

Lipase Activity of Endophytic Actinobacteria from Medicinal Plants

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ABSTRACT

Endophytic bacteria are known to reside within host plant tissue without giving a harmful effect. The endophytes may play an important role, as they may produce similar bioactive compounds as produced by the host plant. Various medicinal plants have long been used to cure diseases. Traditionally, leaves extract of *Guazuma ulmifolia*, *Psidium guajava*, or the rhizome of *Curcuma xanthorrhiza* can be used to treat disease, e.g. hyperlipidemic. The mechanism can be through lipase activity, where the lipase catalyzes the hydrolysis of triacylglycerol to fatty acids and acylglycerol. The objective of this research was to assess potency of endophytic bacteria as anti-hyperlipidemic compounds producer through their lipase activity. Sixty nine endophytic bacteria which comprised of 22, 27 and 20 isolates were isolated from the leaves of *G. ulmifolia*, *P. guajava*, and the rhizome of *C. xanthorrhiza*, respectively. Eight out of the 69 isolates showed lipase activity, and the two selected isolates, i.e. DPG 3(2) and AJB 4(4) were considered as good lipase producers. The highest specific lipase activity of DPG 3(2) isolate was observed for 0.874 units per mg at 38 h, whereas AJB 4(4) isolates had the specific lipase activity at 1.139 units per mg after 72 h observation. These data indicate that the two selected isolates have the potency as antihyperlipidemic compounds producer through their lipase activity.

1. Introduction

Changing in lifestyle can lead to a shift in food consumption patterns to products that contain more fat and less fiber, which can cause the problem of obesity. Overweight refers to a situation of excess weight due to increase concentrations of blood lipids which characterised by the increasing of triglycerides, low density lipoprotein (LDL), total cholesterol in the blood exceed normal limits (>200 mg/dL), and decreasing concentrations of high density lipoprotein (HDL) (<40 mg/dL) (Forti and Diament 2006). This condition is known as hyperlipidemic, which can trigger the emergence of various diseases such as atherosclerosis, which is a cause of coronary heart disease (CHD) and stroke. Hyperlipidemic can be caused by changes in diet, like consuming a lot of fat, especially saturated fat, sugar, alcohol, salt in the daily diet, lack of exercise, genetic disorders, emotional stress, and metabolic disorders. Some conventional

way to solve the problem of hyperlipidemic is by doing physical exercise, low cholesterol diet and consuming antiobesity drugs. Alternatively, person with obesity can use of natural ingredients such as medicinal plant extract to reduce the concentration of fat in the body.

Several medicinal plants, e.g. leaves extracts of *G. ulmifolia*, *P. guajava*, and the extract of rhizome of *C. xanthorrhiza*, traditionally and empirically can be used to lower the lipid concentration of obesity person. Some research data indicated that giving the plant extracts could decrease the concentration of cholesterol in hyperlipidemic mouse liver (Rahayu 2007). Several bioactive compounds produced by endophytic bacteria can function as antitumor, anti-inflammatory, antioxidant (Firaikovai *et al.* 2007), antimicrobes and enzyme inhibitor (Lestari 2006; Pujiyanto *et al.* 2012; Lestari *et al.* 2015). The ethanol extract of *G. ulmifolia* leaves, has the ability as an anti-obesity compound through the mechanism of pancreatic lipase inhibitory activity (Silitonga 2008). Lipase (triacylglycerol hydrolase, EC 3.1.1.3) can hydrolyze triacylglycerol compounds into fatty acids and acylglyserol (Litthauer *et al.* 2002).

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Various commercial antihyperlipidemic can be chemically synthesized, e.g. amphetamines, phenylpropanolamine, fenflur-amine and dex-fenfluramine; or naturally it can be produced by *Streptomyces toxytricini*, e.g. lipstatin that works as an inhibitor of pancreatic lipase enzyme (Weibel *et al.* 1987). Antihyperlipidemic commercial product is known as Orlistat (tetrahydrolipstatin, Xenical®), and another relatively new antihyperlipidemic compounds is known from the bioconversion of compound FR177391 by *Serratia liquefaciens* (Kobayashi *et al.* 2005). These data indicate that the microbes are able to produce bioactive compounds that can serve as antihyperlipidemic.

Endophytic bacteria that colonize plant tissues play an important role, and may produce similar bioactive compounds as produced by the host plant. The endophytes obtain nutrients and protection from the host plant. The role of endophytes of medicinal plants which traditionally used for lowering cholesterol, thus it can function as antihyperlipidemic is rarely reported. Scientific data about antihyperlipidemic compound still limited to the properties contained within the plant, while the antihyperlipidemic compound produced by endophytic microbes is rarely explored. This work aimed to assess lipase activity of endophytic bacteria from several medicinal plants with traditionally used as antihyperlipidemic. The results from this study contributes to the deepening understanding regarding the potency of endophytic bacteria from medicinal plants which has antihyperlipidemic activity.

2. Materials and Methods

2.1. Screening of Lipase Activity of Endophytic Bacteria

There were sixty nine of endophytic bacteria belonged to actinobacteria and non-actinobacteria isolated from medicinal plants were assessed for their lipase activity. The non actinobacteria was inoculated (0.8 cm in diameter of inoculum) on Triton Glucose Yeast Extract (TGY) medium containing (per L): 5 g tryptone, 5 g yeast extract, 1 g dextrose, and 1 g K_2HPO_4 at pH 7.2, whereas the actinobacteria were inoculated on to International Streptomyces Project no. 2 (ISP2) media. Each of those media supplemented with 3% of olive oil and 2 mL of 0.1% Rhodamine B dye that has been sterilized using millipore (Swinnex) 0.2 μ m. The non actinobacteria were incubated at 30°C for 2 days, while the actinobacteria were incubated at 25°C for 5 days. Lipase activity in the screening media was detected under UV light as fluorescent orange zone.

2.2. Pattern of Lipase Activity

The selected isolates that have high lipase activity were inoculated in to 50 mL Tryptone Soy Broth (TSB) media, and incubated at 50°C for 24 hours. The bacterial starter culture were then inoculated in to 50 mL of production medium (per L) contained 1% olive oil, 0.02% $CaCl_2 \cdot 2H_2O$, 0.01% $MgSO_4 \cdot 7H_2O$, and 0.04% $FeCl_3 \cdot 6H_2O$. The non actinobacterial culture was incubated at 50°C in a shaker incubator of 150 rpm. Every 12 hours, bacterial sample was collected to measure cell density using 620 nm wave length, which lasted for 4 days. Meanwhile, the actinobacterial culture was taken every 24 hours for 10 days. The cell culture was centrifuged for 10 min at 4°C, 10000 rpm to separate cell biomass and supernatant. The obtained supernatant was treated as crude extract containing lipase and then measured for its lipase activity.

2.3. Lipase Activity and Protein Content

The crude extract containing enzyme was measured for its lipase activity using the method of Kwon and Rhee (1986). One mL of sample was mixed into a solution containing 2.5 mL of olive oil, 0.005 M phosphate buffer (1: 1), and 20 mL $CaCl_2$. The solution was placed in a shaker incubator, 200 rpm at 60°C for 30 min. Then adding 1 mL of 6 N HCl and 5 mL iso-octane, followed by homogenizing using a vortex for 1 min.

Four mL of top layer of iso-octane was transferred into a new tube, and added with 1 mL cupric acetate-pyridine reagent, then homogenized using a vortex for 1 min. After that the mixed solution was measured for its absorbance using a spectrophotometer at 715 nm wavelength. One unit of lipase activity was defined as the amount of enzyme that release 1 mol of fatty acids per mL/min at 60°C. Measurement of lipase activity was done by using margaric acid (heptadecanoic acid), as standard curve, measured the absorbance at 715 nm.

Margaric acid is one type of saturated fatty acid, as a result of hydrolysis of triglycerides by lipase enzyme. Margaric acid is also reacted with cupric acetate-pyridine reagents. The amount of lipase activity characterized by the number of fatty acid released per minute. Therefore, the use of margaric acid in this study was aimed to measure the approximate amount of fatty acids released from the hydrolysis of triglycerides (olive oil) by the crude extract containing lipase (sample). The protein content was measured by the method of Bradford (1976) using the standard bovine serum albumin (BSA).

2.4. Measuring of Specific Lipase Activity

The specific lipase activity was measured by using the following formula:

$$\text{Lipase Specific activity (units/mg)} = \frac{\text{lipase activity (units/ml)}}{\text{protein content (ml/mg)}}$$

The specific lipase activity were then synchronized with the concentration (ppm) of lipase type VII as a positive control.

3. Results

3.1. Lipase Inhibitory Activity of Endophytic Bacteria

Eight out of 69 isolates of endophytic bacteria showed lipase activity indicating by orange luminescence zone on agar medium containing Rhodamine B.

Two selected isolates e.g. DPG 3 (2) and AJB 4 (4) produced strong orange luminescence zone, indicating high lipase activity (Figure 1). The DPG 3 (2), a non actinobacteria, was isolated from leaves of *P. guajava*, while AJB 4 (4), an actinobacteria, was isolated from the roots of *G. ulmifolia*.

3.2. Pattern of Lipase Inhibitory Activity

The highest specific lipase activity produced by DPG 3 (2) was obtained after 36 h incubation, which was equal to 4.405 log cfu. At that stage, it produced about 0.874 units per mg of specific lipase activity (Figure 2). At 48 h measurement and after, the cell number was slightly decreased, in line with slightly decrease in specific lipase activity.

For AJB 4(4), the highest specific lipase activity was obtained at 72 h incubation that was 1.139 units per mg, after that the specific activity declined (Figure 3).

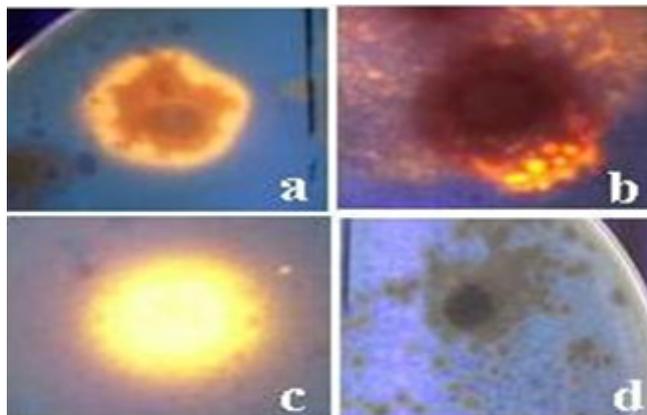


Figure 1. Lipase activity of DPG 3(2) (a), AJB 4(4) (b) grown in screening Rhodamine B media, compared with a positive control, type VII lipase (1000 ppm) (c), meanwhile the AJB 4(3) (d) showing no lipase activity

The specific lipase activity of both DPG 3 (2) and AJB 4 (4) was compared with the concentration of lipase type VII (Sigma chemical Co.), as the positive control.

As shown in Tables 1 and 2, the highest specific lipase activity of DPG 3 (2) was equivalent to 172

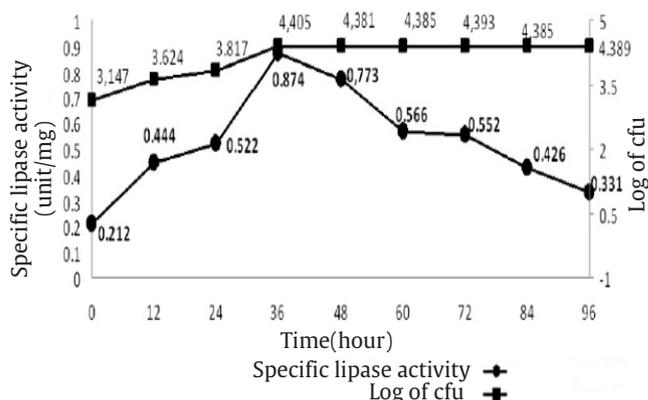


Figure 2. Pattern of specific lipase activity of DPG 3(2) in the medium containing 1% of olive oil

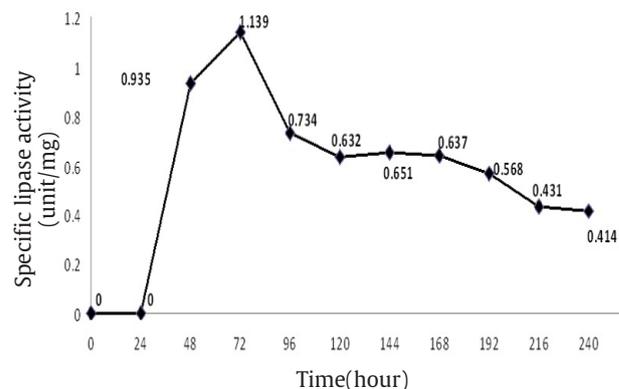


Figure 3. Specific lipase activity of AJB 4(4) in the medium containing 1% of olive oil

Table 1. Comparison of specific lipase activity of DPG 3(2) with the lipase type VII concentration

Time (hour)	Specific lipase activity (unit/mg)	Concentration of lipase type VII (ppm)
0	0.212	2.479
12	0.444	5.177
24	0.522	6.080
36	0.874	10.172
48	0.773	8.995
60	0.566	6.592
72	0.552	6.434
84	0.426	4.970
96	0.331	3.826

Table 2. Comparison of specific lipase activity of AJB 4(4) with the lipase type VII concentration

Time (hour)	Specific lipase activity (unit/mg)	Concentration of lipase type VII (ppm)
0	0	0
24	0	0
48	0.935	10.888
72	1.139	13.260
96	0.734	8.552
120	0.632	7.363
144	0.651	7.577
168	0.637	7.415
192	0.568	6.616
216	0.431	5.022
240	0.414	4.823

ppm the concentration of lipase type VII; meanwhile for the AJB 4 (4) was equivalent to 13.260 ppm the concentration of lipase type VII.

4. Discussion

Screening of lipase-producing bacteria on an agar medium is usually done by using tributyrin or Tween 80 as the substrate. However, such substrates are not suitable for detecting the actual lipase because the compound can also be hydrolyzed by esterase (Peled and Krenz 1981). Lipase also plays a role in the metabolism of lipoproteins in the blood by hydrolyzing triglycerides and phospholipids contained in chylomicrons, intermediate density lipoprotein (IDL) and HDL (Dugi *et al.* 2000). Lipase can be visualized on agar containing trioleoylglycerol and a fluorescent Rhodamine B dye (Hofelmann *et al.* 1983). In this study, 3% of olive oil was used to substitute the trioleoylglycerol. Eight of the 69 isolates isolation results are already known to have lipase activity in the screening media containing 3% of olive oil and Rhodamine B dye.

Rhodamine B bind specifically with fatty acids and mono- or diglycerides, this process is highly sensitive. The formation of complexes between Rhodamine B dimer fatty acids and mono- or diglycerides, thus produce luminescence that can be detected under UV light (Kouker and Jaeger 1987). The specificity of this method is shown when esterase is tested on screening media containing 3% olive oil and Rhodamine B dye, no fluorescent zones generated. The sensitivity of this method has been proven by the fact that the lipase activity can be detected up to 60 nmol of fatty acid released per minute. Meanwhile, screening for lipase activity using titrimetric method can detect lipase activity when reaching 1200 nmol of fatty acids released per minute. Based on that evidences, the eight endophytic bacterial colonies

(2 isolates of actinobacteria and 6 isolates belonged to non actinobacteria) that have been grown in a medium containing 1% olive oil and Rhodamine B dye can be confirmed that those isolates able to produce lipase. Both DPG 3(2) and AJB 4(4) showed to produce strong fluorescence orange zone compared with the other six tested isolates. The strength of orange fluorescence can indicate the more capable of the bacteria in producing lipase (Hou and Johnston 1992).

The DPG 3 (2) is a non actinobacteria bacteria, that can secrete proteins with lipase activity in the production media added with 1% of olive oil. Similarly, *Bacillus* sp. 42 strain also has a lipase activity in the media containing 1% olive oil (Eltaweel *et al.* 2005). The highest specific lipase activity of DPG 3 (2) obtained at the later stage of log phase, when cell density reached the highest number (4.405 log of cfu) at 36 h incubation and the lipase activity equal to 0.874 units per mg. It has been reported that indigenous *Streptomyces* sp. SKK1-8 was able to produce xylanase (Meryandini *et al.* 2006). Some isolates of *Streptomyces* are also known to produce hydrolytic enzymes such as cellulases, hemicellulase, chitinase, amylase, and glucanase (Hasegawa *et al.* 2006).

The AJB 4 (4) produced the highest specific lipase activity after incubated for 72 h which its lipase activity equal to 1.139 units per mg. The specific lipase activity value of both DPG 3 (2) and AJB 4 (4) is positively correlated with various concentrations of lipase type VII used as a positive control. It showed from the highest specific lipase activity value of DPG 3 (2) after 36 h incubation is equivalent to 10.172 ppm lipase type VII. Similarly, the highest specific lipase activity of AJB 4 (4) reached after 72 h incubation, which was equivalent to 13.260 ppm lipase type VII. Endophytic bacteria from *G. ulmifolia* and *P. guajava* can be further studied for their lipase activity such as its roles in lipolysis of reserved fat that accumulate in adipose tissue, thus can effectively cause weight loss.

Conflict of Interest

The authors declares that there is no conflict of interest.

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