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Activity Assay and Identification of Lipolytic Bacteria from Wastewater Fish Industry at District Muncar, Banyuwangi Indonesia

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Abstract: Fish processing industry at Muncar, Banyuwangi, produced wastewater containing fish oil and could be degraded into fatty acids and glycerol by lipase-producing bacteria. Aims of this research were to determinethe activity and identify bacteria that have the highest lipase activity based on 16S rDNA sequences derived from fish mill wastewater at Muncar, Banyuwangi. Bacteria were isolated using simple mineral media, obtained isolates were assayed qualitatively based on the clear zone diameter by using 2 % olive oil as substrate. Clear zone diameter on the 7th day of incubation were analyzed by One-Way ANOVA(p=0,05). The lipase activity of four isolates with the highest diameter, 1me (4.08 ± 0.21 mm), 3ma(3.94 ± 0.03 mm) 3mb (3.99 ± 3.15 mm), and 3mc (4.02 ± 0.15 mm) were assayed simultaneously with the growth curve of bacteria. The highest lipase activity of 4.05 U/ml was produced by 3mc at the 34th hours incubation. Bacterial 3mc isolate were identified based on 16S rDNA sequence with 27F and 1492F primers. The identification results showed that 3mc has the 100 % similarity 16S rDNAsequence with *Staphylococcus aureus* B3.
Keywords: lipase activity, bacteria, 16S rDNA.

Introduction

Banyuwangi, Indonesia, has a high potential for marine industry. One of central fish processing industry at Banyuwangi is located at Muncar District had at least 67 macro and micro industries of fish processing industry, with a total capacity of production more than1,209 tons per day[22]. Fish industry waste water consists of nitrate (NO_3), phosphate (PO_4), sulfide (H_2S), ammonia (NH_3-N), chlorine-free[19]and the most compound lipid[22]. These compounds had polluted environment and waste treatment ponds are suitable natural habitats for lipid degrading bacteria to live solitary, competitively, or formed a consortium with other microbes[4].

Microbial ability to produce lipase enzyme is related lipid degrading activity. Lipase (triacylglycerol lipase) is soluble enzyme that could hydrolyze triacylglycerol to release free fatty acids and glycerol. Lipase has a large application in biotechnology. Bacterial lipase has been widely used in the food processing industry to hydrolyze the milk, fat milk, cheese ripening, improved synthetic flavors, and the process of lipolysis in the manufacture of cheese and cream[6]. Lipase had been also used in the detergent industry as an additive in powdered cleanser[7], in the textile industry to improve the absorbance of the product[23], in the synthesis of compounds or biodegradable polymers[16]and differentiation trans-esterification reaction[8]. This enzyme was used as a catalyst for the production of cosmetics[6], the paper industry[2], the synthesis of biodiesel[18], synthetic leather manufacturing industry, and the pharmaceutical industry[10].

Lipase has been isolated and purified from molds, yeasts, bacteria, plants, and animals[14].Lipases derived from bacteria more valuable economically and more stable[25]. Lipase-producing microbes could be found in habitats that support them such as industrial waste, oil processing plants, and oil-contaminated soil[13], such as local environmental disposal of wastewater fish factory in Muncar. Therefore, bacterial lipase activity assay and identification is required to determine its potency in order to obtain excellent isolates that could be applied in the remediation as well as in the field of biotechnology.

Experimental

Sampling, isolation, and characterization of lipolytic bacteria.

Samples were taken from PT Multi Agro, PT Maya Muncar, and PT Sari Laut and were isolated using a serial dilution of 10^{-1} - 10^{-7}. The isolation medium consist of (g / l) $NaNO_3$ 7 g; K_2HPO_4 2 g; KCl 0.1 g; $MgSO_4$.7H2O 0.5 g; $CaCl_2$ 0.025 g; $FeSO_4.7H_2O$ 0.025 g; and yeast extract 1 g (pH 7.8)[8]. Media was added with a trace element components (g / l) H_3BO_3 0.26 g; $Na_2MoO_4.2H_2O$ 0.06 g; $CuSO_4.5H_2O$ 0.50 g; $ZnSO_4.5H_2O$ 0.70 g, 0.50 g $MnSO_4.H_2O$; 0.5 ml per liter of media (0.05%) and 10% olive oil. The bacteria culture were incubated at 37 °C for 24-48 hours. Afterwards, the bacterial colonies were purified by spread plate method then stored into new slant medium contains Trypticase Soy Agar (TSA) with the addition of Tween-80.

Qualitative lipolytic assay.

Each bacterial isolateswere taken 1 loopfuloose and cultured for 24 hours in 5 ml liquid medium with a composition of 1% NaCl, 1% yeast extract, 2 %peptone, 1% Tween-80, and2%sterile olive oil[8]. Paper disc (d = 5 mm) was dipped into bacterial cultures media for 10-15 min and put onto the surface of the solid media in the Petri dish with the composition (g/l) peptone 10 g, 5 g NaCl, 0.1 g $CaCl.2H_2O$, Agar 20 g, Tween-80 2.5%, and 5% olive oil, and 0.01% methyl red. The cultures were incubated for 7 x 24 hours. The clear zone diameter was measured based on clear zone divided with diameter of bacterial colonies.

Quantitative assay of lipase activity.

Lipase activity assay was conducted simultaneously with the growth curve of bacteria using the same brothmedium of qualitative lipolytic assay. Each sampling, 4 ml of the culture was centrifuged at 4°C, 8000 rpm, 10 min. The supernatant was filtered to obtained crude extract enzyme. Crude extract enzyme was taken 1 ml added with 1 ml of sterile olive oil, and 2 ml phosphate buffer pH 7 and then incubated at 40°C, 90 min. After incubation, it was added with a solution of acetone: ethanol (1: 1) 5 ml and 2 drops of phenolphthalein indicator (pp) then titrated with 0.03 N NaOH until the color change to pale pink. Lipase activity can be calculated as equation (1)[24]. One lipase unit (U) is defined as the amount of lipase per minute needs to liberate 1 mol of fatty acid of olive oil as the substrate[24].

Lipase activity (U / ml) = ((AB) x N NaOH x 1000) / (VE x 90) ..... (1).
Description :
A = Volume (ml) of NaOH for titration of the sample.
B = Volume (ml) NaOH for blank titration.
1000 = conversion to mmol to mol.
90 = incubation time.
VE = total volume of the mixture of crude extract.

Identification based on 16S rDNA.

The excellent lipolytic of lipolytic bacteria was grown in LB medium (Luria Bertani) broth and incubated at 30° C overnight, then 1.5 ml centrifuged at 4 °C, 5000 rpm , 5 min. Pellets were suspended with 567 mL TE buffer, 30 mL of 10% SDS, and 10 mL proteinase K. It was added with 100 mL of NaCl 5M, 50 mL lysozim, 80 mL CTAB. The PCI solution (phenol: chloroform: isoamylethanol) 700 mL was added and suspension on the top layer was taken and added with isopropanol and centrifuged at 4 °C, 5000 rpm for 5 min to obtain DNA precipitate. DNA was added with 0.5 ml of 70% ethanol, centrifuged at 4 °C, 10,000 rpm, 20 min. DNA pellets were suspended in 50 mL TE buffer pH 7.6 and stored at minus 20 °C.

Amplification of 16S Rdna was done using PCR (Polymerase Chain Reaction) thermocycle rAmplitron®-1 by using universal primers 27F (5 '-GAG AGT TTG CTG GCT ATC CAG- 3') as a forward primer and 1492R (5 '-CTA CGG CTA TAC TGT CCT GA- 3 ') as a reverse primer [27]. Amplicon of 16S rRNA purified and sequenced in First Base, Malaysia. BLAST alignment results then performed and matched the nucleotide sequence in GenBank (http://www.ncbi.nlm.nih.gov). Phylogenetic tree was constructed using the program MEGA ver. 5.1.

Results

Lipolytic activity assay clear zone diameter.

Olive oil is used as a lipid substrate as well as a carbon source for bacterial growth. Media screening or selection lipolytic microbes must contain a lipid or Tween-80 as the sole carbon source[17]. The microbes will produce the enzyme lipase metabolism by degrading the substrate contained in the media. Tween-80 has proven to be an analog of lipid compounds and has been used as an emulsifier even as a substrate for studies related to the lipase enzyme. Tween-80 could stimulate the synthesis of lipase by increasing the permeability of cells and facilitate the export of certain compounds through the cell membrane to increase the availability of substrates for the microbes[9].

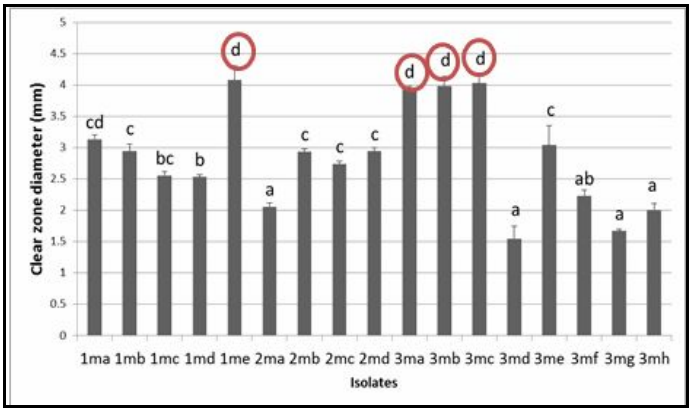

Fig 1. Qualitative lipolytic assay

Seventeen isolates was found from the isolation and the lipolytic assay was determinedbased on the diameter of clear zone. Among 17 isolates, 4 isolates were have highest clear zone diameter($p < 0.05$) (Fig 1). Isolates with the highest diameter were 1me, 3ma, 2mb, and 3mcwhere the clear zone diameterwas 4.08 ± 0.21 mm; 3.94 ± 0.03 mm; 3.99 ± 3.15 mm; and 3MC 4.02 ± 0.15 mm, respectively. The diameter of clear zone produced in the medium indicated the different capability from each lipase-producing bacteria.(Fig 2).

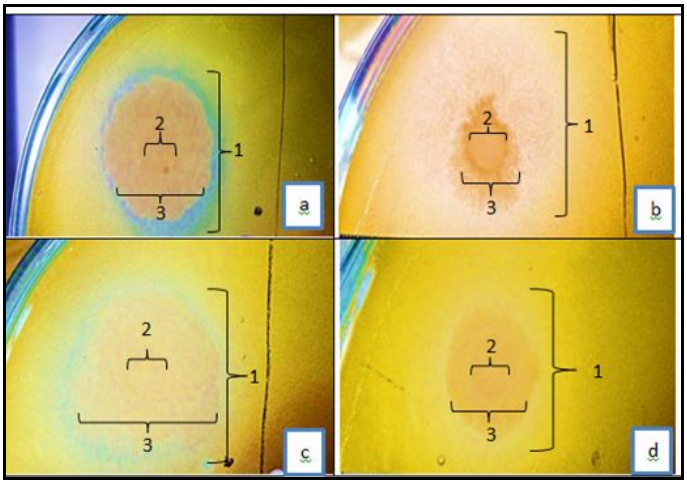

Fig 2.Lipolytic activity clear zone. (a) 1me, (b) 3ma, (c) 3mb, dan (d) 3mc (1) Clear zone, (2) paper disk, (3) bacterial colony.

The growth curve and lipolytic activity of bacteria.

Lipase activity was measured simultaneously with the growth of bacterial cells which aimed to determine the phase of bacteria growth that obtained highest lipolytic activity. The logarithmic generation time of 1me, 3ma, 3mb, and 3mc, respectively was 3.7 hours, 13.3 hours, 5.4 hours, and 4 hours (Fig 3). The lipase activity of 1me, 3ma, 3mb, and 3mcrespectively3.52 U/ml, (24 h), 3.98 U/ml (30 h), 3.74 U/ml(34 h), and 4.05 U/ml(34 h). Therefore, 3mc showed highest optimum lipase activity compared to other isolates. The optimum lipase activity of four isolates happened at different times. However it was still within the range of the final phase of logarithmic growth phase, except 3mb, which had the highest activity in the early stationary phase (Fig 3c). Bacterial isolates showed lipase activity by following the pattern of bacterial growth. Therefore, the lipolytic activity was probably being part of the primary metabolite activity of bacterial isolates. The primary metabolite activity occurred following microbial growth phases, increased optimal in the late phase of logarithmic or early stationary phase, and might decreased with decreasing microbial activity and reduced nutrition as a substrate[15].

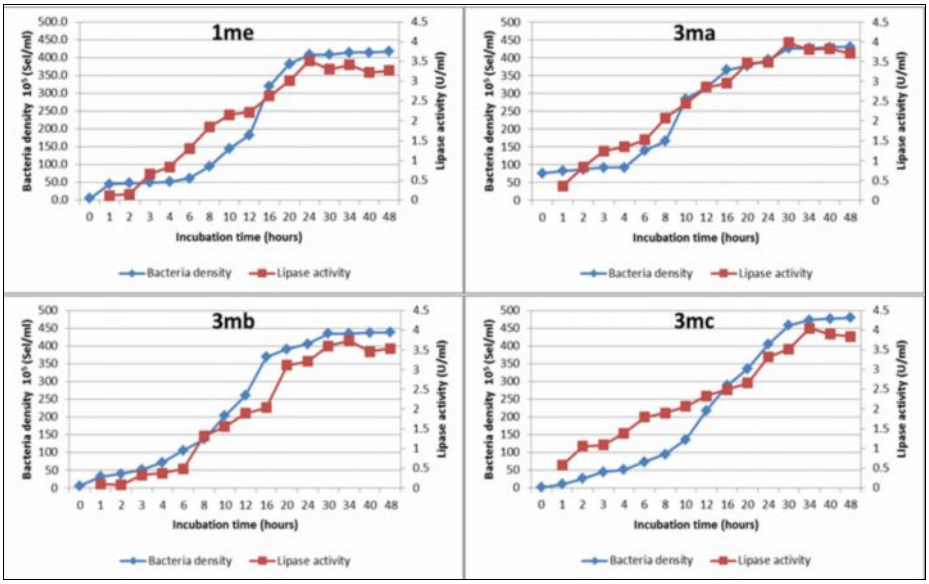

Fig 3. Growth curve and lipase acticity isolate 1me (a), 3ma (b), 3mb (c), and 3mc (d)

Haba *et al*. (2000) found the bacteria *Staphylococcus aureus* CECT 9 grown in a medium containing 2% olive oil had the highest lipase activity 0.7 U/ml at 72 hours incubation. Haba *et al*. (2009) also assayed the lipase activity using a substrate2 % waste sunflower oil as to *Staphylococcus aureus* CECT 9 obtained the lipase activity 2.4 U/ml at 144 hours incubation. Examples of other substrates is *Staphylococcus epidermidis* bacteria CMST-Pi 1 was cultured using 2.5% cod liver oil and castor oil, respectively produced optimum lipase activity of 14.8 U/ml and 13.7 U/ml at 48 hoursincubation[5].Lipase activity is influenced by the type of microbes, substrate concentration, temperature, and incubation time associated with microbial growth phase. Thus, the further research on optimization of lipase activity of various parameters is required.

Identification based on 16S rDNA

DNA sample of 3mc produced amplicon 1500 bp. Based on 16S rDNA sequence matching of lipolytic bacteria in BLAST website, it showed that 3mc was belonged to the *Staphylococcus* Genus. Isolate 3mc had similar 16S rDNAsequence of the *Staphylococcus aureus*, *Staphylococcus warneri*, and *Staphylococcus pasteuri*. It showed that 3mc have evolutionary distance closest to *Staphylococcus aureus* B3 with 100% similarity (Fig 4).In general, *Staphylococcus aureus* was commonly pathogens, including *S. aureus* B3. It could be found in oil-contaminated soil wherein it was able to produced lipase[1] to infect and destroy the host tissue in the pathogenesis mechanism[12]. According Horchani *et al*. (2007),*S. aureus* produced lipase optimally in the alkaline pH range 8-10, which had tetrametric protein lipase structure[11]. *Staphylococcus aureus* lipase (SAL) had high stability and performance was slightly affected by the short length of carbon chain of the substrate and the addition of surfactants and detergents, such as NaDC or Triton X-100. This was evident from the research Horchani *et al*. (2007) that the performance of the SAL in control (untreated) and treated with surfactants and

detergents continued to show optimal performance (100%) compared with *S. xylosus* lipase (SXL) and *S. simulans* lipase (SSL).

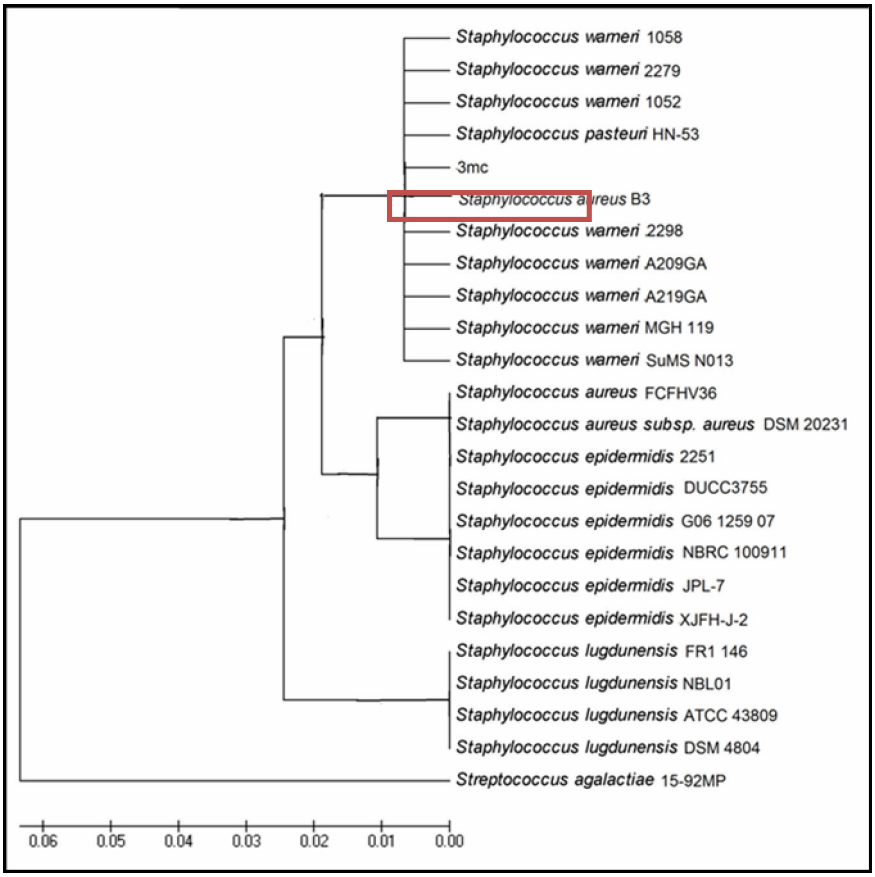

Figure4. Phylogenic tree based on sequence aligment 16S rDNA 3mc and references isolates

Generally, *Staphylococcus* is potentially good lipase producer as it could produce stable lipase in a large amount relatively. However, mostly this genus is pathogenic to human, including *S. aureus* B3. Bacteria *Staphylococcus aureus*is commonly found in contaminated environments and could also be the natural microflora in the human skin. Mechanisms of the pathogenesis of *S. aureus* will occur when the natural human immune system is weaken. Bacteria *S. aureus* isolated from infected human skin had a higher lipase activity than the lipase obtained from *S. aureus* isolated from the environment[20].

SAL has important role directly in the virulent process[26]. Lipids as the main target of lipase has many roles in human. Aside from being a reserve-forming foods energy and structural components of the cell membrane, lipid also acts as a biological effector. Lipids plays an important role in signal transduction, intracellular transport system, and gene transcription. The process of lipid hydrolysis of triacylglycerol by lipases caused the formation ofdiacylglycerol and free fatty acid (FFA).Both the end product of the of lipids degradation are still useful in a variety processes. Diacylglycerol is second messenger compound and triggers various signal transduction cascade reactions, while the FFA acts as a bioactive substance. For example, the FFA may play a role in the process of phospholipase, ion channel, ATP-ase, G-protein and protein kinase that could regulate phospoinocytide and spingomyelin cycle, signal transduction hormones, and even gene transcription. However, both of these products interfere with some functions of the immune system. It is well known that the FFA is an inhibitor of T-cell proliferation[3]. This is allows that the SAL purified to give some negative impact on the human immune system. The presence of SAL could be evidence as virulent agents, which detects the presence of antibodies IgG anti-lipase in patients suffering *S. aureus* infection[21].

Hence, although *S. aureus* has good potency to degrade lipid or oil and could produce lipase enzyme large quantities, the utilizing of *S. aureus* as bioremediation agents must be rethought wisely in the case it pathogen ability in human. Although it could not perform remediation freely, but still could be done cultivation, production, and purification lipase in the laboratory and industrial scale with supervision and strict production control.

Conclusion

From the 17 isolates, there were 4 isolates that had the highest lipolytic clear zone diameter, 1me (4.08 ± 0.21 mm), 3mA (3.94 ± 0.03 mm) 3mb (3.99 ± 3.15 mm), and 3MC (4.02 ± 0.15 mm). The 3mc was excellent isolate compared to others whereas it produced highest optimum lipase activity of 4.05 U/ml at the 34hours incubation. Based in 16S rDNA sequences, 3mc identified has 100 % similarity with *Staphylococcus aureus* B3.

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