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Characterization and Identification of Thermophilic Lipase Producing Bacteria from Thermogenic Compost

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Thermophilic lipase producing microorganisms were isolated from Pasir Impun thermogenic compost at temperature between 50-70° C. From 40 isolates tested, 10 isolates showed lipolytic activity on agar plate media containing olive oil and rhodamine B. The isolates showed lipolytic activity in the range of 15 – 315 U/L on p-nitrophenyl palmitate (pNPP) as substrate. Two isolates, namely PI6A and PI13C, were further characterized and showed maximum specific activity at a mid exponential of growth phase. Identification of the isolate was performed through ribotyping method. Chromosomal DNA from these isolates was extracted and the 16S rRNA gene fragments were amplified, resulting fragment length at around 1.5 kb on agarose gel. Further analysis of the 16S rRNA gene sequences using homological and phylogentetical methods showed that the PI6A and PI13C were close to *Bacillus licheniformic* and *Bacillus subtilis* respectively.

Key words: Lipase, thermophiles, 16S rRNA, compost, Pasir Impun.

Lipases or acylglycerol hydrolases (E.C. 3.1.1.3) are enzymes that catalyze the hydrolysis of long chain triacylglycerides with the formation of diacylglyceride, monoglyceride, glycerol and free fatty acids at the interface between the insoluble substrate and water through interfacial activation.[1,2] Apart from their natural substrates, lipases catalyze the enantio- and regio-selective-hydrolysis and synthesis of a broad range of natural and non-natural esters[3].

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Microbial lipases have been widely used for biotechnological application in fat, food ingredients, detergents, dairy, textile industries, production surfactants, and oil processing.[4,5,6] Most biocatalys are inherently labile; therefore, their operational stability is of paramount important for any bioprocess.[7] Enzymes derived from thermophilic bacteria are more resistant to denaturation than their mesophilic counterpart.[8] Most of lipases are mesophilic enzymes, which hydrolyzing a substrate that exits in solid form at room temperature. Thermophilic lipases show higher thermostability, higher activity at elevated temperatures, and often shows more resistance to chemical denaturation. This makes them ideal tools in industrial and chemical processes where

relatively high reaction temperatures and/ or organic solvent are used. Furthermore, running bioprocess at elevated temperatures lead to high diffusion rate, increased solubility of lipids and other hydrophobic substrates in water and reduced risk of contamination[9].

A small number of thermophilic lipase producing bacteria have been described in the last decades[10,11,12,13]. As each industrial application may require specific properties of biocatalysts, there is still an interest in finding new lipases that could create novel application[14].

In the last decades composting process is believed as one of the alternative to overcome domestic waste which is very rich of protein, lipid and carbohydrate complex. The process is decomposition of organic material by community of microbes in humid, aerobic and thermogenic condition.[15] This process consists of 3 steps, mesophilic, thermophilic and maturation processes[16]. In the early of the process the mesophilic microbes use organic materials as carbon and nitrogen sources.[17] On the thermogenic process (40-60^{o}C) degradation of protein, lipid and carbohydrate carry out very fast[18].

In this report, we describe isolation, characterization and identification of thermophilic lipase producing bacteria from thermogenic compost. Identification of thermophilic strains was based on ribotyping method through homological and phylogenetic analysis.

MATERIAL AND METHODS

Sampling of compost

Sample of compost was taken from TPA Pasir Impun, Bandung with the distance at around 10 km from the Laboratory. The composting process use domestic waste with traditional process. 3 -4 kg of thermogenic phase of compost at temperature at around 50-70°C was collected and used for this study.

Isolation and cultivation of microbes

Approximately 30 gram of compost was diluted in 250 mL sterile H2O with temperature at 50°C, and filtered. The filtrate was added into twice concentration of Nutrient Broth (1% Beef extract, 1% Peptone, 0,5% NaCl). The cultures were incubated at 50 and 60°C for 48-72 hours with shaking at 150 rpm. The pure culture was made by serial dilution and growth on agar plate.

Identification of lypolitic activity

The presence of lipase producing isolates were determined by growing the culture onto agar plates media containing olive oil 1% and rhodamine B as described by Kouker and Jaeger.[19] Lipase producing isolates were monitored by irradiating the plates with UV light at 350 nm.

Quantitative assay of lipase

The isolates were grown in 250 mL medium with composition of *olive oil* 1%, yeast extract 0.2%, pepton 0.1%, $(NH_4)_2SO_4$ 0.1%, $MgSO_4$ 0.1%, Na_2HPO_4 0.1%, NaCl 3%. [20] Extracelluler lipase was isolated by centrifugation of culture for 30 minutes at 15.000 g. Supernatant was collected and stored in -20°C.

Lipase activity was measured by spectrophotometric assay as described by Lee *et al.*[21] with *p*-nitrophenyl palmitate as substrate. One unit of lipase is defined as the amount of enzyme releasing 1 mmol PNP per min under the assay conditions. The molar absorption coeficient of PNP at 405 nm was determined to be 1.457×10^5 cm^2 mol^{-1}.

Isolation of chromosomal DNA

The pellet cells were suspended in 10 mM Tris HCl buffer (pH 8.0) containing 8 mg/ mL of lysozyme and incubated at 37°C for 1 h. The cells were lysed by adding lysis buffer containing 2% SDS, 0.8 mg/mL proteinase K and 200 mM EDTA pH 8.0. The lysis process was carried out by incubation at 50°C for 30 min. Ice cold potassium acetate and acetic acid glacial mixed solution were added. The denatured proteins were precipitated by centrifugation. Supernatants were mixed with an equal volume of chloroform isoamylalcohol (24:1, v/v). The aqueous phase was recovered by centrifugation and precipitated with 0.6 volume of isopropanol at room temperature for 1 h. The pellet of crude nucleic acids were obtained by centrifugation at 16.000 g for 20 min at room temperature, washed twice with cold 70% ethanol, and resuspended in sterile deionized water.

Amplification and sequencing of 16S rRNA gene

The amplification of 16S rRNA gene was carried out as described previously by Baker *et al.*[22] The 16S rRNA genes were amplified using a

fluorescence was variation among the colonies. Two colonies showed strongest intensity of fluorescence, namely PI6A and PI13C were further characterized. The lypolytic activity of PI13C was observed since the first day while isolate of PI6A showed lypolytic activity on the 5th day of incubation. PI13C showed stronger lipolytic activity compared to that the PI6A isolate.

In both isolates the lipases were produced associated with the growth of the isolates, however, the highest activity was shown at the middle of exponential phase (Fig. 2 and 3). The quantitative of lipolytic activity was assayed based on hydrolysis of p-nitrophenyl palmitate.

Chromosomal and 16S rRNA genes of pasir impun isolates

The 16S rRNA genes were amplified from total chromosomal DNA of the isolates respectively. The agarose gel electrophoresis showed that the sized of the amplicons were at around 1.5 kb as expected (Fig. 4). For further characterization the amplicons were purified and exposed for direct sequencing. The sequences of the amplicons were deposit in the GenBank database under accession number EU998983 and EU998984 for PI6A and PI13C respectively.

Homology of 16S rDNA fragment

The complete sequence of the fragments were compared to available sequences data on the GenBank and the result showed that the sequences have close homology to all 16S rRNA genes. Homological analysis of isolate PI6A showed that the isolate close to *B. licheniformis* (Table 1) with 99% homologous. Meanwhile, PI13C showed 99% homolog to *B. subtilis* (Table 2). Phylogenetic analysis of the sequences was conducted through comparison with other 20 best homologous sequences (Fig. 5). The result confirmed that the PI6A and PI13C were closed to *B. licheniformis* and *B. subtilis* respectively. Further analysis by comparing 16S rRNA gene sequences among the highest homology showed that the PI6A isolate showed 5 nucleotide difference with the consensus sequences, two of

Table 1

Accession Number	Description	Max ident
EU050709	Uncultured *Bacillus*	99 %
DQ981800	Uncultured bacterium	99 %
EU257697	*Bacillus licheniformis*	99 %
EU256501	*B. licheniformis*	99 %
EU256500	*B. licheniformis*	99 %
AY871102	*B. licheniformis*	99 %
EU344793	*B. licheniformis*	99 %
AY786999	*B. licheniformis*	99 %
AY750906	*B. licheniformis*	99 %
AY071857	*B. licheniformis*	99 %

Table 2

Accession Number	Description	Max ident
AM981261	*Bacillus* sp	99 %
EU581710	*Bacillus subtilis*	99 %
EU571106	*B. subtilis*	99 %
EU144043	*B. subtilis*	99 %
EU557030	*B. subtilis*	99 %
EU835569	*Bacillus* sp	99 %
EU262980	*B. subtilis*	99 %
EU251191	*B. subtilis*	99 %
AB244447	*Bacillus* sp	99 %
EU047884	*B. subtilis*	99 %

Table 3

Base Number	Nucleotide sequence Consensus	PI6A	Region
166	C	T	variable
194	A	G	variable
637	G	A	conserved
701	G	A	conserved
731	G	A	conserved

Table 4

Base Number	Nucleotide sequence Consensus	PI6A	Region
234	C	T	variable
919	T	G	variable
1385	A	G	variable

them on the variable region of the gene, however three nucleotide differences with the consensus lied at the conserved regions (Table 3). Meanwhile for the isolate PI13C only showed three nucleotides differences to the consensus sequences at variable region (Table 4).

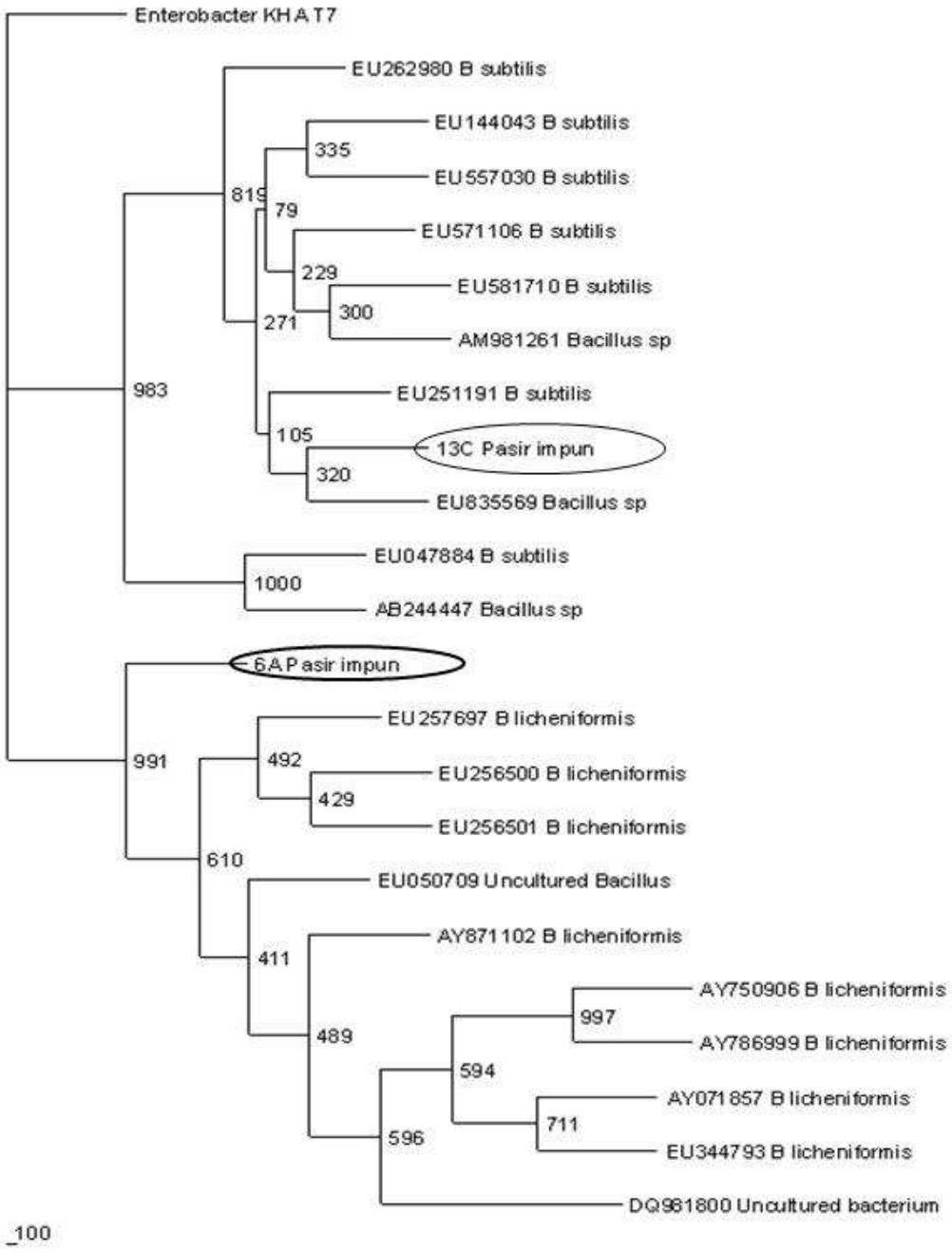

Fig. 5.

DISCUSSION

Lipase is one of the enzyme that have been long interested to detergent industry for their ability to aid in the removal of lipid stains and to deliver unique benefit that can not otherwise be obtained with the conventional detergent technologies. The main objective of the present work was to broaden the sources of lipase by screening, characterization and identification of thermophilic lipase producing microorganisms.

Ten lipase producing isolates have been identified from thermogenic domestic waste compost. The choice to use domestic waste compost was due to that the domestic waste is rich of lipid content.[27] Two of the best producing isolates, namely PI6A and PI13C, were identified close to *B. lichenoformis* and *B. subtilis* (Fig. 5). Some of lipase producing *Bacillus* have been reported.[12,5] Lipase is one of the enzymes that catalysing hydrolysis of esters formed glycerol and long fatty acids[28] and thus involved on the primary metabolite. This type of enzyme usually expressed on the exponential phase of bacterial growth. The result on this work confirmed that the best expression of lipase in both isolates occurred at the mid logarithmic phase (Fig. 2 & 3).

Identification of the isolates was carried out based on the ribotyping method through the sequences of 16S rRNA gene. Analysis using the BLAST Program[23] resulted that the sequences had high homology to all 16S rRNA genes available on the GenBank. The result confirmed that the amplicons were part of the 16S rRNA gene. 10 highest homology of each sequence showed that the PI6A has 99% homolog to *B. lichenoformis* (Table 1), while PI13C has 99% homolog to *B subtilis*. The highest homology of the isolates to the genus of *Bacillus* was also supported by morphological identification (data not shown). A large collection of rod shaped bacteria[29,30] may be due to the media or technique used on the sampling and the treatment of the compost sample during cultivation in laboratories. *Bacillus* was known as dominance bacteria on the environment due to its ability to form endospore, while other bacteria need very specific condition to struggle for growth.[31]

Phylogenetic trees were constructed by Clustal W, using 20 nucleotide sequences that displayed close homology from the BLAST program. Pasir Impun isolates, PI6A and PI13C, are closet to *B. licheniformis* (EU 257697) and *B. subtilis* (EU251191). However, for the PI6A isolate, the closest homology did not occur in the same branch as PI13C. Further analysis by detail comparison of the 16S rRNA sequences of the isolates to the GenBank showed that PI13C only has 3 nucleotide variations in the variable regions, meanwhile PI6A has more nucleotide variation and some of them in the conserved regions. All of the data suggested that PI13C is most probably as *B. subtilis*, meanwhile PI6A was unlikely as known *B. licheniformis* but rather as unique *B. licheniformis* or other new spesies of *Bacillus*. The last proposal was supported by the detail homological results and phylogenetic analysis which showed variation on the conserved regions and made new branch with other known *B. licheniformus*.

CONCLUSION

Two of lipase producing isolates, namely PI6A and PI13C, have been identified from thermogenic compost. The highest lipolytic activity of the isolates showed at mid exponential of growth phase. Based on 16S rRNA sequences analysis, the PI13C was belong to *B. substilis*. While PI6A was close to *B. licheniformis*, however based on homological and phylogenetic analysis, PI13C might be belong to unique *B. licheniformis* or other spesies of *Bacillus*.

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