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Characterization and Lipase Production of *Micrococcus* sp. L69 Isolated from Palm Oil-contaminated Soil

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Abstract: This research aims to characterise and study the lipase production of *Micrococcus* sp. L69 isolated from palm oil-contaminated soil. Lipase production was carried out by cultivating the bacteria in the medium containing vegetable oils. The lipase activity was determined by spectrophotometric method toward *p*-nitrophenyl palmitate as a substrate. The results of this research showed that the bacteria isolate L69 was a unique lipolytic bacteria. Based on sequence of 16S rRNA gene, the bacteria had low similarity level ($\leq$ 93%) to sequences data listed in GenBank. Olive oil and coconut oil are good carbon sources for bacterial growth and lipase production. The highest lipase activity achieved at temperature of 50°C and pH 7.0. The addition of 7 mM Ca^{2+} and Fe^{++} enhanced the lipase activity for 1.95- and 2.26-fold, respectively.

Key words: Lipase, *Micrococcus*, palm oil-contaminated soil.

Introduction

Lipase is one of the industrial enzymes which is involved in a variety of important reactions in aqueous media as well as non-aqueous media. Microbial lipases are preferred as biocatalysts in various industrial processes because of their ability to use various substrates, high activity and stability in organic solvents, regio- and/or enantioselectivity (Anobom et al., 2014; Kumar et al., 2016).

Various industrial applications of microbial lipases are included such as fat and oleo-chemical, detergent, pulp and paper industry, production of biodegradable polymer, food processing, flavour development, medical and pharmaceutical, biosensors, waste treatments, cosmetics, perfumery and biodiesel (Choudhury and Bhunia, 2015).

In previous study, several lipolytic bacteria had been isolated from palm oil-contaminated soils, including *Lactococcus garvieae* (Sumarsih et al., 2018), and isolate L69 whose morphology and physiology were identified as *Micrococcus* sp. (Sumarsih et al., 2019).

Lipase production seems to be constitutive and independent of the addition of lipid substrates to the culture medium. However, their presence can enhance the level of produced lipase activity. Lipases are generally produced in the presence of a lipid such as oil, triacylglycerols or any other inductor, such as fatty acids, Tween 20, Tween 80, hexadecane, tributyrin and tripalmitin. Lipidic carbon sources seem to be essential for obtaining a high lipase yield. Generally, lipase production increases when the relative percentage of C18:n fatty acid esters in the respective vegetable oil is increased (Zarevúcka, 2012).

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In the present research, molecular identification was carried out to reveal the bacterial species of *Micrococcus* sp. L69, based on sequence of 16S ribosomal RNA gene. The effects of vegetable oil on growth and lipase production were studied by cultivating the bacteria in the medium containing different vegetables oils. The lipase activity was determined in various temperature, pH and metal ions.

Materials and Methods

Microorganism and Medium Composition

Microorganism used in this study was a lipolytic bacteria *Micrococcus* sp. strain L69 isolated from palm oil-contaminated soil. The bacteria was periodically sub-cultured in Luria Bertani (LB) medium, consisted of (w/v): 1% tryptone, 0.5% yeast extract, 1% NaCl and 2% bacto agar.

Modified medium for lipase production consisted of 0.5% yeast extract, 0.5% sea salt, 1% ammonium sulfate, and 2% vegetable oil (virgin olive oil and virgin coconut oil). The other materials used in this study were Tris (hydroxymethyl) aminomethane, *p*-nitro phenylpalmitate (*p*NPP), *p*-nitrophenol (*p*NP), ethanol, acetone, isopropanol, HCl, Na_2CO_3, NaCl, $CaCl_2$, $FeSO_4$, $MgSO_4$, $MgCl_2$, $FeCl_2$ and Na_2EDTA.

Bacteria Identification

The bacteria was identified based on 16S rRNA gene sequence. Amplification of bacterial 16S rRNA gene was carried out using GoTaq® Green Master Mix using a pair of universal primers P0 (F): 5'-GAGAGTTTGATCCTGGCTCAG-3' and P6 (R): 5'-CTACGGCTACCTTGTTACGA-3'.

Lipase Production

Lipase production was carried out by cultivating bacterial cell in medium containing various concentration of vegetable oils. Overnight bacterial cultures (OD_{600}= 0.5) were inoculated in 500 mL flasks containing 100 mL of production medium and incubated at 37˚C on a shaker incubator 150 rpm. The bacterial growth and lipase production were monitored every 2 hours. Bacterial growth was observed by measuring the optical density (OD) at λ = 600 nm. Enzyme production was observed based on the lipase activity.

Assay of Lipase Activity

Lipase activity was determined by the spectrophotometric method toward *p*-nitro phenylpalmitate (Sumarsih et al., 2019). The reaction mixture consisted of 0.1 mL enzyme extract, 0.8 mL of 0.05 M Tris buffer (pH 8) and 0.1 mL of 0.01 M of p-nitrophenyl palmitate (disolved in isopropanol). The reaction mixture was incubated at 37°C for 15 minutes. The reaction was stopped by adding 0.25 mL of 0.1 M Na_2CO_3. The reaction mixture was centrifuged at 11,000 rpm for 15 min and the absorbance was measured by spectrophotometer UV-Vis at 410 nm. The enzyme activity expressed in units/ mL (U/ mL). One unit of lipase activity was defined as the amount of enzyme which liberated 1 μmol of p-nitrophenol per minute.

Effect of Metal Ions on the Lipase Activity

The lipase activity was determined in presence of different ions Na^+, Mg^{++}. Fe^{++}, and Ca^{++}. Various concentration of NaCl, $MgCl_2$, $FeCl_2$, $CaCl_2$, $NaCO_3$ and Na_2EDTA that is 1 mM, 3 mM, 5 mM and 7 mM, respectively, were added into the reaction mixture. Reaction mixture without metal ion was used as a control.

Results and Discussion

Bacteria Identification

Nucleotide sequence of PCR product was determined by the Sanger method using primer P0(F) and P6(R). The sequence of 16S rRNA gene fragment about 1440 bases was obtained by pairwise alignment method using the Bioedit program version 7.2.6.1. The sequence of 16S rRNA gene of bacteria isolate L69 was compared with reported 16S rRNA sequences in GenBank database using NCBI Basic Local Alignment Search Tool (BLAST) retrieved from website https://www.ncbi.nlm.nih.gov/BLAST. The result showed that the lipolytic bacteria isolate L69 were unique bacteria that had low similarity level (< 93%) to sequences data listed in GenBank. The bacteria isolate L69 is most closely related to *Micrococcus luteus* strain Amic_9 (93.31%).

The phylogenetic tree neighbour joining based 16S rRNA gene sequence of *Micrococcus* sp. L69 to bacteria listed in GenBank was constructed by Molecular Evolutionary Genetics Analysis (MEGA) X software (Figure 1).

Lipase Production

Lipase production was expressed as the activity of the lipase produced. The results of the study showed that 1-15% vegetable oils that is both olive oil and coconut oil were good carbon source for lipase production from *Micrococcus* sp. L69 (Figure 2). The crude enzyme

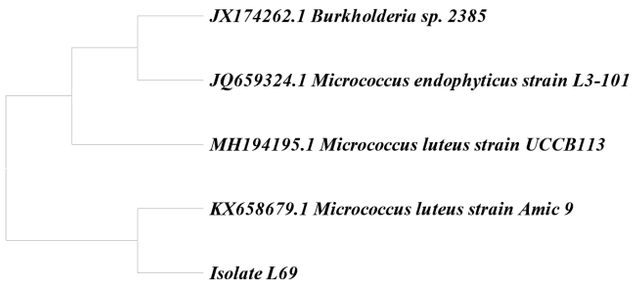

Figure 1: Phylogenetic tree neighbour joining based 16S rRNA gene sequence of isolate L69.

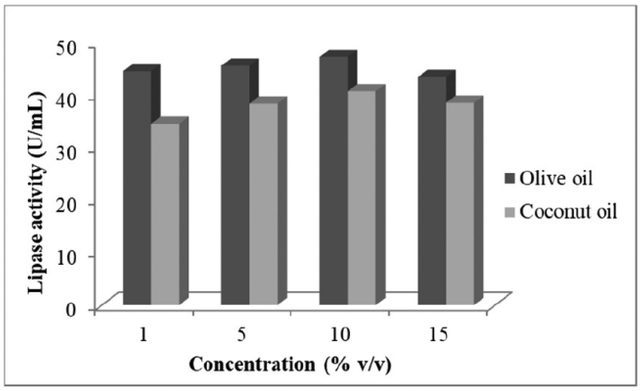

Figure 2: Effect of vegetable oil on lipase activity.

showed lipase activity of 43.36-47.20 U/ mL (olive oil) and 34.50-40.66 U/mL (coconut oil).

The induction process in lipase production can be done by adding vegetable oils to the fermentation medium. Olive oil has been referred as one of the best inductors of lipase production in several microorganisms such as *Aspergillus niger, Bacillus subtilis, Candida rugosa*. Generally, the activity of intra and extracellular lipases increases with increasing lipid concentrations, although excessive levels in the growth medium may be cytotoxic (Zarevúcka, 2012).

Effect of Vegetable Oil on Bacterial Growth and Lipase Production

Growth and lipase production profile of *Micrococcus luteus* L69 cultivated in the medium containing olive oil and coconut oil is shown in Figure 3.

The bacteria were grown at medium containing olive oil, which has been specified to find out the optimum incubation period. Maximum lipase activity of 43.45 U/ mL was observed on 20 hours of production after going through the stationary phase to the death phase. On the 20 hours cultivation, a decline in growth of the bacteria and maximum lipase activity was observed.

The decrease of bacteria growth may be due to overpopulated culture and a fixed amount of nutrients

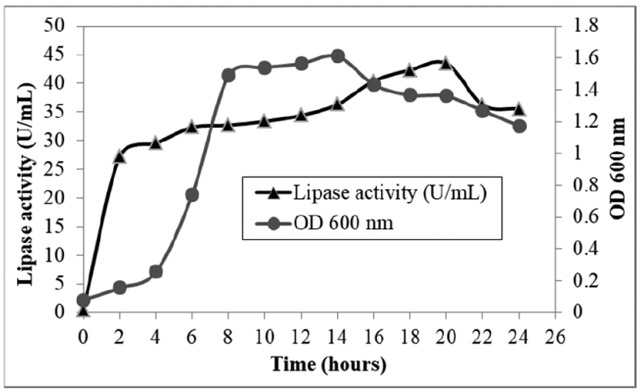

Figure 3: Time course of bacterial growth and lipase production.

with which the organism starts liberating proteolytic enzymes. The decrease of lipase production at the later stage could be possibly due to pH inactivation, proteolysis, or both (Suci et al., 2017).

Lipase Characterisation

The lipase produced by *Micrococcus* sp. L69 showed highest activity at temperature of 50°C and pH 7.0.

The graph (Figure 4) showed that different metal ions have different effect on lipase activity. For Na^{+} ion at concentration of 1-7 mM, there was a relative activity profile similar to the control (no metal ion). However, the other ions: Ca^{++}, Fe^{++} with concentration of 7 mM enhanced the lipase activity by approximately 95%, and 126%, respectively. However, Mg^{++} ion decreases lipase activity.

Katiyar and Ali (2013) reported that Fe^{++} and Ca^{++} ions enhanced lipase activity of *Candida rugosa*. Lipase of *Chromohalobacter japonicus* BK=AB18 was enhanced significantly by addition of Ca^{++} ion (Hertadi and Widhyastuti, 2015). Anions of Na^{+} gave different effect on the lipase activity. The addition of NaCl mixture did not affect the lipase activity, but there was a slight increase in activity (5.896%) on the addition

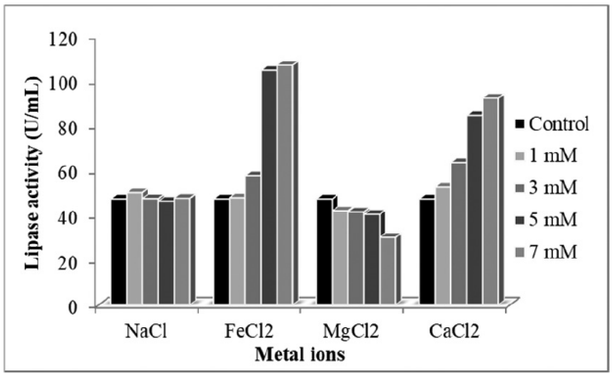

Figure 4: Effect of metal ions on lipase activity.

of 1 mM NaCl. The addition of 7 mM Na_2CO_3 and Na_2EDTA inhibited lipase activity by 24.949% and 33.121%, respectively. This was similar to the work reported by Tripathi et al. (2014), the addition of $MgCl_2$ and EDTA decreased lipase activity by 14% and 49.3%, respectively.

Conclusion

Based on partial sequence of 16S rRNA gene, the bacteria showed low similarity to *Micrococcus luteus* strain Amic_9 (93.31%). Vegetable oils (olive oil and coconut oil) are good carbon source for the bacterial growth and lipase production. Temperature, pH and metal ions influenced in different effect to the lipase activity.

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