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Lysinibacillus sphaericus Isolated from Palm Oil Waste Land as Lipase Producer

Sri Sumarsih[1*], Sofijan Hadi[1], Fatimah[2], Rizka Diah Fitri[2], Gilva Illavi[2]

[1]*Chemistry Departement, Faculty of Sciences and Technology, Universitas Airlangga, Kampus C Unair Mulyorejo, Surabaya 60115, Indonesia*
[2]*Department of Biology, Faculty of Sciences and Technology, Universitas Airlangga, Kampus C Unair Mulyorejo, Surabaya 60115, Indonesia*

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ABSTRACT

In this research, lipolyic bacteria have been isolated from palm oil waste land for the production of lipase. Species of potential lipolytic bacteria were identified based on their morphology and sequences of 16 rRNA gene. Enzymes are produced by growing bacteria in a medium with various vegetable oils and nitrogen sources. The enzyme produced by the bacteria measured its lipolytic activity against the substrate para-nitrophenylpalmitate. The lipolytic bacteria was recognized as *Lysinibacillus sphaericus* L49a based on morphological and phylogenetic analysis. Mineral media with different vegetable oils as carbon sources, and different nitrogen sources were suitable for growth and production of lipase enzymes of *L. sphaericus* L49a. Culltivation of *L. sphaericus* L49a in medium containing ammonium sulfate and olive oil produced lipase with the highest lipolytic activity.

1. Introduction

Lipase (triacylglycerol acylhydrolase) is an important industrial enzyme, especially because of its ability to catalyze various reactions of hydrolysis, esterification, interesterification, aminolysis and alcoholysis, which are very important reactions supporting industrial processes. Microbial enzymes are considered more beneficial than lipases from plants and animals. This is because microbial lipases have several advantages, including showing varied activities, easy to genetically modified, easily available in abundance, the production process does not depend on the season, growing fast and being able to use cheap media sources. Various strains of bacteria and fungi that have been studied show a high ability to produce lipase (Guerand 2017; Chandra *et al.* 2020).

Lipase from microorganisms plays an important role in various industries, including the oleo chemical, detergent, textiles, polymers, flavors, medicines, paper and pulp, cosmetics and perfume, and biodiesel. Besides that, lipase is also used in leather tanning, and waste treatment (Andulema and Gessesse 2012; Choudhury and Bhunia 2015). The widespread applications of lipase increase the demand for enzymes in the market, especially in the industrial sector, thus encouraging the exploration of microbial lipase producers, and unique novel enzymes. Lipase-producing microorganisms have been isolated from various sources or environments, including household and industrial wastes, vegetable oil factories, rotten food, compost, hot springs, and oil-contaminated soil (Thakur 2012).

Different conditions that influence the microbial enzyme production are composition of media, pH, temperature and dissolved oxygen. The presence of a substrate in the medium greatly influences the production of an inductive enzyme. The addition of lipids, triacylglycerols, fatty acids, tween, bile salt, glycerol or esters into the media will increase the production of the lipase enzyme. In any case, micronutrients and nitrogen sources moreover have to be considered to optimize the growth and enzymes production of microorganisms (Sharma *et al.* 2001). This study focused in isolation of lipolytic bacteria from palm oil waste land, and identified based on

* Corresponding Author
E-mail Address: sri-sumarsih@fst.unair.ac.id

morphology, biochemistry and phylogenetic analysis. Lipase enzymes were produced by growing bacteria in a medium with various vegetable oils and nitrogen sources.

2. Materials and Methods

2.1. Sample and Culture Media Composition

Lipolytic bacteria was isolated from palm oil waste land, at a palm oil mill in Central Kalimantan, Indonesia and screened in plate agar media containing rhodamine B and olive oil (Sumarsih *et al.* 2018).

Pre-culture medium was Luria Bertani medium, compost of NaCl 1%, tryptone 1%, and yeast extract 0.5%. Bacterial culturing was done by incubating for 16 hours at 30°C in a shaker incubator. The compositions of modified medium for lipase production were (g/L): $MgSO_4 \bullet 7H_2O$ (0.3), Na_2HPO_4 (12), $CaCl_2$ (0.25), KH_2PO_4 (2), peptone (2), and vegetable oils 2% (v/v). In this study, peptone was replaced with different nitrogen sources (urea and $(NH_4)_2SO_4$). Four different vegetable oils namely, coconut, olive, sunflower, and mustard oil were added to media as the main carbon sources, that also function as inducers for lipase production. Based on carbon and nitrogen sources used, there are 12 different medium variations.

2.2. Characterization of Isolate L49a

Isolates L49a characterization included morphological observations through Gram staining and biochemical properties using a commercial identification kit Microbact™ GNB 12A/B/E, 24E. The targeted lipolytic bacterial species is determined by aligning its 16S rRNA gene sequence with other 16S rRNA sequences stored in Genbank that have the highest similarity.

The genomic DNA was extracted and purified from bacterial cell pellet according to the instructions of kit manufacturer. Thermal cycler machine was used for amplifying 16S rRNA gene from the isolate L49a genomic DNA, generated with Master Mix of pGoTaq® Green using universal primers pair: 5'-GAGAGTTTGATCCTGGCTCAG-3' (F), and 5'-CTACGGCTACCTTGTTACGA-3' (R) (Cello *et al.* 1997). The 16S rRNA gene was amplified in a thermal cycler for a total of 30 cycles, initiated with a denaturation process at 95°C for 2 min. The conditions of each cycle were: denaturation 95°C for 30 s, annealing 55°C for 1 min, extension 72°C for 2 min and the final extension 72°C for 5 min. The PCR product obtained was then purified and analyzed by agarose gel electrophoresis. The purified PCR product obtained were then sequenced using the dideoxy (Sanger) approach using PCR primers (Dorit *et al.* 2003). The sequence of DNA fragments was aligned to other 16S rRNA sequences of various microorganisms in GenBank database at http://www.ncbi.nlm.nih.gov/blast.

2.3. Lipase Production in Different Carbon and Nitrogen Sources

Optimization of carbon and nitrogen sources was carried out to obtain the best conditions for bacterial growth and high productivity in producing lipase with high activity. The bacterial cell cultures were grown in 12 different medium types with various sources of nitrogen such as peptone, $(NH_4)_2SO_4$ and urea with various carbon sources consisting various vegetable oil. The cultivation of bacteria was performed by shaking at speeds of 150 rpm at room temperature. Turbidity growth of cells culture were measured using UV/Vis spectrophotometer at λ = 600 nm in interval 4 h. Crude enzyme was separated from cell biomass by centrifugation for 15 min at 8,000 xg. The production of lipase enzyme was studied by measuring its lipolytic activity toward substrate p-nitrophenyl palmitate.

2.4. Lipolytic Activity Test

Lipolytic activity of crude enzyme was measured p-NPP (para-nitro phenylpalmitate) as a substrate using UV/Vis spectrophotometer (Tripathi *et al.* 2014). The mixture of enzyme reaction that comprised of 800 µl Tris buffer (0.05 M, pH 8), 100 µl crude enzyme, and 100 µl substrate (0.01 M p-NPP in isopropyl alcohol) was placed in water incubator 37°C for 20 min. The enzymatic process was ended by adding 0.1 M Na_2CO_3 the reaction mixture. The supernatant containing product of enzymatic reaction was separated by centrifugation 8,000 xg for 15 min, and then the absorbance was measured using spectrophotometer at λ = 410 nm. The amount of enzyme that liberate 1 µmol product p-nitrophenol per minute expressed in units activity.

3. Results

3.1. Morphologic of Isolate L49a

Microscopic characterization results using Gram staining showed that isolate L49a was Gram positive (+) bacteria, rod-shaped and endospore-forming. The bacterial colonies were pale-yellow, circular, rod shaped and 4 µm in size. Spores were spherical at terminal position (Figure 1).

Cells were positive for urease and oxidase Cells fermented glucose, mannose, and xylose. Based on the morphologic and biochemical characteristics data obtained, isolate L49a is considered as *Bacillus sphaericus* (78%). Based on morphological, biochemical and molecular characterisation the isolated microorganism was found to be *Lysinobacillus sphaericus* L49a strain.

3.2. Molecular Identification of Bacteria Isolate L49a

DNA fragment of 16S rRNA gene was successfully amplified from genomic DNA of isolate L49a using a pair of universal primers, as shown at Figure 2, the presence of a single band with a size of 1,000-1,500 bp.

The sequencing of PCR product were carried out by Sanger approach using PCR primers. The sequence of DNA fragments could be obtained by performing pairwise alignment in the Bioedit program. The DNA fragment composed 1,431 bp. The 16S rRNA gene sequence of bacterial strain, *Lysinibacillus sphaericus* strain L49a was deposited in the GenBank database with the accession number MH879783.1.

The nucleotide sequence of target 16S rRNA gene was aligned with 16S rRNA gene of different microorganisms at https://www.ncbi.nlm.nih.gov/BLAST. There were 10 strains of *Lysinibacillus* listed in GenBank database showed high sequence similarity (99%) with the 16S rRNA sequence of isolate L49a, including species *Lysinibacillus sphaericus, Lysinibacillus fusiformis, Lysiniacillus tabacifolii*, and *Lysinibacillus mangiferihumi*.

The phylogenetic tree of the strains was performed by MEGA X program was a representation of the bacteria isolate and its closest relatives 16S rRNA gene sequences of *Lysinibacillus* strains obtained by NCBI/BLAST (Figure 3). Phylogenetic tree listed at Figure 3 showed that bacteria isolate L49a was most closely to strain *Lysinibacillus sphaericus* DSM 28 (99%) and strain *Lysinibacillus sphaericus* ATCC 14577 (99%). Hence, the proposed name for bacteria isolate from palm oil mill waste land was *Lysinibacillus sphaericus* strain L49a. This bacterium has been registered in GenBank with accession no. MH879783.1.

There was a difference in species names for bacteria isolate 49a. Based on the microscopic and biochemical characteristics data obtained, bacteria isolate L49a

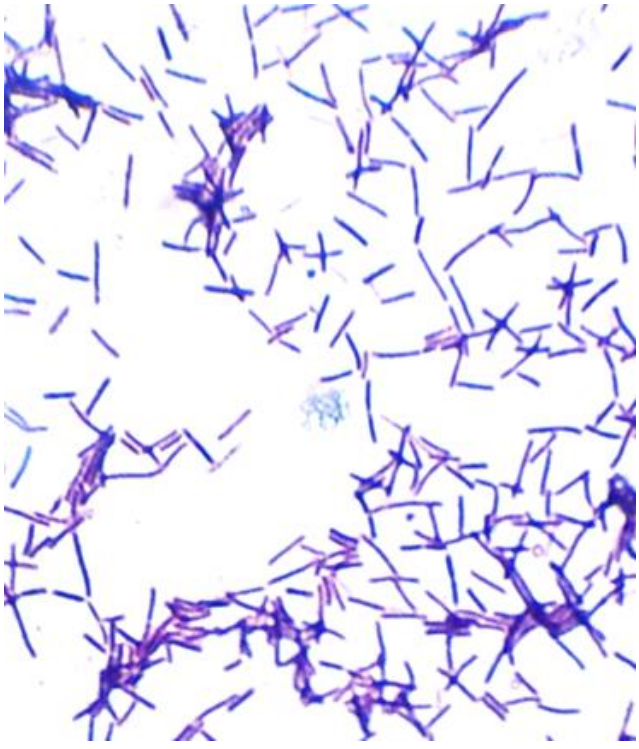

Figure 1. Microscopic characteristic of bacteria isolate L49a (1,000x)

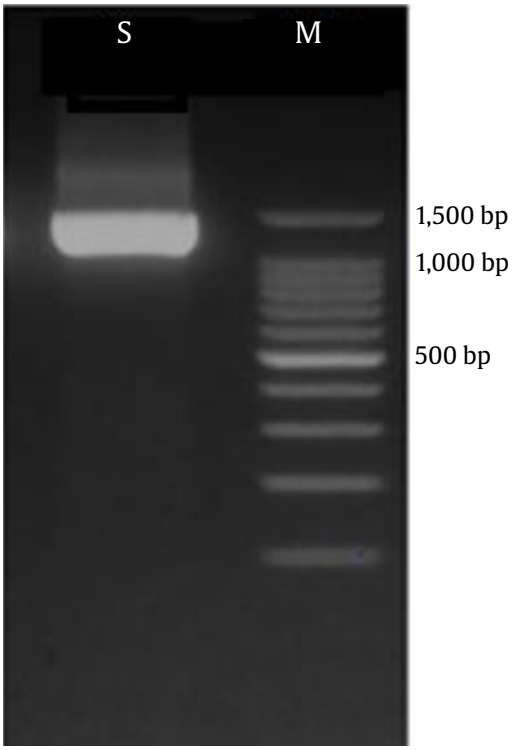

Figure 2. Electrophoregram of PCR product S = sample, M = marker DNA (100 bp)

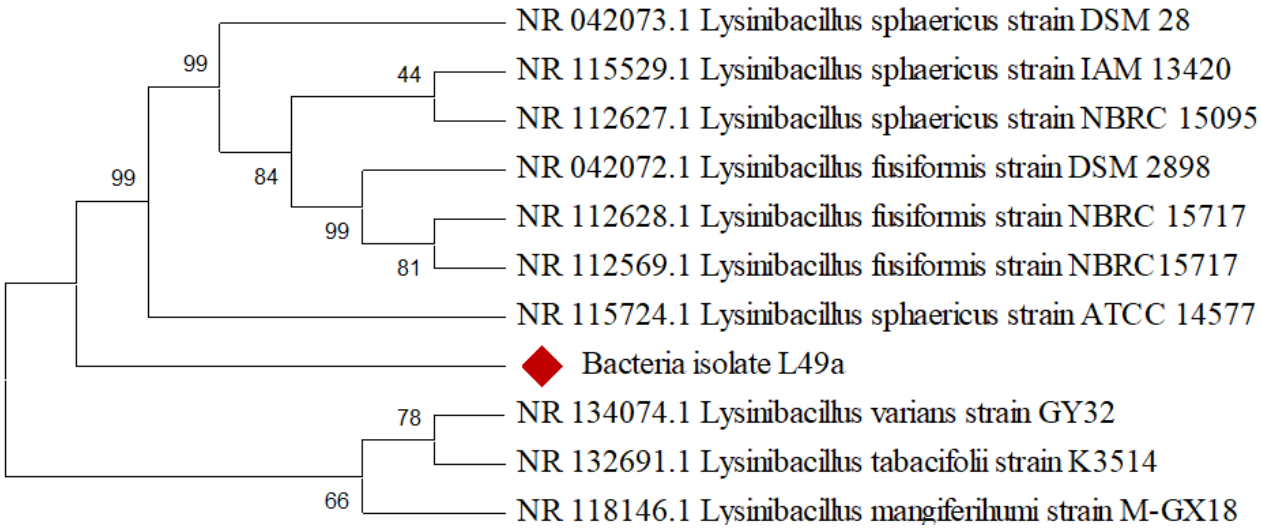

Figure 3. Phylogenetic tree of bacteria isolate L49a to other *Lysinibacillus*

was considered as *Bacillus sphaericus,* but molecularly identified as *Lysinibacillus sphaericus*. Basically, both names are of the same species. *Bacillus sphaericus* was the previous name of *Lysinibacillus sphaericus*, because of the distinctive peptidoglycan composition, and physiology differ to the genus *Bacillus*.

3.3. Optimization of Lipase Production of *L. sphaericus* L49a

Optimization of lipase production from bacteria was conducted to know the best conditions for the lipase production, that produce enzymes with the highest lipolytic activity. The lipase production was performed using a modified medium containing mineral salt with various carbon sources (sunflower, olive, mustard, and coconut oil) and nitrogen sources (urea, ammonium sulfate and peptone). The influence of nitrogen sources and vegetable oils in the medium on growth of *L. sphaericus* L49a can be seen on the graphs in Figure 4-6. The study results show that among 12 different media types containing tested vegetable oils and nitrogen sources were good for growth and enzyme production of *L. sphaericus* L49a.

The graph in Figure 7. showed lipase activity produced by *L. sphaericus* L49a after 20 h cultivation in medium containing different nitrogen sources and vegetable oils.

As shown in figure 6 the addition of vegetable oils into mineral media was more influential on lipase production. Lipase with higher activity was obtained in addition of olive oil than mustard, sunflower and coconut oil. Olive oil in the media increased production of lipase enzyme. The produced enzyme showed lipolytic activity of 69.944, 55.082, 47.236, and 47.389 U/ml, respectively. The combination of olive oil and ammonium sulfate gave the best result in lipase production.

4. Discussion

Lysinibacillus sphaericus L49a have been isolated from various habitats showing different activities. In the present study, *Lysinibacillus sphaericus* strain L49a isolated from palm oil-contaminated soil showed lipase activity. Several bacterial strains have been reported to be capable of producing lipase, including bacteria *L. sphaericus* from oil-rich water (Aderiya and Sulaimon 2017), *L. sphaericus* C3-41 isolated from soil of paddy ecosystem, showed high toxicity to instar larvae of *Culex quinquefasciatus*, and *Anopheles subpictus* (Kalmath *et al.* 2014). Strain *L. sphaericus* ZA 9 produced bioactive compounds for stimulation of plant growth and bio-control to phytopatogenic fungi (Naureen *et al.* 2017), *L. sphaericus* strain PTB isolated from the Arabian Sea showed resistance to lower concentration of antibiotics, and showed lipase activity of up to 50.58 U/ml (Tallur *et al.* 2016).

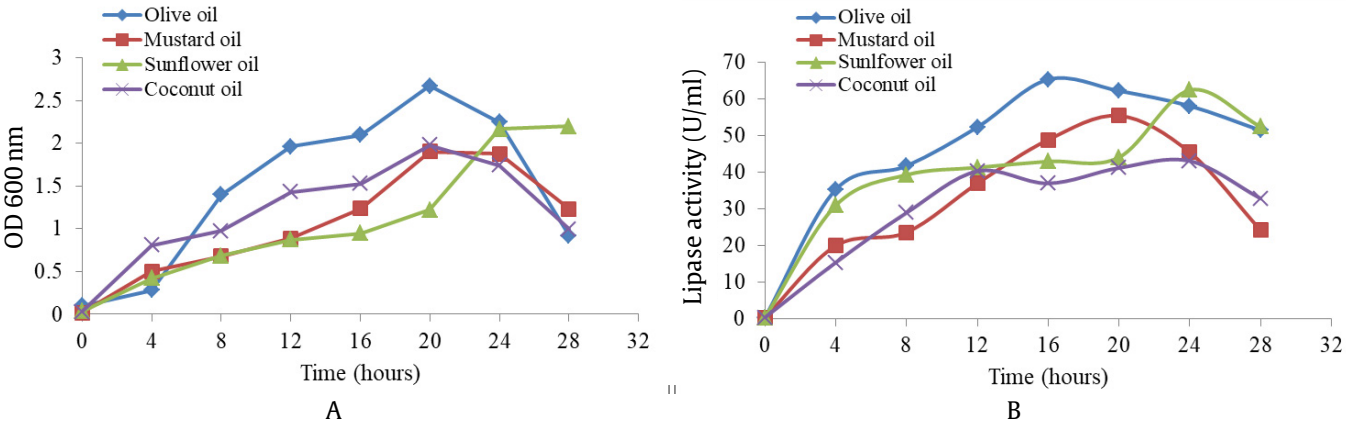

Figure 4. Growth turbidity (A) and lipase production (B) curves of *L. sphaericus* L49a cultivated in modified medium containing peptone and vegetable oils

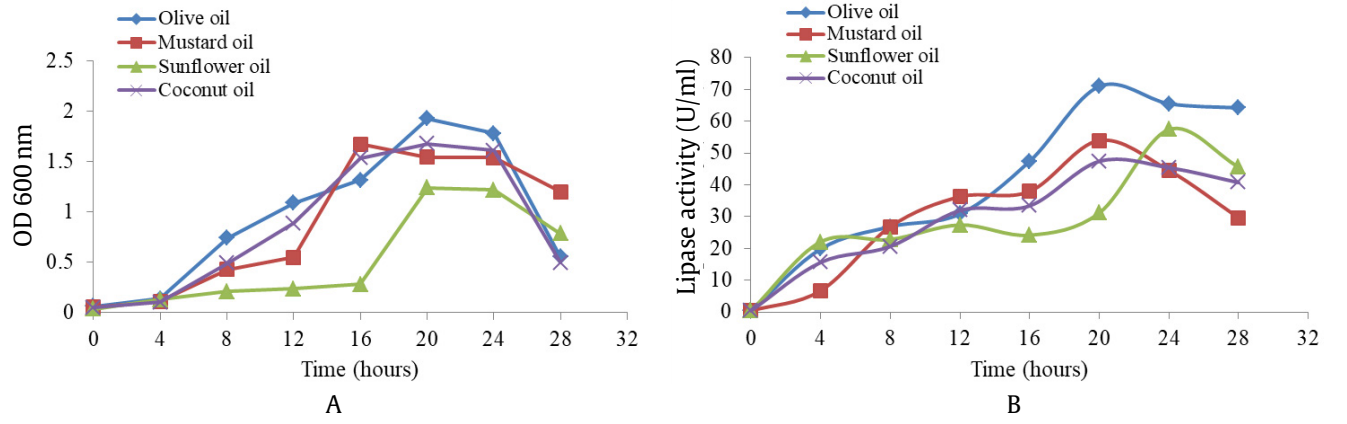

Figure 5. Growth turbidity (A) and lipase production (B) curves of *L. sphaericus* L49a cultivated in modified medium containing ammonium sulfate and vegetable oils

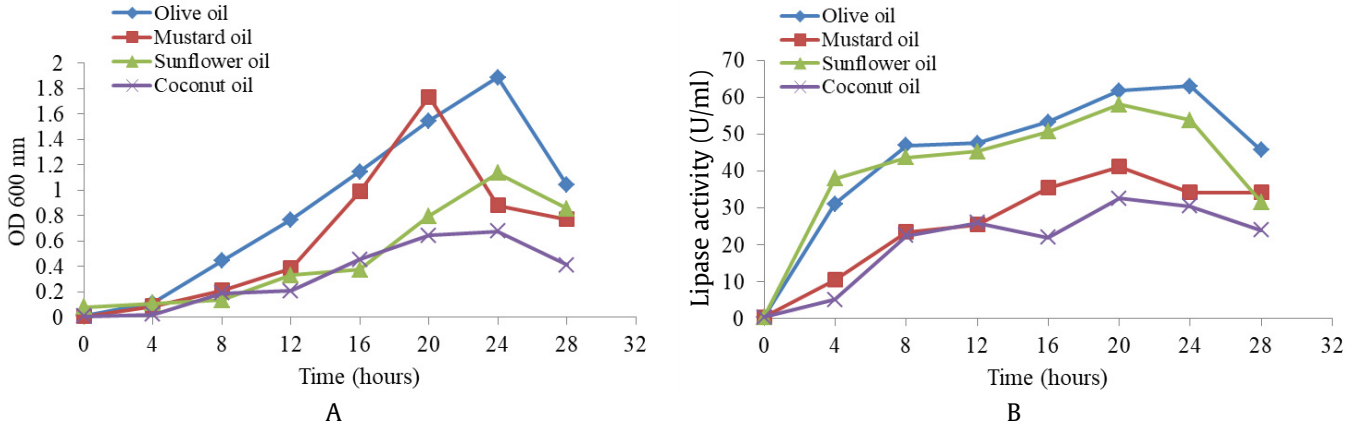

Figure 6. Growth turbidity (A) and lipase production (B) curves of *L. sphaericus* L49a cultivated in modified medium containing urea and vegetable oils

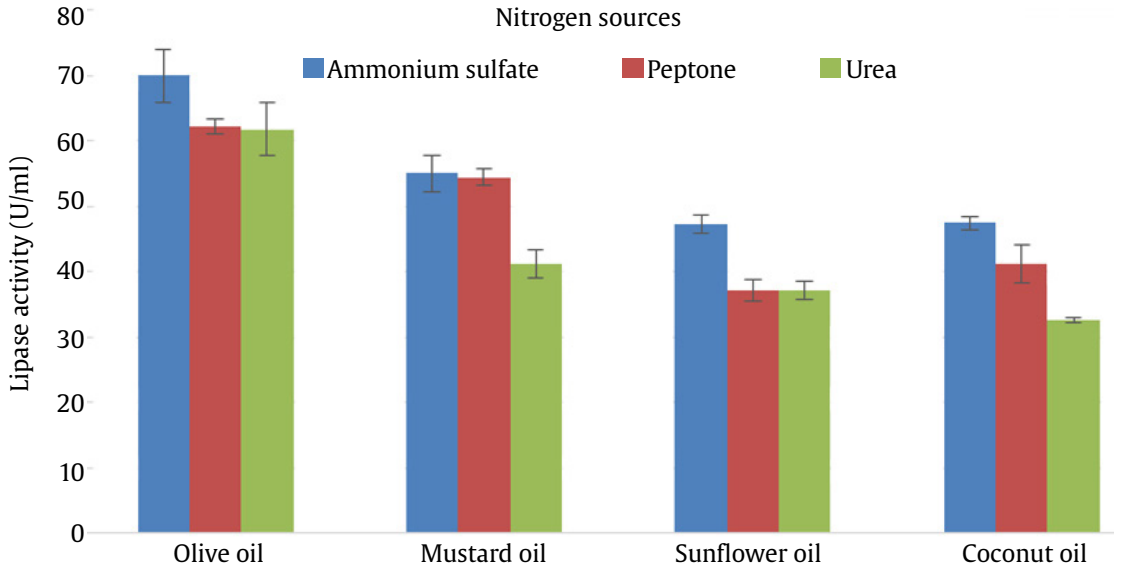

Figure 7. Lipase activity of *L. sphaericus* L49a produced after 20 hours cultivation in medium containing different nitrogen sources and vegetable oils

The combination of olive oil and ammonium sulfate in the mineral media could improved the lipase production *Yarrowia lipolytica* (Lopes *et al.* 2016) obtained the same result, the addition of between ammonium sulfate and olive oil increased of lipase production.

Other researchers have also proven that vegetable oils such as olive oil was a good nutrient for microbial lipase production, including: *Lactococcus garvieae* (Sumarsih *et al.* 2018), *Bacillus sphaericus* MTCC 7542 (Tamilarasan and Kumar 2011), and *Aneurinibacillus thermoaerophilus* (Masomian *et al.* 2010). The lipase production decreased/inhibited if olive oil was replaced with glucose, mannitol, lactose, fructose, arabinose, galactose, maltose, or sucrose (Veerapagu *et al.* 2013).

Extracellular lipases are generally produced in very small quantity without any substrates in production media. Lipases are inducible enzyme, the addition of inducers improve the lipase production. This means that lipase production can be increased by the presence of inducers in the production medium. Lipid as substrates for lipase, are very essential for improving lipase production. The presence of lipids, for example vegetable oils, glycerol, triacylglycerol, fatty acids or tweens, oil industry wastes in the medium increased the production of lipase (Andulema and Gessesse 2012; Veerapagu *et al.* 2013).

Substrate play the role as inducer have a significant influence on lipase production (Kumar *et al.* 2012). The increasing of percentage of C18-unsaturated fatty acid in vegetable oil plays the role in the enzymes synthesis and secretion (Zarevucha 2012). The research results of Pham *et al.* (2021) reported that the induction of lipase production by soil bacteria. The ability of soil bacteria to produce lipase can be increased by incubating the soil for 1 month in an artificially polluted medium containing 10% olive oil. In this study, there were two potential lipolytic bacteria that could be obtained from artificially contaminated soil, namely *Lysinibacillus* PL33 and *Lysinibacillus* PL35, which produced lipase with the highest activity of 480 U/ml and 420 U/ml respectively (Pham *et al.* 2021).

Organic and inorganic nitrogen play the different role in the cell. Organic nitrogen are sources of amino acids, and serve as growth factors. Inorganic nitrogen can be quickly used by cells. Ammonia has certain role in the stage of enzyme induction, and regulate pH. The ammonium ion will be released into the fermentation broth, influence in cell growth, and expression of foreign protein. Protease production can be prevented by NH_4^+ in the fermentation broth, and increase the specific activity of extracellular lipase (Yu *et al.* 2013).

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