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Isolation, Characterization and Identification of Lipolytic Thermophiles with Methanol Tolerance from Domestic Compost

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Five thermophilic bacteria with lipolytic activity were isolated from domestic compost namely AL17, AL59, AL64, AL89, and AL96. All of the isolates showed rod shape and gram negative microbes. The lipase activity among the isolates are varies when grown in medium with pH 7.0 and pH 9.0. AL17 and AL89 isolates showed best activity in medium with pH 7.0. Meanwhile AL64 and AL96 showed less activity in both type of the media, in addition AL59 was found to be mostly active when cultivated in pH 9.0. Methanol tolerant properties of the isolates was investigated through addition of 3% methanol in medium. Growth rate measurement of the isolates showed that AL96 was better methanol tolerant properties compared to others. The 16S rRNAs genes of all five bacteria have been sequenced and deposited to GenBank. Phylogenetic analysis of the 16S rRNA genes showed that four isolates close to genus *Pseudoxanthomonas*, while the other was highly similar to *Brevibacillus thermoruber*.

Key words: Lipase, 16S rRNA, Methanol Tolerant Bacteria.

Extreme microbes were classified into three types based on temperature of the environment, i.e psychrophile (-3 to 20°C), mesophile (13 to 45°C) and thermophile (45 to 122°C) [1]. A thermophile is an organism that thrives at relatively high temperature, between 41°C up to 122 °C [2]. Thermophiles are classified into facultative (50 to 65°C), obligate (65 to 70°C), and extreme thermophiles (higher than 70°C) [3]. Thermophilic bacteria have the ability to produce thermozyme (thermostable enzyme). Lipase is one of the largest group of industrial enzymes and commonly used to catalyze hydrolysis and ester synthesis reactions. It can be used as biocatalyst in food, pharmaceutical, detergent, and chemical industries [4]. Lipase is also able to catalyze interesterification, acidolysis, esterification, alcoholysis, aminolysis, and transesterification reactions [5].

Previous research were successfully isolated thermophilic microorganism from many area in Indonesia such as Cimanggu Hot Spring, Papandayan Crater in Garut, Domas Crater in Tangkuban Perahu Bandung, West Java [6]; Wayang Crater [7], and Gedongsongo Hot Spring [8]. Thermophilic microorganisms are also found in high temperature environment such as compost or waste processing unit [9]. Decomposition of organic wastes in the composting process was carried out by succession of microbial communities [10]. It consist three major phases: mesophilic, thermophilic and maturation stages. Study by Madayanti *et. al.*, (2008) have succesfully cultivated and collected 10 isolates from thermogenic phases (50-70°C) during composting process which showed lipolytic activity [11]. Tiquia (2002), found that microorganism from compost are able to produce lipase-esterase with moderate activity during the thermogenic phase. Composting process are also involving decomposition of carbohydrates into alcohol by microorganism, hence the possibility to find alcohol tolerance microorganism is very promising [12]. Several research has been succesfully isolated dan cultivated microorganism from compost environment [13, 11], but only few studies

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concentrated on alcohol tolerance microorganism.

The objective of this research is to isolate novel methanol tolerant and thermostable lipase producing microbes. In this report we would like to present the isolation and identification of alcohol-tolerant microbes. Phylogenetic analysis was employed to identify the screened lipolytic bacteria based on their 16S rRNA gene sequences. By combining the phylogenetic profile with the data of cells morphology, here we identified a few lipolytic alcohol tolerant microbes.

MATERIALS AND METHODS

All chemicals with pro analysis grade were purchased from Merck (Germany) and Sigma-Aldrich (USA), bacterial growth media such as lab lemco, yeast extract obtained from Bio Basic (Canada), biochemical reagents such as dNTPs, PCR Buffer, Taq DNA Polymerase were purchased from Fermentas (USA) and Kapa Biosystems (USA), and oligonucleotides (primers) were provide from Macrogen (South Korea).

Isolation of Microbes

Sample were collected from domestic compost at the thermogenic phase (72°C). Five grams of sample was diluted in 50 mL sterile normal saline (0.9%), and shaken at 150 rpm for 30 min at 55°C. After shaking, 1% of the suspension was transferred into a 250-mL Erlenmeyer flask containing 50 mL of medium. The medium containing 0.5% yeast extract, 0.1% NaCl, 0.1% $CaCl_2$ and 0.5% Lab Lemco were suspended using aquadest (pH 7.0). The culture was incubated overnight at 55°C under 150 rpm agitation. Serial dilution was conducted to the culture, followed by direct spreading into media with 2% bacto agar. The incubation take place for 24 hour at 55°C. Isolation of bacteria were carried out by *4-quadrant streak plate* method using the same media. Single colonies of various bacteria were obtained by replica plating method repeated at least four times.

Screening of Lipase-Producing Bacteria

Screening of lipase-producing bacteria was carried out using rhodamine B-olive oil agar plate method [14]. The culture was incubated at 55 °C for 2 days in agar plate contains 1% (v/v) olive oil, 0.5% (v/v) tween 80, 0.3% aquabidest and 0.2% (v/v) rodhamine B. The lipase-producing bacteria were identified by the orange fluorescent halos around the colonies when the plates were irradiated with UV illuminator.

Screening of Alcohol-Tolerant Bacteria

Bacteria isolates were grown in medium as stated before with addition of 3% (v/v) of methanol at 55°C with shaking at 150 rpm. The cells were then incubated at 55°C with shaking at 150 rpm. The number of cells were determined as optical density (OD) at 600nm.

Morphological analysis

Single colonies of various bacteria were obtained by replica plating method repeated at least four times. A single colony confirmation was undertaken by observing cell's shape morphology and Gram's staining [15].

Lipolytic activity assay

Lipolytic activity was measured using spectrophotometric assay by Lee *et. al.*, (1999) with p-nitrophenyl laurate (PNPL) dissolved in acetonitrile at concentration of 10 mM as substrate [16]. Subsequently, ethanol and potassium phosphate buffer (50 mM; pH 8.0) were added to final composition of 1:4:95 (v/v/v) of acetonitrile:ethanol:buffer, respectively. The cell-free supernatant (0.3 mL) was added to the substrate solution (0.9 mL), then incubated at 55°C. After 15 min, enzyme activity was measured by monitoring the absorbance at 405 nm, representing the amount of p-nitrophenol (PNP) released. One unit of lipase activity is defined as the amount of enzyme producing 1 µmol PNP per min under the assay conditions.

DNA extraction

Chromosomal DNA from microorganisms were isolated using Klijn *et. al.*, (1991) method with some modification [17]. Cell pellet was resuspended in 250 µL of 0.1M Tris HCl buffer (pH 8.0) containing 2.5 mg of lysozyme and incubation at 37°C for 1 hour. The cells were lysed by addition of 200µL lysis buffer (40 µL of 10% sodium dodecyl sulfate; 80 µL of 0.5M sodium EDTA pH 8.0; 8 µL of 2% proteinase K; and 72 µL of dd H_2O), and followed by incubation at 50°C for 30 minutes. The tube containing the mixture was then cooled on ice and the DNA was precipitated by addition of 150 µL 5 M sodium acetate and 25 µL acetic acid glacial. After centrifugation for 20 min at 12000 g, the supernatant was separated from debris and transferred into a new sterile micro tube. The

supernatant was then added with 300 µL of isopropanol and incubated for 1 hour at room temperature. The mixture was centrifuged at 10000 g at 4°C for 15 min. The pellet was then washed with 300 µL of 70% ethanol and centrifuged at 8000 g, 4°C for 5 min. DNA pellets were separated from the supernatant and dried with concentrator followed with resuspension by 25-50 mL nuclease free water and stored at 4°C. The obtained DNA solution is ready to use for PCR amplification.

Amplification and Sequencing of 16S rRNA Gene

The 16S rRNA genes of the five isolates were amplified by *Polymerase Chain Reaction* (PCR) technique [18]. The 16S rRNA gene of each bacteria was amplified using a pair of universal primers, namely UniB1 (Univ1492R: 5'-GGTTAC(G/C)-TTGTTACGACTT-3') and BacF1 (Bac27F: 5'-AGAGTTTGA-TC(A/C)TGGCTCAG-3') (22-23). A typical PCR mixture (30 µL in volume) was prepared with the following components: 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM KCl, 0.08% (v/v) Nonidet P40, 2.5 mM $MgCl_2$, a 250 µM of each deoxynucleoside triphosphate, 0.25 µM of each primers, and 1 U of *Taq* DNA polymerase (Thermo scientific). The PCR were set at: an initial denaturation temperature at 94°C for 4 min, followed by 30 cycles of denaturation @30 s at 94°C; annealing temperature was set at 48°C for 1 min; polimerization at 72°C for 2 min; while final extention was programmed at 72°C for 5 min. The PCR products were verified by electrophoresis that conducted on 1% agarose in buffer TAE 1x (diluted from 1 L buffer solution stock of TAE 50x containing 242 g Tris Base, 57.1 mL acetic acid glacial and 50 mM EDTA pH 8.0), using submerged horizontal electrophoresis cell (BioRad) for 45 minute at 70 volt. In order to obtain complete sequences of 16S rRNA genes in each samples, an automatic DNA sequencer (Macrogen, Korea) was employed based on direct sequencing method from PCR products using four pairs of PCR primers.

Phylogenetic analysis of the 16S rRNA gene

The electrophoregram data from the sequencing results were firstly analyzed by Seqman program. Homology of each sequence was analyzed using online software "NCBI-Blast", via the NCBI website (http://blast.ncbi.nlm. nih.gov/Blast.cgi). About hundred of high similarity sequences were found by Blast program and these sequences will be used to generate phylogenetic profile. MEGA 6 program was employed to generate phylogenetic profile based on the neighbor joining clustering method [19]. In order to generate such profile, initially multiple sequence alignment between sequence of each isolate together with their sequences similarity (99% identity) obtained from Blast program was undertaken using Clustal W2 program. The resulted alignment file then used as input for MEGA 6 program to generate phylogenetic profile.

RESULTS AND DISCUSSION

Isolation and Morphological Test of Isolates

Sampling was carried out randomly from Composting Unit at Institut Teknologi Bandung, West Java. The temperature of compost sample was at 72°C and pH 7.4. Using cultivation method, we found 50 viable isolates. Five lipolytic bacteria were isolated based on the orange fluorescence emitted by the colony under ultraviolet light. The fluorescence was occurred due to the complex formation between cationic Rhodamin B and free fatty acids resulted from the hydrolysis of lipid substrates by lipase [20]. The five isolates were then further sub-cultured to obtain pure isolated bacteria. The isolates were assigned as AL17, AL59, AL64, AL89, and AL96. The morphology observation towards the isolates using gram staining revealed that all of isolates are Gram negative microorganism. Using optical microscope with 1600x magnification were found as rod shape microbes.

Lipolytic Activity and Methanol Tolerance of the Isolates

The assay was used two different medium (0.5% yeast extract, 0.1% NaCl, 0.1% $CaCl_2$ and 0.5% Lab Lemco) of growth, one with pH 7.0 and the other with pH 9.0. The extracellular lipase from each isolate was tested based on lipolytic activity assay using p-nitro phenyl laurate as substrate. The result showed that lipolytic activity among the isolates are varies (Fig. 1). AL17 and AL89 has higher activity in medium at pH 7.0 compared to that at pH 9.0. Meanwhile, AL59 showed extremely higher activity at pH 9.0 compared to that at pH 7.0. In addition, AL64 and AL96 showed less activity in both media.

The methanol tolerance of the isolates was assayed based on the growth of the isolates

in medium containing 0% and 3% methanol. Addition of methanol to the culture was conducted at the early stage of incubation. Growth rate was measure based on optical density of the isolates at the logarithmic phase. The result showed that by addition of 3% methanol decreased the growth rate of all isolates (Fig2). AL 96 showed the most tolerance in medium with 3% methanol. The addition of methanol decreased only 10% of growth rate of the strain compared to that the control. Meanwhile AL17, AL59, AL96, and AL89 showed less methanol tolerance properties. Addition of methanol decreased the growth rate of 34, 22, 23, and 18% of the respective isolates. The result suggested that AL64 has better methanol tolerance properties compared to the other isolates. Previous reports stated that the solvent toxicity toward cells is related not only to the solvent hydrophobicity[21], but also the characteristic of the cell membranes [22]. In this research the addition of methanol slightly decreased the growth rate of the isolates.

Table 1. Similarity of 16S rRNA gene of AL17, AL64, AL89, and AL96 isolate with other 16S rRNA genes in the GenBank.

Organism	Accession Number	Identity (%) of the Isolates AL17	AL64	AL89	AL96
Pseudoxanthomonas taiwanensis strain NBRC 101072	NR_113974.1	99	99	99	99
Pseudoxanthomonas taiwanensis strain CB-226	NR_025198.1	99	99	99	99
Pseudoxanthomonas suwonensis strain 4M1	NR_043276.1	97	96	96	97
Pseudoxanthomonas suwonensis strain 11-1	NR_074771.1	97	96	96	97
Pseudoxanthomonas broegbernensis strain NBRC 101013	NR_113970.1	97	96	96	96
Pseudoxanthomonas daejeonensis strain NBRC 101159	NR_113984.1	96	96	96	96
Pseudoxanthomonas daejeonensis strain TR6-08	NR_042973.1	96	96	96	96
Pseudoxanthomonas broegbernensis strain B1616/1	NR_025306.1	97	95	96	96
Pseudoxanthomonas koreensis strain T7-09	NR_042972.1	97	96	96	96
Pseudoxanthomonas kaohsiungensis strain J36	NR_043070.1	96	96	96	96
Pseudoxanthomonas mexicana strain AMX 26B	NR_025105.1	96	95	95	95
Xanthomonas campestris strain ATCC 33913	NR_074936.1	96	95	95	95
Xanthomonas gardneri strain DSM 19127	NR_104793.1	96	95	95	95
Xanthomonas axonopodis pv. citri str.306 strain 306	NR_074937.1	96	95	95	95

Table 2. Similarity of 16S rRNA gene of AL59 with the other 16S rRNA genes in the GenBank.

Organism	Accession Number	Identity (%) of the Isolate
Brevibacillus thermoruber strain DSM7064	NR_112213.1	99
Brevibacillus thermoruber strain BT2	NR_026514.1	99
Brevibacillus aydinogluensis strainPDF25	NR_117986.1	99
Brevibacillus fulvus strain K2814	NR_125456.1	97
Brevibacillus levickii strain LMG 22481	NR_114928.1	97
Brevibacillus borstelensis strain NBRC 15714	NR_113799.1	97
Brevibacillus borstelensis strain DSM6347	NR_040984.1	97
Brevibacillus borstelensis strain LoganB4029	NR_029131.1	97
Brevibacillus panacihumi strain DCY35	NR_044485.1	96
Brevibacillus massiliensis strain phR	NR_118322.1	96
Brevibacillus invocatus strainLMG 18962	NR_041836.1	96
Brevibacillus invocatus strainNCIMB 13772	NR_112210.1	96
Brevibacillus limnophilus strainDSM 6472	NR_024822.1	95
Brevibacillus fluminis strain CJ71	NR_116293.1	96
Brevibacillus centrosporus strainDSM 8445	NR_112211.1	95

The sequence and Phylogenetic of the Isolates

PCR amplification of 16S rRNA gene using BacF1-UniB1 pairs of primers have successfully amplified full-sized of 16S rRNA gene with the length at about 1500 bp (Fig. 3). All 16S rRNA gene sequences are deposited to GenBank database (https://www.ncbi.nlm.nih.gov) with accesion number ID of KR 263159 (AL17), KR 263160 (AL64),

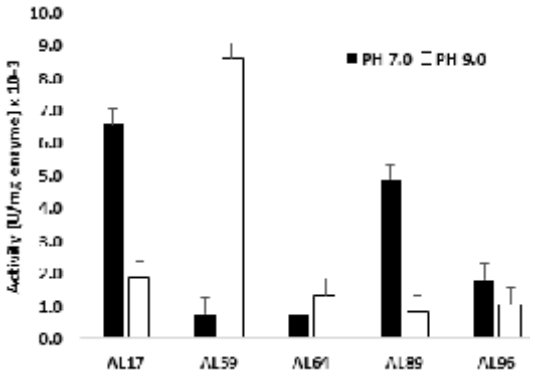

Fig. 1. Lipolytic activity of the isolates using 2 type of medium. % for pH 7.0 and ¡% for pH 9.0.

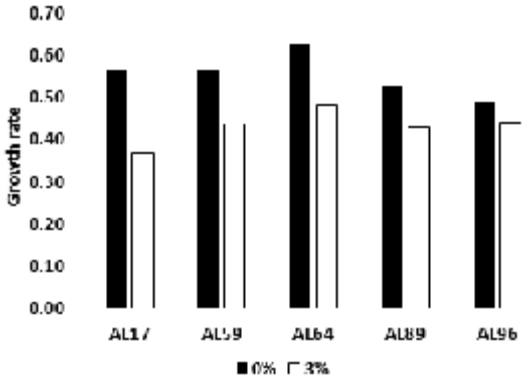

Fig. 2. Growth rate of the isolates during log phase. % for 0% methanol and ¡% for 3% methanol

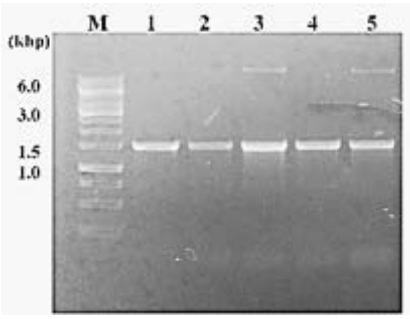

Fig. 3. Amplicon of 16S rRNA gene of the five isolates. The assigned lane number is as follow: DNA marker (M), AL17 (1), AL59 (2), AL64 (3), AL89 (4), and AL96 (5)

KR 263161 (AL89), KR 263162 (AL96), KR 263163 (AL59). Respectively, the sequence alignment of each 16S rRNA gene was carried out by BlastN program to generate the list of known bacteria in the GenBank with high similarity (99–100% identities) to our samples. Alignment of the gene showed that four bacteria have high similarity to the genus of *Pseudoxanthomonas* (Table 1.), while AL59 was closed to *Brevibacillus thermoruber* (Table 2.). The Blast alignment results were subsequently used as an input data for phylogenetic analysis. Based on the phylogenetic tree analysis showed that AL17 and AL64 were closely homolog to *Pseudoxanthomonas taiwanensis* (Fig. 4). Meanwhile AL89 and AL96

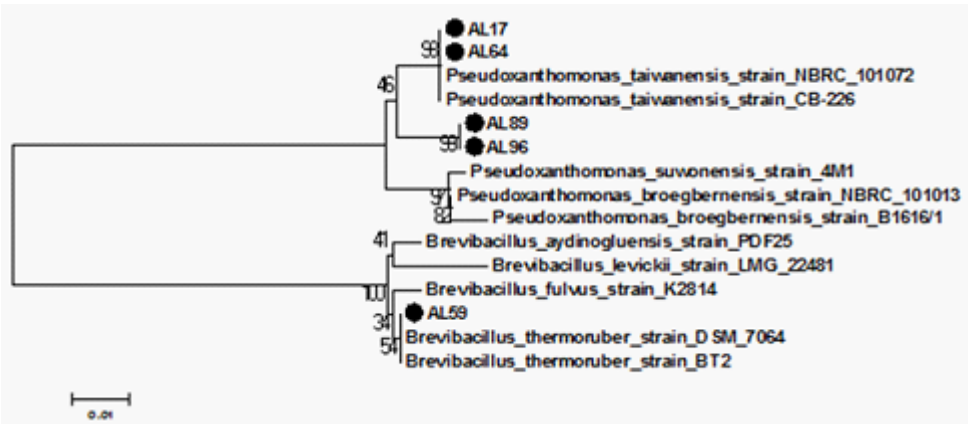

Fig. 4. Phylogenetic tree of 16S rRNA gene of the isolates using MEGA6 programme with 1000 bootstrap replication

formed different branch with other *Pseudoxanthomonas* and different sequence compared to that *Pseudoxanthomonas broegbernensis*. In addition AL59 showed closely homolog to genus of *Brevibacillus*.

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