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Characterization of *Candida cylindracea* lipase produced from Palm oil mill effluent based medium

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Abstract

Production of *Candida cylindracea* lipase was optimized and highest lipase production was achieved after 36 hr of fermentation using palm oil mill effluent supplemented medium. The extracellular lipase was purified by DEAE-Sepharose and Sephacryl S-200 exhibiting a single band with molecular mass of 58 kDa by SDS-PAGE and gel filtration. The temperature and pH optima of the enzyme were found to be 35°C and 8, respectively. The enzyme was stable at pH 7.0 and 8.0 for 12 hr and also at temperature range of 25° to 35°C for 1 hr. Among the divalent cation salts tested on the lipase activity, Ca^{2+} and Mn^{2+} activated the enzyme most, while Cu^{2+} and Fe^{2+} inhibited it. Non-ionic (Tween-80 and Tween-20 and Triton X-100) and ionic detergents (SDS) at 1% appeared to have stimulatory and inhibitory effects on lipase activity respectively. In case of stability in organic solvents, water miscible solvents are concentration dependent; where their low concentrations showed some stimulatory effects, with marked inhibition at higher concentrations. It can be concluded that knowledge of these properties of the enzyme as described in this study could go a long way in its effective utilization for industrial application processes.

Key words: *Candida cylindracea*, Palm oil mill effluent, Extracellular lipase, Purification

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1. Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are important industrial enzyme due to their versatile applications [1]. Lipases catalyze a variety of reactions, such as partial or complete hydrolysis of triacylglycerols and reactions of esterification, transesterification and inter-esterification of lipids [2]. The recent interest in the production of lipases is associated with their applications as additives in food, fine chemicals, detergent, waste water treatment, cosmetics, pharmaceuticals, biomedical assays and leather processing [3, 4]. Lipases can be produced by all biological systems, viz. animals, plants and microorganisms. However, microbial lipases are receiving more attention because of the cheaper cost of production and use of renewable agricultural resources. Palm oil mill effluent (which is abundant in Malaysia), a by-product from palm oil industry has been used as a potential low-cost fermentation medium for the production of lipases. While *Candida cylindracea* recognized as GRAS (generally regarded as safe) was used as a lipase producing strain [5].

The availability of a specific lipase with all the satisfactory characteristics in terms of hydrolysis and synthesis is rare in nature. Since each industrial application requires specific properties of the enzyme. Based on this, most industrial applications are often hampered by limited stability of the enzyme at various temperatures, pH and organic solvents, and this merits the selection of potential microorganisms capable of producing lipases with physicochemical properties suitable for industrial applications [6, 7].

The purpose of the present study was to purify and characterize the lipase produced by *C. cylindracea* with respect to pH and temperature as well as stability, and to evaluate the effects of organic solvents, divalent cations and surfactants on the enzyme activity. This may aid in understanding the biotechnological applications that the produced enzyme can be utilized.

2. Materials and methods

2.1. Sample collection

Palm oil mill effluent (POME) was collected from West Oil Mill of Sime Darby Sdn. Bhd. Carey Island, Malaysia in clean containers and immediately brought to the

laboratory and stored at 4°C. All other chemicals and reagents used were of analytical grade.

2.2. Microbial culture

Candida cylindracea ATCC 14830 was obtained from American Type culture collection. The strain was grown on the potato dextrose agar (PDA) plates at 28°C for 4 days and sub-cultured every two weeks.

2.3. Fermentation of POME based medium

The fermentation medium used in this study was based on our previous study [5], containing POME sample of 1.0% (w/v) total suspended solids (TSS) supplemented with 0.2% (v/v) olive oil, 0.65% (v/v) Tween-80 and 0.45% (w/v) peptone, adjusting the initial pH to 6.0. The fermentation was carried out in 2-L Biostat (Sartorius BBI Systems) bioreactor filled with 1-L of POME based medium and sterilized in situ at 121°C and 15 psi for 15 min. The bioreactor was inoculated with 2.2% (v/v) of actively growing cells of *C. cylindracea* (10^8 cells/ml) from 48 hr-Erlenmeyer flask cultures. Standard operating conditions (temperature, agitation and aeration) were set as developed by Salihu, et al. [8] using an incubation period of 36 hrs.

2.4. Partial purification

Following the fermentation process, the cell free filtrate was obtained after centrifuging the fermentation broth at 5000 × *g* for 10 min and the resultant supernatant was used as the starting material for lipase purification. The supernatant was concentrated to one-third its original volume by ultrafiltration using hollow fiber membrane cartridge in QuickStand™ bench top system. A pre-packed DEAE-Sepharose equilibrated with Tris-HCl buffer (pH=8.0; 20 mmol/L) was employed for the anion exchange chromatographic step. The enzyme solution was dialyzed overnight using the Snakeskin® pleated dialysis tube (ThermoScientific) equilibrated with Tris-HCl buffer system. Fast protein liquid chromatography (FPLC) system was used at a flow rate of 1 ml/min. Fractions were collected and assayed for the enzyme activity and total protein. The active fractions were pooled and used for the gel filtration chromatographic step using Sephacryl S-200.

2.5. Assay for lipase activity and total protein

This was carried out according to the method reported by Gopinathet al.[9]using *p*-nitrophenylpalmitate (*p*NPP) as the substrate. Ten millilitres of isopropanol containing 30 mg of *p*NPP was mixed with 90 ml of 0.05 M phosphate buffer (pH 8.0), containing 207 mg sodium deoxycholate and 100 mg gum arabic. A total amount of 2.4 ml of freshly prepared substrate solution was dispensed into each test tube. Thereafter, 0.02 ml of enzyme solution (using appropriate dilution) was added to initiate hydrolysis. After 15 min of incubation at 37°C, the optical density at 410 nm was measured against an enzyme free control. One lipase unit (U) was defined as the amount of enzyme that liberated 1 µmol*p*-nitrophenol per milliliter per minute under the standard assay conditions.

Protein determination was performed according to Bradford method [10], which utilizes CoomassieBrillant Blue G-250 dye. The dye binds to a protein to form a complex which can be detected at 595 nm. Bovine serum albumin (BSA) was used as the standard.

2.6. Determination of pH and temperature effects on lipase activity

The optimal pH for lipase activity was measured by incubating the enzyme substrate mixture at various pHs (50 mM citrate buffer, (pH 5.0 and 6.0) and 50 mM phosphate buffer, pH 7.0 – 9.0). Also, the effect of storage pH on lipase activity was measured at pH 6.0 – 9.0 for 6, 12 and 24 hrs. Residual activity was assayed at pH 8.0 and expressed in terms of percentage. The optimal temperature for lipase activity was determined quantitatively by incubating the enzyme substrate mixture at temperatures ranging from 15° to 65°C, pH 8.0 using 50 mM phosphate buffer. In the second set of experiments, the temperature stability was determined by pre-incubating the enzyme and then measuring the remaining activity for 20, 40 and 60 min at temperatures between 25° and 55°C. Residual activity was calculated by taking the initial activity as 100%.

2.7. Stability in organic solvents

To test the lipase stability in the presence of organic solvents; the enzyme (0.1 ml) was incubated in 0.1 ml of different concentrations (20%, 50% and 80%) of methanol, ethanol, isopropanol, acetone and hexane for 60 min. Control was incubated instead with phosphate buffer (50 mM, pH 8). Residual activity was measured by taking the activity of the control as 100%.

2.8. Effects of divalent cations, detergents and other chemicals

For determining the effect of divalent cations, detergents and other chemicals on lipase activity, the enzyme solution was pre-incubated with 10 mM of $CaCl_2$, $CoCl_2$, $NiCl_2$, $CuCl_2$, $MnCl_2$, $BaCl_2$, $MgCl_2$, $FeCl_2$, EDTA, β-marcaptoethanol and 1% (v/v) detergents (Triton X-100, SDS, tween-80 and tween-20) for 60 min at 35°C and then the residual activity was determined.

3. Results and Discussion

The enzyme purification was carried out using anion-exchange and size exclusion chromatography. Sephacryl S-200 and DEAE-Sepharose were used as size exclusion column and anion exchanger respectively using Fast Protein Liquid Chromatographic technique (FPLC). After fractionation by FPLC, molecular mass of the protein contained in the active fractions was determined by SDS-PAGE. A summary of purification profile of lipase from C. cylindracea was shown in Table 1.

The results of purification profile reported in Table 1 described a two-step purification procedure, with DEAE-Sepharose as an initial step where the lipase activity was recovered with a purification fold of 1.23. This indicates that some level of purification was achieved considering the increase in the specific activity of the enzyme when compared with the crude enzyme. In the last step, a larger part of contaminating proteins was removed by Sephacryl S-200 with increase in specific activity and purification fold of 1.69. There was significant loss in total activity of the

enzyme, attributable to loss of some of the enzyme activity during the course of the purification.

The analytical SDS-PAGE of the purified enzyme following the anion and gel filtration chromatography was shown in Figure 1, indicating a single protein band with molecular weight of about 58 kDa. Lipases from different microbial species show various molecular weight ranges as reported in the literature. Hiol et al. [11] reported that extracellular lipase from *Mucorhiemalis f. hiemalis* had a molecular weight of 49 kDa using gel filtration on Sephadex G75 and SDS-PAGE.In case of *Mucor*sp.isolated from palm fruit, the extracellular lipase was purified by ammonium sulfate precipitation, carboxymethyl–sephadex and Sephadex G75 to a final specific activity of 6600 U/mg. The apparent molecular weight was found to be 42 kDa by gel filtration and SDS–PAGE [12]. More so, two distinct lipases produced by *C. rugosa*were identified and separated by a high resolution anion-exchange column (Mono Q) after an ethanol extraction of the crude lipase. Both proteins showed an apparent molecular weight of 58,000 Da by SDS-PAGE [13].

3.1. Effect of pH on lipase activity

The pH activity dependence curve indicates that the lipase activity increases from pH 5.0–8.0 and then decreases. Thus, the enzyme optimum pH was found to be 8.0 in 50 mM phosphate buffer (Figure 2) and no activity was detected at pH below 5. Consistently, the activity remained almost unaffected at pH 8.0 after 24 hrs of incubation. While about 90% and 60% activity were retained at pH 7.0 and pH 9.0 respectively. Similar trend was observed by Bussamara et al. [14] for yeast, *Pseudozymahubeiensis* HB85A where 100% of its original activity was retained at mild alkaline pH. Most lipases reported in the literature have optimal activity at neutral or slightly basic pH values [14]. Lipase produced by *Cunninghamellaverticillata* isolated from oilmill waste was reported to be stable between a pH range of 7.5–9.0 for 24 hrs, with pH optimum of 7.5 [15]. Also, pH profile of three distinct forms (Lip A, Lip B and Lip C) of extracellular lipases produced by *C. rugosa* indicated that Lip B and Lip C were optimally active at slightly alkaline pH of 7.5–8.0 and pH 7.0–7.5 respectively. While Lip Apreferred neutral pH (7.0) as the optimum [16]. Thus, it can be inferred that the lipase produced by *C. cylindracea* in POME based medium is pH dependent as evidenced from the absence and or gradual decline in the activity beyond the optimum.

3.2. Effects of temperature on lipase activity

The effect of temperature range of 15^{o}C to 65°C was studied on the enzyme activity. The produced lipase was found to be mesophilic as indicated by the optimum temperature of 35°C (Figure 3). After that the activity decreased with increase in temperature. Most microbial lipases exhibit optimal temperatures in the range of 25^{o} to 40°C. Lipase from *Mucor hiemalis f. hiemalis*, a major contaminant of Cameroonian palm fruit had an optimum temperature of 40°C [11], while the optimum for *R. oryzae* was 35°C and retained 65% of its activity at 45°C [17]. Also 35^{o}C was found to be the optimum for lipases from *P. candidum* [18]. In case of *P. chrysogenum* 9' and two isoforms from *C. rugosa* lipase B, maximum activity was found at 30^{o}C [19, 20].

In case of temperature stability, the lipase from *C. cylindracea* was stable at 25°–35°C, retaining its full activity after 60 min of incubation (Figure 4). At 45^{o}C only 50% of its activity was retained while 55^{o}C led to almost complete loss of the enzyme activity. The stability of this lipase was comparable to that reported by Liu, et al. [21] on *Aureobasidium pullulans* HN2.3 which showed good stability at lower temperature and inactivated totally at 50^{o}C within 1 hr. Benjamin and Pandey [22] indicated that the optimum and stability of all the three forms of lipases purified from the supernatant of *C. rugosa* DMS 2031 range from 35^{o} to 40^{o}C. Stability of *P. candidum* lipase was tested after incubation at different temperatures for 10 min. The enzyme retained its full activity at temperatures below 25°C. At 35°C the lipase retained 70% of its activity; while at 75°C complete loss of activity was observed [18].

3.3. Lipase stability in organic solvents

Lipases are able to work in both organic solvents and in aqueous solutions. Their stability in organic solvents is desirable especially in reactions involving synthesis. Various water-miscible and water-immiscible solvents were tested for their effects on lipase activity and stability (Table 2). A marked stimulation of the enzyme was observed upon addition of hexane up to 80% to the assay mixture. In contrast, ethanol, methanol and isopropanol inhibited the enzyme at concentrations of 50% and above but no effects were observed at 20% in all the solvents.

Thus, water miscible solvents (ethanol, acetone and isopropanol) appeared to be concentration dependent, where low concentration of these solvents showed some stimulatory effects on the lipase activity. Similar trend in stability was observed in *P. chrysogenum* 9' lipase, where the enzyme was more than 90% stable in the presence of hexane, 1,4-dioxane and almost 70% in the presence of cyclohexane. Butanol, pentanol and xylene inhibited the enzyme activity completely at higher concentrations [19]. Sztajer et al. [23] showed that *P. simplicissimum* lipase has low stability in water-miscible organic solvents with good stability in water immiscible solvents such as hexane, heptane, benzene and isooctane.

3.4. Effects of divalent cations, detergents and other chemicals

All the divalent cations tested on the lipase activity showed some stimulatory effects except Cu^{2+} and Fe^{2+} where 10% and 28% residual activity respectively (equivalent to 90% and 72% inhibition) were observed after 60 min incubation at 35^{o}C. Among the cations, Ca^{2+} and Mn^{2+} activated the enzyme most (Table 3). It is not surprising that Ca^{2+} and other metal ions appeared to have stimulatory effect on the lipase; this is because metals contribute to the stabilization of enzyme tertiary structure by acting as cofactors, where their presence enhances the catalytic efficiency of the enzyme [24]. Also, β-mercaptoethanol had little or no effect on the enzyme activity (Table 3).

However, EDTA (ethylene diamine tetraacetic acid) as metal chelator inhibited the enzyme activity by 81% (19% residual activity), suggesting that the produced lipase could be metalloprotein. In contrast to our result, Bussamara

et al. [14] showed that the lipase from yeast *P. hubeiensis* was enhanced up to 123.4% by EDTA, while Ca^{2+} showed inhibitory effects on the enzyme activity. In case of *C. verticillata* lipase, the enzyme activity was strongly inhibited by $AgNO_3$, $NiCl_2$, $HgCl_2$, $CdCl_2$ and EDTA; while the presence of Ca^{2+}, Mn^{2+} and Ba^{2+} ions enhanced the activity of the enzyme [15].

The non-ionic-detergents, tween-80 and tween-20 stimulated the lipase activity by 112% and 118% respectively while ionic detergent SDS strongly inhibited the enzyme. The stimulation of lipases by non-ionic detergents could be due to their functions as stabilizers of interfacial area facilitating the access of the substrate to the enzyme, since the catalytic reactions of lipases occur at an oil-water interface [25].

Abbas et al. [12] and Hiol et al. [17] showed that there was full inhibition by surfactants (Triton X-100 and SDS at 0.1%) on lipase activity of *Mucor* sp. strain and *R. oryzea* isolated from palm fruit. However, in case of *P. candidum* lipase activity, all detergents had a positive effect except tween-80 and sodium cholate, which have a negative influence on the activity [18].

Table 1. Purification table of *C. cyindracea* lipase

Purification step	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold
Crude	0.58	41.46	71.48	1
DEAE-sepharose	0.08	7.02	87.75	1.23
Sephacryl S-200	0.035	4.23	120.85	1.69

Table 2. Effect of different concentrations of organic solvents on lipase activity

Organic solvents	Concentration (% v/v)	Relative activity (%)
Methanol	20	90.28±5.21
	50	7.62±0.03
	80	4.42±0.04
Ethanol	20	108.75±6.02
	50	10.37±0.02
	80	5.65±0.01
Acetone	20	108.43±2.05
	50	103.26±4.03
	80	5.41±0.17
Isopropanol	20	100.77±0.08
	50	6.87±1.09
	80	4.11±0.11
Hexane	20	106.26±2.25
	50	110.99±3.16
	80	103.37±0.19
Control*		100

*Control contains the enzyme and buffer at pH 8

Table 3. Effect of diverse chemicals and detergents on lipase activity

Compounds	Concentrations	Relative activity (%)
$MgCl_2$	10 mM	148.40±8.77
$CaCl_2$	10 mM	176.31±3.95
$CoCl_2$	10 mM	105.32±1.04
$CuCl_2$	10 mM	10.11±0.06
$FeCl_2$	10 mM	28.01±3.33
$MnCl_2$	10 mM	159.95±4.88
$NiCl_2$	10 mM	158.12±6.89
$BaCl_2$	10 mM	151.73±0.97
EDTA	10 mM	19.43±0.01
β-marcaptoethanol	10 mM	102.03±2.37
SDS	1%	9.75±4.08
Triton X-100	1%	97.60±2.13
Tween-80	1%	112.61±5.09
Tween-20	1%	118.13±0.12
Control*		100

*Control contains the enzyme and buffer at pH 8

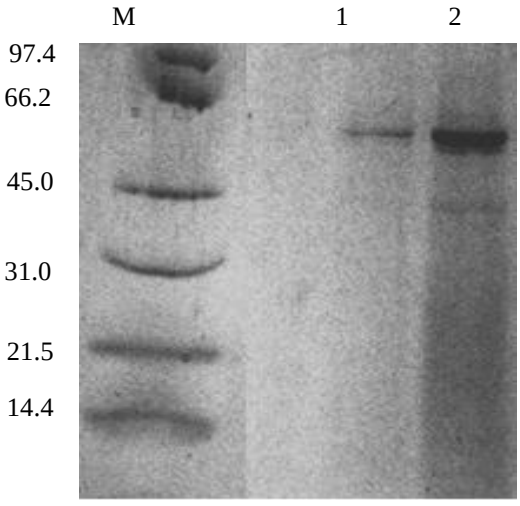

Figure 1. SDS–PAGE analysis of purified *C. cylindracea* lipase. Lane M: molecular weight markers; lane 1 & 2: concentrated purified lipase following gel filtration and anion exchange chromatography respectively.

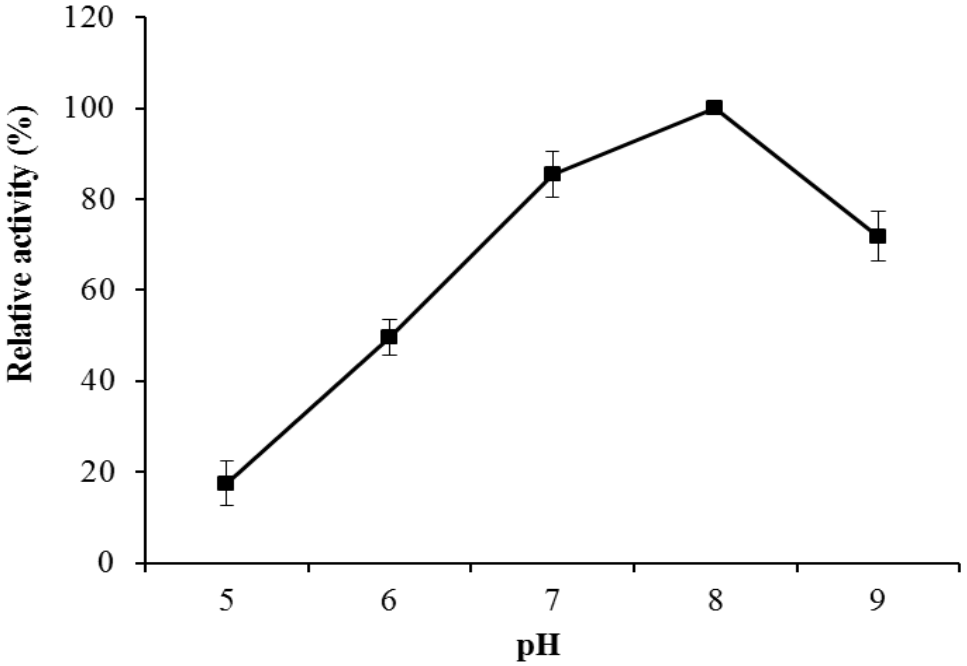

Figure 2. pH profile of *C. cylindracea* lipase

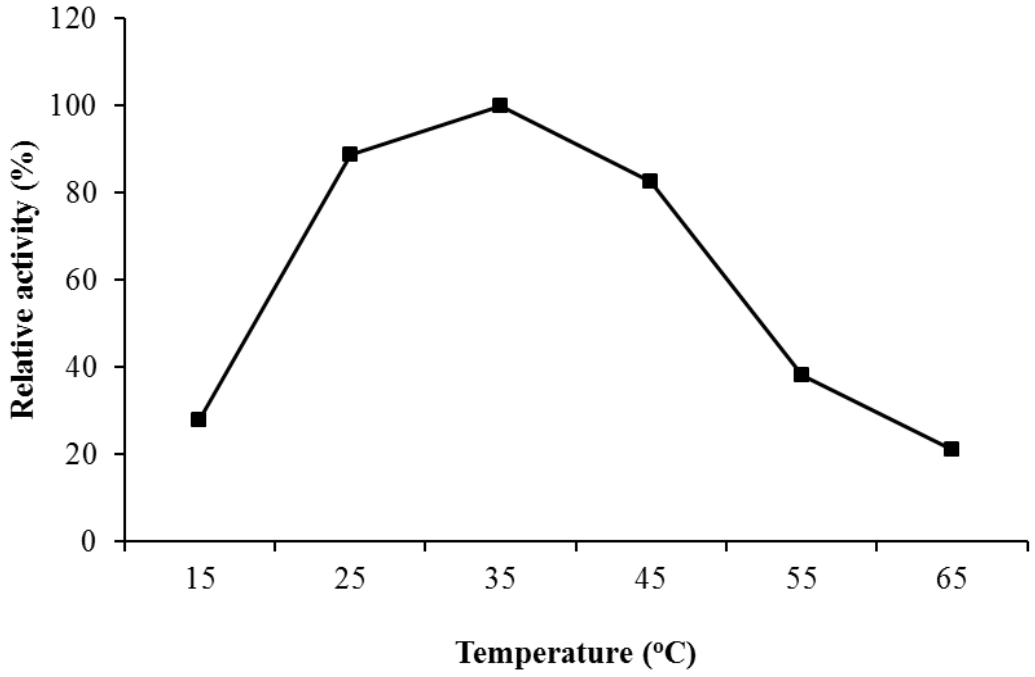

Figure 3. Effects of temperature on *C. cylindracea* lipase

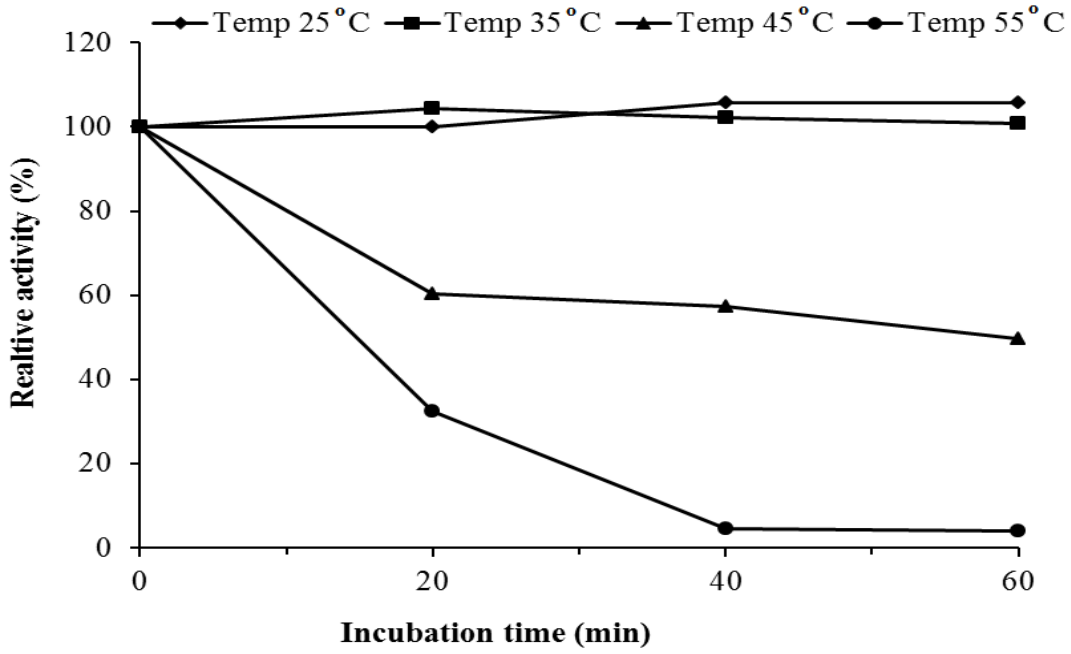

Figure 4. Temperature stability of *C. cylindracea* lipase

4. Conclusion

The produced lipase was purified and characterized, targeting some potential industrial applications. The enzyme has a good stability in organic solvents as well as under mild alkaline conditions with optimum activity at 35°C. Knowledge of these properties of the enzyme as described in this study could go a long way in its effective utilization for the industrial application processes.

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