

Characterization of Lipases from *Geobacillus stearothermophilus* and *Anoxybacillus flavithermus* Lysates

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Abstract

The aim of this study was to characterize lipases from two thermophilic bacteria, *Geobacillus stearothermophilus* (GS) and *Anoxybacillus flavithermus* (AF) in heat treated cell lysates. The pH optimum, pH stability, temperature stability and substrate kinetics and specificity of the lipases were determined. Optimum activity of the lipase from GS (LGS) was observed at pH 7.5, and the optimum activity of the lipase from AF (LAF) was at pH 8.0. LGS was stable up to 70°C after 12 hrs while LAF was stable up to 90°C after 12 hrs. Both enzymes were stable at a pH range of 6 to 8 over 12 h at 4°C. LGS had a highest V_{max} value of 22 mM·min⁻¹·mg⁻¹ with p-nitrophenyl acetate while the lowest K_m was 0.8 mM with p-nitrophenyl laurate. The highest V_{max} of LAF was 2.5 mM·min⁻¹·mg⁻¹ with p-nitrophenyl myristate, and the lowest K_m was 0.4 mM with p-nitrophenyl octanoate. LGS preferentially hydrolyzed p-nitrophenyl acetate and p-nitrophenyl octanoate while LAF preferentially hydrolyzed p-nitrophenyl myristate and p-nitrophenyldodecanoate. Lipases from both GS and AF showed characteristics that would be beneficial in food processing.

Keywords

Geobacillus, Anoxybacillus, Thermostable, Lipase

1. Introduction

Lipases (EC 3.1.1.3) are widely used in food processing, as an additive to detergents, in animal feed and for biofuels production. The microbial lipase market is estimated to be 425 million USD in 2018 and predicted to reach 590 USD by 2023 [1]. Lipases catalyze the hydrolysis of triacylglycerols to fatty acids, diacyl-

glycereols and monoacylglycerols in aqueous environments. Lipases display interesterification and esterification activities in nonaqueous environments. Microbial lipases have advantages over lipases from plants or animals including high yield, absence of seasonal fluctuations, regular supply, and economical growth conditions [1].

Microbial lipases from different sources including *Thermomyces lanuginosa*, *Rhizopusoryzae*, *Asperigillus* sp., *Candida* sp. and *Bacillus* sp. are available commercially [2]. These species have advantages such as fast lipase production, and the lipases have high biotechnological potential due to their stability at high temperatures and in organic solvents. The genera *Geobacillus* and *Anoxybacillus* are thermophilic bacilli and are distinct from the genus *Bacillus* [3] [4]. These microbes survive at the 37°C to 75°C temperature range. *Geobacillus* are considered as a “generally regarded as safe” (GRAS) strain, and some novel hydrolases have been isolated from *Geobacillus* and are used in food processing [5].

Thermostable lipases are advantageous to the food industry because of their stability at high temperatures. Lipases from thermostable bacteria have high heat tolerance which is necessary for the use in oils or fats for interesterification reactions to keep the substrates soluble. An example is in the case of cocoa butter substitutes, where thermostable immobilized lipases catalyze the interesterification reaction of different vegetable oils (such as soy bean oil and sunflower oils) to produce fats having a composition and properties comparable to those of cocoa butter (such as melting behavior) [6]. Cocoa butter has a melting point of approximately 37°C which dissolves in the mouth and is considered a high-value fat. Lipases are also used in place of hydrogenation to change the fatty acid profile of fats, such as soybean oil, to have higher melting points [1]. These industrial processes require the use of enzymes that have the ability to withstand temperatures up to 70°C [7].

There have been limited studies on the activity of lipases from *G. stearothermophilus* [2] [8] or *A. flavithermus* [9] especially with respect to thermal and pH stability. Lipases from GS and AF have not been fully characterized for their potential use in the food industry. In this study, we characterized the lipases from these organisms in heat treated cell lysates. The pH optimum, pH stability, temperature stability, substrate kinetics and substrate specificity of the lipases were determined.

2. Materials and Methods

2.1. Growth of Microorganisms

GS culture was prepared by adding 0.1 mL of a stock solution obtained from NAMS *G. stearothermophilus* cell line 7943 spore suspension (2.4×10^6 spores/0.1 mL, VWR, Atlanta GA, USA) to 10 mL of sterile water. This diluted stock was incubated in a water bath (ISOTEMP 210, Fisher Scientific, Dexter MI, USA) for 10 min at 80°C. 1 mL of this was added to 25 mL of tryptic soy broth (TSB, VWR, Atlanta GA, USA) media in a sterile 250 mL Erlenmeyer flask. The

flask was covered with sterile foil and the sample incubated at 55°C for 24 h in a shaker at 100 rpm (New Brunswick Scientific Series 25, Incubator Shaker, New Jersey, USA). The optical density (OD) of bacterial growth was measured using a dual beam spectrophotometer (Shimadzu, BioSpec-1601, Kyoto, Japan) at 600 nm [10].

A. flavithermus TNO-09.006 culture was a gift from RemcoKort (Vrije University, Amsterdam, The Netherlands) in the form of slant tubes. One loop from the slant tube was used to make a streak plate on tryptic soy agar (VWR, Atlanta GA, USA) and was incubated at 55°C for 48 h. A subculture was grown by inoculating 25 mL of TSB with one loop of the culture grown on the streak plate in a sterile 250 mL Erlenmeyer flask covered with sterile foil. Cells were grown with aerobic condition at 55°C in a shaker at 100 rpm for 16 - 18 h. Growth of AF was confirmed by measuring OD 600 nm using a spectrophotometer [11]. Frozen stocks were prepared by inoculating 2 mL of overnight culture into 20 ml of a 30% glycerol solution (Mallinckrodt Specialty Chemicals Co, St. Louis, MO USA) (glycerol/TSB, w/v) and stored in 2.0 mL cyro-vials at -20°C. Frozen stocks (0.1 mL) of each organism was added to 25 mL TSB in a sterile 250 mL Erlenmeyer flasks, then covered with sterile foil and incubated at 55°C for 16 - 18 h in a shaker at 100 rpm [11]. The 25 mL growth of bacteria was added to 1 L of TSB and cells were grown at 55°C at 100 rpm until the OD at 600 nm was between 0.7 to 0.9.

The bacteria were separated from the medium by centrifuging at 3000 ×g for 10 min at 10°C. The cells were washed in 10 ml 50 mM Tris-HCl (tris hydrochloride, Fisher Scientific, Waltham, MA USA) buffer pH 7.5 and centrifuged again as described above. After centrifugation, the cells were mixed with 5 - 10 mL Tris-HCl buffer (50 mM Tris-HCl, pH 7.5), and this mixture was sonicated at 40% amplitude for 1 min using a 4.5 mm microtip and a QsonicaSonicator power source (500 W power; QSonica Q500, Newtown, CT). The sonicated solution was centrifuged at 5000 ×g at 0°C for 20 min and the lysate was filtered using a 1.0 µm microfilter (GlassfiberPrefilter, Tullagreen, Carrigtwohill Co. Ireland).

The cell lysates from GS and AF were adjusted to 0.95 mg/mL with Tris-HCl buffer, then heated at 70°C for 10 min in a water bath then placed in an ice bath to cool. The protein content was determined using a protein determination kit (BCA kit, Thermo Scientific, Waltham, MA, USA). The samples were centrifuged at 10,000 ×g for 30 min at 4°C to precipitate denatured proteins [12]. The supernatants were dried using a VIRTIS sentry freeze-dryer overnight.

2.2. Total Protein and Enzyme Activity

Cell lysates were assayed using p-nitrophenyl acetate (PNPC2) (Sigma-Aldrich, MO, USA) as the substrate. 5 mL of substrate stock solutions (20 mM of PNPC2) were prepared using an acetonitrile and isopropanol mixture (1:4 v/v) as described by [13]. 1 mL of the substrate stock was diluted with 20 mL of assay buf-

fer (50 mM Tris HCl, pH 7.5 containing 1 mM CaCl₂ (Thermo Fisher Scientific, Waltham, MA, USA) and 0.3% Triton X100 (Mallinckrodt Specialty Chemicals Co, St. Louis, MO, USA) for a final concentration of 0.055 mM substrate solution. For the standard lipase assay, 900 µL of substrate solution was pipetted in 1.5 mL centrifuge tube (VWR, Atlanta GA, USA) and mixed with 100 µL of enzyme solution (50 mM Tris HCl, pH 7.5 containing 1 mM CaCl₂) for a final volume of 1 ml. After 1 min at 20°C, the sample was transferred to 1.5 mL disposable plastic cuvette (VWR Atlanta GA, USA). Absorbance at 410 nm was recorded every 5 seconds over 1 min using a dual beam spectrophotometer at room temperature. The blank consisted of 900 µL of substrate solution and 100 µL of water. One unit of lipase activity (U) was defined as 1 µmol substrate hydrolyzed per minute under the assay conditions. This is referred to as the standard lipase assay. Specific activity was determined by calculating the ratio of the lipase activity (U) and protein concentration (mg) and expressed as U/mg. The protein content was determined using a protein determination kit (BCA kit, Thermo Scientific, Waltham, MA, USA).

2.3. Effect of pH on Enzyme Activity and Stability

To determinate the optimal pH, lipase activity was assayed in the range of pH 4 to 12. The buffers were 50 mM sodium acetate buffer (pH 4 to 5), 50 mM Tris-HCl buffer (pH 6 to 8), and 50 mM glycine-NaOH buffer (pH 9 to 12). Standard activity assay using PNPC2 as the substrate in the reactions for each pH solution buffer and reading the absorbance at 410 nm at 20°C.

The pH stability was tested after incubation of 50 µL of the lipase sample with 100 µL of various pH buffers at pH values of 5 to 12 for 24 h at 4°C. After incubation, 100 µL of samples were diluted into 900 µL of assay buffer. Lipase activity was determined using the standard lipase assay. The data were graphed using the relationship of pH and incubation time with the relative activity of lipases. The relative activity (%) was calculated using the highest activity obtained and set at 100% using the standard enzyme assay. Relative activity (%) is the ratio between the highest enzyme activity and activity of lipase at various pH values and therefore expressed a percentage.

2.4. Effect of Temperature on Enzyme Activity and Stability

Thermostability of lipases was determined by measuring the residual activity after incubating the enzyme solutions (50 mM Tris HCl, pH 7.5) at 20°C to 90°C for 24 h in 1 mL microcentrifuge tubes. At the end of incubation, the tubes were cooled down in an ice bath and were assayed by added 100 µL of enzyme solution to 900 µL of the assay buffer solution using the standard lipase assay at room temperature. The data were graphed using the relationship of various temperatures and incubation times with the relative activity of the lipases. The relative activity (%) was calculated in relation to values obtained with the highest activity of the enzyme. Relative activity (%) is the ratio between the highest en-

zyme activity and activity of lipase for various temperatures and therefore expressed as a percentage.

2.5. Determination of Kinetics and Substrate Specificity of Lipases

The kinetic parameters including maximum reaction rate (V_{max}) and Michaelis-Menten constant (K_m) were calculated using different p-nitrophenyl esters (p-nitrophenyl acetate (PNPC2), p-nitrophenyl octanoate (PNPC8), p-nitrophenyl laurate (PNPC12), p-nitrophenyl myristate (PNPC14), and p-nitrophenyl palmitate (PNPC16) (Sigma-Aldrich, MO, USA)). Initial rate measurements with lipases at a constant temperature of 20°C and 50 mM Tris HCl, pH 7.5 with increasing substrate concentrations (0.06 - 1.2 mM), were performed to determine the kinetic parameters. Substrate solutions containing PNPC12, PNPC14, and PNPC16 were placed in a 60°C water bath (ISOTEMP 210, Fisher Scientific, Waltham, MA, USA) until substrate solutions were clear before use. Substrate solutions containing PNPC2 and PNPC8 did not need heating to form a clear solution. Absorbance at 410 nm for 1 min reaction times was used to determine the initial enzyme activity. Lineweaver-Burk plots were used to determine the maximum velocity (V_{max}) and Michaelis-Menten constants (V_{max}) by linear regression analysis for each experiment using Microsoft Excel (2016 Office 365). The reciprocal of velocity (v) was graphed vs. the reciprocal of substrate concentration [S] and the best-fit line was obtained. Values of V_{max} and K_m were determined using the equation:

$$\frac{1}{v} = \frac{K_m}{V_{max} [S]} + \frac{1}{V_{max}}$$

The catalytic constant (K_{cat}) was calculated using V_{max} , molecular weight, and concentration of the enzyme.

Lipase activity was measured spectrophotometrically at 410 nm by mixing 100 μ L of the enzyme with 900 μ L substrate buffer for a 1 min reaction time. The highest activity values for p-nitrophenyl esters are reported as 100%. Relative activity (reported in %) was expressed as the ratio of enzyme activity for different p-nitrophenyl ester substrates compared to the highest activity value recorded amongst the p-nitrophenyl ester substrates. For the lipase from GS, PNPC2 was the control substrate to calculate the relative activity, while PNPC14 was the control substrate to calculate the relative activity of lipases from AF.

2.6. Statistical Analysis

Three replicates were used to grow the GS and AF cultures and produce heat treated lysates. Analysis was done in triplicate for each replicate. The values are presented as means and standard deviation (SD) as calculated in Microsoft Excel 2016, Office 365.

3. Results and Discussion

In this study, we characterized the lipases from GS and AF in heat treated cell

lysates for characteristics (such as thermostability) what would be beneficial to the food industry. The purity of the enzyme varies for food use. An example is xylose isomerase use for the production of high fructose corn syrup from glucose which uses lysed or homogenized whole cells from *Bacillus coagulans* crosslinked to an inorganic carrier [7]. Lysates can be heat treated before crosslinking to inactivate proteases which would reduce the xylose isomerase activity. The lipases in GS and AF lysates were identified previously as 42 kDa and 33 kDa enzymes [14]. The lipase specific activity in crude cell lysates was 37.67 for GS and 6.86 U/mg for AF. After heat treatment, lipase activity was 42.91 U/mg from GS and 9.65 U/mg from AF, which resulted in 1.14 and 1.41 fold enrichment, respectively.

3.1. Effect of pH on Enzyme Activity and Stability

LGS and LAF were assayed using PNPC2 as a substrate to determine the optimum pH and the pH-stability at 20 °C with a pH range of 4 to 12. Relative activity (%) is the ratio between the activity at the optimum pH and the activity at various pH values and is therefore expressed as a percentage. The relative activity (%) was calculated as a percentage of the enzyme activity measured at pH 7.5 for GS and pH 8.0 for AF. The results show (Figure 1(a)) that the optimum pH of LGS was 7.5; however, the enzyme still had up to 60% activity at pH 9. Also, the result show the highest activity for LAF was between pH 8 to 9 and at pH 10, 40% of the activity remained (Figure 1(b)). The optimum pH for both lipases was observed in alkaline conditions when the pH increased from 7 to 9. The lipase activity decreased in acidic conditions with the enzyme activity decreasing to 20% at pH 6 and lower for GS and less than 10% activity for AF at pH values of 7.5 and less (Figure 1(a) and Figure 1(b)). Previous studies reported that *G. stearothermophilus* est 30 and est 55 have optimal activity between pH 8 to 9 [8], and *G. stearothermophilus* JC has optimal activity at pH of 9 [15]. *Anoxybacillus gonensis* G2 and *Anoxybacillus* sp. PDF1 reported a similar observation of optimum activity at pH 7.5 and 8 [16] [17].

The pH stability profiles of LGS and LAF are shown in Figure 2(a) and Figure 2(b). The pH-stability profile shows high enzyme activity between pH 6 to 11, but less stability at pH 5. Also, the enzyme activity was relatively stable at all

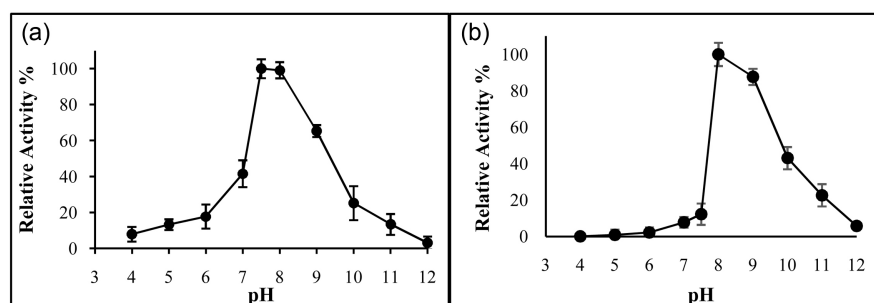


Figure 1. Optimum pH of lipases from *G. stearothermophilus* (a) and *A. flavithermus* (b). The values are presented as mean \pm S.D.

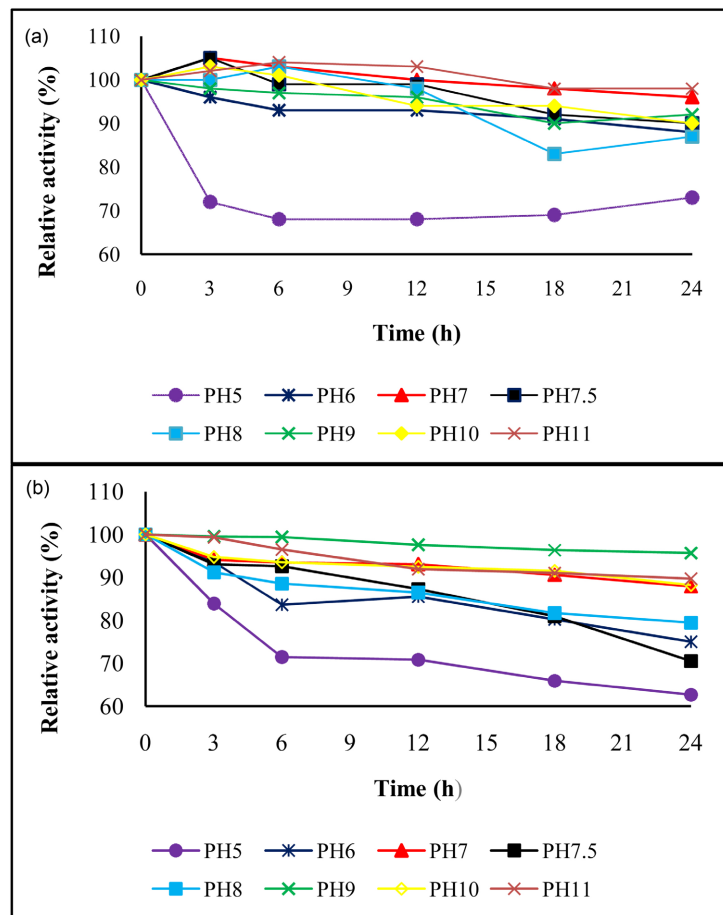


Figure 2. Relative activity at different pH values of lipases from *G. stearothermophilus* (a) and *A. flavithermus* (b). The values are presented as mean \pm S.D.

pH values except at pH 5 for 24 hours. The result show that more than 80% of LGS activity could be retained at pH values between 6 and 11, but the enzyme activity decreased at pH 5 (**Figure 2(a)**). LAF showed the highest stability at pH 9 and the activity was 70% at pH 6 to 8 and 90% at pH 10 to 11. At pH 5, only 60% activity was seen for LAF compared to pH 9 (**Figure 2(b)**). Both lipases showed higher stability with a pH range of 6 to 11 which indicates the ability of both enzymes to work in alkaline conditions compared to acidic. Previous reports stated similar stability profiles of lipases from different strains of *Geobacillus* and *Anoxybacillus*. For example, a lipase from *Geobacillus thermodenitrificans* PS01 had stability at pH 6 to 9 over 30 days at 4°C [18], *Geobacillus stearothermophilus* AH22 lipase was stable in the pH range of 4 to 10 at 4°C [2], *Anoxybacillus gonensis* A4lipase had stability at pH 6 at 4°C for 24 h [19], and *A. flavithermus* HBB 134 lipase was stabilize at pH 6 to pH 11 at 25°C, 40°C, and 50°C for 24 h [9].

Lipases which have high stability in neutral to acidic ranges can be used in the oleochemicals sector where the hydrolysis of FFA occurs and requires acidification of the formed soaps to obtain fatty acids [20]. Interesterification of oil by

enzymatic reactions utilizes lipases that have high activity in the pH range of 6 to 7 [21]. Lipases which have higher stability in alkaline pH conditions can be used in laundry and household detergents [2].

3.2. Effect of Temperature on Enzyme Stability

The thermostability of LGS and LAF was determined by incubation of enzymes at 20°C to 90°C, at pH 7.5 without substrate for a time course up to 24 h, and the results are shown in **Figure 3(a)** for LGS and **Figure 3(b)** for LAF. The relative activity (%) was calculated in relation to values obtained upon incubation at 24 h and 20°C. The activity of LGS was relatively stable between 20°C to 60°C with the relative activity being between 75% to 100% after 24 h of incubation. A decrease in the relative activity of approximately 55% was observed with an increase in temperature to 70°C and incubation time of 12 h. LGS activity was significantly reduced at 70°C after 6 h, and after 12 h the activity was 46%. A drop in activity was induced by incubation at temperatures above 70°C. Interestingly,

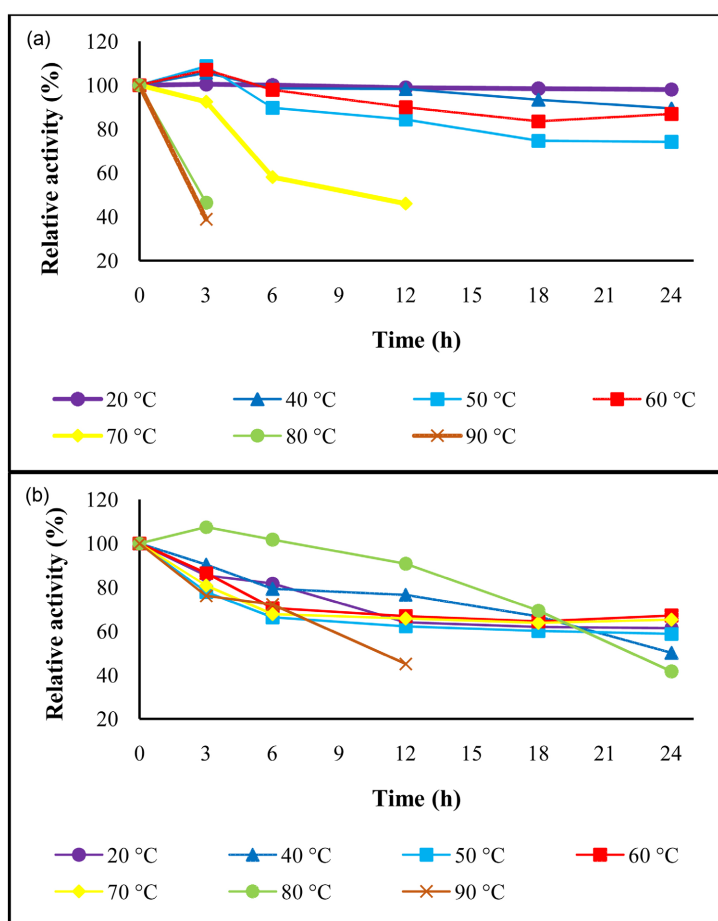


Figure 3. Thermal stability of lipases from *G. stearothermophilus* (a) and *A. flavithermus* (b). Samples were incubated at each temperature for 24 hrs before being assayed at 20°C. The values are presented as mean \pm S.D. The percentage of residual activity was compared with control enzyme activity (enzyme without thermal treatment).

LGS activity was higher at 60°C than at 50°C. Using the data from this study, LGS can retain stable activity at 70°C for 6 h and would operate well at 50°C. LGS activity was below 40% after 6 h at 90°C.

LAF showed 60% activity between approximately 20°C to 80°C for 24 h (Figure 3(b)). At 90°C, LAF activity was retained for 6 h. LAF showed remarkable thermal stability at 80°C compared to LGS and would operate well at this temperature. Results showed that LGS and LAF activity was stable at 20°C to 60°C with LAF being more thermostable at temperatures greater than 60°C. Also, the lipases have the ability to be active at 70°C for 6 h and LAF can retain 50% of its activity at 80°C after 24 hrs.

The thermostability data for both enzymes suggests that LGS was more stable at temperatures less than 70°C while LAF is more stable at 70°C and 80°C. In this study, the thermostability of LGS and LAF were within the industry application range (50°C to 60°C) for dairy processing such as cheese flavor production [1]. LAF would be suitable for the interesterification of oils which required lipases to have activity at 50°C to 70°C [7] [22]. These results are similar to a lipase from *A. flavithermus* HBB 134 which was thermostable at 25°C to 50°C after 24 h [9] and a lipase from *Anoxybacillus* sp. PDF1 was stable after a 30 min incubation at 60°C [16]. Based on the data, LGS was stable at 25°C to 60°C after a 24 h incubation and retained 50% of initial activity at 70°C after 12 h. When comparing to previous studies, LGS was more stable than a lipase from *Geobacillus* sp. strain ARM, which was stable after 150 min at 50°C [23]. A lipase from *Geobacillus* sp. SBS-4S was active at 50°C after 80 min of incubation [24]. When comparing to commercial lipases, the thermostability of LGS and LAF was similar to lipase produced from *Thermus thermophilus*, which is active in the temperature range of 65°C to 70°C [25].

3.3. Lipase Kinetics and Substrate Specificity

Lipases have different types of specificity including substrate chain length and positional which can be triacylglycerol stereospecific or non-specific. This research investigated the substrate chain length specificity of LGS and LAF. LGS and LAF kinetic constants were determined with different concentrations of various p-nitrophenyl esters (Table 1). With the standard lipase activity conditions (pH 7.5, at 20°C, and 1 min reaction time), the Michaelis-Menten kinetic constants of K_m and V_{max} were determined by Lineweaver-Burk plots. Turnover numbers (k_{cat}) and the catalytic efficiency (k_{cat}/K_m) were used to compare the differences between LGS and LAF (Table 1). LGS showed the highest V_{max} with PNPC2, corresponding to 22 mM/min/mg followed by 2.6 mM/min/mg with PNPC12. The lowest K_m of 0.8 mM was obtained using PNPC12 and this substrate also showed the highest K_{cat}/K_m value. The highest K_{cat} was with PNPC2, corresponding to $9.3 \times 10^8 \text{ s}^{-1}$ and K_{cat}/K_m was $1.5 \times 10^{10} \text{ mM}^{-1}\cdot\text{s}^{-1}$ (Table 1).

For LAF, the kinetic parameters V_{max} , K_m , K_{cat} , and K_{cat}/K_m of LAF varied between various chain length esters, but the highest V_{max} was with PNPC14 and

Table 1. Kinetic analysis of the reaction rates for lipases with various substrates.

Lipase sources	Substrate	Vmax (mM·min ⁻¹ ·mg ⁻¹)	Km (mM)	Kcat (s ⁻¹)	Kcat/Km (mM ⁻¹ ·s ⁻¹)
<i>G. stearothermophilus</i>	PNPC2	22 ± 2.6	64 ± 10	9.3 ± 1.1 × 10 ⁸	1.5 ± 0.1 × 10 ¹⁰
	PNPC8	2.3 ± 0.3	14.6 ± 2	9.7 ± 0.6 × 10 ⁷	6.7 ± 1.0 × 10 ¹⁰
	PNPC12	2.6 ± 0.4	0.8 ± 0.0	1.1 ± 0.2 × 10 ⁸	1.4 ± 0.3 × 10 ¹¹
	PNPC14	2 ± 0.1	2.3 ± 0.1	8.3 ± 1.4 × 10 ⁷	3.6 ± 0.3 × 10 ¹⁰
	PNPC16	2 ± 0.0	1.9 ± 0.2	8.6 ± 0.8 × 10 ⁷	4.7 ± 0.1 × 10 ¹⁰
<i>A. flavithermus</i>	PNPC2	1.1 ± 0.1	2.4 ± 0.1	3.5 ± 0.5 × 10 ⁷	1.5 ± 0.0 × 10 ¹⁰
	PNPC8	1.4 ± 0.2	0.4 ± 0.0	4.6 ± 0.8 × 10 ⁷	1.1 ± 0.0 × 10 ¹¹
	PNPC12	0.9 ± 0.1	1.1 ± 0.1	3.1 ± 0.6 × 10 ⁷	2.7 ± 0.2 × 10 ¹⁰
	PNPC14	2.5 ± 0.2	6.6 ± 0.5	8.2 ± 0.9 × 10 ⁷	1.2 ± 0.2 × 10 ¹⁰
	PNPC16	1.1 ± 0.0	1.5 ± 0.4	3.5 ± 0.3 × 10 ⁷	2.3 ± 0.9 × 10 ¹⁰

PNPC2 is p-nitrophenyl acetate (C2), PNPC8 is p-nitrophenyl octanoate (C8), PNPC12 is p-nitrophenyl laurate (C12), PNPC14 is p-nitrophenyl myristate (C14), and PNPC16 is p-nitrophenyl palmitate (C16). Samples were assayed at 410 nm with 20 °C and 7.5 pH value. The values are presented as mean ± S.D.

PNPC8 and the lowest K_m was with PNPC8 which also resulted in the highest K_{cat}/K_m value (Table 1). The kinetics results showed that the K_m value was low for both enzymes when the substrates had chain length esters between C8 to C16 which indicated that both enzymes had an excellent affinity for p-nitrophenyl esters [26]. The results showed that the kinetic parameter values were close to many lipases for the various substrates from different sources. Lipases from *G. stearothermophilus* (Est55 and Est 30) showed affinity towards a PNPC8 substrate, and with this substrate Est55 showed a K_m of 0.5 μM and K_{cat} 39,758 s^{-1} , while Est30 showed a K_m of 2.16 μM and K_{cat} 38 s^{-1} respectively [8]. The V_{max} and K_m of a lipase from *Geobacillus* sp. SBS-4S using a PNPC2 substrate was 2273 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ and 3.8 mM respectively [24]. Previous research showed a lipase from *A. gonensis* A4 had activities with a PNPC10 substrate and the K_m and V_{max} were 176.5 μM and 800 U/L [19].

The substrate specificities of LGS and LAF were examined with different chain length esters (Figure 4). Relative activity (%) of lipases was evaluated between the highest activity values of the enzyme with other activities using different substrates with the same conditions. The control substrate used to calculate relative activity for LGS was PNPC2 and for LAF was PNPC14 which were set at 100% relative activity. The enzyme specificity of LGS was higher towards the short chain fatty acid esters PNPC2 and PNPC8 compared to the longer chain PNPC14 and PNPC16. The results revealed that LGS had the highest activity of 100% with PNPC2, 61% with PNPC8, and 36%, 14%, and 7% with PNPC12, PNPC14, and PNPC16 substrates, respectively (Figure 4). The results were different with LAF, and the enzyme activity was highest with PNPC14 while the lowest enzyme activity was PNPC16. The relative activity of LAF was 12%, 100%,

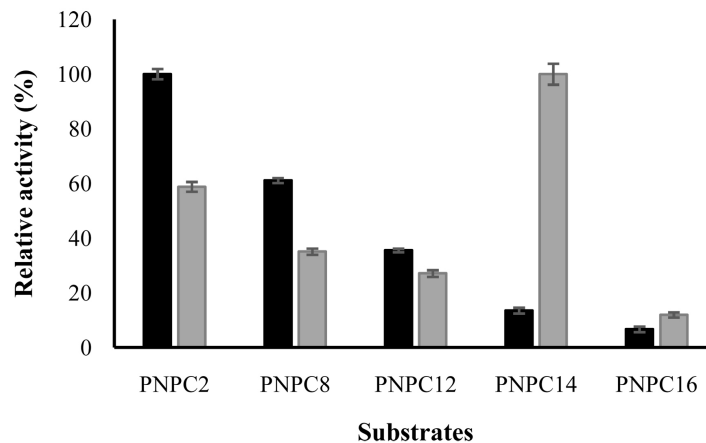


Figure 4. Comparison of lipase activities from *G. stearothermophilus* (black bars) and *A. flavithermus* (grey bars) with different substrates. The values are presented as mean \pm S.D. PNPC2 is p-nitrophenyl acetate, PNPC8 is p-nitrophenyl octanoate, PNPC12 is p-nitrophenyl laurate, PNPC14 is p-nitrophenyl myristate, and PNPC16 is p-nitrophenyl palmitate. Samples were assayed at 410 nm at 20°C and at pH 7.5. The values are presented as mean \pm S.D. The highest activity values of the enzyme for p-nitrophenyl esters were taken as 100%. The highest activity of LGS was calculated with PNPC2 while the highest activity of LAF was calculated with PNPC14.

27%, 35%, and 59% for PNPC16, PNPC14, PNPC12, PNPC8, and PNPC2, respectively (Figure 4). *Geobacillus* sp. SBS-4S lipases showed higher specificity toward a PNPC12 substrate than PNPC16 and PNPC20 substrates [24].

Lipases from *G. stearothermophilus* (Est55 and Est 30) more efficiently catalyzed the hydrolysis of esters of PNPC4 and PNPC6, with relative hydrolysis rates of 100%, 97% respectively compared to PNPC2 [8]. A lipase from *Anoxybacillus* sp. PDF1 had a higher catalytic efficiency with PNPC4 with a relative activity of 100% [16]. The reported substrate specificity observed in lipases from GS and AF agrees with the kinetic parameters. LGS has a specificity towards short chain fatty acid esters, while LAF has a high affinity to a 14 carbon chain fatty acid ester. Both enzymes, with their different specificity can be used in the food industry especially in the hydrolysis of milk fat, cheese ripening, modification of butter, and flavor development in meat and fish. Also, oils can be modified by enzymatic interesterification through lipases which catalyze medium and long chain fatty acid reactions [9] [18] [27].

4. Conclusion

Lipases were enriched in cell lysates from GS and AF using heat treatment (70°C). The lipases were the most active in neutral and alkaline pH of 7.5 for LGS and 8.0 for LAF. LGS was thermostable between 20°C and 60°C and between 20°C and 80°C for LAF. LGS hydrolyzed PNPC2, PNPC8 and PNPC12 substrates at higher rates than PNPC14 and PNPC16 substrates, while LAF hydrolyzed PNPC14 substrates at higher rates than PNPC12 and PNPC8 sub-

strates. In our study, the highest K_{cat}/K_m value for LGS was with PNPC12 and was with PNPC8 with LAF.

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Conflicts of Interest

The authors declare no conflict of interest regarding this manuscript.

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