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Carbon and Nitrogen Sources for Lipase Production of *Micrococcus* sp. Isolated from Palm Oil Mill Effluent-Contaminated Soil

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Abstract. This research aim to optimize the lipase production of *Micrococcus* sp. isolated from oil palm contaminated soil. The production of lipase enzymes is carried out in modified medium containing olive oil, sea salt, yeast extract with various types of carbon source (glucose, sucrose, glycerol) and nitrogen sources (tryptone, ammonium phosphate, urea and ammonium nitrate). The activity of lipase enzyme was determined by UV-Vis spectrophotometric method toward p-nitrophenylpalmitate (p-NPP) as a substrate. The results showed that glucose 1% was better carbon source compared with sucrose and glycerol. Ammonium phosphate, urea and ammonium nitrate were good nitrogen sources for lipase production of *Micrococcus* sp., although tryptone was the best nitrogen source.

Keyword: Lipase, carbon source, nitrogen source, *Micrococcus* sp., soil,

1. Introduction

Lipases occur widely in nature, but microbial lipases are commercially significant because of low production cost. Most of the industrial microbial lipase is derived from fungi and bacteria. The majority of enzymes used in food industry are for food processing, mainly for modification and breakdown biomaterials. Most of the commercial lipases produced are utilized for many industries, for examples dairy, fat and oil, flavor enhancement, and bakery industry. Lipases are also used in pharmaceuticals, cosmetics, leather, paper industry [1,2,3], and bio-energy production, especially biodiesel [4]. Lipases are important biocatalyst in organic synthesis, such as, esterification, and trans-esterification [5]. Microbial lipases are biotechnological potential, because of stable in organic solvent, do not need cofactor, broad substrate specificity, stabil in organic solvents, high specificity of the substrate, and show high-enantioselectivity [2,3].

Microorganisms with potentials for producing lipases can be obtained from soil contaminated by oil, industrial waste, organic waste, hot springs and other habitats [6]. Some of the best microorganisms producing extracellular lipase enzymes are *Candida rugosa*, *Pseudomonas*, *Rhizopus* and *Geotrichum* sp. [7]. Several studies have been conducted to obtain enzymes with high activity including media optimization and production conditions. The production media used is modified modification media where components such as carbon, nitrogen and mineral sources can be optimized.

2. Experimental Method

Bacteria used in this research was isolated from palm oil mill effluent-contaminated soil which is a collection of the Biochemistry Laboratory of the Faculty of Science and Technology, Universitas Airlangga. Media used in this study were Luria Bertani (LB) medium was consisted of (v/v): 1% tryptone, 0.5% yeast extract and 1% NaCl for bacteria growth, rhodamine B-LB agar plate containing olive oil for lipolytic bacteria screening, modified medium for lipase production containing yeast extract, mineral salt, olive oil, various carbon sources (glucose, sucrose, glycerol) and various nitrogen sources



(tryptone, ammonium phosphate, ammonium nitrate, urea). The other materials were bacto agar, Tris, HCl, Na₂CO₃, mineral salt (sea salt), *p*-nitrophenylpalmitate (p-NPP), para-nitrophenol (pNP), ethanol, acetone, isopropanol,

Screening of lipolytic bacteria was carried out by inoculating bacteria cells on agar-rhodamine B medium containing olive oil. The culture was incubated at 37°C for 48 hours. Lipolytic activity is detected by the halo zone around orange colonies with UV radiation [8,9].

Bacterial characterization was observed macroscopically and microscopically. Bacterial characteristics were observed macroscopic and microscopic characteristics were observed by Gram staining. Observation of bacterial physiological characteristics was carried out using microbact™ GNB 12A / B / E, 24 E Identification kit.

Optimization of carbon sources for lipase production was carried out in a medium containing sea salt, yeast extract, olive oil and various carbon sources (glucose, sucrose, glycerol) and incubated at 37°C by shaking at 150 rpm.

Optimization of nitrogen sources for lipase production was carried out in a modified containing sea salt, yeast extract, olive oil and various nitrogen sources (tryptone, ammonium nitrate, urea, ammonium phosphate) and incubated at 37°C by shaking at 150 rpm. The influence of carbon sources and nitrogen sources on bacterial growth was observed by measuring the optical density of the culture medium with a UV-Vis spectrophotometer at 600 nm. The lipase activity of crude extract was determined toward *p*-nitrophenylpalmitate as a substrate.

Lipolytic activity was determined by a spectrophotometric method toward *p*-nitrophenylpalmitate as a substrate [4]. 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 0.01M of p-NPP (dissolved in isopropanol). The reaction mixture was incubated at 37°C for 10 min. the reaction was added with 0.25ml of 0.1M mixture of cold acetone-ethanol (1:1) Na₂CO₃ to stop the reaction. The reaction mixture was centrifuged at 11,000 g for 15 min and the absorbance was measured by spectrophotometer UV-Vis at 410 nm. The enzyme activity expressed in units of 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.

The lipase activity was determined in various substrate concentration *p*-nitrophenylpalmitate (0.5 mM, 0.75 mM, 1 mM, 1.25 mM and 1.5 mM) and various enzyme concentration.

3. Results and discussion

The screening of lipolytic bacteria of isolates bacteria from oil-contaminated soil, obtained 15 isolates of 150 bacterial collection were lipase-producing bacteria with various hydrolysis index.

Figure 1 shows the lipolytic activity of isolate L69 grown in agar-rhodamine B medium containing 1% olive oil. The color formation of the orange zone around bacterial colonies seen under UV light indicating the presence of lipolytic activity.

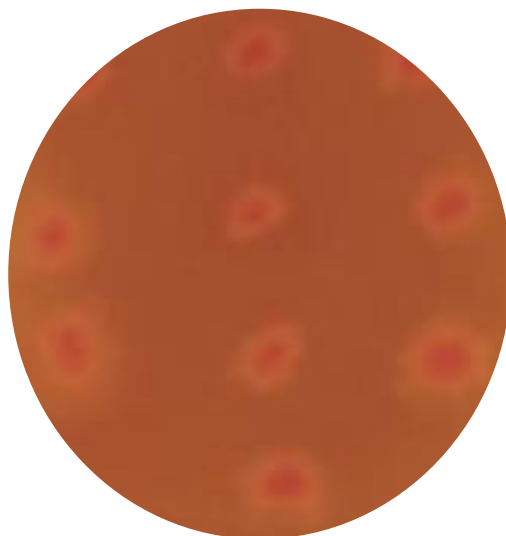


Figure 1. Lipolytic activity of isolate L69 grown in agar-rhodamine B medium containing 1% olive oil

In previous research, a lipolytic bacteria molecularly identified as *Lactococcus garvieae* showed good lipolytic activity [8]. The basic principle in this method involves the interaction between olive oil substrate hydrolysis products and rhodamine B on the medium to produce a fluorescent orange color. Lipases produced by bacteria hydrolyze olive oil in the medium. Free fatty acids released are detected on the agar plate which forms a complex with rhodamine B, when exposed to UV-A light, forms an orange fluorescence [9,10,11].

Figure 2 shows morphological characteristic of isolate L69 using Gram staining, the isolate L69 was a round-shaped Gram positive bacteria (+).

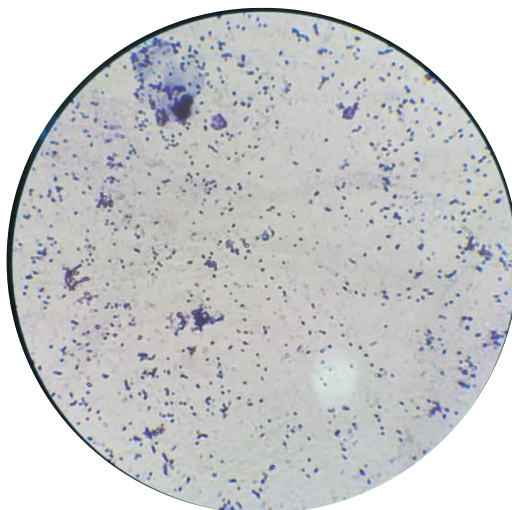


Figure 2. Microscopic characteristic of isolate L69

Table 1 lists physiological characteristics of bacterial cell isolate L69 using Microbact Identification Kits 24E. Based on the physiological characteristics data obtained, it can be concluded that bacterial isolate L69 belong to the genus *Micrococcus* sp.[12].

Table 1. Physiology characteristics of isolate L69

Physiological characteristic	Observation result	Physiological characteristic	Observation result
Lysine	-	Sorbitol	+
Ornitin	-	Rhamose	+
H ₂ S	-	Sucrose	+
Glukosa	+	Lactose	+
Manosa	-	Arabinose	-
Xylosa	-	Adonitol	-
ONPG	-	Rafinose	-
Indol	-	Salisin	-
Urease	-	Arginin	-
VP	-	Motilitas	-
Sitrat	-	Amylum	-
TOA	-	Esculine	-
Gelatin	-	Nitrat	-
Malonat	+	Oxidase	+
Inositol	+		

The improvement of lipase production was performed by cultivating bacteria in modified medium containing yeast extract, mineral salts, olive oil with various carbon sources and nitrogen sources.

Figure 3 shows *Micrococcus* sp. growth on during cultivation in modified medium containing various carbon sources. The bacterial cultivation with glucose carbon sources has a higher lipase enzyme activity of 67.714 U/ mL. This is due to the rapid adaptability of *Micrococcus* sp on media containing carbon glucose sources. Glucose is used by bacteria in cell metabolism through the cycle of glycolysis where there is a change in glucose to pyruvic acid along with the production of ATP and NADH, which can occur aerobically or anaerobically in the cytosol. The pyruvic acid produced will be converted to lactic acid and used for *Micrococcus* sp. growth in the fermentation process [13].

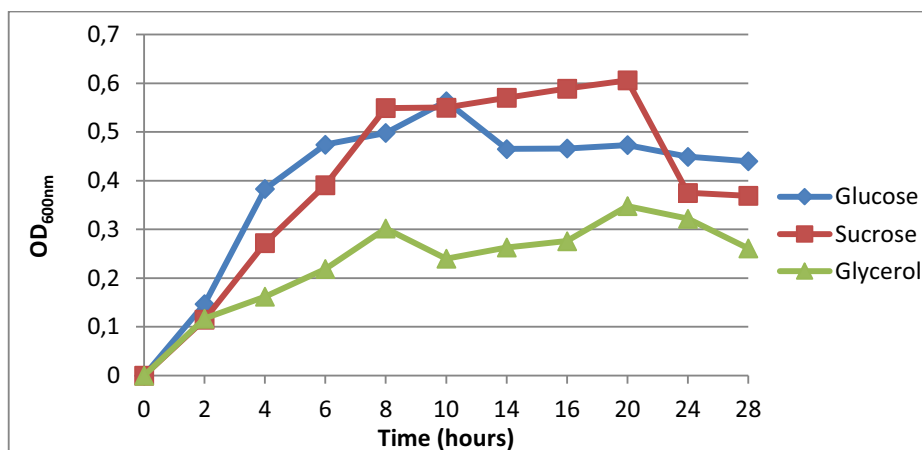


Figure 3. *Micrococcus* sp. growth on during cultivation in modified medium containing various carbon sources

Figure 4 shows lipase production of *Micrococcus* sp. during cultivated in modified medium containing various carbon sources. The increasing of lipase activity is proportional to glucose

concentration. The highest lipase activity 70.857 U/ mL achieved when *Micrococcus* sp. cultivated in modified medium containing 1% glucose. Glucose concentration more than 1.5% could inhibit enzyme production.

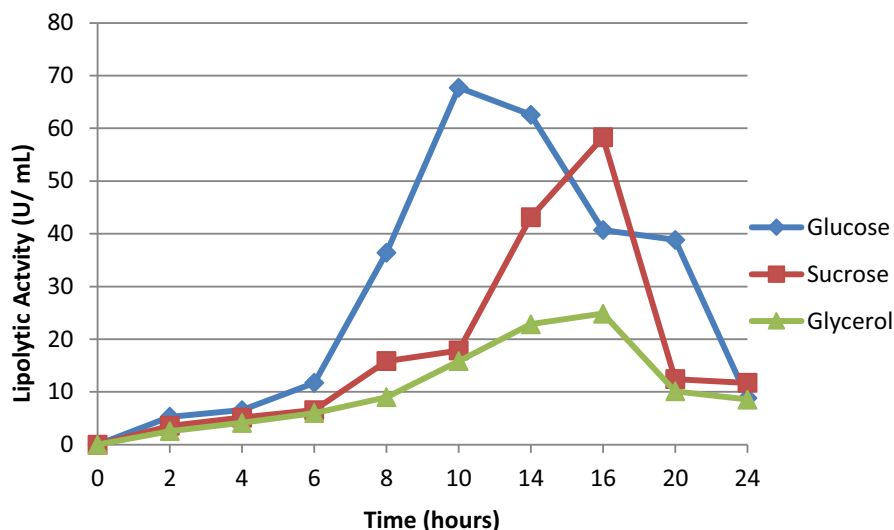


Figure 4. Lipase production of *Micrococcus* sp. during cultivated in modified medium containing various carbon sources

Figure 5 shows *Micrococcus* sp. growth on during cultivated in modified medium containing various nitrogen sources. Ammonium phosphate, urea and ammonium nitrate were good nitrogen sources for *Micrococcus* sp. growth, although tryptone was the best nitrogen source.

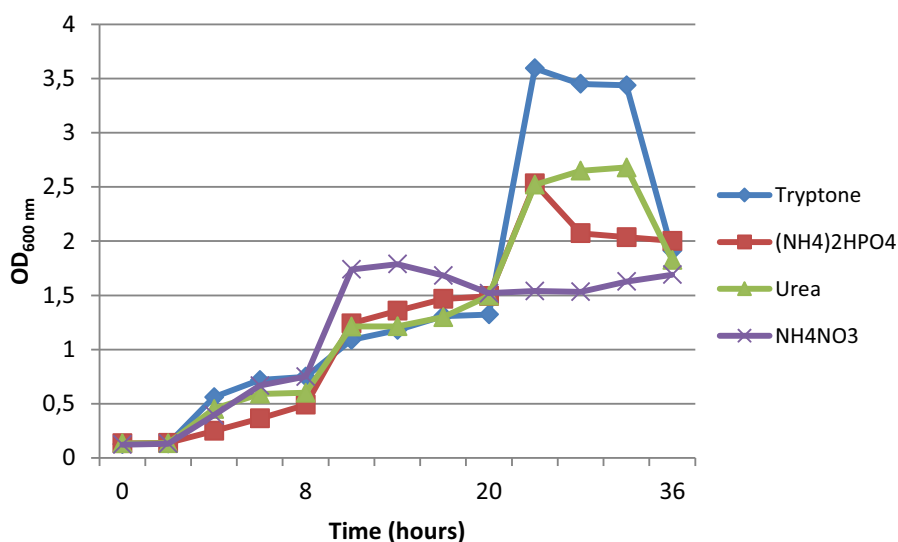


Figure 5. *Micrococcus* sp. growth on during cultivated in modified medium containing various nitrogen sources

Figure 6 shows the lipase production of *Micrococcus* sp. during cultivated in modified medium containing various nitrogen sources. Ammonium phosphate, urea and ammonium nitrate were good nitrogen sources for lipase production of *Micrococcus* sp., although tryptone was the best nitrogen source.

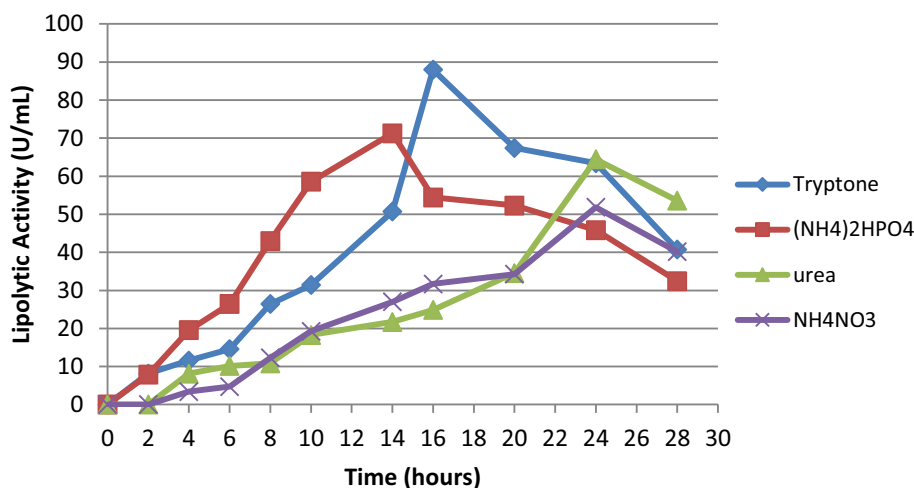


Figure 6. Lipase production of *Micrococcus* sp. growth on during cultivated in modified medium containing various nitrogen sources

Figure 7 shows the effect of enzyme concentrations to lipase activity. Increasing enzyme concentration will increase the rate of reaction, as more enzymes will be colliding with substrate molecules. The increase in lipase volume from 50 μ L to 100 μ L causes an increase in lipase activity. The highest lipase activity was shown in a 100 μ L lipase volume of 72.286 U / mL.

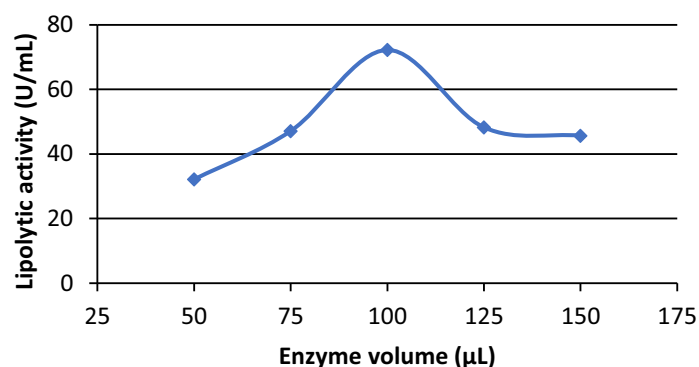


Figure 7. Effect of enzyme concentrations on lipase activity

Figure 8 shows the effect of substrate concentration on lipase activity. The graph shows an increase in lipolytic activity along with an increase in pNPP substrate concentration. The lipase activity has increased with increasing concentration of *p*-nitrophenylpalmitate substrate (0.5-0.75 mM). Increasing substrate concentration increases the rate of reaction. This is because more substrate molecules will be colliding with enzyme molecules, so more product will be formed. However, after a certain concentration of pNPP substrate (≥ 1 mM), any increase will have little effect on the rate of reaction. The lipase activity of 87.480 U/ mL was reached at a substrate concentration of 1.5 mM.

Increasing concentration will increase the rate of reaction up to a point, after which any increase will not affect the rate of reaction. As a reaction proceeds, the rate of reaction will decrease, since the substrate will get used up.

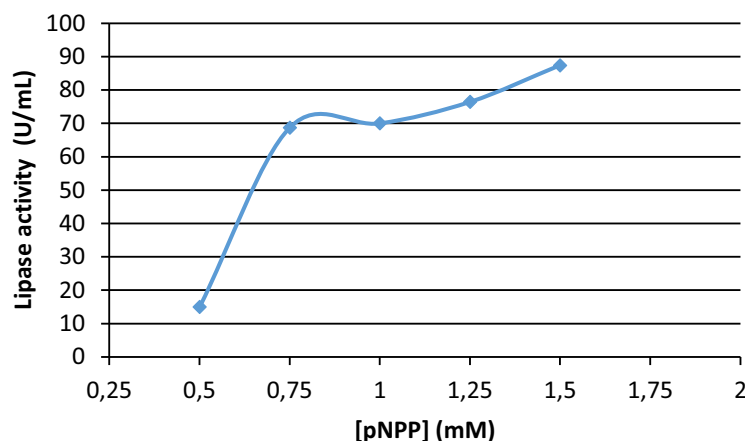


Figure 8. The effect of substrate concentration on lipase activity

4. Conclusion

The conclusion of this research were lipolytic bacteria isolate L69 belong to the genus *Micrococcus* sp. Glucose 1% was better carbon source compared with sucrose and glycerol. Ammonium phosphate, urea and ammonium nitrate were good nitrogen sources for lipase production of *Micrococcus* sp., although tryptone was the best nitrogen source.

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