belonging to the species of *Serratia marcescens*.

Keywords: lipolytic bacteria, *wajik*, *jenang*, 16S rRNA gene sequence

INTRODUCTION

Lipase (triacylglycerol acyl hydrolases, EC 3.1.1.3) is an enzyme that serves as a catalyst and synthesis of ester hydrolysis. Apart from being the catalyst for the hydrolysis reaction and the synthesis of ester (esterification), lipase can also catalyze the Trans-esterification process which is useful in industrial oleochemicals and biodiesel (Gupta et al. 2011). Trans-esterification is a reaction process of triglycerides in vegetable oil or animal fat with short-chain alcohol such as methanol or ethanol to produce fatty acid methyl esters (Fatty Acids Methyl Esters/FAME) or biodiesel and glycerol (glycerin) as the by-products (Hikmah and Zuliyana 2010). Biodiesel produced in the transesterification process can be used as a substitute for diesel oil. This biodiesel also has some advantages such as renewable, non-toxic, biodegradable and can improve local air quality by reducing emissions of harmful gases contributing to global warmings such as SOx, NOx, and CO_2 (Pratt et al. 2009). Lipase production can also be applied in food industries (modifying tastes), chemicals substance production industries (synthetic ester), detergent industries (hydrolysis of fats), wastewater treatment (decomposition and removal of oily substances), cosmetics industries (removal of lipids), medicine (digestion of oils and fats in the foods), leather processing (removal of fat from animal skin) and biomedical tests (blood triglycerides) (Salihu et al. 2012).

Due to the importance of lipase enzyme in the biodiesel and other industries, various efforts to find a new source of lipase enzyme has been conducted. Most of lipase was isolated and purified from a variety of sources such as fungi, bacteria, plants and animals (Hasan et al. 2006). However, lipase derived from bacteria is known to be more economical and stable when it is used on an industrial scale both pharmaceutical industry and agriculture (Snellman et al.2002). Lipolytic bacteria can be isolated from foods containing fat or vegetable oil. One of examples of these foods is *wajik* and *jenang*. *Wajik* and *jenang* are traditional Indonesian cake made from steamed glutinous (sticky) rice and further cooked in palm sugar containing coconut milk. The coconut milk in this cake is favorable for the growth of lipolytic bacteria. Based on the research by Pratiwi (2014) wajik contains lots of fat, i.e., 8.78%. *Wajik* and *jenang* stored for a long time will undergo rancidity. Rancidity is a decomposition process of fat in which free fatty acids will be produced. Hence, rancidity is a sign of lipolytic activity. The aim of this study was to obtain lipolytic bacteria and to identify species of the lipolytic bacteria based on the 16S rRNA encoding gene from traditional sticky rice cake *wajik* and *jenang*.

MATERIALS AND METHODS

Samples of *wajik* and *jenang*

Wajik samples obtained from wajik-producing center in Janti village, Klaten, Central Java, were stored for 21 days

in a plastic container and sealed properly. During the storage period, on day 7th, 14th and 21st samples were taken. Both *jenang manten* and *jenang alot* were obtained from Kenep village in Sukoharjo Regency, Central Java. *Jenang manten* was stored for 8 days and *jenang alot* for 21, 28, and 35 days.

Isolation of lipolytic bacteria

One gram of each sample was weighed, crushed and dissolved in 9 mL of 0.85% salt solution (physiological solution). Subsequently, a serial dilution was made up to 10^{-4}. Minimum media contains $MgSO_4.7H_2O$ 0.03% (w/v), K_2HPO_4 0.005% (w/v), $(NH_4)SO_4$ 0.5% (w/v) enriched with olive oil 2% (v/v) was used during isolation of lipolytic bacteria.The diluted solution was spread onto plate with duplication and incubated for 48-72 hours at 27°C (Susanty et al. 2013). Each grown colony having different morphology in shape, color and size were selected and purified as candidates for lipolytic bacteria. Pure cultures were stored at 4°C.

Lipolytic activity test

Lipolytic activity test was done using media containing nutrient broth 0.008% (w/v), NaCl 0.004-0.02% (w/v), olive oil (31.25 mL/L), and 10 mL/L rhodamine B (1 mg/mL). One inoculation loop of each isolate was streak on the olive oil-rhodamine B media and incubated at 27°C for 48 hours. A positive result could be observed from orange color glows of colonies under UV (Ultra Violet) light with a wavelength of 350 nm (Kumar et al. 2012). This color appearance resulted from hydrolysis of olive oil into a fatty acid, interacted with rhodamine B. □

Observations of colony and cell morphology of the lipolytic bacteria

Morphological identification bacterial colonies grown on nutrient agar medium including shape, color, elevation, and the edge of colonies were examined (Hadioetomo 1993). Furthermore, bacterial classification was also performed by Gram staining method which could distinguish between two major groups of bacteria (Gram positive and Gram negative) and also determined the shape of the cell (Irianto 2006).

Amplification of 16S rRNA gene using PCR method

Lipolytic bacterial genomic DNAs were extracted using Presto TM Mini gDNA Bacteria Kit (Genaids). The 16S rRNA gene of the lipolytic bacteria was amplified using forward primer (63F: 5 'CAGGCCTAACACATGCAAGTC-3') and reverse primer (1387r: 5'-GGGCGGAWGTGTACAAGGC-3 '). Polymerase Chain Reaction (PCR) mixture consisted of 12.5 µl Kapa 2G Fast Ready Mix DNA polymerase, 1.25 µl of each primer (10 pmol), 1 µl of DNA template (100 ng), and 9µl of ddH_2O. Thermocycling conditions include pre-denaturation at 95°C for 3 minutes, and 30 cycles of denaturation at 95°C for 15 seconds, annealing at 56°C for 15 seconds, elongation 72°C for 30 minutes. Finalizing process was performed at 72°C for 2 minutes (Marchesi et al. 1998).

Table 1. Bacterial isolates obtained from *wajik* and *jenang* stored for several days

Type of traditional food/cake	Duration of storage (days)	Number of bacteria strains isolates	Number of bacteria with lipolytic activity
Wajik	7	14	1
Wajik	14	6	3
Wajik	21	4	1
Jenang manten	8	16	2
Jenang alot	21	6	-
Jenang alot	28	4	-
Jenang alot	35	2	-
Number of isolates		52	7

Sequencing

PCR products of 16S rRNA gene were then sequenced by sending it to 1st Base Singapore. The 16S rRNA gene sequences were analyzed using BLAST Nucleotide software on NCBI website (www.blast.ncbi.nlm.nih.gov/blast.cgi) in order to identify the species of the lipolytic bacteria based on the sequence similarity with the GenBank database (Waturangi et al. 2008).

RESULTS AND DISCUSSION

Lipolytic bacteria isolated from *wajik* and *jenang* experiencing with rancidity

According to Swandi et al. (2015), lipolytic bacteria can be found in many places that contain oils or fats. Environments containing oil or fat are good substrates for lipolytic bacterial growth. Thus, we believe lipolytic bacteria can also be isolated from traditional food, i.e., *wajik, jenang manten* and *jenang alot*. The selection of *wajik* and *jenang* as sources of lipolytic bacteria was because these foods contain fat (emulsion fat) derived from coconut milk, which could trigger the growth of lipolytic bacteria. In the long-stored periods, these foods will be rancidly caused by the activity of lipid-breaking bacteria. During this experiment, we successfully isolated 52 bacteria strains as candidates of lipolytic bacteria (Table 1). Many bacterial isolates found in this study that indicate lipolytic bacteria. These isolates can grow on minimum medium supplemented with olive oil as a source of carbon. The bacterial isolates use olive oil as a carbon source. In further lipolytic activity testing, only 7 bacterial isolates showed strong lipolytic activity with the onset of orange luminescence on rhodamin B media. □

Lipolytic bacteria can grow well if nutrients in media are appropriate for the growth and one of the crucial components in media is oil that will be used as a carbon source by these bacteria (Swandi et al. 2015). Therefore, the isolation of lipolytic bacteria in this research was conducted using minimum media enriched with olive oil as a sole carbon source. These selected media were used to limit the carbon type consumption of bacteria, which were specifically derived only from olive oil so that during the isolation the specific bacteria will be obtained. Olive oil

had been selected because it is easily obtained and used by bacteria (Carissimi et al.2007). Lipase-producing bacteria can grow on tributyrin agar medium containing tributyrin substrate as well as olive oil because the short-chain fatty acids (4 atom C) from those substrates are easily hydrolyzed by bacteria (Susanty et al. 2013).

Lipolytic activity

Lipase-producing bacteria will produce orange fluorescence color on rhodamine B media when they were exposed to UV light at wavelength 350 nm (Telussa 2013). Rhodamine B is a lipase sensitive fluorescence indicator. Orange fluorescence color occurs based on the changes of acid from Rhodamine B to the form of cations and complex formation with uranylfatty acid ions. It is inversely proportional between the long-chain fatty acids and orange fluorescence excitation at 350 nm (Carissimi et al. 2007). Bacterial isolates giving positive response of lipolytic activity with orange luminescence in rhodamine B media are shown in Figure 1.

Seven bacterial isolates showed positive response for lipolytic activity (Table 1). Those bacterial strains were labeled as W203, W204, W205, W111 and W303 which are derived from *wajik* and JM8.O and JM8.P are from *jenang manten*. This result showed that lipolytic bacteria found in *wajik* and *jenang manten* have been experienced with rancidity. Rancidification is the decomposition of fats, oils and other lipids by hydrolysis or oxidation, or both. Hydrolysis will split fatty acid chains away from the glycerol backbone in glycerides. These free fatty acids can then undergo further auto-oxidation. Oxidation primarily occurs with unsaturated fats by a free radical-mediated process. These chemical processes generate highly reactive molecules in rancid foods and oils, which are responsible for producing unpleasant and noxious odors and flavors. Lipolysis is the enzymic hydrolysis lipids to free fatty acids and partial glycerides. The enzymes have responsible to cause detrimental effects of lipolysis in those microbial origin. Therefore, rancidity indicates the presence of lipolysis activity by lipolytic bacteria. No lipolytic bacteria found from *jenang alot*. All of isolates isolated from *jenang alot* did not show lipolytic activity on rhodamin B screening media under UV light exposure. Isolates with no growth performances potentially had lipolytic activity because they are esterase-producing bacteria observed from pink fluorescence on rhodamine B screening media (Niyozima and Sunil 2013). Screening using rhodamine B could not be used to test bacterial cultures producing fatty acid metabolites because of the absence of orange luminescence, which might happen due to two reasons, i.e., bacteria that consuming the fatty acids that have been produced and the absence of lipase activity (Telussa 2013).

Morphological characteristics of lipolytic bacteria

Morphological seven strains of bacteria having positive lipolytic activity were observed. Results of these observations were presented in Table 2. Colony morphology of lipolytic bacteria showed slight different in colors, edges, and elevations indicating differences isolates. Gram staining bacteria produce bacterial cells that are red. This shows that the five lipolytic bacteria are gram-negative bacteria, with the shape of coccus and *Bacillus*. Several bacteria are reported to produce lipases from Gram-negative or Gram-positive groups, namely *Acinetobacter sp.*, *Achromobacter lipolyticum*, *Aeromonas hydrophila*, *Photobacterium lipolyticum*, *Moraxella sp.*, *Pseudomonas fluorescens*, *Pseudomonas fragi*, *Psychrobacter okhotskensis*, *Serratia marcescens* and *Staphylococcus epidermidis*, and *Bacillus sphaericus* (Joseph et al. 2007). However, in this study only groups of Gram-negative bacteria were found.

Lipolytic bacteria identity based on 16S rRNA gene sequences

Lipolytic bacterial isolates were identified molecularly based on sequences of genes encoding 16S rRNA. Bacterial genomic DNAs were amplified by PCR method with primers 63F and 1387r. Marchesi et al. (1998) discovered that those primers could amplify 16S rRNA gene sequences with a size of about 1300 base pairs. PCR products from each of the 7 lipolytic bacterial strains showed almost the same size, which was about 1300 base pairs. Visualization of PCR products could be seen in Figure 2.

Several pair combinations of forward and reverse universal primers used to amplify gene encoding 16S rRNA of bacteria such as 23f, 24f, 1392r and 1492r. Marchesi et al. (1998) have designed and evaluated 63f and 1387r primer for amplification gene encoding 16S rRNA of the bacterial domain. The primer pairs can amplify 16S rRNA gene with the size of 1300 base pairs and able to identify and produce 43 genera and 88 species.

Table 2. Colony and cell morphology of lipolytic bacteria isolated from traditional food of wajik and jenang, grown on nutrient agar

Name of isolates	Colony shape	Edge	Color	Elevation	Cell shape	Gram staining
W203	Irregular	Undulate	White	Flat	Coccus	Negative
W204	Irregular	Undulate	White	Flat	Coccus	Negative
W205	Irregular	Undulate	White	Flat	Coccus	Negative
W111	Circular	Entire	Yellowish	Convex	Coccus	Negative
W303	Irregular	Undulate	White	Flat	Coccus	Negative
JM8.O	Circular	Entire	White (may be changed to pink)	Convex	*Bacillus*	Negative
JM8.P	Circular	Entire	White (may be changed to pink)	convex	*Bacillus*	Negative

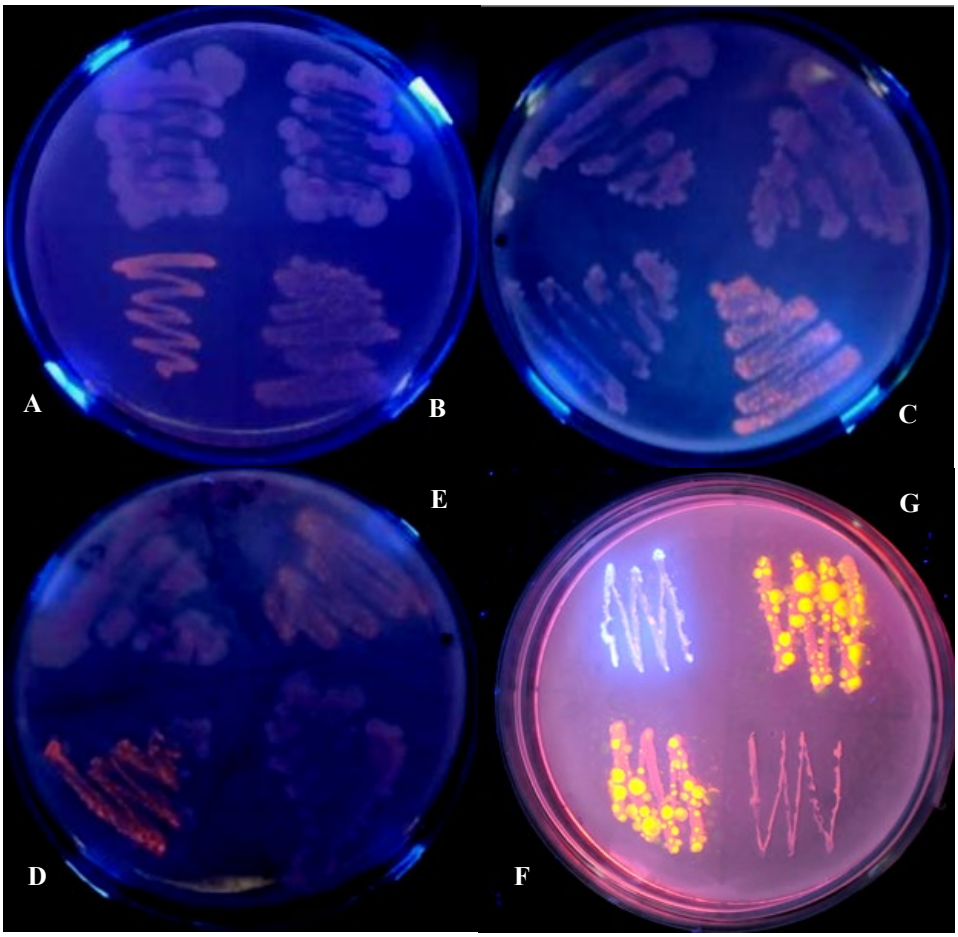

Figure 1. Streak of lipolytic bacteria isolated from traditional foods of *wajik* and *jenang* on rhodamine B media. Isolates W203 (A), W204 (B), W205 (C), W111 (D), W303 (E) JM8.O (F) and JM8 (G) showed orange fluorescent under UV light at 350 nm which indicates lipolytic activity. Other isolates did not show lipolytic activity

Based on BLASTN analysis, 7 lipolytic bacteria had the similarity percentage ranging between 97-98% within the existing database on GenBank (Table 3). The degree of similarity was determined from the obtained value identity. The higher value of identity, the more similarity with the reference sequence contained in GenBank (Hall 2001). Isolates W203, W204, W205 and W303 had similarities with *Acinetobacter ursingii* with 97% identity for W203 and 98% identity for three remaining isolates. Morphologically, those four isolates also share the same characteristics. However, another isolate from *wajik* sample, W111, produced 97% similarity to *Pseudomonas*. Different species were found from two lipolytic bacteria of *jenang manten*. Both isolates, JM8.O and JM8.P, represented 98% and 99% similarity, respectively to *Serrata marcescens*. High-level similarity of those isolates compared to GenBank data confirmed that these isolates belonged to the same genus/species as mention in database. According to Drancourt et al. (2000), based on comparison sequences of genes encoding 16S rRNA in GenBank, two bacteria that had the maximum degree of similarity (Maximum identity) ≥99% indicates that the species being compared are the same species, while the maximum rate ≥97% similarity can be stated that the isolates are in the same genus and similarities between 89-93% showing different family.

Species belong to *Acinetobacter* genus are widely distributed in nature and they are often found in soil, water and dry environment. Research by Musa and Bukola (2012), stated that *Acinetobacter* sp. could be isolated from contaminated soils, the substance of food that had been damaged and the rest of materials. In addition, the research also showed that *Acinetobacter* sp. had supreme lipase activity for 7 hours of incubation compared to other bacteria namely *Yersinia* sp., *Bifidobacterium* sp., *Arthrobacter* sp., *Brevibacterium* sp., *Staphylococcus* sp. *Streptococcus* sp., *Lactobacillus* sp., *Citrobacter* sp., *Serratia marcescens, Bacillus* sp ., *Acetobacterium* sp., *Acidomonas* sp., and *Aeromonas hydrophila.* The average rate of lipase production was known to be 1.25-8.65 U/mL, and *Acinetobacter* sp. ranks first for the bacteria that produce the highest lipase.

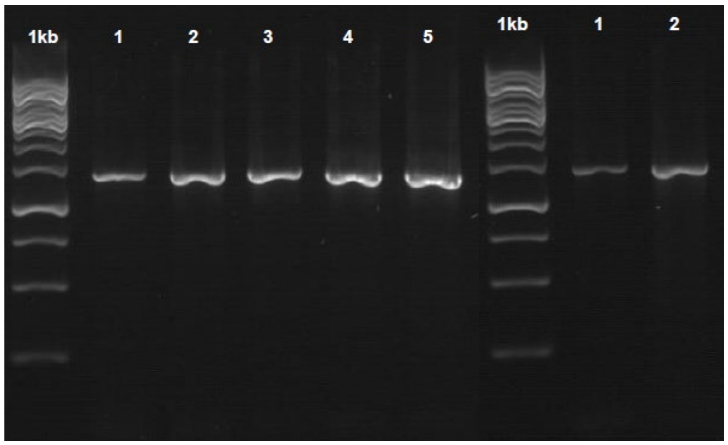

Figure 2. Agarose gel electropherogram of 16S rRNA gene amplification of lipolytic bacteria. Amplification products size were about 1300 bp. Description: M = Marker 1 kb, (A): 1 = Isolate W203, 2 = Isolate W204, 3 = Isolate W204, 4 = Isolate W111 and Isolate 5 = W303. (B) 1 =Isolate JM8.O , 2 = Isolate JM8.P. Condition: 0.8% agarose gel. Amount of DNA ladder loaded per lane: 0.2ug each Volume of sample loaded per lane: 1uL each 1kb DNA Ladder (bp): 250, 500, 750, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 5000, 6000, 8000, 10000 1kb DNA Ladder (ng/0.2ug): 10, 10, 10, 24, 10, 10, 10, 28, 12, 12, 12, 28, 12, 12

Table 3. Identity of lipolytic bacteria from traditional foods of wajik and jenang based on sequences of gene encoding 16S rRNA

Isolates names	Identity%	Query cover%	Closest species identity	Accession number
W203	97	98	*Acinetobacter ursingii* 505	KM281506.1
W204	98	99	*Acinetobacter ursingii* 505	KM281506.1
W205	98	99	*Acinetobacter ursingii* NBRC 110 605	LC014147.1
W111	97	99	*Pseudomonas* sp. SW-114	KY382819.1
W303	98	98	*Acinetobacter ursingii* 505	KM281506.1
JM8.O	99	98	*Serratia marcescens* strain R12 Root	KM099142.1
JM8.P	99	99	*Serratia marcescens* train 40	KX058478.1

Pseudomonas genus is Gram-negative, rod-shaped *(rods)* or cocci *(*coccus*),* obligate aerobe, motility by polar flagellum bacteria. This bacterium is oxidase positive, catalase positive, no fermenter and grow well at 4°C or below 43°C. *Pseudomonas* was found in soil, plants, and water (Suyono and Salahudin 2011). *Pseudomonas* is a genus of bacteria known as good extracellular lipase producer (Lokre and Kadam 2014). According to Haba et al. (2000), *Pseudomonas* genus produces greater lipase activities among *Bacillus, Rhodococcus* and *Staphylococcus* genus that was equal to 1,703 U/L. Traditional foods *wajik* and *jenang* contain coconut milk which is a fatty emulsion that can trigger the growth of lipolytic bacteria. This traditional fatty food is a local source of various species of bacteria that have high lipolytic activity. Therefore, the bacteria are potential to be developed as lipase-producing bacteria. Characterization and activity of these lipolytic enzymes need to be done in subsequent research.

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