

# Lipase-producing ability of bacteria from inasua (fish fermented product) from Central Moluccas, Indonesia

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**Abstract.** Anggriani L, Budiarti S, Mubarik NR. 2020. Lipase-producing ability of bacteria from inasua (fish fermented product) from Central Moluccas, Indonesia. *Biodiversitas* 21: 622-628. One of fish preservation techniques through fermentation is Inasua which occurs spontaneously and involves various types of microorganisms. Isolates from Inasua are believed to have the ability to produce lipolytic enzymes. The purpose of this study was to identify potential bacterial isolates isolated from Inasua and to determine their lipase producing ability. The confirmation test of lipase-producing ability was conducted by inoculating the microbes into media containing Nutrient agar (NA), olive oil, and rhodamin-B. Lipolytic bacteria were detected using a screening method with UV light. From the results, there were 5 isolates of bacteria that showed lipolytic activity as IG 3.1, IG 6, IG 10, IG 12, IG 66. Lipase assay was determined using spectrophotometric method with p-nitrophenyl palmitate (pNPP) as a substrate and titration method with olive oil as a substrate. The enzyme showed maximum activity at pNPP as a substrate. IG 6 and IG 12 gave the highest activity from lipase assay. Growth and production curves were created before partial purification was carried out. The results of the curves showed that the optimum activity of IG 6 and IG 12 were in the stationary phase with value 38.16 U.mg<sup>-1</sup> protein and 7.01 U.mg<sup>-1</sup> protein, respectively. After ammonium sulfate precipitation, the activity of the lipase IG 6 and IG 12 were found to be 39.65 U.mL<sup>-1</sup> and 37.05 U.mL<sup>-1</sup>, respectively. During optimization, the enzyme showed maximum activity at pH 8/70°C temperature. Molecular identification by using the 16S rRNA gene and phylogenetic analysis showed that isolates IG 6 and IG12 are closely related to *Bacillus paramycoides* and *Bacillus kochii*, respectively.

**Keywords:** 16S rRNA gene, *Bacillus*, fish fermented, lipase-producing

## INTRODUCTION

Lipase is a group of hydrolase enzymes that performs a multitude of roles in daily life. These enzymes are used extensively in food industry (Muthumari et al. 2016). The human body uses lipase to break down fat during digestion of food in the digestive system (Kumar et al. 2017). Lipases are produced by animals, humans, plants, and even microorganisms (Thakur 2012). The microorganism lipases are more useful because its can be produced in large amounts in shorter periods of time (Gopinath et al. 2013). *Aspergillus niger* (Salihu et al. 2016), *A. flavus*, *Alternaria* sp., *Penicillium* sp. (Wadia and Jain 2017), *Bacillus subtilis* (Chaturvedi et al. 2010), *GeoBacillus stearothermophilus* (Ekinici et al. 2015), and *Lactobacillus* sp. (Rashmi and Gayathri 2014) have ability to produce lipase.

Microorganisms from Thai fermented fish (Pekasam) (Daroopunt et al. 2018) and shrimp paste (Ka-pi) (Daroopunt et al. 2019) have been reported as lipolytic bacteria. In Indonesia, there are many kinds of fermented foods. One of fermented foods from Indonesia is Inasua made up of fermented fish from Central Moluccas, Indonesia. Inasua is processed using salt coconut sap without any inoculum (Nara et al. 2013). The fish species typically used are oil fish (*Ruvettus pretiosus* Cocco). The skin of this fish is thick and oily (Ling et al. 2009). Some bacteria have been successfully isolated from Inasua,

including *L. plantarum* IN05, *L. rhamnosus* IN13, IG 3.1, IG 6, IG 10, IG 12, and IG 66 (Mahulette et al. 2016; Amaliah 2017). Jaeger et al. (1994) said that lipolytic bacteria can be found in oily environments, so that isolates from Inasua have the potential to produce lipases. The biodiversity of microorganisms involved in the Inasua needs to explore the beneficial health effect. The aim of this research was to identify the isolates potential bacteria from inasua and to determine their lipase producing ability.

## MATERIALS AND METHODS

### The selection of lipase-producing bacteria

Seven isolates in this study were selected based on previous studies, including *L. plantarum* IN05, *L. rhamnosus* IN13, IG 3.1, IG 6, IG 10, IG 12, and IG 66 (Mahulette et al. 2016; Amaliah 2017). Bacterial isolates were inoculated in Nutrient Agar (NA) and de Man Ragosa Agar (MRSA) media for 24 hours. After that, the bacterial culture was inoculated into media with 1% Rhodamine B and 1% olive oil + 2% PVA (1:3) (Kouker and Jaeger 1987), then incubated for 24-48 hours at 37°C temperature. After 24-48 hours, it was observed under UV light (365 nm).

### Crude enzyme extract production

One loop of isolates was inoculated into 20 mL of Nutrient Broth (NB) medium and then incubated for 24-48 h at 27 °C temperature. Inoculum was inoculated into 100 mL of NB medium with 1% olive oil then incubated for 24 h at 27 °C temperature. The cultures yielded were centrifuged at 10000 rpm (595 g) for 10 minutes at 4 °C temperature to obtain supernatant. The crude enzyme-containing supernatant was removed into Falcon conical tube for lipase unit activity measurement.

### Lipase assay

Lipase activity test was carried out through titration and spectrophotometry methods. In titration method was done by employing olive oil was employed as a substrate. 4 ml of the substrate was mixed with 2 ml of phosphate buffer 0.1 M pH 7, 2.5 ml distilled water, 0.5 ml 0.1 M CaCl<sub>2</sub>, and 1 ml of crude extract enzyme which then incubated for 15 minutes in a shaking incubator at 27 °C temperature. The reaction was stopped by adding 1 ml of absolute ethanol 96%. The mixture was titrated with 0.05 M NaOH using phenolphthalein indicator. One unit of lipase activity was defined by the number of enzymes that produce 1 µmol of fatty acid per minute (Xu et al. 2002).

In spectrophotometry method p-nitrophenyl palmitate (pNPP) was used as a substrate. 30 mg pNPP was dissolved in 10 ml of isopropanol (Solution A), after that, solution B (phosphate buffer pH 7, 0.1 M) was prepared. Solution A is then mixed with solution B as much as 90 ml (substrate). 0.5 ml crude extract enzyme was reacted with 2 ml substrate and incubated for 30 minutes at 27 °C temperature. After 30 minutes, 50 µl Triton X-100 was added. The absorbance of the solution was measured using a spectrophotometer (410 nm) (Gupta et al. 2003).

### Establishment of growth and production curves

Two loops of bacterial culture from NA slant were grown on 40 mL NB medium with olive oil. The culture was incubated for 7 h (absorbance = 0.8) in shaking incubator at 27 °C temperature. Lipase was produced by inserting 1% of bacterial inoculum from the culture into 200 mL NB medium with olive oil. Cell density was measured at a wavelength of 600 nm with a spectrophotometer every 3 h for 51 h to establish a growth curve. Enzyme was produced in every time segment to establish a production curve. The lipase activity was then measured by spectrophotometry.

### Partial purification of lipase with ammonium sulfate

For purification precipitation was conducted to determine the optimum fraction with saturation of ammonium sulfate (Scopes, 1994). An ammonium sulfate concentration series from 0% to 100% was used in crude enzyme purification. The purified lipase was stored at 4 °C temperature for 24 h then centrifuged at 6000 rpm for 30 min. The precipitate yielded was solved in 0.1 M phosphate buffer pH 7 in a volume of 2 mL. Afterward, the lipase activity and protein content were subjected to testing by Bradford's method (Bradford 1976).

### Lipase characterization

Lipase characterization covered optimum pH and temperature. The optimum pH was determined by reacting the enzyme with pNPP substrate at pH 3-9, while the optimum temperature was determined by testing at 10-80 °C. In every optimization test, the lipase activity was measured by spectrophotometry.

### Identification of 16S rRNA gene

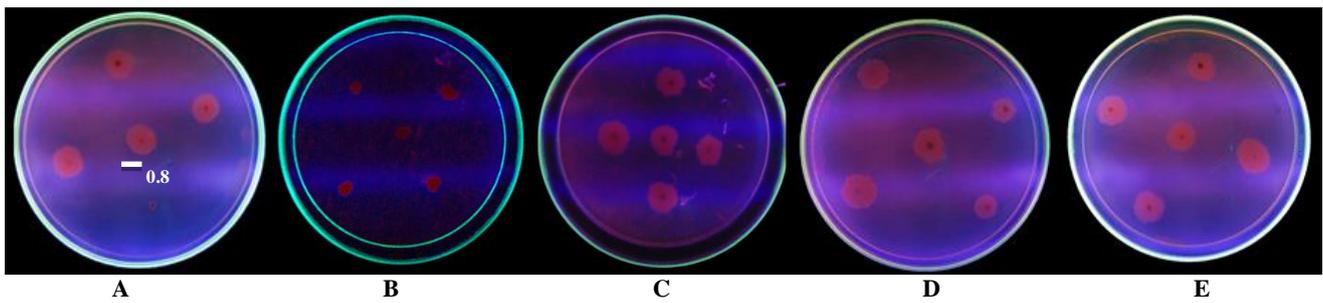
Two isolates with the highest lipase activity were inoculated in (1 loop) a tube containing NB. Later, the culture was developed for 24 hours at room temperature using a shaker. The developed culture was then transferred to a 1.5 mL tube and centrifuged twice at 13000 rpm for 1 minute. The formed supernatant was removed and DNA extraction was carried out on a settled pellet using a Geneaid kit. After that, 5 µL GoTaq Green Master Mix2x was added to 1 pmol DNA sample, 1 pmol 63F or 38F primer and 1 pmol 1387R, respectively. The identification process was carried out by amplifying the 16S rRNA encoding gene using a 63F primer (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387R (5'-GGG CGG WGT GTA CAA GGC-3') on PCR machines (Marchesi et al. 1998). The first stage of PCR process was (pre-denaturation) processed with a temperature of 94 °C for 4 minutes, followed by 35 cycles consisting of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 45 seconds, and elongation process at 72 °C for 1 minute 30 seconds. PCR process was completed by post-elongation at 72 °C for 10 minutes and cooling at 4 °C for 5 minutes. The PCR product was electrophoresed on 1% agarose gel in a TAE 1x buffer with a 90 V voltage for 60 minutes. DNA bands were stained with Ethidium Bromide (EtBr) 5 µg/mL and visualized using UV light. PCR products would be sequenced using services from 1<sup>st</sup> Base Malaysia.

The DNA sequences obtained were analyzed and determined homologically in *GenBank* through the Basic Local Alignment Search Tool (BLAST) program at (<http://www.ncbi.nlm.nih.gov>). By attaching a reference sequence from the results of BLAST, a phylogenetic tree was constructed using MEGA X application. The phylogenetic tree is made using the maximum likelihood method and the best fit model with bootstrap (BS) 1000x. Isolates with the same identity as taxons in one group with BS values ≥ 70 showed close kinship. Then the DNA sequence is submitted to the GenBank database to get the access number.

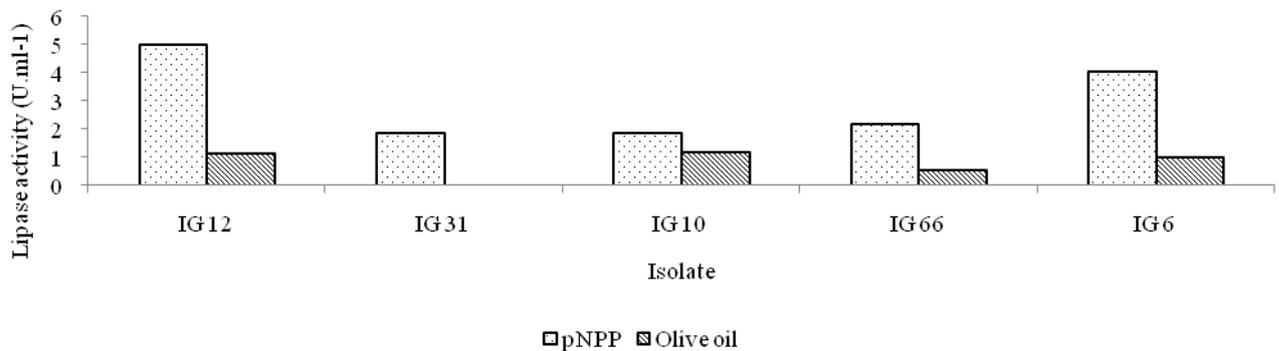
## RESULTS AND DISCUSSION

### Lipolytic bacteria selection with olive oil substrate and cell characterization

Lipolytic bacteria are able to produce lipase. These bacteria can be detected by observation of cell luminescence on the substrate medium that contained rhodamine-B and olive oil. Bacteria with lipase production capability will luminate in UV light (Kouker and Jaeger 1987) (Figure 1).



**Figure 1.** Lipolytic activity of IG 3.1 (A), IG 6 (B), IG 10 (C), IG 12 (D), IG 66 (E) on the Rhodamin-B-containing olive oil substrate on UV light



**Figure 2.** Lipase activity of the crude extract of every lipolytic isolate on the pNPP and olive oil substrates

It was found that 24-hour incubation at room temperature, 70% of the isolates collected from *Inasua* exhibited lipolytic activity as marked with a glow on the rhodamin-B medium when observed under UV light at wavelength of 365 nm. All of the bacteria that demonstrated lipolytic activity were selected by measuring enzyme activity with spectrophotometry and titration because the methods used to measure lipolytic activity in this research lack the ability to yield activity value in quantity.

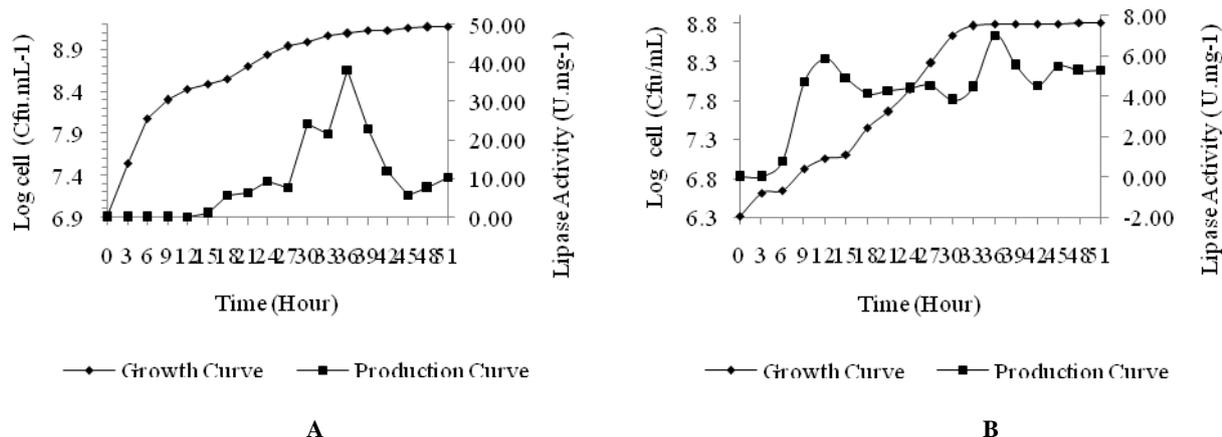
#### Lipase activity in lipase crude extract

Lipase activity testing was conducted to obtain bacterial isolates with the highest potential. This research employed methods of titration and spectrophotometry to measure the enzyme activity in the crude extract. Isolates IG6 and IG12 had the greatest potential for having scored the highest in the enzyme activity in the crude extract (Figure 2). Both isolates scored the highest in both methods used. The titration method showed a change in color into pinkish with addition of phenolphthalein indicator. When NaOH was added, nothing was left to bind to fatty acids, which are weak acids, hence the solution pH increase (Xu et al. 2002).

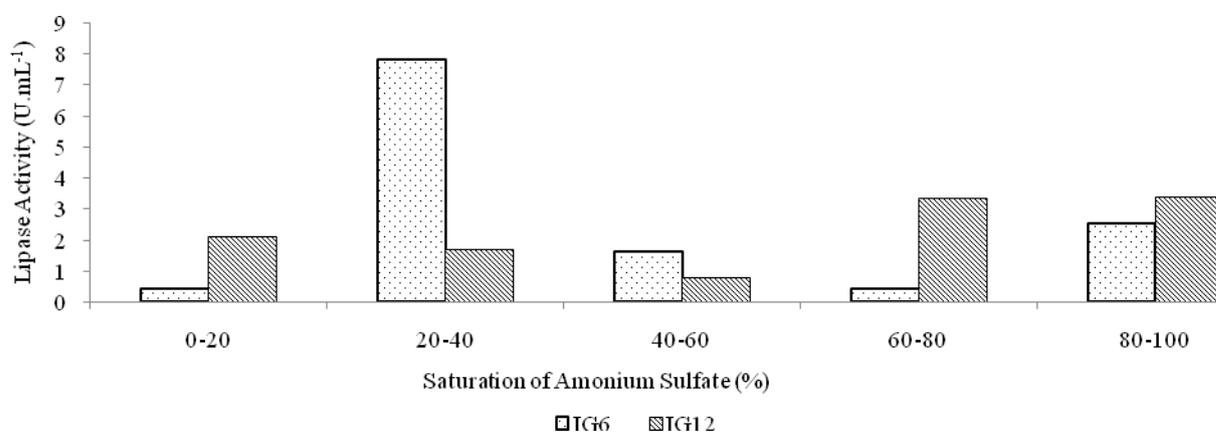
In the spectrophotometry method, meanwhile, lipase activity was measured with pNPP substrate that was incubated for 30 min at 37 °C temperature prior to spectrophotometric visualization (410 nm). The absorbance rate measured was converted using a standard p-nitrophenol (pNP) curve for lipase activity. Lipase activity is defined as  $\mu\text{mol}$  of pNP formed per minute (Becker et al. 1997). The paranitrophenol produced turned the solution into yellow, indicating that the solution's pH shifted alkaline-bound (Pencreac'h and Baratti 1996).

#### Establishment of growth and production curves

Cell growth and lipase production curves of the two isolates were established at pH 7 at ambient temperature to find out about the optimum lipase production time. The two isolates with the highest lipase activity were selected for optimum lipase activity testing. The results showed that isolates IG12 and IG 6 (Figure 3) displayed optimum lipase activity at the 36<sup>th</sup>-hour of incubation with values of 7.01 and 38.16  $\text{U.mg}^{-1}\text{protein}$ , respectively. This suggests that the most optimum of both isolates' activity was apparent at the 36<sup>th</sup> hour of incubation.



**Figure 3.** Growth and lipase enzyme production curves of isolates IG6 (A) and IG12 (B) at 27 °C temperature, pH 7 during 51 hours under shaking



**Figure 4.** Lipase partial purification of IG6 and IG12 with ammonium sulfate

**Table 1.** Fold purification enzyme IG 6 and IG 12

Source	Volume (mL)	Total protein (mg)	Enzyme activity (Unit)	Specific activity (U.mg <sup>-1</sup> )	Fold purification	Yield (%)
Crude enzyme	200	20.07	276.8	13.79	1	100
	40%	10	1.18	77.94	66.12	4.79
Crude enzyme	200	20.68	92.72	4.48	1	100
	100%	10	1.004	33.64	33.52	7.49

**Partial purification of lipase with ammonium sulfate**

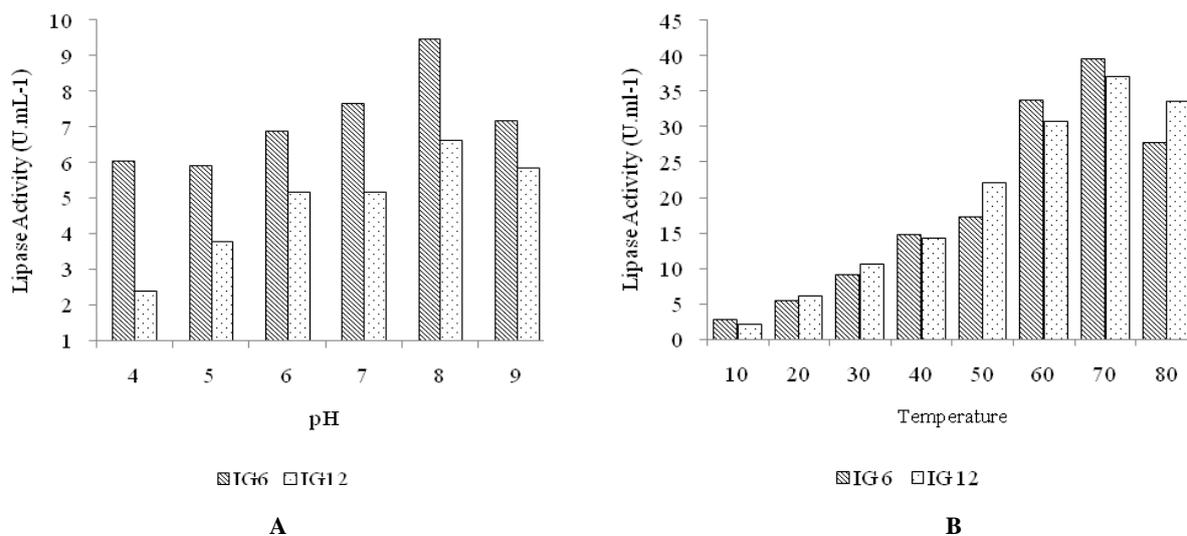
Enzyme precipitation with ammonium sulfate was performed to purify the enzyme produced. The precipitate from the crude enzyme extract took the form of supernatant from production medium. From the ammonium-sulfate-assisted enzyme precipitation, it was found that the lipase produced had the highest precipitation activity at 40% ammonium sulfate saturation for IG6 lipase and 100% for IG12 lipase (Figure 4).

Enzyme activity has increased after partial purification (Table 1). The specific activity of IG 6 lipase in the 40% fraction reached 66.12 U.mg<sup>-1</sup> with a purity level of 4.79

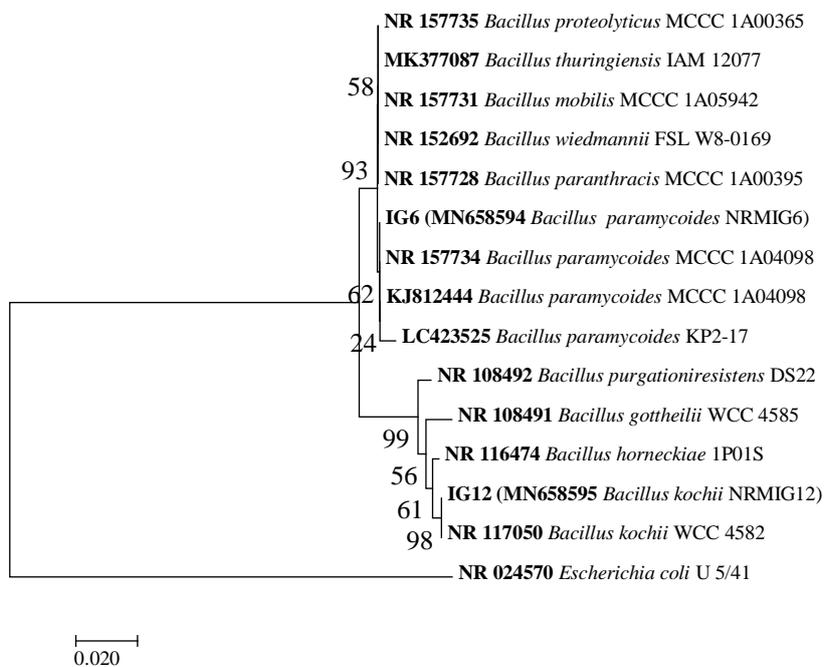
times that of the crude enzyme. Lipase IG 12 has a lipase activity value of 33.52 U.mg<sup>-1</sup> at 100% fraction with a purity level of 7.49 times that of the crude extract enzyme.

**pH and temperature optimum**

The optimum pH and temperature were determined by purification with highest precipitation activity. The purification IG 6 and IG 12 lipase showed optimum activity at pH 8 and 70°C temperature (Figure 5). IG 6 and IG 12 lipase reached the maximum lipase activity 39.65 U/mL<sup>-1</sup> and 37.05 U.mL<sup>-1</sup>, respectively, at 70 °C temperature and pH 8.



**Figure 5.** Lipase activity of isolates IG6 and IG12 at variable pH (Citrate buffer of pH 4, phosphate buffer of pH 5-7, Tris-HCL buffer of pH 8-9) (A) and temperatures at pH 8 (B)



**Figure 6.** Phylogenetic tree base on 16S rRNA genes of the IG 6 and IG 12. The percentage of bootstrap values was shown by the number at each branch point.

**Table 2.** Bioinformatics analysis from NCBI of isolates IG6 and IG12 by 16S rRNA gene sequences

Isolate	Species	Strain	Similarity	Accession number
IG 6	<i>Bacillus paramycoides</i>	MCCC 1A04098	99.82%	NR 157734
	<i>Bacillus thuringiensis</i>	IAM12077	99.64%	MK377087
	<i>Bacillus paranthracis</i>	MCCC1A00395	99.64%	NR 157728
	<i>Bacillus wiedmannii</i>	FSLW80169	99.55%	NR 152692
	<i>Bacillus mobilis</i>	MCCC1A05942	99.55%	NR 157731
IG 12	<i>Bacillus kochii</i>	WCC 4582	99.72%	NR 117050
	<i>Bacillus gottheilii</i>	WCC 4585	98.59%	NR 108491
	<i>Bacillus horneckiae</i>	1P01S	99.43%	NR 116474
	<i>Bacillus purgationiresistens</i>	DS22	99.15%	NR 108492

### 16S rRNA gene sequencing

Two selected isolates were identified using the 16S rRNA genes. The amplified 16S rRNA genes were verified by electrophoresis, showing 1300 bp DNA strands. PCR amplification of isolates IG6 and IG12 used primers 63F and 1387R. The DNA strands of the two isolates that were visible in UV light during the electrophoresis process belonged to the 1000-1500-bp range. The resulted PCR was then sequenced using 1<sup>st</sup> Base Malaysia's service. The best analysis results showed that isolates IG6 and IG12 compare with another species bore 99.82% similarity to *B. paramycoides* and 99.72% to *B. kochii*, respectively (Table 2). The results of submitting DNA sequences obtained access numbers and strain isolates. IG 6 is a species of *B. paramycoides* strain NRMIG6 with access number MN658594. IG 12 is a *B. kochii* strain NRMIG12 species with access number MN658595.

The Phylogenetic tree was constructed by the neighbor-joining method and Kimura-2 Parameter model in MEGA X application. Bootstrap values base on 1000 replications (Figure 6).

### Discussion

The olive oil present in the medium was hydrolyzed by lipase and bound to Rhodamine-B, causing the bacteria to illuminate in UV light (Kouker and Jaeger 1987). Lipid generally induced lipase production (Rathi et al. 2001). Olive oil was used in this study as inducer for lipase production. Olive oil, according to Zouaoui and Bouziane (2011), is one of the best inducers for lipase production by bacteria. Isolates IG6 and IG12 gave the highest activity from lipase assay. From the measurement results, it can be seen that the IG 6 and IG 12 yielded the highest lipase activity in the stationary phase. Base on 16S rRNA gene sequence similarity, the isolate IG6 showed that 99.82% similarity with *B. paramycoides* MCCC 1A04098, while the isolate IG12 was closely related to *B. kochii* WCC 4582 with 99.72 %.

The highest activity for IG6 lipase partial purification reached 39.65 U.mL<sup>-1</sup> at pH 8/70 °C temperature. The IG12 lipase partial purification reached the highest activity of 37.05 U.ML<sup>-1</sup> at pH 8 and 70 °C temperature. The optimum pH and temperature for the lipase from *Bacillus* sp. THL027 nearly resembles those of IG6 and IG12 lipase. *Bacillus* sp. THL027 has the optimum crude enzyme activity of 7 U.mL<sup>-1</sup> at 70 °C temperature and pH 7 (Dharmsthiti and Luchai 1999). This also accords with previous research, which showed that *B. Paramycoides* from Thai prawn paste has lipase activity of 22 U.mL<sup>-1</sup> (Daroopunt et al. 2019). *B. kochii* was only reported by Seiler et al. (2012) as producing fatty acids from esterase lipase (C8) process. However, the result of lipase-producing ability of *B. kochii* has not previously been described.

Ammonium sulfate's role in the precipitation process was to separate protein compounds from non-protein ones. The protein in the substrate was precipitated by ammonium sulfate as protein molecules were bigger in size. Besides, this precipitation process would also maximize the enzyme's concentration. Protein precipitated to different

salt concentrations. Hydrophobic proteins precipitated to lower salt concentrations, while hydrophilic proteins required higher salt concentrations (Scopes 1994). Purification IG 6 and IG 12 lipase showed maximum activity at 70 °C temperature and pH 8 (Tris-HCL buffer). The IG 6 and IG 12 lipase activity showed lower results compared to lipase activity from *Pseudomonas* sp. ADT3 with an activity value reached 1433.8 U.mg<sup>-1</sup> on the 80% ammonium sulfate with fold purification 2.9 from the crude extract enzyme (Dey et al. 2014). The lipase activity decreased significantly at 10 °C temperature. It was also highly active in 20-80 °C temperature. According to Janssen et al. (1994), thermal stability is one of the bacterial lipases outplay characteristics because pancreatic lipase loses its activity at a temperature above 40 °C temperature. This is nearly close to lipase reported from *Bacillus* sp. (Dharmsthiti and Luchai 1999) and *B. thermoleovorans* ID-1 (Lee et al. 1999). Every bacterial lipase has unique optimum pH and temperature. The optimum pH, which is influenced by the charges of amino acid side chains, allows a suitable condition, accelerating the reaction an enzyme is undergoing (Sumarlin et al. 2013). Bacterial lipase activity traditionally remains stable within pH range 6 through 8 and declined if the pH exceeds 8 (Handayani et al. 2011). Lestari et al. (2009) stated that the lipase activity outside the optimum pH remained lower because the enzyme is denaturated.

Environmental factors like temperature influences the lipase production. As explained by Handayani et al. (2011), lipase activity goes down if lipase is incubated at temperatures below or above the optimum ones. Low temperatures lower the enzyme-substrate complex formation speed, limiting the complex, and the product, production to only small amounts. By contrast, temperature increase up to the optimum one can increase the enzyme-substrate complex formation, thus also elevating the enzyme activity. The enzyme-substrate complex formation speed is highly effective at the optimum temperature, and as a result, the product produced increase in quantity as well. If the temperature keeps increasing beyond the optimum one, the enzyme activity again lowered down. The enzyme undergoes denaturation and changes in its three-dimensional structure, causing difficulty for the substrate to bind the active side of the enzyme and, thus, lowered enzyme activity.

Five bacterial isolates isolated from Inasua showing the ability to produce lipase. IG 6 and IG 12 were the potential isolates because of their highest activity. Base up-on the 16S rRNA gene sequence similarity, IG 6 was closely related to *B. paramycoides* with 99.82% and IG 12 showed that similarity 99.72% with *B. kochii*.

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