



Production and Activity Characterization of Lipase from *Bacillus flexus* InaCC-B486

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Abstract: Lipases are widely used in a variety of industries, both to develop products and to improve process efficiency. The need for lipase increased along with the wider application of this enzyme. Therefore, studies related to the search for potential lipase-producing microbes that answer the needs of the industry are required to be carried out continuously. Enzymes produced by microbes are preferred because they can be produced quickly compared to other sources. *Bacillus flexus* InaCC-B486 was used to produce lipase in this study with olive oil as substrate. This research aimed to observe the production of lipase from *B. flexus* InaCC-B486 and characterize its activity. The result shows that the production of *B. flexus* InaCC-B486 lipase was optimal at day-4 which was 11.983 ± 0.101 U/mL. The activity of *B. flexus* InaCC-B486 lipase was optimal at an incubation time of 15 minutes (2.810 U/mL), pH of 8.0 (3.173 U/mL), and a temperature of 35 °C (3.173 U/mL). These findings can be used for further applications, both in research and industry, that use *B. flexus* InaCC-B486 as a resource for lipase production or any related applications.

Keywords: *Bacillus flexus* InaCC-B486, lipase enzyme, production optimization.

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1. INTRODUCTION

The touch of biotechnology has contributed to the development of industry. One application in the field of biotechnology is the use of enzymes in industry, both in the process of making a product and in the use of enzymes as ingredients of a product. There are potential industries using enzymes, such as food and beverages (dairy, bakery, fruit juices, beer, and wine) (1), cosmetics, detergents, and oil-mining industries (2). Moreover, the enzyme has already been applied in the ecological services industry as an agent to eliminate pollutants. One enzyme widely applied is lipase. Lipase works to break down the ester bond in acyl-glycerols to glycerol and free fatty acids by hydrolysis process. This versatile enzyme enhances flavors, pharmaceuticals, and cosmetics. Moreover, it serves as an essential component in wastewater treatment, including oil-

pollutant removal, detergent cleaning, and other water-wastes elimination (3,4).

Research on lipase, especially in production, has been conducted for a long time and is still interesting today. Lipase production must be efficient in process and cost so that it can be more applicable. Lipase is naturally present in the human stomach and pancreas, as well as in other animal species, to digest fats and lipids. Other than that, lipase is also produced by microbes such as bacteria, yeast, and fungi. We can use microbial as the source of lipase to enhance and optimize production. Microbial enzymes contribute to approximately 90% of the global lipase market and industry. Moreover, the use of microbes tends to support sustainability since the microbes are relatively easy to grow and reproduce (5–7). Many microorganisms have been reported to produce lipase, such as *Bacillus*, *Burkholderia*,

Corynebacterium, *Geobacillus*, *Idiomarina*, *Oceanobacillus*, *Pseudomonas*, *Staphylococcus*, and *Virgibacillus* (8). *Bacillus* sp. were found to be predominant in the production of enzymes because of their shorter generation time, and the ease of genetic and environmental manipulation (9). *Bacillus* sp. is known to produce extracellular enzymes such as amylase, lipase, and protease (10,11). Roy et al. (10), reported that *Bacillus flexus* is a potential source of lipase, protease, amylase, and cellulase. The strain *B. flexus* KP1-14 cultivated in NB medium supplemented with 1% (v/v) Tween 80 was reported to have the highest lipase activity ($29.68 \pm 0.80 \text{ U mL}^{-1}$) by using p-nitrophenyl butyrate (8). The pathogenicity test performed in blood agar did not show any halo zone formation, thus indicating that *B. flexus* is non-pathogenic (10). So, lipase produced by *B. flexus* is safe to be used in the food and beverage industry as well as for other commercial needs. Other than that, the activity optimization of lipase needs to be conducted since the artificial environment applied, such as temperature, pH, activator, inhibitor, or other factors, can significantly influence the work of the enzyme itself.

The research on lipase from *B. flexus* needs to be enriched to fill the knowledge gap so that the further use of lipase could be complete and comprehensive enough to be applied in industry. Furthermore, the various collections of *B. flexus* exist and can be obtained from any culture collection organization. One of them is the collection of *B. flexus* from the Indonesian Culture Collection (InaCC), Indonesia. There was no report regarding the production and activity characterization of lipase from *B. flexus* collection from InaCC. Only a few isolates of *B. flexus* from Indonesia have been reported to have lipolytic activity, namely strains C₁₃ and C₁₄ (12) as well as SS5 (13). However, all these isolates have not been studied further regarding their lipase activity. Therefore, this research was designed to investigate the production and characterize the activity of lipase produced by *Bacillus flexus* InaCC-B486. The lipase production was monitored daily for a 7-day period. Subsequently, activity characterization was systematically conducted, focusing on some parameters, including incubation time, pH, and temperature.

2. EXPERIMENTAL

2.1. Materials

The materials employed in this research included glucose, peptone, yeast extract, NaCl, K₂HPO₄, MgSO₄·7H₂O, (NH₄)₂SO₄, p-nitrophenyl palmitate (p-NPP), triton X-100, Arabic gum, acetone, ethanol, olive oil, aquadest, and a bacterial isolate of *B. flexus* InaCC-B486, obtained from the collection of the Indonesia Culture Collection (InaCC).

2.2. Media Preparation

Specific media was prepared for the growth of *B. flexus* InaCC-B486, following Soleymani et al., (14). The media was formulated to support lipase

production. The media was made for 1 L and consisted of glucose and peptone (10 g), yeast extract and NaCl (5 g), K₂HPO₄ (1 g), CaCl₂ (2 g), MgSO₄·7H₂O (0.2 g), (NH₄)₂SO₄ (2 g). The pH was adjusted to 7.0 and then sterilized using an autoclave at 121°C for 15 minutes. A total of 20 mL of olive oil that had been sterilized by filtering was added afterward to the media.

2.3. Lipase Production

The 24-hour starter of *B. flexus* InaCC-B486 inoculated in nutrient broth was added to the prepared media as much as 1% (v/v). The mixture was then incubated in a rotary shaker with an agitation time of 110 rpm at room temperature. The production was conducted in triplicate. The lipase was obtained by centrifugating the culture at 4 °C, 5000 rpm, and 20 minutes. The supernatant was collected since the lipase was soluble in the supernatant. The lipase was collected each day within 7 days for testing the activity of lipase. The incubation time with the highest lipase activity value is used as the production time for the next stage, which is the effect of reaction incubation time, pH, and temperature.

2.4. Effect of Incubation Time, pH, and Temperature on Lipase Activity Assay

Lipase obtained from the production process was used to characterize the lipase activity in several parameters. These observations were conducted sequentially for incubation time, pH, and temperature. The base temperature and pH were 35°C and 7.0. The incubation time varied between 0 and 60 minutes, with an interval of 15 minutes. After gaining the highest lipase activity during the incubation time, the effect of pH was observed. The pH was varied between 5 and 10, with an interval of 1 scale. In the third step, the effect of temperature on lipase activity was observed. The highest lipase activity of incubation time and pH was used to observe the effect of temperature. The temperature varied between 25°C and 50°C with an interval of 5°C. Experiments to determine each parameter were carried out in triplicate.

2.5. Lipase Activity Assay

The lipase activity was measured using p-nitrophenyl palmitate (p-NPP) as a substrate. The substrate was prepared by dissolving 6.6 mg of p-nitrophenyl palmitate (p-NPP) in 1 mL of isopropanol, which was then added to a detergent solution. The detergent solution was prepared from 200 µL Triton X-100 dissolved in a 20 mL buffer to which 11 mg of Arabic gum had been added. The reaction solution consisted of 250 µL of substrate and 500 µL of crude lipase (culture-free supernatant). The reaction solution was homogenized using a vortex and then incubated at 35°C for 15 minutes. The reaction was stopped with 250 µL of acetone: ethanol (1:1) solution. The absorbance of the sample was read at a wavelength of 410 nm (15). Lipase activity was determined by measuring the concentration of p-nitrophenol (p-NP) released during the hydrolysis reaction, utilizing the standard curve of p-NP. One unit of enzyme

activity using this method was defined as the amount of enzyme liberating 1 μmol of p-nitrophenol per minute.

2.6. Standard Curve of p-Nitrophenol (p-NP)

Standard series of p-NP were prepared in concentrations of 10, 20, 30, 40, 50, and 60 μmol . The absorbance was measured at a wavelength of 410 nm. The linear curve was drawn with the y-axis as absorbance and the x-axis as the concentration of p-NP. The formula and linear regression of the curve were determined to calculate the lipase activity (15).

2.7. Data Analysis

Data were analyzed using one-way ANOVA, and if the results were significant, they were then continued with Duncan's Multiple Range Test (DMRT) at a 95% confidence level. The data was processed using Microsoft Excel 2013.

3. RESULTS AND DISCUSSION

Bacillus is well known as the best lipase-producing source (1,3). The microbial lipases are mostly extracellular enzymes secreted into the lipase production medium. Submerged fermentation (liquid fermentation system) was widely used as a technique to produce lipase enzymes from microorganisms. This is a method of cultivation of microorganisms in a liquid broth medium that

breaks down the supplied nutrients into a compound (3). In this study, olive oil was used as a lipidic substrate. Zarevúcka (16) reported that the use of olive oil in lipase production can enhance the lipase yield compared to other oil sources.

3.1. Lipase Production Time

Lipase production from *B. flexus* InaCC-B486 using olive oil as a substrate gave the best results on day 4, with a value of 11.983 ± 0.101 U/mL, which was a significant difference compared to the three first days and day 5. Lipase production was first detected on the first day of incubation and continued to increase until the fourth day of incubation. Lipase production began to decrease on the fifth day. On the sixth and seventh day, there was a slight increase in lipase production, but it was not significantly different from the previous day. (Fig. 1). Another study also reported that the optimum production of lipase time by *B. subtilis* PCSIR NL-38 was found on day 4, but with an activity value of 8.8 U/mL at 32°C (17). Meanwhile, the highest lipase production from *B. cereus* ATA179 was obtained on day 2 with a value of 6.6 U/mL (18). The decrease in lipase activity observed after a longer incubation time could be attributed to decreased nutrient availability (19). Furthermore, the presence of proteolytic activity, which can decompose enzyme proteins, caused a decrease in enzyme activity (20,21).

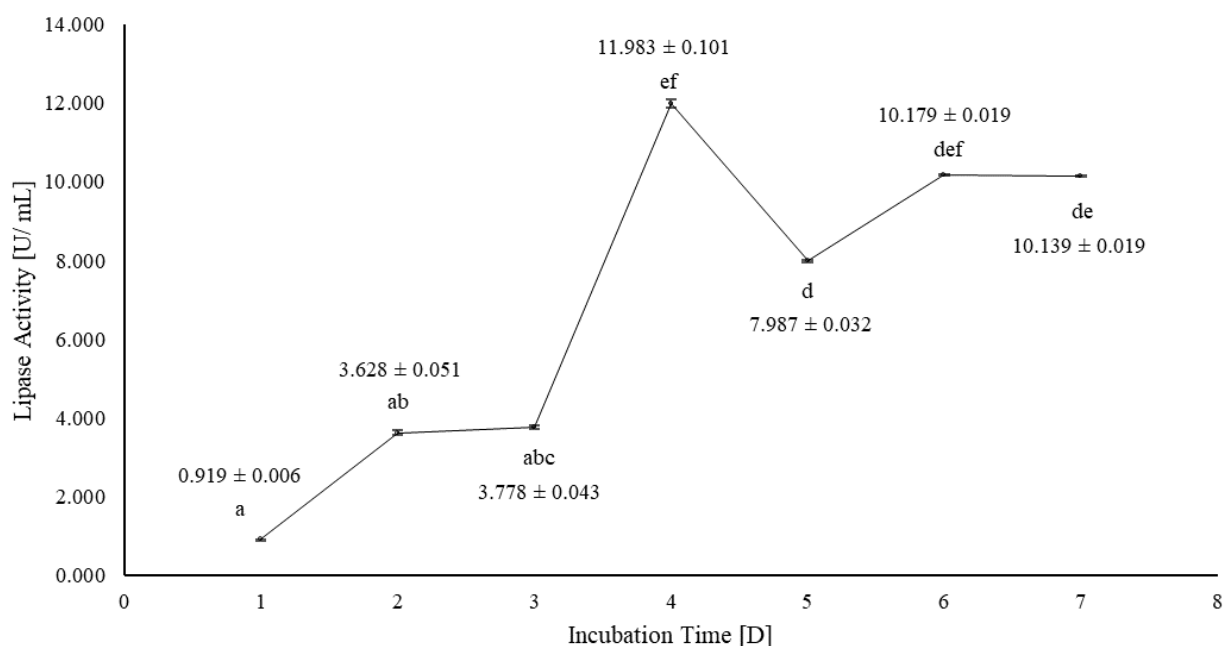


Figure 1. Lipase production time from *Bacillus flexus* InaCC-B486 on lipase activity.

The difference in lowercase symbols between treatments indicated a significantly different ($P < 0.05$) of lipase activity value according to Duncan's Multiple Range Test (DMRT).

3.2. Effect of Incubation Time, pH, and Temperature on Lipase Activity Assay

The lipase activity assay in this study followed the spectrophotometric method using p-nitrophenyl palmitate (p-NPP) as the substrate. The assay was carried out based on the colorimetric principle with

the hydrolysis of the ester substrate from palmitate by lipolytic enzymes, which had an effect on the release of the para-nitrophenol (p-NP) chromogenic product (22). This reaction produces the yellow-colored product of p-NP, which is measurable spectrophotometrically at 410 nm. One unit of

enzyme activity was defined as the amount of enzyme liberating 1 μmol of p-nitrophenol per minute. This method was advantageous because of its short reaction time and the result can be read using spectrophotometric analyses (23). The standard p-NP curve in this study was linear in the range of 0.147–0.819 μmol , with a determination

coefficient (R^2) of 99.95% (Fig. 2). The equation of this standard curve will be able to predict the concentration of p-NP products released by the reaction that occurs, which then the lipase activity can be calculated.

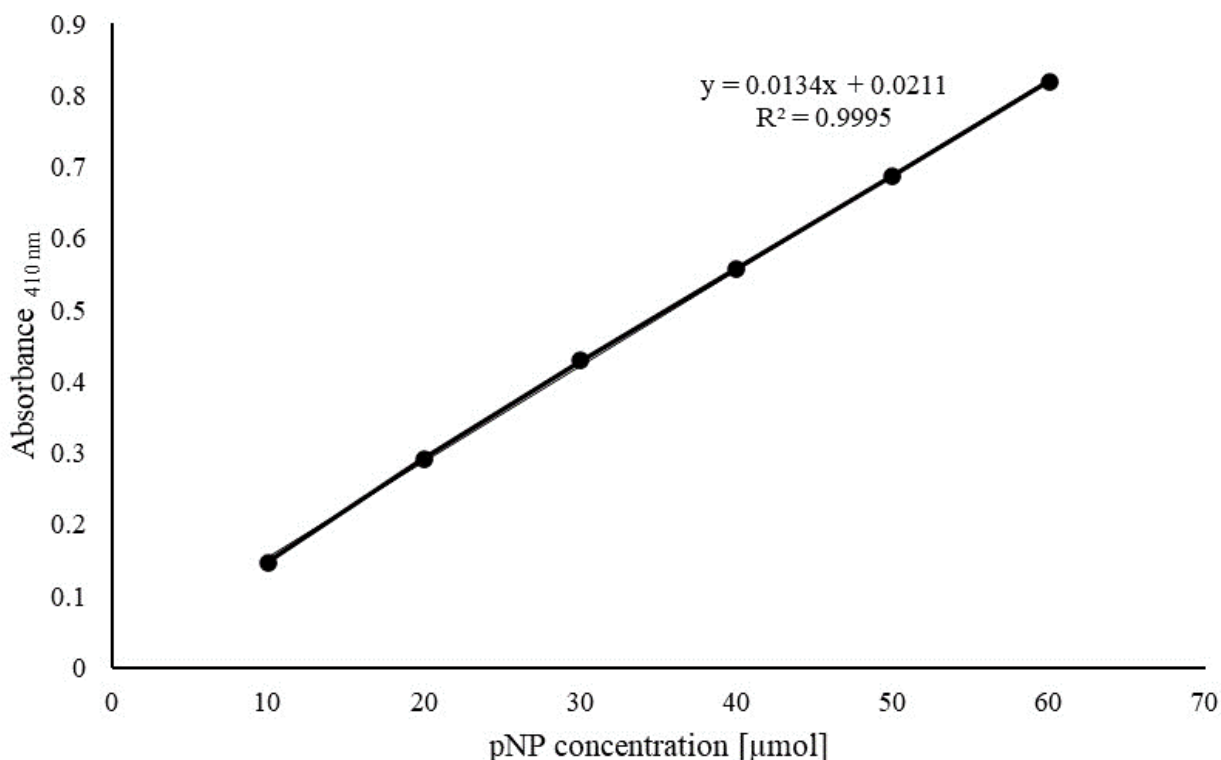


Fig. 2. Standard curve of p-NP.

Mostly, the active sites of bacterial lipases are covered by an alpha-helical flexible “flap” that can change from closed to open conformation when the lipase enzyme is absorbed by the lipid–water interface. The phenomenon of increased lipase activity at the lipid–water interface is referred to as interfacial activation (24–26).

The characterization of lipase activity began with the search for the best reaction time. The data obtained showed that the reaction had not occurred

initially (minute 0) and the best lipase activity with a value of 2.810 ± 0.106 U/mL was found at the 15th, minute which was significantly different from the incubation time of other observation times. The lipase activity after 15 minutes of incubation continuously decreases until 60 minutes of observation (Fig. 3). The decrease in enzyme activity can be caused by denaturation, structural modification, or dwindling substrate availability, which eventually triggers the repression of enzyme action (27).

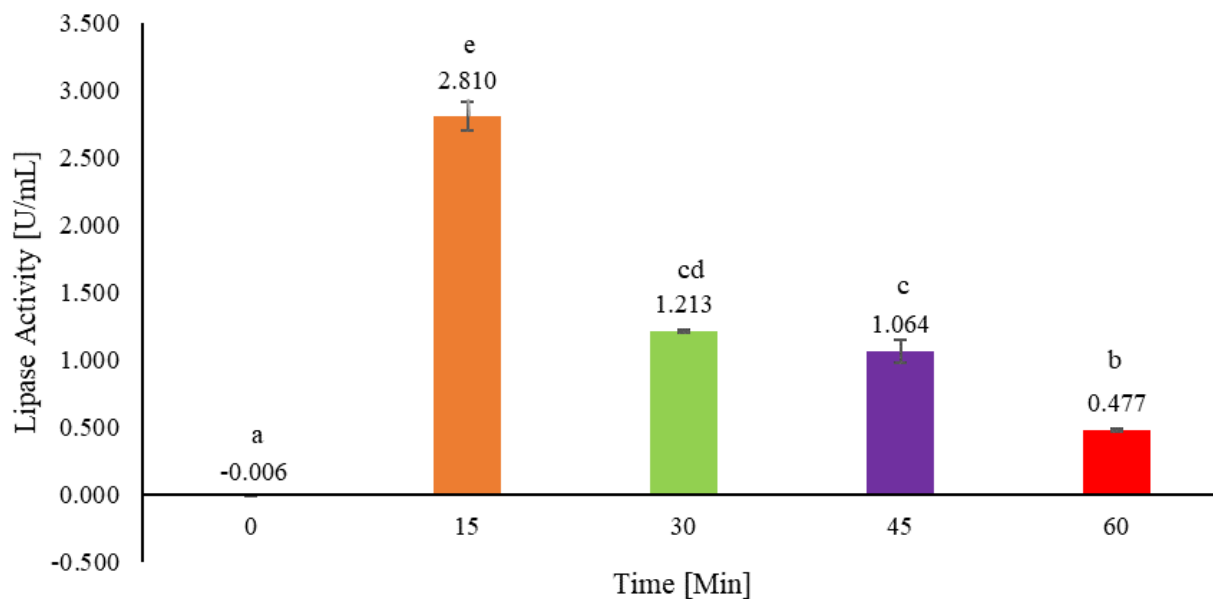


Fig. 3. Effect of reaction time conditions of lipase activity assay.

The difference in lowercase symbols between treatments indicated a significantly different ($P < 0.05$) of lipase activity value according to Duncan's Multiple Range Test (DMRT).

Subsequently, the effect of pH on lipase activity was observed. The highest lipase activity was obtained at pH 8, with a value of 3.173 ± 0.070 U/mL, which was significantly different from the other pH conditions tested (Fig. 4). This finding indicates that the lipase enzyme produced by *B. flexus* InaCC-B486 is of alkaline nature (alkaliphilic). The ionization of groups at the enzyme's active site and on the substrate can change as the pH changes, influencing the rate of substrate binding to the active site (28). Different *B. flexus* strains also showed the highest activity at alkaline pH, which was even higher at 10 (21). Generally, lipase enzymes produced from bacteria have good yields if produced under alkaline and neutral pH conditions (3). However, the lipases produced by fungi have an optimum yield if produced at acidic pH conditions (29,30). Tambekar et al. (11) reported that *B. flexus* isolated from Lonar Lake was able to produce extracellular lipase enzymes, and the optimum activity was recorded at pH 9. Lipase from *Bacillus cereus* NC7401 was also reported to be optimal at pH 8.0 and stable in the pH range of 5–10 (31). *B. subtilis* DR8806 that has been reported, exhibiting a pH optimum of 8.0 (32). Another alkaliphilic bacterial lipase has been reported from *Cohnella* sp. A01 exhibited maximum activity at pH 8.5 and was stable in the pH range of 8.5–10.0 (25). The alkaline lipases have great potential for use in food and beverage, detergent, flavoring, leather processing, pharmaceutical, and cosmetic industries (33). Alkaline lipases from bacteria are now popularly explored for a large variety of industrial purposes (34,35).

The optimum temperature that showed the highest lipase activity was found at 35°C (Fig. 5). The lipase

activity decreased dramatically when temperature increased to 40°C and 45°C. Interestingly, lipase activity was seen to increase again at 50°C but was lower than at 35°C. Increasing temperature enhances the rate of reaction at a certain point. On the other hand, higher temperatures denature enzyme proteins that change the shape of the active site of the enzyme, reducing or eliminating its activity (36). Another study found that lipase purified from *B. methylotrophicus* PS3 showed maximum activity at 55°C and was stable at 35°C (37). Bora et al. (38) reported that alkaline lipase has optimum activity at 30°C–65°C. The low optimum temperature can save an industry's production costs because it does not require high energy. According to its optimum temperature, the lipase produced by *B. flexus* InaCC-B486 belongs to the mesophilic family of enzymes. Mesophilic lipase has been used in several industrial applications, such as detergents, food processing, and waste treatment (39).

The results of the study in this article are limited to obtaining a determination of lipase activity from *Bacillus flexus* InaCC-B486, which was produced using olive oil as a substrate, by observing the influence of incubation time, pH, and temperature using p-nitrophenyl palmitate (p-NPP) during the reaction. The use of olive oil with the main components being oleic acid (C_{18}) and p-NPP (C_{16}) can provide an overview of the work of the lipase enzyme in breaking down fats with ester bonds from long chain fatty acids, in contrast to esterase, which prefers short chain fatty acids (40). Further research needs to be carried out, including enzyme purification and characterization, and then it can be tested for specific application purposes.

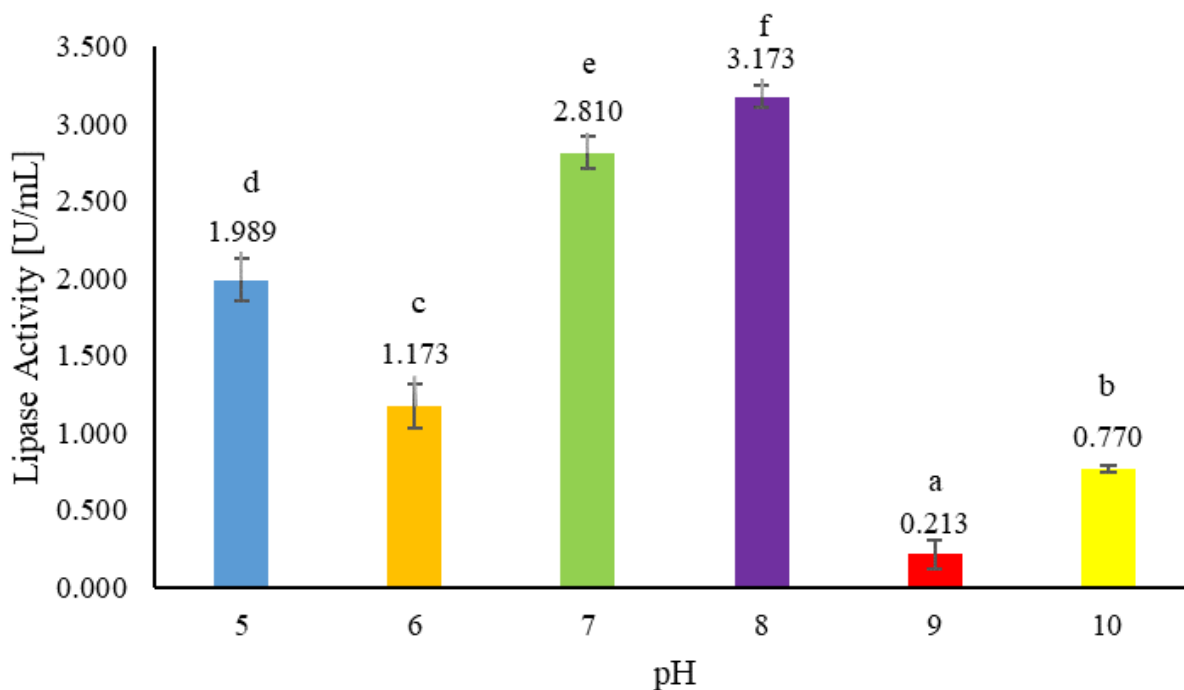


Fig. 4. Effect of reaction pH solutions of lipase activity assay.

The difference in lowercase symbols between treatments indicated a significantly different ($P < 0.05$) of lipase activity value according to Duncan's Multiple Range Test (DMRT).

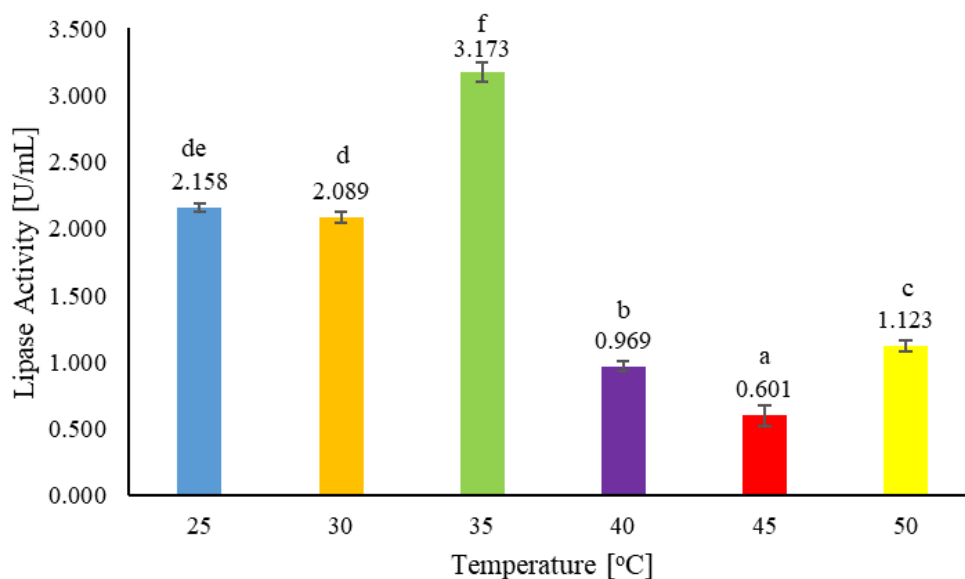


Fig. 5. Effect of reaction temperature of lipase activity assay.

The difference in lowercase symbols between treatments indicated a significantly different ($P < 0.05$) of lipase activity value according to Duncan's Multiple Range Test (DMRT).

4. CONCLUSION

Lipase production from *Bacillus flexus* InaCC-B486 was successfully achieved, reaching its optimum on day 4 with the addition of olive oil to the growth medium. We found that the lipase from *B. flexus*, InaCC-B486, has optimum activity at the first 15 minutes of incubation, a pH of 8, and a temperature of 35°C. These findings can hopefully be used as a reference for further research or industry

requirements, mainly in the use of lipase from *B. flexus* InaCC-B486.

5. CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

6. ACKNOWLEDGMENTS

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