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Case Report

Characterization of a thermostable-organic solvent-tolerant lipase from thermotolerant *Rhizopus* sp. strain *PKC12B2* isolated from palm kernel cake

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ABSTRACT

In this work, a new thermostable and organic solvent-tolerant lipase produced from *Rhizopus* sp. strain PKC12B2 was characterized to evaluate its activity and stability toward pH, temperature, organic solvents, and surfactants. The catalytic efficiency of the produced enzyme was also analyzed through enzyme kinetics study. Produced lipase exhibits remarkable stability at pH 8.0 to pH 10.0 and shows optimum activity at pH 9.0, demonstrating alkali lipase properties. Lipase has an optimum temperature of 45 °C and possesses significant thermal stability in the temperature range between 45 and 55 °C. Exceptional enzyme stability was also demonstrated in the presence of both polar solvents, such as glycerol, isopropanol and acetone, as well as non-polar solvents, such as cyclohexane and heptane up to 30 % (v/v) solvent concentration, where the enzyme retained more than 80 % activity after 1-h pre-incubation. For methanol, ethanol, and *n*-octanol, the relative activity of lipase was highly stable in the presence of up to 20 % (v/v) solvent concentration. Na^{+}, Zn^{2+}, and Fe^{3+} ions significantly stimulate the lipase activity at a final concentration of 10 mM. Lipase also demonstrated stimulatory effects towards a broad concentration range (0.2–1 %) of surfactants Tween 80 and Triton X-100. The Linearized Michaelis-Menten model in the form of Lineweaver-Burk ($R^2 = 0.995$) and Hanes-Woolf ($R^2 = 0.996$) models was found to successfully display the enzyme kinetics, in which the former generated Km and V_{max} of 1.965 mM and 4.51 mM/min respectively, while the latter generated Km and V_{max} of 1.295 mM and 3.389 mM/min, respectively. These new enzyme attributes demonstrate its great potential in biotechnology applications, such as for detergent formulations, biodiesel production and organic synthesis industries.

1. Introduction

Lipase (EC 3.1.1.3) is a biocatalyst that has a pivotal role in the utilization of lipid compounds. The enzyme works by assisting in the breakdown and mobilization of lipids within the organism cells and in the transfer of lipids from one organism to another. Lipase is a triacylglycerol acylhydrolase enzyme, where it has the ability to catalyze the hydrolysis of long-chain acylglycerols in aqueous solutions and several synthesis reactions in organic media, such as esterification and transesterification of lipids. These properties of lipase catalyze multiple reactions and selectivity make the enzyme highly appealing from a commercial perspective.

Recent developments in lipase have heightened the need for well-characterized lipase, particularly those possessing high thermal stability as well as organic solvent tolerance. Lipase-catalyzed reactions, such as those in the synthesis of fine chemicals, biodiesel, and biodegradable polymer production, as well as in the textile and detergent industry, require enzymes with good characteristics [1]. Lipase possessing thermal stability and organic solvent tolerance are believed to enhance lipase-catalyzed reactions of industrial importance [2,3]. A

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thermostable enzyme offers a wide range of applications as it minimizes the possibility of contamination by fast growing mesophilic microorganisms [4,5]. In addition, operating reactions at high temperatures will decrease substrate viscosity while increasing its diffusion coefficient, therefore facilitating the thermostable enzyme to catalyze at a higher reaction rate. Using thermostable enzymes can also achieve higher process yield since the substrate and product solubility increase at elevated temperatures. Additionally, several studies reported that the structural properties of enzymes that confer thermal stability also offer stability towards organic solvents [6–8]. Biocatalysts with organic solvent-tolerant properties are reported to have several potential benefits over those in aqueous media, as they improve the solubility of the organic compounds in non-aqueous media and are capable of performing new reactions that are thermodynamically or kinetically unfavourable in water [9].

One of the greatest challenges is that enzymes tend to lose their activity and stability when exposed to elevated temperatures and organic solvents. Many chemical alterations occur as the result of reactions at elevated temperatures. Most lipases readily denature in organic solvents and consequently lose their catalytic activities. Recent evidence suggests that when an organic solvent like methanol is used in the transesterification reaction of biodiesel production, the lipase employed often gets severely inactivated due to the inhibitory effect of the organic solvent present in the medium [10]. Previously, the thermophilic lipolytic bacterium *Aneurinibacillus thermoaerophilus* strain HZ was used to produce thermostable and organic solvent-tolerant lipase [11]. Other thermophilic lipolytic bacterium *Geobacillus* sp. [12], *Aneurinibacillus thermoaerophilus* strain AFNA [13], *Staphylococcus warneri* [14], and *Acinetobacter* sp. AU07 [15] were also studied to secrete lipase with thermostable and organic solvent-tolerant properties.

So far, however, very few studies have explored the production of thermostable and organic solvent-tolerant lipase secreted from fungi. The most related study was reported by Adachi, D. et al. [6], who developed a robust whole-cell thermostable and solvent tolerant biocatalyst by introducing a thermostable lipase from *Geobacillus thermocatenulatus* (BTL2) into an *Aspergillus oryzae* whole-cell biocatalyst. In fact, among the microorganisms, fungal lipase has gained more industrial attraction due to its cheap extraction, high thermal and pH stability, broad substrate specificity and better activity in organic solvents [16]. Besides, Manpreet et al. [17] also stated that filamentous fungi could spread over and penetrate inside the solid substrate during fermentation and cause the mycelia to synthesize and produce large amounts of extracellular hydrolytic enzymes.

In a recent study, a thermotolerant fungi, *Rhizopus* sp. strain PKC12B2, was locally isolated from palm kernel cake [18], which secreted a thermostable and organic solvent-tolerant lipase under solid-state fermentation (SSF). Maximum crude lipase activity was obtained at 45 °C and an initial pH of 10.0. Under the optimum lipase production, the yield showed a significant improvement of activity by almost two-fold compared to the initial screening production [19]. Therefore, in the present study, the biochemical characteristics of the newly thermostable organic solvent-tolerant lipase produced by thermotolerant *Rhizopus* sp. strain PKC12B2 were further studied. The effect of temperature, pH, organic solvents, metal ions and surfactants were analyzed on the activity and stability of the newly produced enzyme. Enzyme kinetics was also performed to understand how the produced enzyme functioned. The findings of this study will be promising as a biocatalyst for industrial processes.

2. Materials and methods

2.1. Substrates and chemicals

para-Nitrophenyl palmitate (*p*-NPP) was purchased from Sigma-Aldrich (USA). All other chemicals were of analytical grade and were purchased from Hmb GLOBAL Chemical (Germany), Merck (Germany), Fisher scientific (UK) and Bumipharma Sdn. Bhd (Malaysia).

2.2. Microorganism and lipase production

Thermo-solvent tolerant *Rhizopus* sp. strain PKC12B2 locally isolated from palm kernel cake was used in this study. The strain was cultivated *via* SSF in a medium that was previously optimized nutritionally and physically for lipase production [19], which comprises of 2 % (v/w) inoculum size, 20 g/L olive oil, 6 g/L peptone, 20 g/L ethanol, 70 % moisture content and initial pH 10.0. Cultivation was performed at 45 °C for 72 h.

2.3. Measurement of lipase activity and protein

Lipase activity was determined spectrophotometrically based on the modified method described by Gopinath et al. [20] using (*p*-NPP) as the substrate. The total 1 mL reaction solution consisted of 940 μL of phosphate buffer (50 mM, pH 8.0), 10 μL of *p*-NPP (100 mM, a 1 mM substrate final concentration), 40 μL of ethanol and 10 μL of the enzyme solution. Unless otherwise stated, the enzyme reaction was maintained at pH 8.0 and incubated at 45 °C for 15 min. The reaction was stopped by chilling on ice. Enzyme absorbance was measured using spectroscopy at 410 nm against an enzyme-free control. One unit of lipase (U) was expressed as the amount of enzyme releasing one μmol *para*-nitrophenol per millilitre per minute under the standard assay conditions. Enzyme activity was taken as Unit/gram of the initial dry substrate (U/gds). All the enzyme assays were conducted in triplicate. The protein content of the crude lipase extract was analyzed using the method of Lowry et al. [21] using bovine serum albumin (BSA) as standard and was expressed as mg of total proteins per gram of dry palm kernel cake.

2.4. Effect of pH on lipase activity and stability

The optimum pH for enzyme activity was carried out at pH ranging from 6.0 to 12.0 using *p*-nitrophenyl palmitate (*p*-NPP) as the substrate by incubating at 45 °C for 15 min. The effect of pH on lipase stability was determined by the same method after pre-incubation of crude enzyme in 50mM buffer at the specific pH for 0, 30, 60, 90, 120, 150 and 180 min at 45 °C. The pH stability was also studied at pH 7.0–10.0 by measuring the residual activities at optimum pH as control (100 % of relative activity). Buffer systems used were: 50 mM phosphate buffer (pH 6.0–8.0), 50 mM Glycine-NaOH buffer (pH 9.0–10.0) and 50 mM Na_2HPO_4–NaOH buffer (pH 11.0–12.0) [22].

2.5. Effect of temperature on lipase activity and stability

The effect of temperature on the lipase activity was measured by enzyme assaying at various temperatures ranging from 35 to 85 °C (10 °C interval) for 15 min. The highest enzyme activity was used as the temperature control and taken as 100 % activity. As for temperature stability, the enzyme was incubated at temperatures from 45 to 65 °C for different incubation time from 0 to 90 min (15 min interval). After each period of incubation, the enzyme was immediately cooled in an ice bath for 15 min and the residual activity was determined [23].

2.6. Effect of organic solvent, surfactants and metal ions on lipase stability

The effects of organic solvents and surfactants on lipase activity were investigated by pre-incubating the crude enzyme with different concentrations (15 %v/v, 20 %v/v, 25 %v/v and 30 %v/v) of various organic solvents (Table 1) and different concentrations (0.1 %v/v or 1 % v/v) of various detergent (Table 2). The reaction was performed at 45 °C with shaking at 180 rpm for 1 h. Reactions containing 10 mM, 6 mM and 2 mM metal ions (Na^+, Zn^{2+}, Ca^{2+}, Fe^{3+}, K^+) were pre-incubated in similar conditions. After these incubations, residual lipase activities

Table 1
Effects of various organic solvents on the activity of lipase.

Organic solvent	Log P	Relative activity (%) at different concentrations			
		15 %	20 %	25 %	30 %
Control		100	100	100	100
Glycerol	−3.03	118.71 ± 0.044	95.54 ± 0.011	88.46 ± 0.013	89.00 ± 0.010
Isopropanol	−0.77	119.24 ± 0.008	108.54 ± 0.011	93.20 ± 0.013	93.99 ± 0.009
Methanol	−0.76	102.92 ± 0.031	92.54 ± 0.014	69.68 ± 0.014	64.64 ± 0.008
Ethanol	−0.24	120.19 ± 0.033	91.27 ± 0.011	74.78 ± 0.020	64.85 ± 0.011
Acetone	−0.23	109.41 ± 0.017	76.16 ± 0.004	81.58 ± 0.008	85.90 ± 0.003
N-Octanol	2.9	92.04 ± 0.026	92.31 ± 0.006	60.25 ± 0.006	76.70 ± 0.005
Cyclohexane	3.2	102.75 ± 0.024	111.55 ± 0.040	81.22 ± 0.008	87.88 ± 0.004
Heptane	4	109.69 ± 0.032	98.18 ± 0.017	80.64 ± 0.001	91.91 ± 0.032

Table 2
Effects of various surfactants on the activity of thermotolerant *Rhizopus* sp. Strain PKC12B2 lipase.

Surfactant	Relative activity (%)				
	0.2 %	0.4 %	0.6 %	0.8 %	1 %
Control	100	100	100	100	100
Tween 80	111.89 ± 0.013	101.56 ± 0.003	119.95 ± 0.032	122.61 ± 0.017	121.13 ± 0.002
Triton X100	110.80 ± 0.004	115.65 ± 0.007	119.41 ± 0.025	119.80 ± 0.005	111.27 ± 0.002

were assayed in 50 mM Glycine-NaOH (pH 9.0) at 45 °C for 15 min. The enzyme activity determined in the buffer with no additives was set as 100 %. The relative activity percentage was measured relative to the control. In all cases, each analysis was measured in triplicates [22].

2.7. Enzyme kinetic

Enzyme kinetic was performed by assaying the crude enzyme at different concentrations: 0.1, 0.3, 0.5, 1, 2, 3 and 10 mM of *p*-NPP as substrate in 50 mM Glycine-NaOH (pH 9.0) at 45 °C for 15 min. Kinetic parameters (Km and Vmax) were determined graphically based on the linearized Michaelis-Menten equation in the form of Lineweaver-Burk, Eadie-Hofstee and Hanes-Woolf plots by measuring the velocity of enzyme-catalyzed reaction, V at different concentrations of substrate, S.

2.8. Statistical analysis

All experiments were carried out in triplicate. IBM Statistical Program for Social Science (SPSS) 26 was used for statistical analysis of one-way Analysis of variance (ANOVA) with a 95 % confidence level to see the significance value on the effect of various parameters (temperature, pH, organic solvents, metal ions and surfactants) on the activity and stability of the lipase produced.

3. Results and discussion

3.1. Effect of the fermentation time on lipase production

In this study, maximum extracellular lipase production (58.63 U/gds) of thermotolerant *Rhizopus* sp. strain PKC12B2 was attained after 72 h of growth and started to decrease with longer fermentation time (Fig. 1). The decrease in lipase activity might be due to the release of product inhibition, such as the production of protease enzyme during the post exponential growth phase of the microorganism or due to the accumulation of fatty acids, which could interfere with the metabolism of the microorganism [24]. Chander, H et al. [25] also reported that the inhibition of lipase production might be due to a reduction in mold growth resulting from non-utilization or less availability of lipids and carbon sources by the microorganism.

Biomass is fundamental in the characterization of microbial growth. Thus, the protein contents were also measured for the indirect estimation of fungal biomass. The protein concentration (Fig. 1) was initially increased when the fermentation time increased and reached its maximum at 48 h with about 130 mg/g PKC and decreased slightly to 124.4 mg/g PKC after 72 h growth, where maximum extracellular lipase was produced. The protein content was found to further decrease until 120 h of fermentation, which might be due to the depletion of medium components containing proteins by the microorganism for its growth as well as metabolic formation. The increase in protein concentration at longer fungal growth from 120 h to 168 h may be due to the secretion and accumulation of various proteins and enzymes in the medium. These results are in agreement with the previous studies [26,27], where the lipase activity was demonstrated to start soon after the beginning of incubation, and the highest crude enzyme extract was obtained after 72 hs. Similarly, a decrease in lipase activity was observed at longer fermentation times, which might be due to the presence of proteases in the culture medium.

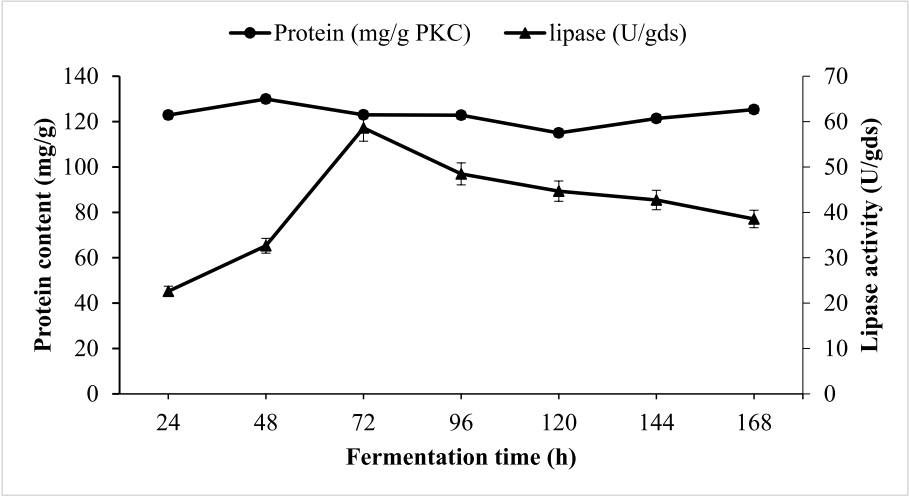

Fig. 1. Time profile of lipase production and protein content for the thermotolerant *Rhizopus* sp. strain PKC12B2 grown in palm kernel cake under SSF conditions.

3.2. Effect of pH on lipase activity and stability

The lipase produced was examined in different buffers to evaluate its effect at various pH. The effect of pH on the lipase activity at 45 °C using pNPP as substrate was examined at different pH values. The enzyme was active in the pH range of 7.0–10.0, and the optimal pH was shown to be 9.0 in 0.05 M Glycine-NaOH buffer (Fig. 2).

The results demonstrated that lipase activity decreased sharply when the pH was below 8.0 or over 10.0. In the previous study, thermoactive and alkaline lipase from *Talaromyces thermophiles* fungus had an optimum pH of 9.5 [28], and alkaline thermostable lipase from *Aspergillus carneus* [29] had with optimum pH of 9.0. This showed that the produced lipase is considered an alkaline lipase.

The stability of the produced lipase was determined by pre-incubating the enzyme for 120 min at different pH buffers. The result (Fig. 3) demonstrated that the lipase remained stable at pH 8.0 and 9.0 for 120 min, retaining 92.0 % and 95.5 % of the activity at 120 min for pH 8.0 and pH 9.0, respectively. At lower pH, the activity of lipase retained 75.6 % of the residual activity at 60 min pre-incubation period before it finally dropped to 56.5 % after 120 min. At pH 10.0, although more than 50 % of the activity was lost at 120 min pre-incubation, like at pH 7, the enzyme was relatively more stable at shorter pre-incubation time, retaining about 81.4 % activity in 60 min. The remarkable stability of the produced lipase at higher pH (pH 8.0–10.0) justifies it to be a potential alkaline lipase.

The pH stability of the produced lipase was slightly inferior to that of highly alkaline-tolerant lipases from *Talaromyces thermophiles* (pH 9.0–11.0) [28]. However, it is still similar or superior to the lipase from *Peziza* sp., which remained stable in the pH range of 8.0–10 after pre-incubation at room temperature for 15 min [30], and LipB from *Lasiodiplodia theobromae* that showed stability over a narrower range, pH 7.0–8.0 [31]. Generally, fungal lipases were relatively stable in the lower pH, ranging between 4.0 and 7.0 and showed lower tolerance to alkaline pH values [32]. Lipase and other enzymes currently used in detergent formulations ought to have tolerance to alkaline conditions because the pH of laundry detergents is commonly in the range of 9.0–11.0 [33]. Hence, the *Rhizopus* sp. strain PKC12B2 lipase produced with alkaline stability possesses the potential for use in detergent formulation.

3.3. Effect of temperature on lipase activity and stability

In this study, the optimum temperature was determined by testing the activities of enzymes incubated at different temperatures between 35 °C and 85 °C (Fig. 4). The enzyme exhibited the highest activity at 45 °C. This implies that the produced lipase is a thermophilic enzyme. The lipase activity increased steeply from 35 °C and reached the maximum at 45 °C. Further increases in temperature decreased the lipase activity.

Generally, an increase in temperature enhanced the number of effective collisions between the enzyme and substrate to form the activated complex and, therefore, increased the rate of reaction. However, there is a limit to the increase in enzyme activity with the increase in temperature. When the rate of enzyme catalyzed reactions is measured at several temperatures, the reaction becomes fastest at a certain optimal temperature. Beyond that temperature, the reaction rate decreases steeply, mainly due to the enzyme denaturation by heat [34]. Thus, finding the temperature optimum is crucial.

The results attained from the stability test (Fig. 5) revealed that the lipase produced from thermotolerant *Rhizopus* sp. strain PKC12B2 was stable at 45 °C, retaining 100 % and about 85 % of its activity after pre-incubation for 30 min and 60–90 min, respectively. At 55 °C and 65 °C, the enzyme lost about 30 % and 60 % of its activity after 30 min of incubation, respectively, retaining approximately 70 % and 42 % activity and gradually declining with longer incubation time, making up to a final residual activity of about 53 % and 31 % respectively after 90 min incubation. These relatively high stabilities of the enzyme at elevated temperature prove the thermostable property of the produced enzyme.

In previous studies, an optimum temperature of 45 °C was reported for fungal lipase *Malbranchea cinnamomea* [35], while some thermostable fungal lipase was found at 40 °C optimum temperature, such as *Hypocrea pseudokoningii* [36] and *Thermomucor indicae seudaticae* N31 [37]. Lipase from *Hypocrea pseudokoningii* was stable at a broad temperature range of 30–60 °C within 2 h of incubation with a half-life of 107 min at 60 °C incubation. On the contrary, *Thermomucor indicae seudaticae* N31 lipase was thermostable at a narrow temperature range of about 40–45 °C.

3.4. Effect of organic solvents on lipase stability

The activity and stability of the enzymes in organic solvents have received much attention as lipases are used in a wide range of biotechnological fields, such as catalysts in organic synthesis and biotransformation [22]. However, the number of fungal-producing organic solvent-tolerant lipase is limited. Since the thermotolerant *Rhizopus* sp. strain PKC12B2 was isolated from PKC by enrichment in 10 % organic solvent (ethanol) [18], the production of solvent stable enzyme was possible. In this study, various polar and non-polar organic solvents were investigated for their effects on the activity and stability of *Rhizopus* sp. strain PKC12B2 lipase.

Table 1 presents the effect of various organic solvents on the relative activity of lipase from thermotolerant *Rhizopus* sp. strain PKC12B2. The enzyme was found to be substantially stable and active in most of the organic solvents when pre-incubated at 45 °C for 1 h. Lipase was

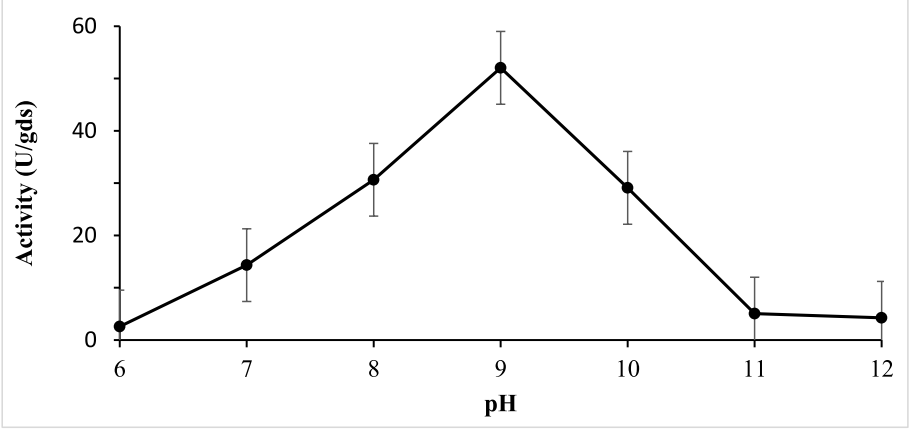

Fig. 2. The effect of pH on the activity of lipase produced.

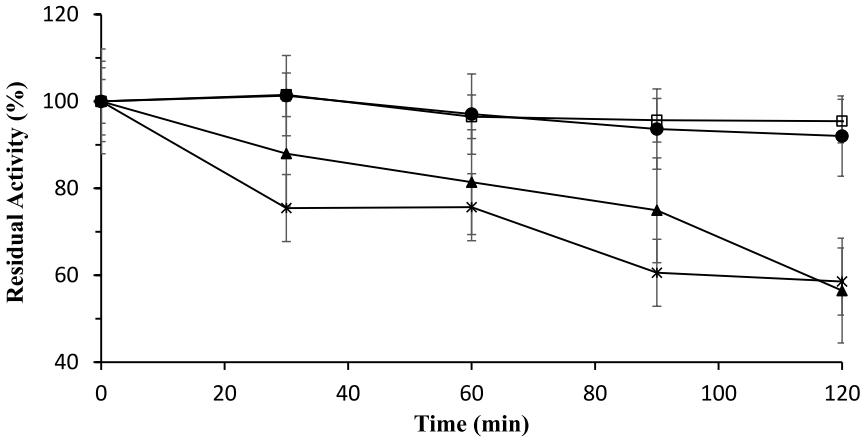

Fig. 3. pH stability of the lipase produced. The enzyme was incubated at Phosphate buffer (pH 7.0, *), Phosphate buffer (pH 8.0, ●), Glycine-NaOH buffer (pH 9.0, □), Glycine-NaOH buffer (pH 10.0, ▲) for the indicated time. The residual activity was measured by a standard assay.

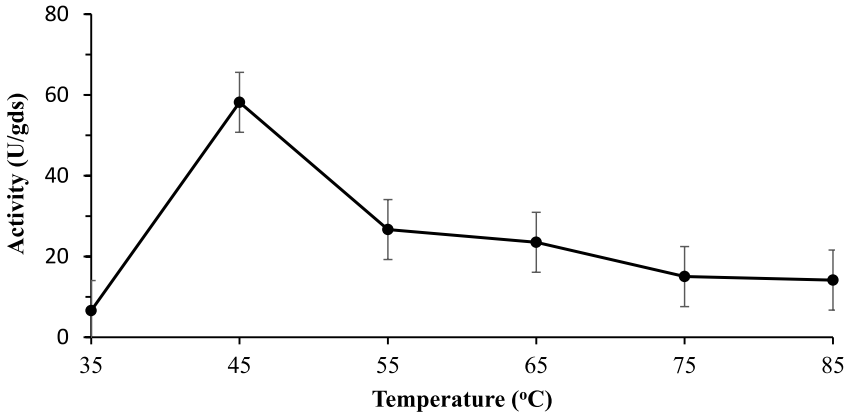

Fig. 4. Effect of temperature on the activity of the lipase produced.

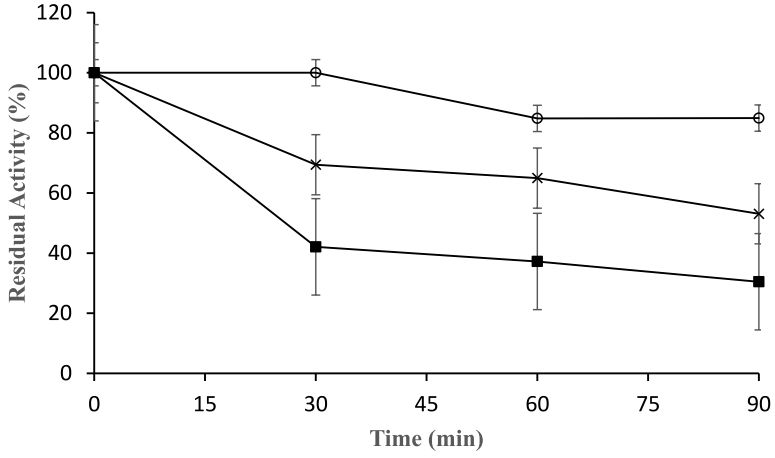

Fig. 5. Thermal stability of the lipase produced. The enzyme was incubated at 45 °C (o), 55 °C (×) and 65 °C (<) for the indicated time.

remarkably stable in the presence of both polar (hydrophilic) solvents (log $P < 0.3$), such as glycerol, isopropanol and acetone, as well as non-polar (hydrophobic) solvents (log $P > 2.0$), such as cyclohexane and heptane up to 30 % (v/v) solvent concentration. Meanwhile, for methanol, ethanol, and *n*-octanol, the relative activity of lipase was highly stable in the presence of up to 20 % (v/v) concentration of these solvents with around 92.54 %, 91.27 %, and 92.31 %, respectively, but it was gradually reduced to about 64.64 %, 64.85 %, and 76.70 %, respectively, when the solvent concentration was increased to 30 % (v/v).

The increase in lipase activation (stimulatory effect) found in some

organic solvents could be due to the diverse sensitivity of the produced lipase toward the nature of the organic solvents. According to Wang et al. [38], there is a certain level of water molecules needed by the enzyme to maintain the natural conformation, which allows them to carry out their full functionality. In the case of the produced lipase, this water could be tightly bound to the enzyme, thereby leaving the biocatalyst in an active state under various solvents, and even some hydrophilic solvents did not strip it. This justification is in agreement with Zaks and Klibanov [39], who found that pancreatic lipase appeared to bind the required water tightly to the enzyme molecules, causing it to be catalytically active under organic solvents.

Although solvent stability has been reported for other fungal lipases, the stability in polar solvents has rarely been observed. Marques et al. [40] reported the production of a thermostable, alkaline and organic solvent-tolerant lipase from *Trichoderma atroviride* 676, in which the enzyme showed a stimulatory effect in 10 % non-polar solvents (kerosene (194.24 %), *n*-dodecane (190.29 %) and hexane (139.17 %), but complete inhibitory effect towards polar solvents (isopropanol, ethanol, acetone and acetonitrile) after 2 h incubation in 30 °C. In another study, Hiol, Jonzo [2] also reported that *Rhizopus oryzae* lipase is stable in 30 % alkanes and long chain alcohols (propanol (61 % relative activity), butanol (87 %), hexane (100 %), heptane (101 %) and cyclohexane (103 %)). However, it is highly denatured in hydrophilic solvents such as acetone (22 %) and short chain (C1–C3) alcohols (methanol (2 %) and ethanol (33 %)) after incubated at 25 °C for 1 h. The stability of *Aspergillus carneus* lipase was also investigated [41] and it was observed that solvents with a log p < 2 were inhibitory, while the lipase was stable in iso-octane, benzene, toluene and xylene. These showed that thermotolerant *Rhizopus* sp. strain PKC12B2 fungal lipase has broader organic solvent stability with the potential to be used in biotechnology. For instance, the short-chain alcohols methanol and ethanol have been widely used in transesterifications. Thus, tolerance to methanol, ethanol and glycerol is a highly desirable feature for biodiesel production. Besides, the above-mentioned non-polar solvents also allowed the produced lipase to remain stable (over 90 % of its initial activity). This could be because these hydrophobic solvents leave the hydration shell of the protein together, thus resulting in only a small redistribution of water, which preserves the natural protein structure and decreases the side reaction in the liquid phase [22]. Hence, these organic solvent-tolerant characteristics of the produced lipase are particularly significant for organic synthesis.

3.5. Effect of metal ions and surfactant on lipase stability

By studying the effect of metal ions (2–10 mM) on the produced lipase activity (Fig. 6), Na^+, Zn^{2+} and Fe^{3+} were found to stimulate the lipase activity significantly, with 107.52 %, 145.46 % and 96.60 % residual activity at a final concentration of 10 mM, respectively. These metal ions might bind to some active sites of the produced lipase and change its conformation, which may also be necessary for its catalytic properties. Metalophilic lipase from *Ralstonia solanacearum* was also stable in the presence of 10 mM Na^+ and Fe^{2+} but slightly denatured in Zn^{2+} [42]. Similarly, thermo-solvent tolerant lipase SL-4 was slightly enhanced in the presence of 10 mM Na^+ [22]. Unlike the produced lipase, Zn^{2+} and Fe^{3+} have been found to dramatically inhibit the activity of halophilic and organic solvent-tolerant lipase KM12 [43] and thermo-solvent tolerant lipase SL-4 [22]. Ca^{2+} ion has been reported to have a positive effect on the activity and stability of lipases [42,44,45]. However, a slight reduction in activity on thermotolerant *Rhizopus* sp. strain PKC12B2 lipase was observed in this study. Ca^{2+} slightly reduced the produced lipase activity to 88.01 % of its original activity at 2 mM concentration, and despite slight fluctuation in the residual activity as the metal ion concentration was increased, the stability could be retained even at a final concentration of 10 mM, maintaining 87.87 % of its original activity. As for K^+, this metal ion demonstrated an inhibitory effect on the activity of the produced lipase, retaining 61.84 % of residual activity at 2 mM. The residual activity produced by K^+ at the studied concentration ranges between 44.89 and 69.50 %. A similar inhibitory effect of K^+ towards lipase stability was previously reported by Refs. [43,45,46].

Moreover, surfactants (non-ionic detergents) such as Triton X-100 and Tween-80 are usually added to lipase substrate emulsions to improve the emulsion quality, making the substrate more accessible. However, depending on the concentration used, surfactants can also cause lipases to denature.

In this study, Tween-80 and Triton X-100 demonstrated stimulatory effects on the produced lipase at all concentrations (0.2–1 %), with Tween 80 owning higher stability (Table 2). The stimulatory effect of these surfactants on lipase might be due to a change in the conformation of the enzyme associated with the binding of these hydrophobic agents that makes the active site more accessible to the substrate [22].

The results are consistent with the previous finding for the stability test of lipase Lip2 from *Yarrowia lipolytica* on various detergents, such as Triton X-100 and Tween 80. Although lower stability was found

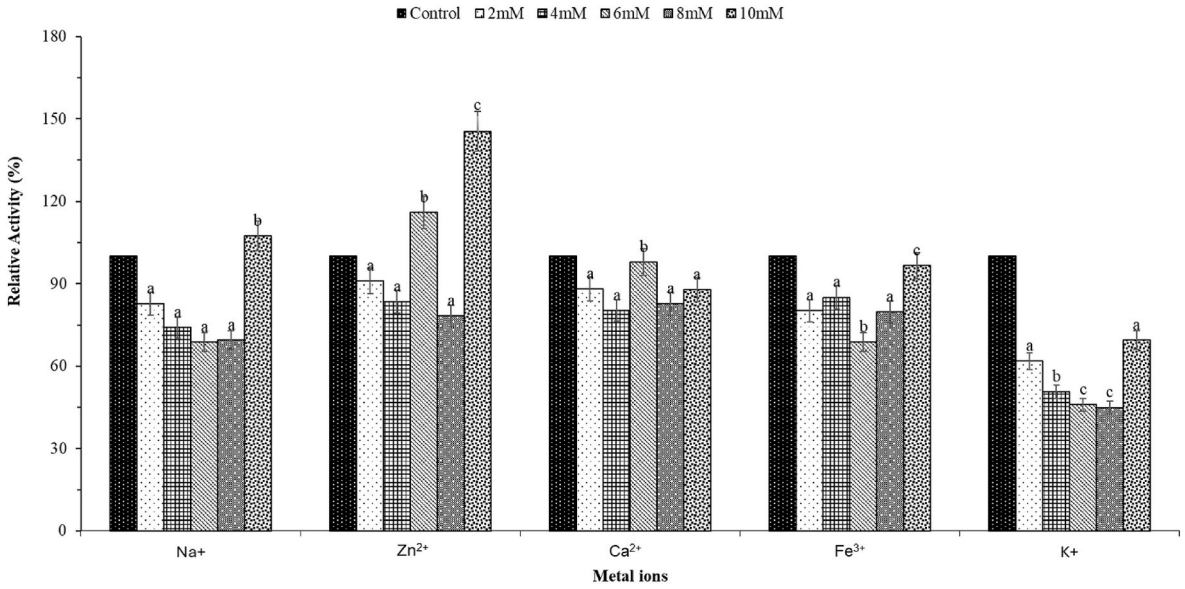

Fig. 6. Effects of various metal ions on the activity of thermotolerant *Rhizopus* sp. strain PKC12B2 lipase.

compared to the produced lipase, 0.1 % Triton X-100 and Tween 80 enhanced Lip2 lipase activity at 30 °C for 30 min of incubation with relative activity of 100.3 % and 105 %, respectively [47]. Pereira et al. [36] and Yoo et al. [48] also reported the stimulatory effect of Triton X-100 and Tween 80 from *Hypocrea pseudokoningii* and *Ralstonia* sp. CS274 lipase, respectively. Lipase sourced from *Mucor hiemalis f. corticola* was found to maintain its stability when placed in a solution containing 0.1 % Tween 80, retaining 101 % of its initial activity. However, when exposed to 0.1 % Triton X-100 and incubated at room temperature for 30 min, its activity was slightly inhibited, with 94 % of its original activity remaining [49]. Lipase from *Mucor* sp was also almost completely inhibited by Triton X-100 at 0.1 % (1 % relative activity) and 1 % (0 %) [50]. Therefore, this exceptional stability of the produced lipase in the presence of these non-ionic detergents, coupled with its alkaline nature and wide organic solvent-tolerant ability, demonstrates that it has enormous potential in biotechnology applications, such as for detergent formulations.

3.6. Kinetics study

The main function of enzymes is to enhance reaction rates so that they are compatible with the need of the organism. Kinetic description of the enzyme activity is required to understand how the produced enzyme functioned. In order to determine the kinetics of the enzyme produced, its secretion pattern was recorded using pNPP as the substrate and subjected to analysis by using linearization of Michaelis-Menten model, so that the kinetics parameters K_m and V_{max} can be determined. Models with highest coefficient of determination (R^2) were regarded as the best fit for the enzyme.

Fig. 7 displays the kinetic models of the produced enzyme for the linearized Michaelis-Menten model and Table 3 summarized the kinetic parameters for each linearized model. Based on the results, Lineweaver-Burk and Hanes-Woolf model, with the R^2 of 0.995 and 0.996, can satisfactorily be used to model the produced lipase. On the other hand, lipase might not fit the Eadie-Hofstee model due to relatively low R^2 value obtained. Comparing the K_m and V_{max} value from lineweaver-Burk and Hanes-Woolf model, lowered value was found when the enzyme was modeled using Hanes-Woolf with 1.295 mM and 3.389 mM/min respectively. The K_m parameter is mainly related to the affinity between the enzyme and the substrate [51]. The lesser the K_m value, the greater the affinity of the enzyme for the substrate. For most enzymes, K_m lies between 10^{-1} and 10^{-7} M. Meanwhile, the V_{max} parameter indicated the maximum initial velocity when all the active sites of the enzyme are occupied by substrate [52].

The K_m and V_{max} values of *Geotrichum candidum* lipase were 465.44 μmol and 0.384 μmol/min, respectively [51]. K_m from the produced lipase was found similar to *Cohnella* sp with 1.077 mM [53], but lower than that from *Botryococcus sudeticus* with 2.02 mM [54], and *Aspergillus niger* F044 with 7.37 mM [55]. The K_m and V_{max} values of the lipase purified from *Bacillus* sp. FH5 were calculated by Lineweaver-Burk plot and it was observed that the lipase has the K_m value as 5.05 mM and V_{max} as 0.416 mM/min [56]. Generally, the rate of reaction increases with an increase in substrate concentrations until the enzyme active sites are filled, at which point the maximum reaction rate is reached [57]. Thus, a lower K_m and higher V_{max} are of great biotechnological and economic importance.

4. Conclusions

The properties of thermostable- organic solvent-tolerant lipase from thermotolerant *Rhizopus* sp. strain PKC12B2 were determined through biochemical characterization. Characterizations of the PKC based produced lipase may show a new enzyme attribute. High activity and stability of the produced lipase in the presence of non-ionic detergents, coupled with its thermal-stability, alkaline nature and diverse organic solvent (polar and non-polar) tolerant ability, demonstrated its great

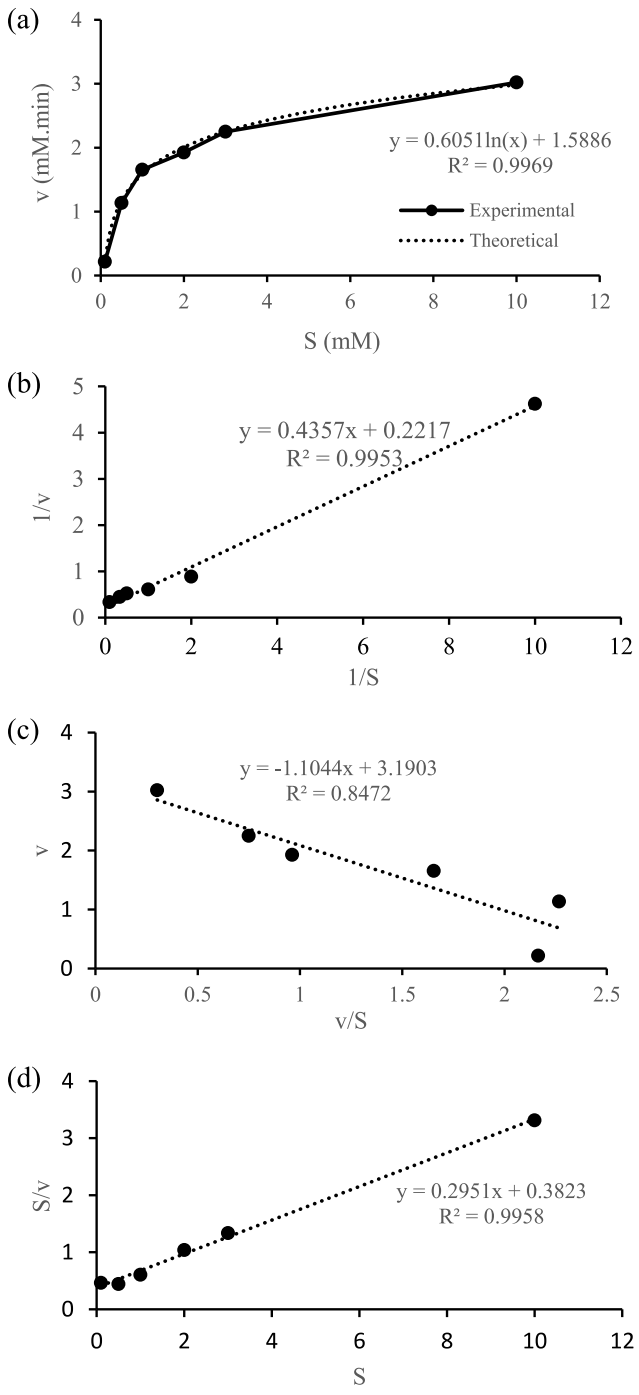

Fig. 7. Kinetic models of (a.) Michaelis-Menten hyperbolic plot (b.) Lineweaver-Burk (c.) Eadie-Hofstee (d.) Hanes-Woolf for the produced *Rhizopus* sp. strain PKC12B2 lipase.

Table 3
Kinetic parameters for lipase produced.

Model	Km (mM)	Vmax (mM/min)	R^2
Lineweaver-Burk	1.965	4.510	0.995
Eadie-Hofstee	1.104	3.190	0.847
Hanes-Woolf	1.295	3.389	0.996

potential in biotechnology applications, such as for detergent formulations, biodiesel production and organic synthesis industries.

CRediT authorship contribution statement

Fatimah Azizah Riyadi: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Md Zahangir Alam:** Writing – review & editing, Supervision, Formal analysis, Conceptualization. **Md Noor Salleh:** Supervision, Funding acquisition. **Hamzah Mohd Salleh:** Writing – review & editing, Supervision, Formal analysis. **Ibnu Maulana Hidayatullah:** Writing – review & editing, Visualization, Funding acquisition. **Hirofumi Hara:** Writing – review & editing, Visualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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